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Human immunodeficiency virus type 1 clade B and C Tat differentially induce indoleamine 2,3-dioxygenase and serotonin in immature dendritic cells: Implications for neuroAIDS

Thangavel Samikkannu,[1] Kurapati VK Rao,[1] Nimisha Gandhi,[1] Shailendra K Saxena,[2] and Madhavan PN Nair[1]

[1]Department of Immunology, Institute of NeuroImmune Pharmacology, College of Medicine, Florida International University, Miami, Florida, USA; and [2]Centers for Cellular and Molecular Biology, Hyderabad, India

Human immunodeficiency virus type 1 (HIV-1) is commonly associated with immune dysfunctions and the suppression of antigen-presenting cells. This results in immune alterations, which could lead to impaired neuronal functions, such as neuroAIDS. The neurotoxic factor kynurenine (KYN), the rate-limiting enzyme indoleamine 2,3-dioxygenase (IDO), serotonin (5-HT), and serotonin transporter (5-HTT) may play a role in tryptophan deficiency and serotogenic dysfunction in neuroAIDS. HIV-1 transactivator regulatory protein (Tat) is known to play a major role in immune dysfunction. Previous studies suggest that HIV-1 B and C clades differentially manifest neuronal dysfunctions in the infected host. In the present study we examine the effect of HIV-1 B and C clade–derived Tat on IDO and 5-HTT gene and protein expressions by dendritic cells as studied by quantitative polymerase chain reaction (qPCR) and Western blot. In addition, the intracellular IDO expression, IDO enzyme activity, and the levels of 5-HT and KYN were also measured. Results indicate that HIV-1 clade B Tat up-regulates IDO and down-regulates 5-HTT gene and protein expressions. Further, HIV-1 clade B Tat caused a reduction of 5-HT with simultaneous increase in KYN levels as compared to HIV-1 clade C Tat. These studies suggest that HIV-1 clade B and C Tat proteins may play a differential role in the neuropathogenesis of HIV-associated dementia (HAD) or HIV-associated neurocognitive disorder (HAND). *Journal of NeuroVirology* (2010) **16,** 255–263.

Keywords: HIV-1 Tat; IDO; immature dendritic cell; kynurenine; serotonin transporter

Introduction

Dendritic cells (DCs) play a significant role in the first line of defense against pathogens, including human immunodeficiency virus type 1 (HIV-1) (Cameron *et al,* 1994; Geijtenbeek *et al,* 2003; Nair *et al,* 2004). HIV-1 directly affects the dendritic cells, causing a deficiency in antigen presentation. This is manifested by a dysregulation of inflammatory cytokines, chemokines, and other factors, such as tryptophan deficiency, indoleamine 2,3-dioxygenase (IDO) (Lichtner *et al,* 2004; Granelli-Piperno *et al,* 1996; Bahr *et al,* 2003), and inflammatory monoamine serotonin (5-HT) (Maneglier *et al,* 2008).

Previous studies indicate that IDO is an intracellular tryptophan-degrading enzyme found in specific DC subsets and involved in the impairment of immune regulation (Munn *et al,* 2002). The rate-limiting enzyme IDO may affect antigenic stimuli by reducing tryptophan availability and suppressing

Address correspondence to Madhavan P. N. Nair, PhD, Professor and Chair of Immunology, Institute of NeuroImmune Pharmacology, Associate Dean of Bio-Medical Research, College of Medicine, Florida International University, University Park, 11200 S.W. 8th Street, Miami, FL 33199, USA.
E-mail: nairm@fiu.edu

