The HIV-1 Tat Protein Selectively Enhances CXCR4 and Inhibits CCR5 Expression in Megakaryocytic K562 Cells

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The hematopoietic compartments act as long-term reservoirs for human immunodeficiency virus type-1 (HIV-1). Although hematopoietic progenitor cells (HPCs) are rarely infectable, HPCs committed to the megakaryocytic lineage can be infected and support a productive infection by both the X4 and R5 strains of HIV-1. Indeed, in contrast to the CD34+ progenitors, the lineage-committed HPCs express high levels of the HIV-1 co-receptors, CXCR4 and CCR5. The HIV-1 transactivator (Tat) protein has been shown to alter co-receptor expression in T lymphocytes and macrophages. We hypothesized that Tat may regulate co-receptor expression in lineage-specific HPCs as well. We have monitored the effects of Tat protein on co-receptor expression and on lineage-specific differentiation, using the HPC cell line, K562. Butyric acid (BA)–induced erythroid differentiation in K562 cells was suppressed by 1–100 ng/ml of Tat, as evident from a 70–80% decrease in hemoglobin (Hb) production and a 10–30-fold decrease in glycophorin-A expression. However, Tat treatment enhanced phorbol myristate acetate (PMA)–induced megakaryocytic differentiation, as evident from a 180–210% increase in ^{3}H-serotonin uptake and a 5–12-fold increase in CD61 expression. Tat did not significantly alter co-receptor expression in erythroid cells. However, Tat co-treatment profoundly effected both CXCR4 and CCR5 gene expression and protein levels in megakaryocytic cells. In PMA-stimulated cells, Tat increased CXCR4 and decreased in CCR5 expression, this was potentiated in cells chronically exposed to Tat. In conclusion, Tat protein suppresses erythroid and facilitates megakaryocytic differentiation of K562 cells. In megakaryocytic cells, Tat differentially effected CXCR4 and CCR5 expression. Because megakaryocytes may play a crucial role in HIV-1 infectivity in viral reservoirs, our findings implicate a role for Tat protein in **dictating co-receptor usage in lineage-committed HPCs.** Exp Biol Med 230:631–644, 2005

Key words: HIV-1; Tat; HPCs; differentiation; CXCR4; CCR5; K562

Introduction

The human immunodeficiency virus type-1 (HIV-1) is the etiologic agent of acquired immune deficiency syndrome (AIDS). Initial infection occurs predominantly in CD4+ T lymphocytes, which serve as short-term viral reservoirs. Infection of CD4+ T helper cell (Th) lymphocytes is cytopathic and results in a precipitous drop in Th immune surveillance function, which eventually leads to the manifestations of AIDS-related complex (ARC; Refs. 1 and 2). Human immunodeficiency virus type-1 can also infect monocytes/macrophages; dendritic cells; and megakaryocytes, where infection is noncytopathic. These myeloid cell types act as long-term reservoirs. The CD4 molecule is the primary HIV-1 receptor in both lymphoid and myeloid cell lineages. The presence of HIV-1 co-receptors, CXCR4 and CCR5, can also dictate susceptibility to infection. The natural receptor for the C-X-C chemokine stroma-derived factor (SDF)-1α is CXCR4, and CCR5 is the predominant receptor for the C-C family of chemokines; regulated on activation, normal T expressed and secreted (RANTES); monocyte inflammatory protein (MIP)-1α and MIP-1β. The T-cell tropic (T-tropic) viruses predominantly recognize CXCR4, and the monocyte/macrophage tropic (M-tropic) viruses recognize CCR5. Preferential co-receptor usage denotes the viral strains as either X4 and R5 viruses, respectively. Although the hematopoietic progenitor cells (HPCs) express low levels of these co-receptors, they are abundantly expressed in the lineage-committed hematopoietic cells (3–5).

Specific co-receptors may be used by HIV-1 to gain entry into cells, which may dictate viral infectivity. The HIV-1 envelope glycoprotein, gp160 (gp120 and gp41 complex), binds to cell-surface receptors resulting in

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membrane fusion and viral entry into host cells (6). After entry and uncoating, viral RNA is reverse transcribed to DNA containing flanking long terminal repeat sequences (LTR). The circularized double-stranded DNA is transported into the nucleus and integrate into host-cell chromosomes to establish the provirus. Proviral activation is controlled by different viral and cellular transcription factors binding to the LTRs. The HIV-1 accessory genes (e.g., *tat, nef, rev, vif,* and *vpu*) are expressed early during the viral replication cycle and play essential roles in regulating viral propagation and infectivity. Expression of late viral transcripts gives rise to the structural proteins, *gag, pol,* and *env,* which eventually dictates a productive infection. The transactivator (Tat) protein has crucial significance in viral transactivation (7–9). Similar transactivators, coded by other lentiviruses, have also been implicated in both viral pathogenesis and the slow progressive disease, characteristic of AIDS (10–13).

Despite highly active anti-retroviral therapy (HAART) the persistence of reservoirs of HIV-1 poses clear obstacles to the eradication of the disease. Although initial viral decay kinetics in plasma had indicated optimistic outcomes of HAART (14), long-term measurements have suggested that mononuclear lymphocytes harbor the virus for prolonged periods (15). Fauci and colleagues (16) had indicated the existence of extravascular reservoirs for HIV-1, which are present in different progenitor cell organs, such as the lymph nodes, brain, and bone marrow. After discontinuation of HAART, a rapid rebound in plasma viremia was documented. The dynamics of the reemerging virus from sequestered progenitor cell reservoirs (17) need to be well understood. Pathologic findings in bone marrow biopsies from patients with AIDS, and frequent manifestations of hematologic dysfunctions in multiple HPC lineages, such as anemia and thrombocytopenia, suggest that productive infection may occur in reservoirs (18). Homing of HIV-1–infected lymphocytes to the bone marrow and lymph nodes has been documented (19), suggesting that viral trafficking from these reservoirs may also occur frequently. Hence, the role of early viral factors in regulating HIV-1 co-receptor expression and in virus reactivation in hematopoietic reservoirs needs to be properly elucidated.