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the T-cell tolerance. The reduced tryptophan synthesis, which could initiate the cell-cycle arrest, produces toxic metabolites, T-cell apoptosis, and altered immune dysfunction in DCs (Mellor and Munn, 2004). The tryptophan deficiency activates IDO and subsequently affects 5-HT (Heyes *et al,* 1992; Wichers and Maes, 2004), which is reciprocally regulated in DC subsets (Agaugue *et al,* 2006; O'Connell *et al,* 2006). The concentration of 5-HT can be regulated via its transport into monocytes and dendritic cells through the activation of 5-HT uptake systems (Mössner and Lesch, 1998; Gordon *et al,* 2003), which are mediated by serotonin transporter (5-HTT). There is evidence that 5-HT is a key player in intercellular inflammatory processes between DC and T-cell signaling (O'Connell *et al,* 2006). The 5-HTT might play a role in the termination of serotonergic neurotransmission by 5-HT uptake, and possibly regulates the magnitude and duration of serotonergic responses (Adham *et al,* 1998). Previous studies have shown that DC plays a wide role in HIV infection that results in increased viral replication, interferon (IFN)-γ–mediated induction of IDO, and subsequently a decreased T-cell response (Munn *et al,* 1999; Hwu *et al,* 2000; Sanders *et al,* 2002; Steinman *et al,* 2003; Duvall *et al,* 2003). In addition, this is suggested to be involved in the progression of HIV-associated dementia (HAD). On the other hand, reduced 5-HT levels have been implicated in depressive and suicidal behaviors in HIV-infected subjects (Kumar *et al,* 2001). It has been reported that the cytokine IFN-γ and the serotonin interactions through IDO may explain the neurodegeneration hypothesis of depression and cognitive impairments (Myint and Kim, 2003).

HIV-1 displays extraordinary genetic variation in geographic clusters and is classified into three groups: M (major), O (outlying), and N (new) (Robertson *et al,* 2000). The M type is the most prevalent, and has nine genetically variant subtypes or clades (A, B, C, D, E, F, G, K, and O). Of these, clades B and C represent the large majority (>86%) of circulating HIV-1 variants (Osmanov *et al,* 2002). Clade B is found in North America, Canada, Brazil, Western Europe and Australia, whereas clade C is found specifically in Africa, Latin America, China, India, and Nepal (Myers *et al,* 1992; Korber *et al,* 1998; Rambaut *et al,* 2001). Global predominant subtypes of HIV-1 clade B and C infections influence the differential effect of immune and neuronal dysfunctions (Mishra *et al,* 2008; Campbell *et al,* 2007). HIV-1 transactivator protein (Tat) is a 14–16-kDa protein and is known to be released in HIV infection and modulate viral replications and various host cellular and immune dysfunctions (Amarapal *et al,* 2005). In addition, Tat plays a significant role in the modulation of various cytokines and chemokines (Izmailova *et al,* 2003; Gandhi *et al,* 2009). Further, previous studies have shown that Tat dysregulates apoptosis and activates the intracellular signaling mechanisms (Bartz and Emerman, 1999; Oshima *et al,* 2000), although the precious role of clade-specific HIV-1 Tat protein on immune dysfunction is not elucidated yet.

In the present study, we hypothesize that the HIV-1 clade B and C Tat differentially induce the IDO gene expression and protein modification as well as kynurenine (KYN), 5-HT, and serotonin transporter (5-HTT) in immature dendritic cells (IDCs), which may provide new insights regarding the immunopathogenic implication of neuroAIDS.

Results

Effect of HIV-1 clade B and C Tat on IDO and 5-HTT gene expression

Because HIV-1 clade B infection is reported to induce more immune dysfunction and secrete neurotoxin kynurenine as compared to HIV-1 clade C infection (Mishra *et al,* 2008; Rao *et al,* 2008; Samikkannu *et al,* 2009), we investigated whether HIV-1 clade B and C Tat differentially modulate the neurotoxin KYN mediated by IDO in immature dendritic cells (IDCs). Data presented in Figure 1A and B show the dose-response (0 to 100 ng/ml) effect of HIV-1 clade B and C Tat on IDO and serotonin transporter gene expression. HIV-1 clade B Tat demonstrates significant up-regulation of IDO gene expression in a dose-dependent manner, whereas 5-HTT gene expression was significantly down-regulated by HIV-1 clade B Tat protein, compared with the control. IDCs treated with HIV-1 clade C Tat showed neither increase nor decrease in IDO and 5-HTT gene expression compared with untreated control. In our kinetic studies (Figure 2A) of 12 to 48 h, a significant increase in IDO gene expression was observed at 24 h ($P < .006$) compared to 12 and 48 h of incubation. Similarly 5-HTT expression (Figure 2B) was significantly down-regulated by HIV-1 clade B Tat protein at 24 h ($P < .01$) compared to 12 and 48 h or compare to HIV-1 C Tat–treated cultures. Based on the dose and kinetics results, we used 25 ng/ml of HIV-1 Tat protein at 24-h period for all our further studies.

HIV-1 B and C Tat reduce the level of serotonin (5-HT)

Serotonin is the major player in the immune modulation functions in DCs. Because no studies have reported on the quantification of 5-HT in dendritic cells by HIV-1 clades, we evaluated the HIV-1 clade B and C Tat–induced 5-HT level in IDC supernatant. Figure 3A indicated that IDCs treated with 25 ng/ml of HIV-1 clade B Tat significantly reduced the level of serotonin (5-HT) ($P < .02$) compared to HIV-1 clade C Tat.

IDO enzyme activation by HIV-1 B and C Tat proteins

Because IDO is one of the major rate limiting enzymes and plays a significant role in tryptophan

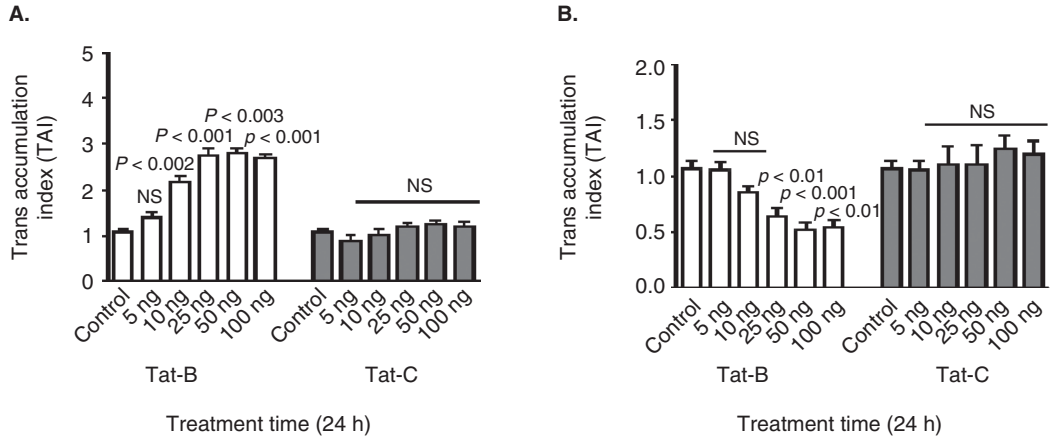

Figure 1 Dose-response effect of HIV-1 clade B and C Tat proteins on IDO (**A**) and serotonin transporter (**B**) gene expression by immature dendritic cells. IDCs (1×10^6 cells/ml) were treated separately with HIV-1 clade B and C Tat at 5 to 100 ng/ml for 24 h and RNA was extracted and reverse transcribed, followed by quantitative real-time PCR using specific primers for IDO, serotonin transporter, and housekeeping β-actin. Data are expressed as mean ± SD of TAI values of three independent experiments compared to control culture.

catabolism, we investigated the role of HIV-1 clade B and C Tat in inducing IDO enzyme activity in IDCs. Data presented in Figure 3B show that treatment of IDCs with 25 ng of HIV-1 clade B Tat ($P < .0009$) up-regulated IDO enzyme activity at 24 h compared to HIV-1 clade C Tat ($P < .01$).

Kynurenine production increased by HIV-1 clade B and C Tat

In order to understand whether IDO gene expression was mediated through kynurenine pathway, we measured KYN concentrations by Ehrlich's assay in the supernatants of HIV-1 clade B and C Tat–treated groups. Data presented in Figure 4 show the dose-response at 24 h of cultures treated with HIV-1 clade B and C Tat proteins (Figure 4A), and kinetic studies at 12, 24, and 48 h (Figure 4B). Our results showed that the KYN levels induced by HIV-1 clade B Tat were significantly higher as compared to HIV-1 clade C Tat and maximum up-regulation was observed at 24 h. Further, the increased level of KYN in the supernatant was concurrent with the increased levels of IDO mRNA expression (Figures 1A, 2A) and IDO enzyme activity (Figure 3B).