The early viral proteins, such as *Tat* and *Rev*, are expressed even in the absence of a productive infection and may affect the infectivity parameters in reservoir cells (20, 21). Although a productive infection is rarely observed in CD34+ HPCs (22, 23), those committed toward the dendritic (24) or the megakaryocytic lineages (25) are frequently infected by both T-tropic (X4) and M-tropic (R5) strains of HIV-1. The dendritic-lineage HPCs express CCR5, are infected by R5-tropic viruses, and play a role in predominance of R5 viruses during the early stages of infection (26, 27). However, the phenomenon observed in the clinical setting, where there is a selective co-receptor usage and a delayed evolution of X4 viruses, is not properly understood. Both CXCR4 and CCR5 are expressed during megakaryocyte maturation, and infection by both X4 and R5 HIV-1 isolates has been observed (28). HIV-1–infected individuals primarily transmit R5 strains to recipients during the early stages of infection, and significant appearance of X4 virus only occurs after long latency periods (29–31). This preferential co-receptor usage has been baffling and a number of possibilities have been entertained in recent studies (26, 32, 33).

Tat protein has a complex secondary structure and diverse array of functional domains (34). It is secreted by infected cells and can have paracrine effects on neighboring uninfected cells (35). The first exon of Tat, especially the arginine-rich core domain and the nuclear localization domain, may alter signaling and transcription factor functions in different cell types (36). Deleterious effects of Tat on hematopoietic differentiation have been documented by our laboratory and others (37–40). Recent studies also showed that Tat could alter co-receptor expression in lymphoid and myeloid cells (41–45). In both monocytes/macrophages and T lymphocytes, Tat increases both CXCR4 and CCR5 expression (41). Interestingly, extracellular Tat protein was shown to be a specific antagonist of CXCR4 and selectively inhibited the entry and replication of X4 in peripheral blood mononuclear cells (PBMCs; Refs. 42 and 43). In resting CD4+ T cells (44) and erythroid cells (45), Tat upregulates CXCR4 but not CCR5. Lineage-specific expression of both CXCR4 and CCR5 at different maturational stages of HPCs has been documented (46), however, the effect of Tat in regulating co-receptor expression in HPCs has not been examined. We monitored the effects of Tat protein on both lineage-specific differentiation and HIV-1 co-receptor expression in the HPC line, K562. Our findings implicate a direct role for Tat protein in regulating lineage-specific differentiation and CXCR4 and CCR5 expression in K562 cells.

Materials and Methods

Reagents. The differentiation inducers, phorbol-13-myristate-12-acetate (PMA) and butyric acid (BA) were purchased from Sigma (St. Louis, MO). The PMA was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 1.0 m*M*. The sodium salt of BA was dissolved in phosphate buffered saline (PBS) to a stock concentration of 2.0 *M*. Recombinant Tat protein was obtained from two different sources, from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD) and from ImmunoDiagnostics (Woburn, MA). Tat protein was dissolved in PBS to a stock concentration of 1 mg/ml, aliquoted, and stored at –70°C. Antibodies were obtained from BD Biosciences (Bedford, MA) and Research Diagnostics, Inc. (Flanders, NJ), stored at 4°C, and used for flow cytometry analysis within 4–6 months. The reverse-transcriptase polymerase chain reaction (RT-PCR) primers for glycophorin-A, CD61 (integrin-β1), integrin-β3, CXCR4, CCR5, and glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) were synthesized from Midland Certified Reagent Company (Midland, TX), reconstituted to a 1 μg/μl concentration in PBS, and stored at −70°C.

Cell Culture and Treatment. The K562 cells were obtained from the American type culture collection (ATCC:CCL-243). Cells were grown in RPMI-1640 medium (Media Tech, Hernden, VA) supplemented with 10% fetal bovine serum (FBS; Alanta Biologicals, Norcross, GA) and 100 μg/ml of the antibiotics, penicillin and streptomycin (Sigma). Experiments were performed using K562 cell cultures within 12–15 passages. To induce erythroid or megakaryocytic differentiation, K562 cells were treated with either 1.0 m*M* BA or 16 n*M* PMA, respectively. Treatment with 1–100 ng/ml of Tat was initiated either by co-treatment with the inducers, or by adding Tat to prestimulated cells. Functional assessments of erythroid or megakaryocytic differentiation were performed at 24, 48, and 72 hrs after treatment by either benzidine staining of the BA-stimulated cells, or ^{3}H-serotonin uptake of the PMA-stimulated cells, respectively. Extent of differentiation was also monitored by analysis of differentiation-specific phenotypic markers. *Glycophorin-A* and *CD61* gene expression were measured by RT-PCR assays using mRNA obtained at 60 hrs after stimulation, and protein levels were monitored at 72 hrs after stimulation by flow cytometry analysis. In each treatment group, CXCR4 and CCR5 gene expression and protein levels were monitored at earlier time points, of 24 hrs after stimulation and 36 hrs after stimulation, respectively.

Functional Assessment of K562 Cell Differentiation. Hemoglobin (Hb) production in BA-stimulated K562 cells was monitored by benzidine staining (39). Stained cells were counted within 10–20 mins using a hemocytometer and an inverted phase-contrast microscope. At each time point (24–72 hrs), percentage of benzidine-positive cells was averaged from three to four replicates. Assessment of megakaryocytic differentiation was carried out by ^{3}H-serotonin uptake (47). The ^{3}H-serotonin uptake levels were measured using a scintillation counter (Tri-Carb 2500; Beckman Instruments, Fullerton, CA) and protein contents in cell extracts were monitored using a protein assay kit (Bio-Rad, Hercules, CA). The cpm values were normalized to protein content of the samples (cpm/milligram of protein).