Effect of HIV-1 clade B and C Tat on IDO and 5-HTT protein expression

In addition, we examined by Western blot whether HIV-1 clade B and C Tat proteins activate differential expression of IDO and 5-HTT proteins in IDCs. Data presented in Figure 5A show that HIV-1 clade B Tat significantly up-regulated IDO protein expression

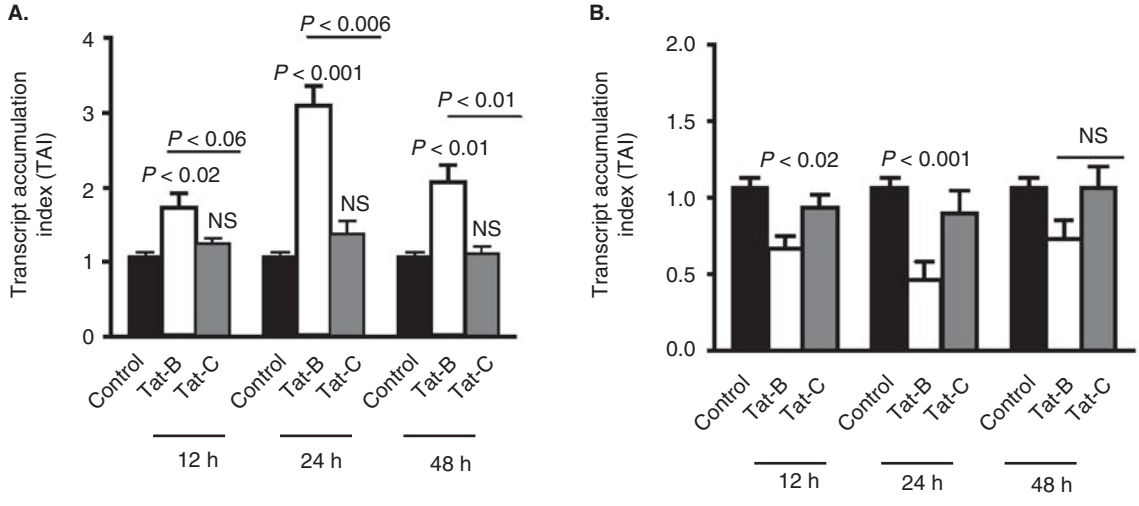

Figure 2 Kinetics studies for the effect of HIV-1 clade B and C Tat proteins on IDO (**A**) and serotonin transporter (**B**) gene expression by immature dendritic cells. IDCs (1×10^6 cells/ml) were treated separately with HIV-1 clade B and C Tat at 25 ng/ml for 12, 24, and 48 h. Data are expressed as mean ± SD of TAI values of three independent experiments.

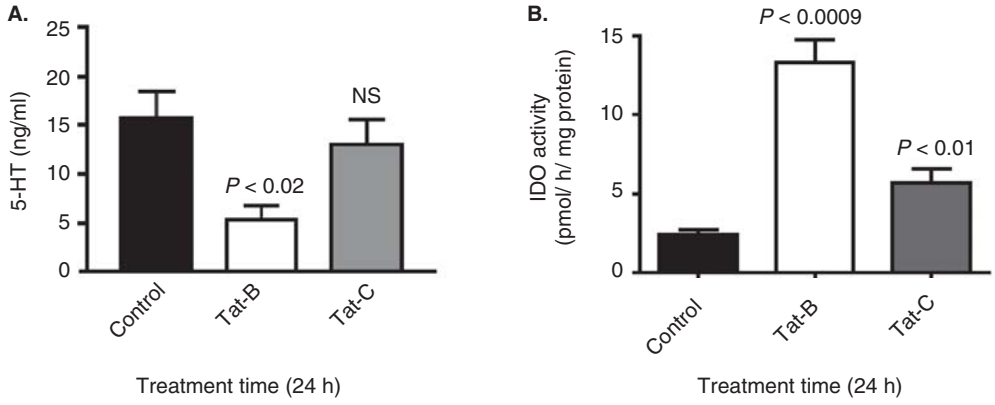

Figure 3 Effect of HIV-1 clade B and C Tat proteins on level of serotonin (**A**) and IDO enzyme activity (**B**) in immature dendritic cells. IDCs (1×10^6 cells/ml) were treated separately with HIV-1 clade B and C Tat at 25 ng/ml for 24 h. The cell supernatants were examined for the level of serotonin by ELISA and the cell lysates were examined for IDO enzyme activity. Data are expressed as mean ± SD of three independent experiments.

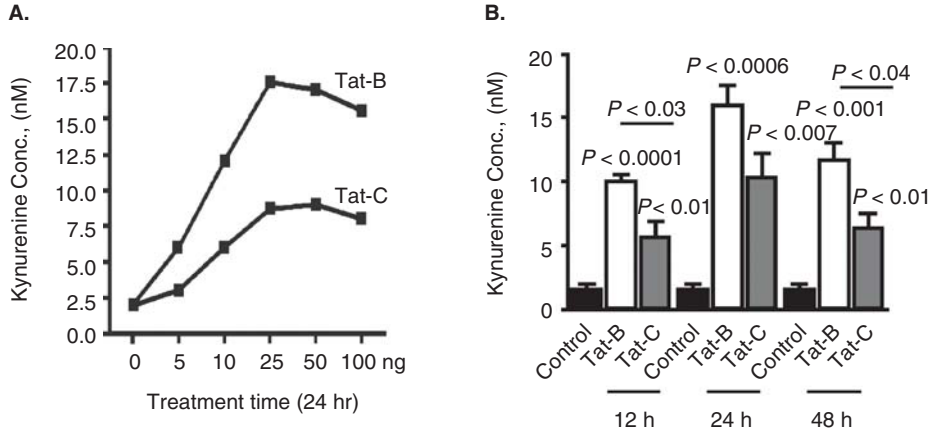

Figure 4 Effect of HIV-1 clade B and C Tat proteins on kynurenine levels by immature dendritic cells. IDCs (1×10^6 cells/ml) were separately treated with different concentrations, from 0 to 100 ng/ml, of HIV-1 clade B and C Tat for 24 h (**A**). Kinetic studies of kynurenine levels in IDCs treated with HIV-1 clade B and C Tat at 25 ng/ml for 12, 24, and 48 h (**B**). Data are expressed as mean ± SD of three independent experiments.

(lane 2; $P < .02$) as compared to HIV-1 clade C Tat (lane 3) or control culture (lane 1). Data presented in Figure 5B shows the densitometric evaluation of IDO protein levels in IDCs treated with HIV-1 clade B Tat compared with IDCs treated with HIV-1 clade C Tat. The results showed that HIV-1 clade B Tat significantly up-regulated IDO protein levels compared to HIV-1 clade C Tat protein. Data presented in Figure 5C and D show that IDCs treated with HIV-1 clade B Tat significantly down-regulated the expression of 5-HTT (lane 2; $P < .01$) compared to HIV-1 clade C Tat (lane 3) or the control culture (lane 1).

Effect of HIV-1 clade B and C Tat on IDO intracellular expression

Further, we confirm the intracellular IDO expression by HIV-1 clade B and C Tat in IDCs. The results show that the increased intracellular IDO expression (Figure 6) by flow cytometry analysis was concurrent with the results of increased KYN level, IDO gene expression, and protein modification. These results suggest that HIV-1 clade B Tat potently activates IDO induction compared to HIV-1 C Tat.

Discussion

Previous studies have shown that HIV infection depletes tryptophan, which is associated with HIV-associated dementia (HAD) or HIV-associated neurocognitive disorder (HAND). Tryptophan is an important factor in the immunoregulatory functions (e.g., immune tolerance) of antigen-presenting cells such as macrophages or dendritic cells (DCs) (Mellor and Munn, 2004). A deficit in tryptophan activated

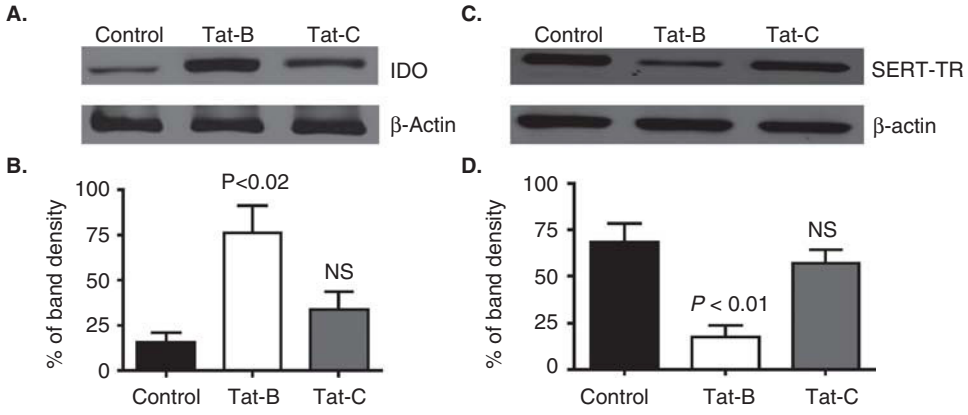

Figure 5 Effect of HIV-1 clade B and C Tat proteins on IDO and 5-HTT protein expression by immature dendritic cells. IDCs (1×10^6 cells/ml) were separately treated with HIV-1 clade B and C Tat at 25 ng/ml for 24 h and analyzed by Western blot. (**A**) A representative experiment on IDO protein expression: lane 1, control; lane 2, clade B Tat treated; lane 3, clade C Tat treated; (**B**) % of band density. (**C**) A representative experiment on 5-HTT protein expression: lane 1, control; lane 2, clade B Tat treated; lane 3, clade C Tat treated; (**D**) % of band density. Data presented as the % of band density are mean ± SD of three independent experiments.