Flow Cytometry Analysis. Phycoerythrin (PE)-conjugated antibodies to human glycophorin-A, and fluorescein isothiocyanate (FITC)–conjugated antibodies to human CD61, clone VIPL2, were used in combination; and PE-conjugated CXCR4 (clone 12-G5) and FITC-conjugated CCR5 (clone 12-G2a) were used in combination. Cells were collected by centrifugation and were co-incubated with 20 μl of the appropriate fluorochrome-conjugated antibodies for 20 mins, and washed twice with PBS. Cells were fixed in buffer containing 2% formaldehyde, and flow cytometric analysis was performed using a BD-Coulter Epic XL flow cytometer (Becton Dickinson, San Jose, CA). Background staining was determined using unstained cells and non-specific binding was determined using isotype-specific anti-mouse antibodies (mouse IgG; BD Biosciences Pharmingen, Franklin Lakes, NJ). In each experiment, cells were gated using unstained cells (region "C") and both percent immunoreactive cells and mean fluorescence intensities (MFI) were monitored in duplicate samples containing approximately 10,000 cells.

RNA Isolation and RT-PCR Assays. Total RNA was isolated by using the Tri-Zol reagent (Promega, Madison, WI) and carried out according to the manufacturer's protocols. The RNA samples were aliquoted and stored at −70°C. The RT-PCR studies were carried out using freshly thawed RNA samples. Briefly, 0.5 μg of total RNA was incubated with 0.5 U of avian-myeloblastosis virus (AMV) RT (Promega) and incubated at 42°C for 15 mins. Equal volumes of complementary DNA (cDNA) were used in PCR amplification, which was carried out using RED-Taq DNA polymerase (0.5 U) and 10 m*M* of each of the dNTPs (dATP, dTTP, dCTP, and dGTP). Each primer pair was amplified according to previous published studies (41, 48–50). Electrophoresis was performed on the PCR products on a 2.0% agarose gel containing ethidium bromide, and the gels were visualized under UV light. The band intensities were quantified by densitometry using a GS-700 densitometer (Bio-Rad) and expressed as normalized densitometric units (D.U.). The RT-PCR primer, reference numbers, product sizes, and sequences for the upstream and downstream primers were as follows: GAPDH (225 base pairs; Ref. 50), 5′-GCCAAAAGGGTCATCATCTC-3′ and 5′-GCCCATCCACAGTCTTCT-3′; glycophorin-A (240 base pairs; Ref. 48), 5′-CGCAAGCTTATGTATGGAAAAATAATCTT-3′ and 5′-GCGGATCCTTCTGGAGGGTAAACAGTCT-3′; CD61/integrin-β_3 (200 base pairs; Ref. 49), 5′-TGCTCATTGGCCTTGCCGCCCTGC-3′ and 5′- ACTATTCGTCAGTAGGAGTCTAGT-3′; integrin-β_1 (300 base pairs; Ref. 49), 5′-AATGGGAACAACGAGGTCATGGTT-3′ and 5′-TTGTGGGATTTGCACGGGCAGTAC-3′; CXCR4 (1023 base pairs; Ref. 41), 5′-GTTACCATGGAGGGGATCAG-3′ and 5′-CAGATGAATGTCACCTCGC-3′; and CCR5 (1115 base pairs; Ref. 41), 5′-GGTGGAACAAGATGGATTAT-3′ and 5′-CATGTGCACAACTCTGACTG-3′.

Statistical Analysis. All statistical analyses were performed with the Instat-2 software (GraphPad, San Diego, CA). Experiments were performed three to five times, and values obtained from two to four replicate samples were averaged in each experiment; data are expressed with the standard error of means (±SEM). Significant changes from control were determined using a two-tailed Student's *t* test and $P < 0.05$ was considered to be significant.

Results

Tat Co-treatment Decreases Hb Production in BA-Stimulated Cells and Enhances Serotonin Up-

take in PMA-Stimulated Cells. To monitor the effect of Tat on erythroid or megakaryocytic differentiation, we used the bipotent HPC line, K562, and monitored differentiation induced by either 1.0 m*M* BA or 16 n*M* PMA at 24–72 hrs after stimulation. The production of Hb in cells was monitored by benzidine staining to assess erythroid differentiation (49), and serotonin uptake was monitored to observe the extent of megakaryocytic differentiation (47). Change in percentage of BA-stimulated benzidine-positive cells or change in PMA-stimulated serotonin uptake was monitored in the presence of increasing concentrations (1–100 ng/ml) of Tat protein.

Figure 1A shows that stimulation with 1.0 m*M* BA increases the percent of benzidine-positive cells. Percent of benzidine-positive cells was 13 ± 2%, 32 ± 5%, and 42 ± 6% at 24, 48, and 72 hrs after BA-stimulation, respectively. However, co-exposure to 1, 10, and 100 ng/ml of Tat protein showed a decrease in percent of benzidine-stained cells, which was dependent on the Tat concentration used. As compared with cells stimulated with BA alone, a 48 ± 7% decrease at 48 hrs and a 69 ± 11% decrease at 72 hrs were observed after Tat co-treatment. At 10 ng/ml and 100 ng/ml, Tat showed significant ($P < 0.01$) suppression in percentage of cells producing Hb. A suppressive effect of Tat protein was observed on erythroid differentiation of K562 cells.