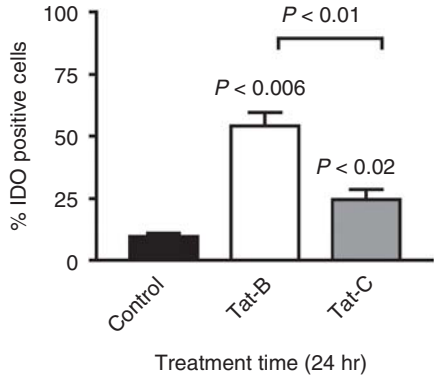

Figure 6 Effect of HIV-1 clade B and C Tat proteins on intracellular IDO expression. Immature dendritic cells (IDCs) (5×10^5 cells/ ml) were treated separately with HIV-1 clade B and C Tat at 25 ng/ml for 24 h and intracellular expression of IDO was analyzed by flow cytometry. Data are expressed as % IDO-positive cells and represent mean ± SD of three independent experiments.

by HIV in IDO mediates an increased secretion of KYN and neurotoxin quinolinic acid (QUIN) production found in cerebrospinal fluid (CSF) of HAD-patients (Sei *et al,* 1995). However, overstimulation of IDO leads to depletion of tryptophan and eventually reduces 5-HT uptake caused by dysfunction of 5-HTT (Heyes *et al,* 1992), which may play a vital role in the termination of serotonergic neurotransmission and lead to the development of depressive, behavioral, and neurocognitive disorders (Wichers *et al,* 2004; Stone and Darlington, 2002; Maes *et al,* 2002) in HAD patients (Martin *et al,* 1992). However, there are no reports on the molecular mechanisms of involvement in IDO expression as well as 5-HT level by HIV-1 clade B and C Tat proteins. The present study provides new insights into the functional role of IDO in L-tryptophan conversion to kynurenine, which subsequently affects serotogenic dysfunction (5-HT and 5-HTT) following HIV-1 clade B and C Tat exposure in DCs. Our *in vitro* studies showed that the clade-specific HIV-1 Tat proteins differentially express KYN, IDO, and 5-HT, which could lead to neuronal dysfunction and are implicated in neuroAIDS.

In the present study, we have shown for the first time that HIV-1 clade B Tat increased IDO and decreased 5-HTT mRNA expression (Figures 1, 2) as well as reduced the level of 5-HT compared with HIV-1 clade C Tat (Figure 3A). In addition, the IDO enzyme activity (Figure 3B) and level of KYN were markedly increased HIV-1 clade B Tat compared to HIV-1 clade C Tat (Figure 4A and B). HIV-1 clade C Tat showed low IDO gene expression, enzyme activity, and KYN level. It is known that IDO is the major player in the secretion of neurotoxic factor QUIN, which suppresses the 5-HT level in HIV-associated dementia and cognitive patients. These studies suggest that patients infected with HIV-1 clade B may have an enhanced role of IDO and neurotoxin KYN (marker of neuropathogenesis) compared to HIV-1 clade C–infected subjects. This is consistent with earlier reports of reduced neurocognitive impairments in HIV-1 clade C–infected subjects (Gupta *et al,* 2007). These studies further confirm that the reduction of 5-HT and the down-regulation of 5-HTT expression in HIV-1 B Tat–treated cells may lead to depression and cognitive disorders in HIV-infected patients.

Further, our results show that HIV-1 clade B Tat–induced activation of IDO protein (Figure 5A and B) was associated with a concomitant elevation of intracellular IDO (Figure 6). The main observation in this report is that both HIV-1 Tat clades B and C increase kynurenine concentrations, but result in different

patterns of IDO gene and protein expression. This suggests that these two different clades may have distinct functional effect. Previous studies indicate that without IDO enzyme activation, the IDO protein was still expressed in mouse splenic cells and human DCs (Mellor and Munn, 2004; Fallarino *et al,* 2002). Thomas *et al* showed that IDO activity and protein expression were not dependent on IDO mRNA expressions but were limited by the cell heme protein availability for IDO activity (Thomas *et al,* 2001). In addition, oxidative stress alters the functional aspect that depends on redox balance in DCs. In our study, IDCs treated with HIV-1 clade B Tat showed a high expression of IDO gene and protein, with significant increase of IDO enzyme activity and KYN concentration, whereas HIV-1 clade C Tat had no significant effect on gene or protein expression. Also, increased functional IDO enzymatic activity in the IDCs could lead to an enhanced production of neurotoxins (Smith *et al,* 2001), which alters immune tolerance and causes imbalance resulting in neuroAIDS.

Overall, the data provide evidence of an association between IDO gene expression, enzyme activation, and formation of KYN concentrations in HIV-1 clade B Tat–exposed cells. Based on these results, it is likely that clade B Tat is more potent in inducing impaired immune function, which may lead to neuropathogenesis. The present studies support molecular basis for increased neuropathogenesis in HIV-1 clade B, which was reported earlier by other investigators (Li *et al,* 2008; Campbell *et al,* 2007).

Materials and methods

Cells, HIV-1 clade B and C Tat recombinant proteins, antibodies, and reagents

The HIV-1 clade B Tat protein used in the present experiments was obtained from the National Institutes of Health AIDS Reference Reagent Program (catalog no. 2222) and HIV-1 clade C Tat was obtained from Diatheva, Fano, Italy. For the purified recombinant Tat proteins, the purities of clade B and clade C were respectively 95% and 90%. The functional properties of clade B and C Tat proteins were confirmed by transactivation assay. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA); mouse monoclonal IDO antibody was purchased from Chemicon International, and rabbit polyclonal IDO antibody was purchased from Alexion International. The mouse monoclonal serotonin transporter antibody was obtained from Abcam (San Francisco, CA). The secondary antibodies, goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology. The phycoerythrin (PE)-conjugated IgG anti-rabbit antibody was obtained from Jackson Immune Laboratory International.

Isolation and generation of IDCs

DCs were prepared from peripheral blood mononuclear cells (PBMCs) as described (Nair *et al,* 2005). Briefly, PBMCs were separated on a density gradient and adhered to plastic culture plates containing medium plus serum. Nonadherent cells were removed after 1 h at 37°C, and adherent cells were cultured in medium containing 500 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF), and 500 U/ml interleukin (IL)-4, and incubated for 5 days at 37°C to differentiate into IDCs. With a change of fresh medium on days 3 and 5, IDCs were removed by gently swirling the plate to resuspend them for use in the experiments. Isolated cells were harvested and washed with phosphate-buffered saline (PBS)/1% fetal calf serum (FCS). Subsequently, cells were stained with anti-human fluorescein-conjugated antibodies, which included the following DC surface markers, CD86, CD40, HLA-DR, DQ, and CD11c, and were characterized by flow cytometry.

Treatment of IDCs with HIV-1 clade B and C Tat proteins

IDCs (1×10^6 cells/ml) were separately treated with HIV proteins (clade B and C Tat, 5 to 100 ng/ml) for dose-response and kinetics (12 to 48 h) studies and subsequently optimized at 25 ng/ml at 24 h. The untreated cells served as control.

RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from 1×10^6 cultured IDCs were extracted using the Qiagen kit (Invitrogen Life Technologies) following the manufacturer's instructions, and the RNA level was determined using a spectrophotometer at 260 nm. Total RNA (5 μg) was used for the synthesis of the first strand of cDNA synthesis using the high-capacity reverse transcriptase (RT) cDNA kit (Applied Biosystems, Foster City, CA). To perform qRT-PCR using Taqman gene expression assays with primers for IDO (Assay ID Hs00158032_ml), housekeeping gene β-actin (Assay ID Hs99999903_m1) (Applied Biosystems), and serotonin transporter forward primer 5′-CACAACCTC CTCCTCCAGTTC and reverse primer 5′-CCCTTC CATCATCAGGCTTAGC obtained from Sigma (St. Louis, MO, USA). Relative expression of mRNA species was calculated using the comparative threshold (C_T) method. All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, β-actin. In addition, results of RNA from treated samples were normalized to results obtained on RNA from the untreated control sample. Briefly, the analysis is performed as follows: for each sample, a difference in C_T values (ΔC_T) was calculated for each mRNA by taking the mean C_T of duplicate tubes and subtracting the mean C_T of the duplicate tubes for the reference RNA (β-actin), which was measured on an aliquot from the same RT reaction.