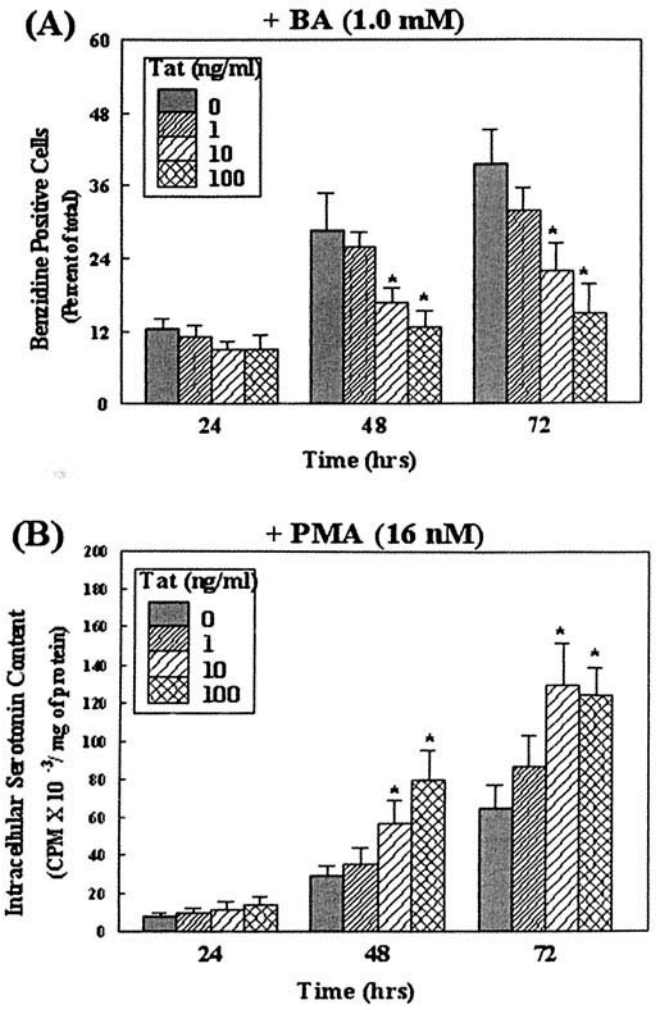


Figure 1. Effect of Tat protein erythroid- and megakaryocyte-specific functional assays in K562 cells. Cells were treated with 1.0 m*M* BA to induce erythroid differentiation or with 16 n*M* PMA to induce megakaryocytic differentiation. Cells were co-stimulated with 1–100 ng/ml of Tat protein and differentiation-specific functions were assayed after 24–72 hrs of benzidine staining (A), or ^{3}H-serotonin uptake (cpm × 10^{-3} /mg of protein; B). Bar graphs represent the average of values ± SEM ($n = 4$). As compared with the BA- or PMA-stimulated control groups, significant changes in erythroid- or megakaryocyte-specific functions in Tat co-treatment groups are represented as *P* values (*, $P < 0.05$).

In Figure 1B, the effect of Tat on PMA stimulated megakaryocytic differentiation was determined by changes in serotonin uptake at 24–72 hrs after stimulation. Stimulation with PMA showed a temporal increase in intracellular ^{3}H-serotonin levels. The normalized values (cpm/milligram of protein) were 6.5 ± 1.5 × 10^3; 28 ± 8 × 10^3; and 79 ± 5 × 10^3 counts at 24, 48, and 72 hrs, respectively. Tat treatment alone did not show any significant differences in active serotonin uptake. However, PMA-induced serotonin uptake was found to be higher in cells co-stimulated with Tat, and a concentration- and time-dependent increase was observed. As compared with PMA alone, a greater than 2-fold increase (2.2–2.5-fold) was evident with 100 ng/ml of Tat, and a 1.5–1.7-fold increase was evident with 10 ng/ml of Tat at 48 hrs after stimulation. At the 72-hr time point, exposure to 10 ng/ml of Tat was as potent as 100 ng/ml of Tat, and showed a 1.75–1.85-fold higher serotonin uptake as compared with PMA-alone groups. These data indicated that Tat co-exposure increases PMA-induced megakaryocytic differentiation in specific functional assays. Cumulatively, these findings suggest that Tat has opposite effects on erythroid and megakaryocytic differentiation of the K562 cells.

Tat Co-treatment Suppresses BA-Induced Glycophorin-A Expression and Enhances PMA-Induced CD61 Expression. The following experiments were carried out to monitor the effect of Tat on lineage-specific molecular markers in BA- or PMA-stimulated cells. Glycophorin-A was used as a marker for erythroid differentiation and CD61 was used as a marker for megakaryocytic differentiation. Expression of glycophorin-A and CD-61 were monitored at both the level of gene expression by RT-PCR (Fig. 2) and at the level of cell-surface protein expression by flow cytometry (Fig. 3). Results showed that BA-stimulated erythroid cells expressed higher levels of glycophorin-A, and PMA-stimulated megakaryocytic cells showed increased expression of CD61. Furthermore, the effect of Tat co-exposure on glycophorin-A and CD61 expression correlated with the differentiation-specific functional assays.

A representative gel picture of the RT-PCR products is shown in Figure 2A. The bands for glycophorin-A and the internal control, GAPDH, are shown in the left panel. The co-amplified PCR products for CD61 (integrin-β3) and the internal control, integrin-β1, are shown in the right panel.