The ΔC_T for the treated sample was then subtracted from the ΔC_T for the untreated control sample to generate a $\Delta\Delta C_T$. The mean of these $\Delta\Delta C_T$ measurements was then used to calculate expression of the test gene relative to the reference gene and normalized to the untreated control as follows: relative expression/transcript accumulation index $(TAI) = 2^{-(\Delta\Delta C_T)}$. This calculation assumes that all PCR reactions are working with 100% efficiency (Shively *et al,* 2003).

Quantification of serotonin (5-HT) by enzyme-linked immunosorbent assay (ELISA)
IDCs (1×10^6 cells/ml) were separately treated with clade B and C Tat proteins at a concentration of 25 ng/ml for 24 h as previously determined. Cell culture supernatants were analyzed for serotonin (5-HT) using commercially available ELISA kits as per the manufacturer's instructions (GenWay Biotech, San Diego, CA).

IDO enzyme assay
IDO activity was assayed by the colorimetric method with minor modifications (Lee *et al,* 2007). Briefly, 2×10^6 cells were disrupted by freezing and thawing, and the lysate (250 μl) was cleared by centrifugation, and an equal amount of 2× IDO buffer (100 mM PBS, pH 6.5, with 40 mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase, and 800 mM L-tryptophan (Sigma-Aldrich, St. Louis, MO) was added. After 30 min of incubation at 37°C, 100 μl of 30% trichloroacetic acid was added to stop the reaction, and incubated for 30 min at 52°C and centrifuged. The supernatant was mixed with an equal amount of Ehrlich's reagent, the color was allowed to develop for 10 min, and then absorbance was read at 490 nm in a spectrophotometer.

Determination of kynurenine
The change in kynurenine levels in the supernatant of HIV-1 clade B and C Tat–treated cultures was measured by spectrophotometer. Briefly, 100 μl of 30% trichloroacetic acid was added to 200 μl of the culture supernatant, vortexed, and then centrifuged at 10,000 rpm for 5 min. A 125-μl volume of the supernatant was added to 125 μl of Ehrlich's reagent (100 mg of *p*-dimethylbenzaldehyde, 5 ml of glacial acetic acid) (Sigma-Aldrich) in a microtiter plate well (96-well format). Samples were read against a reagent blank with a 490-nm filter in a microplate reader (Multiskan MS; Lab Systems, CA). The change in kynurenine concentration was obtained by subtracting the control values from the sample value.

Western blot analysis
To assess the IDO protein activation in IDCs by HIV-1 clade B and C Tat, cells were lysed by lysis buffer (Pierce, IL) with 1× complete cocktail of protease inhibitors. Total cellular protein in equal amounts were resolved by 4% to 15% polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and were incubated with primary mouse monoclonal IDO antibody, mouse monoclonal serotonin transporter antibody, and mouse monoclonal β-actin antibody, followed by corresponding secondary goat anti-mouse IgG antibody. Immunoreactive bands were visualized using a chemiluminescent Western blotting system according to the manufacturer's instructions (Amersham).

Analysis of intracellular IDO expression
The HIV-1 Tat–induced intracellular IDO expressions were analyzed by flow cytometry in fluorescence-activated cell sorting (FACS) calibur (BD Bioscience, San Jose, CA). Briefly, IDCs (5×10^5) were separately treated with HIV-1 clade B and C Tat proteins, 25 ng/ml, for 24 h. At the end of the incubation periods, 10 μg/ml brefeldin A was added to cells to inhibit cytokine secretion 4 h. Cells were harvested and washed twice with PBS. The cells were fixed and permeabilized by Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. Cells were incubated with rabbit anti-IDO antibody (Alexis Biochemicals, San Diego, CA) followed by PE-conjugated IgG anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA).

Statistical analysis
Results presented in the study are representative of three or more independent experiments performed in triplicates. Statistical significance was analyzed with the computer software Graph Pad Prism by use of analysis of variance (ANOVA) or Student's *t* test for unpaired observations. The values are presented as the mean ± SD. A P valve of ≤.05 was considered significant.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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