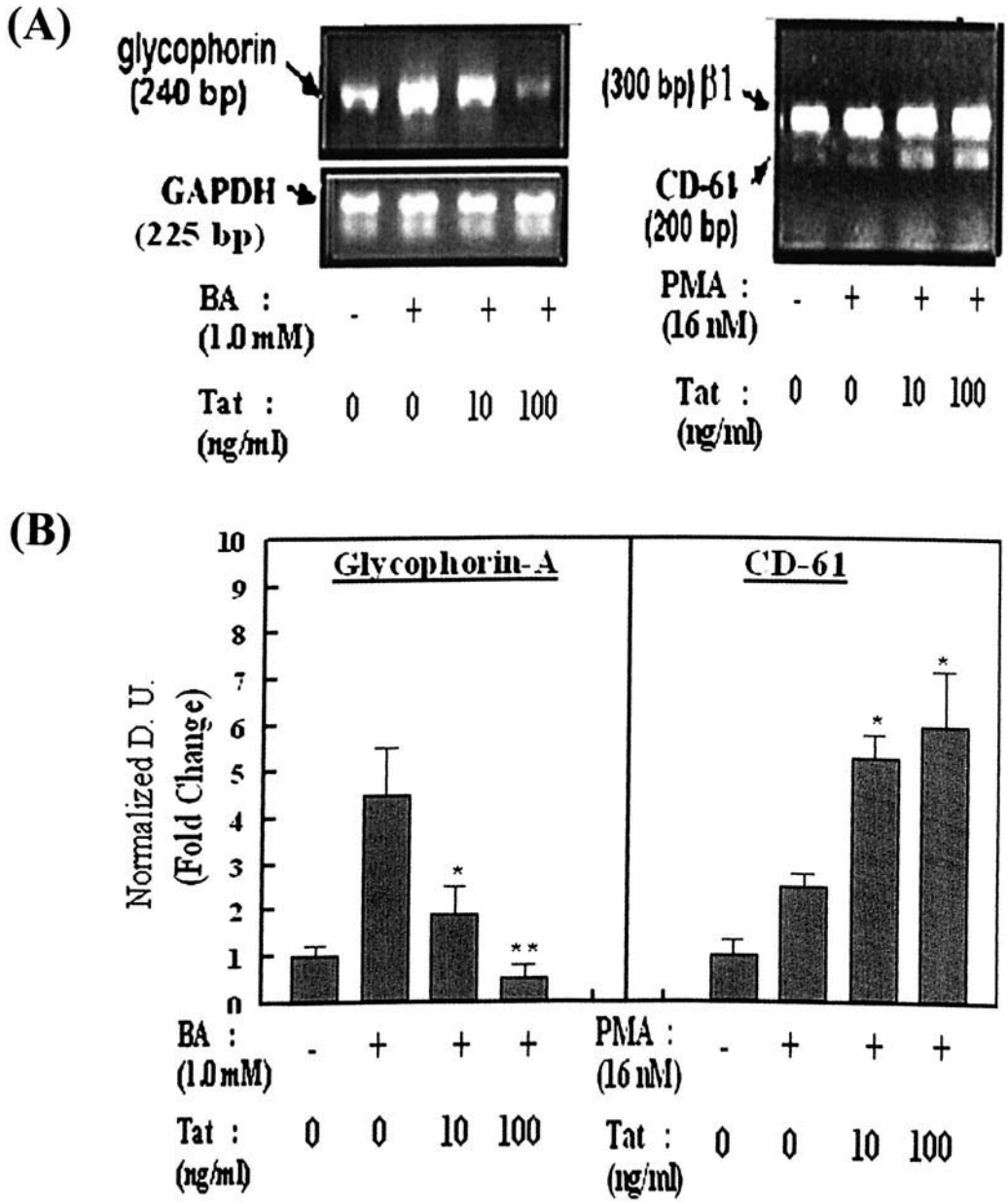


Figure 2. Effect of Tat on *glycophorin-A* and *CD61* gene expression. The message levels for erythroid marker, *glycophorin-A*, and the megakaryocytic marker, *CD61* (*integrin-β3*), were monitored by RT-PCR. Changes in lineage-specific marker expression were compared with internal controls, *GAPDH* or *integrin-β1*. A representative gel picture showing the PCR products (arrows) and their molecular weights (bp) are shown in (A). *Glycophorin-A* expression (Lanes 1–4) and *CD-61* expression (Lanes 5–8) were monitored in unstimulated controls, or in cells stimulated with either 1.0 m*M* BA or 16 n*M* PMA, both alone or in combination with 10 ng/ml or 100 ng/ml of Tat. In (B), normalized densitometric intensities of the PCR products are shown as average fold changes ($n = 3$). The error bars represent ±SEM of values, and significant changes in Tat treated cells as compared with BA- or PMA-stimulation alone, are indicated by *P* values (*, $P < 0.05$).

Bar graphs in Figure 2B show fold changes in normalized densiometric units (D.U.). As compared with the unstimulated controls (Lanes 1 and 5), cells stimulated with BA (Lane 2) showed 4.5 ± 1.6-fold increase in glycophorin-A, and PMA stimulation (Lane 6) showed a 2 ± 0.5-fold increase in CD61 expression. The effects of 10 or 100 ng/ml of Tat in erythroid cells are shown in Lanes 3 and 4; and the effects of Tat in megakaryocytic cells are shown in Lanes 7 and 8. Tat co-treatment dramatically suppressed BA-induced glycophorin-A gene expression, whereas it enhanced PMA-stimulated CD61 gene expression. A 75 ± 5% suppression in glycophorin-A was observed at 10 ng/ml of Tat ($P < 0.05$), and an almost complete suppression after 100 ng/ml Tat co-exposure was documented ($P < 0.01$). In contrast, a 235 ± 15% increase in CD61 mRNA levels was observed after Tat co-exposure ($P < 0.05$). Thus, a suppressive effect of 10–100 ng/ml of Tat on glycophorin-A and an inductive effect on CD61 was observed at the message level (Fig. 2)

Cell-surface protein expression of glycophorin-A and CD61 were monitored by flow cytometric analysis (Fig. 3). In each experiment, PE-conjugated (FL2) anti-human

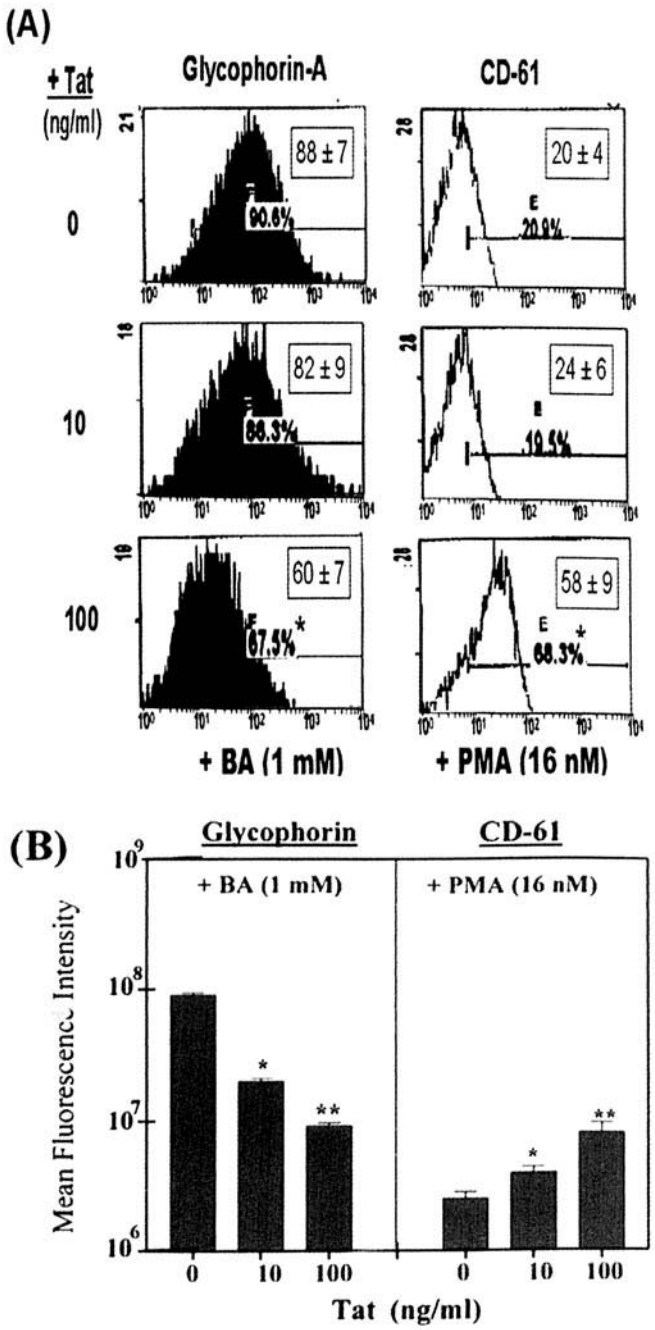


Figure 3. Effect of Tat on glycophorin-A and CD61 protein levels. Lineage-specific cell-surface marker expression was monitored by flow cytometry. Glycophorin-A conjugated to PE and FITC-conjugated CD61 antibodies were used. At 72 hrs after stimulation, the effects of 0–100 ng/ml of Tat protein on glycophorin-A levels in BA-stimulated cells and CD61 levels in PMA-stimulated cells were monitored, and percent change in cells expressing the differentiation-specific markers is shown in (A). A representative FACScan analysis is presented and the average values ($n = 3$) are included in each panel. The MFI for differentiation-specific markers ($n = 4$) are documented in the bar graphs in (B). As compared with BA- or PMA-stimulated cells, significant changes in glycophorin-A or CD61 expression after Tat co-exposure are represented as P values (*, $P < 0.05$; and **, $P < 0.01$).

glycophorin-A (Fig. 3, left panel) or FITC-conjugated (FL1) anti-human CD61 (Fig. 3, right panel) antibodies were used. Changes in percent positive cells are shown as a representative FACScan analysis in Figure 3A. Insets in each panel show the average ($n = 3$) percent positive cells along with the ±SEM of values. In Figure 3B, bar graphs show the mean fluorescence intensity (MFI) as a determinant of total levels of expression of each differentiation marker. We observed that BA stimulation resulted in 90 ± 5% of cells expressing glycophorin-A, and the MFI for glycophorin-A was 1.0 ± 0.3 × 10^8 after BA stimulation for 72 hrs. With PMA stimulation for 72 hrs, there was a 10 ± 2.3-fold increase ($P < 0.05$) in the percent of CD61-positive cells and a 30 ± 10-fold increase in CD61-specific MFI. However, after 10–100 ng/ml of Tat co-treatment, we observed a concentration-dependent decrease in both percent of cells expressing glycophorin-A ($P < 0.05$) and in glycophorin-A–specific MFI ($P < 0.01$). Co-exposure to 10 ng/ml of Tat decreased glycophorin-A expression to 80 ± 5% of cells and 100 ng/ml of Tat decreased the expression to 60 ± 2.5% of cells. Furthermore, a 10–12-fold decrease in glycophorin-A–specific fluorescence was observed with 10 ng/ml of Tat, and a 12–15-fold decrease in MFI was evident with 100 ng/ml of Tat. In the PMA-stimulated cells, however, Tat further increased CD61 protein expression. Although 10 ng/ml of Tat did not significantly upregulate CD61-positive cells, an almost 2-fold increase in CD61-specific fluorescence was observed. Co-stimulation with 100 ng/ml of Tat resulted in a more significant ($P < 0.05$) increase in both percent of CD61-expressing cells (2.5–3.0-fold) and in CD-61-specific MFI (6 ± 2.5-fold). These studies, at both functional and molecular levels, demonstrated opposite effects of Tat on erythroid and megakaryocytic differentiation in the K562 cells.

Tat Co-treatment Alters the Expression of CXCR4 and CCR5 in BA- and PMA-Stimulated Cells. We performed experiments to monitor CXCR4 and CCR5 expression in the lineage-committed K562 cells. Similar to our previous studies, cells were stimulated with either 1 m*M* BA or 16 n*M* PMA and co-exposed to 100 ng/ml of Tat. Co-receptor gene expression was monitored at 24 hrs after co-stimulation by RT-PCR (Fig. 4) and flow cytometry was performed to monitor protein levels at 36 hrs after co-stimulation (Fig. 5).

In Figure 4A, a representative gel picture of the RT-PCR products for *CXCR4*, *CCR5*, and *GAPDH* are documented. Densitometric values obtained for *CXCR4* and *CCR5* were normalized to *GAPDH* levels, which are presented in the bar graphs (Fig. 4B). Results showed that BA stimulation slightly increased ($P < 0.05$) both *CXCR4* (2.8 ± 0.5-fold) and *CCR5* (3.2 ± 0.9-fold) gene expression (Lane 2) and PMA treatment showed a more potent effect on gene expression of both co-receptors (Lane 3) and increased *CCR5* (12–20-fold; $P < 0.01$) and *CXCR4* (3.5–4-fold; $P < 0.05$) mRNA levels. Although treatment with 100 ng/ml of Tat alone did not appreciably change either *CXCR4* or *CCR5* gene expression (Lane 4), Tat significantly affected co-

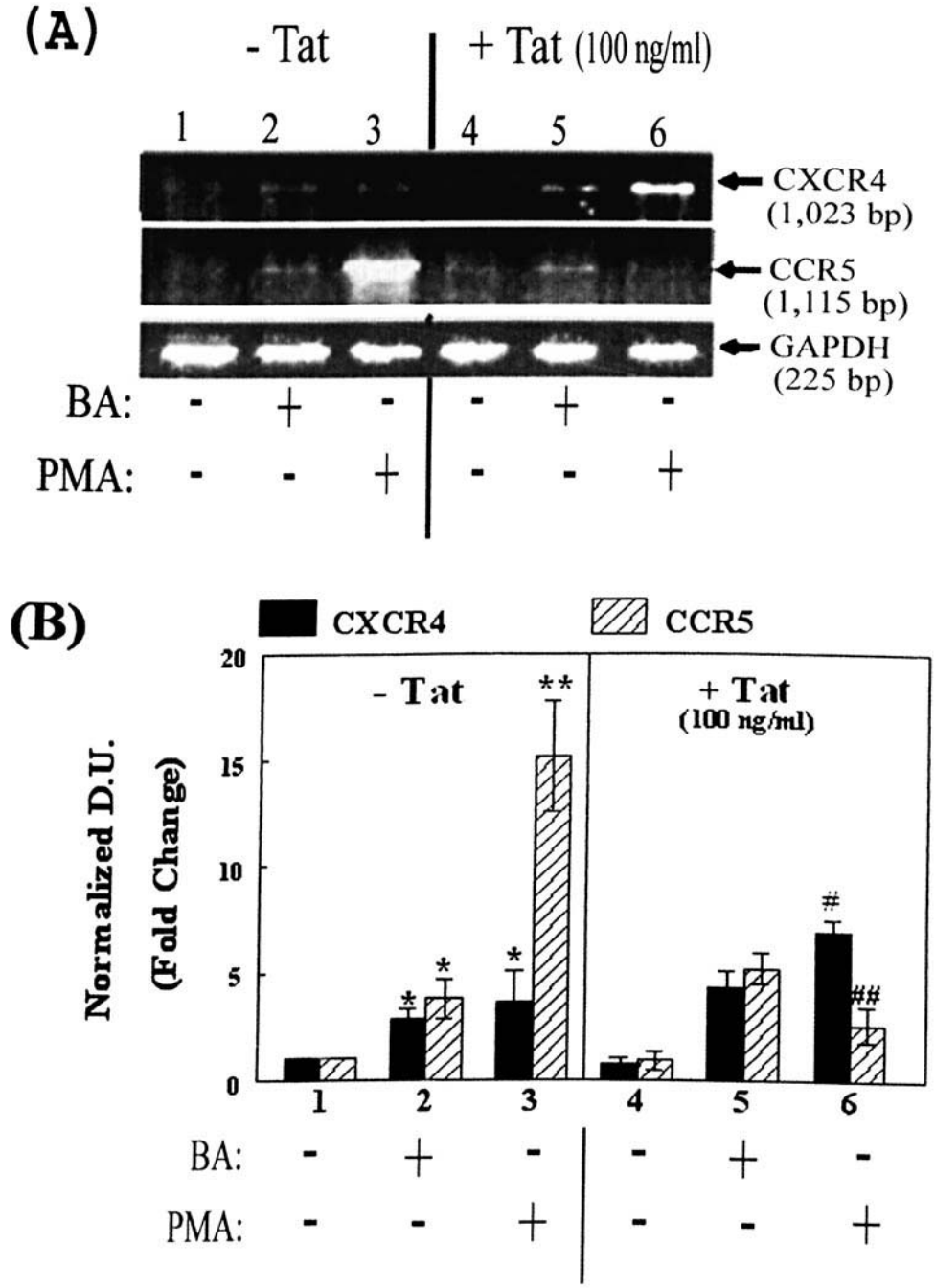


Figure 4. Differential effects of Tat on *CXCR4* and *CCR5* gene expression, in erythroid and megakaryocytic cells. In BA- or PMA-stimulated cells grown in the presence or absence of 100 ng/ml of Tat, *CXCR4* and *CCR5* gene expression were monitored by RT-PCR. A representative gel picture showing the PCR products (arrows) and their molecular weights (bp) are shown in (A). Co-receptor levels in unstimulated control (Lane 1), as wells as in cells treated with either 1.0 m*M* BA (Lane 2) or 16 n*M* PMA (Lane 3) are shown. The effect of 100 ng/ml of Tat alone (Lane 4) or in combination with either BA (Lane 5) or PMA (Lane 6) are also shown. (B). The *GAPDH*-specific mRNA levels were used as internal controls, and values were normalized to the respective *GAPDH* levels. Bar graphs represent fold changes in RT-PCR product intensities. As compared with the unstimulated cells, significant changes ($n = 3$) in co-receptor expression after BA or PMA stimulation are shown as *, $P < 0.05$ and **, $P < 0.01$. As compared with the absence of Tat (–Tat), a significant inductive effect of Tat (+Tat) on *CXCR4* expression (#, $P < 0.05$), and a suppressive effect on *CCR5* (##, $P < 0.01$) expression are also shown.

receptor expression in both BA-stimulated (Lane 5) and PMA-stimulated (Lane 6) cells. Tat treatment dramatically suppressed ($P < 0.01$) PMA-stimulated *CCR5* gene expression (Lane 6; hatched bar) and enhanced ($P < 0.05$) PMA-stimulated *CXCR4* gene expression (Lane 6; solid bar).

In the following experiments, the effect of Tat was also monitored on the cell surface HIV-1 co-receptor levels (Fig. 5). Marked increases in protein levels for both co-receptors were observed after PMA stimulation (Row 5). As much as a 23 ± 2.5-fold increase in CXCR4 levels ($P < 0.01$) and a 35 ± 10-fold increase in CCR5 levels ($P < 0.01$) were observed. However, Tat showed a 60–80% suppression on

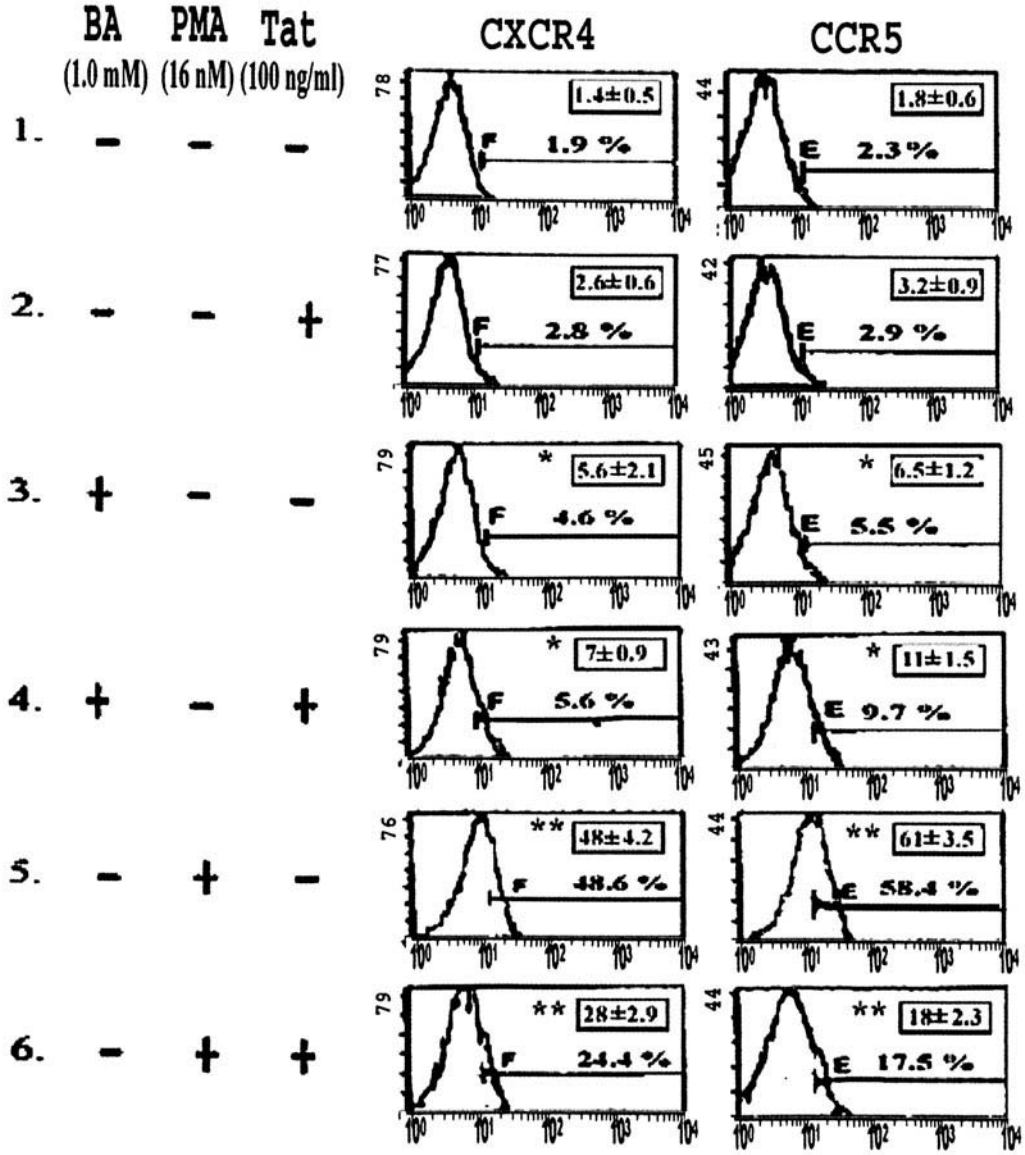
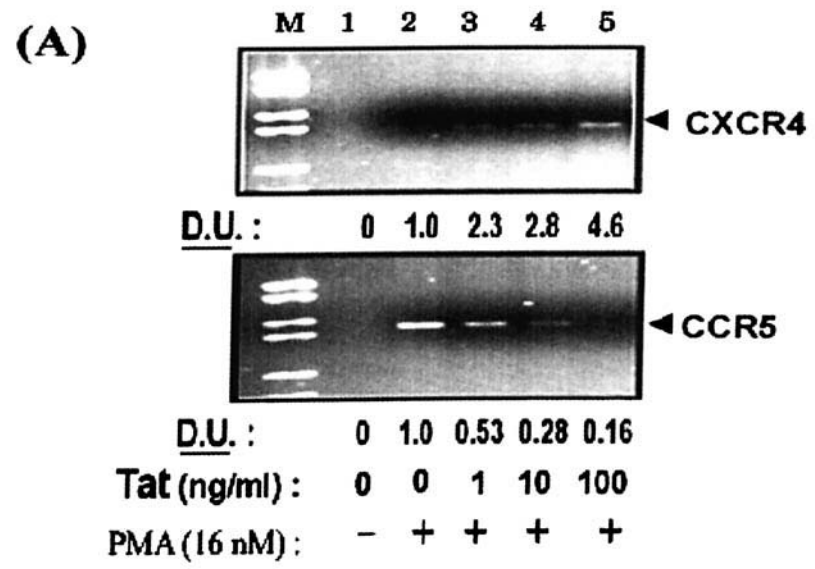


Figure 5. Effect of Tat on CXCR4 and CCR5 protein expression in erythroid or megakaryocytic cells. The representative FACScan shows co-receptor levels in unstimulated cells (Row 1), in cells stimulated with 100 ng/ml of Tat alone (Row 2); stimulated with 1.0 m*M* BA (Row 3) or 16 n*M* PMA (Row 4) alone; and in combination with Tat (Row 5 and Row 6). Percent of fluorescence-positive (PE or FITC) cells are represented, and average values ($n=3$) are also included along with the ±SEM of values. Significant changes in CXCR4 and CCR5 protein levels are shown as *P* values (*, $P < 0.05$; **, $P < 0.01$).

PMA-induced CCR5 protein levels, similar to the data obtained with our RT-PCR studies. However, in contrast to the inductive effect of Tat on *CXCR4* gene expression in the PMA-stimulated cells, we documented a 50 ± 5% decrease after Tat co-treatment. This clearly indicated that Tat may differentially regulate co-receptor expression in megakaryocytic cells at both mRNA and protein levels.

The Differential Co-receptor Expression by Tat Is Potentiated After Chronic Stimulation of Cells Precommitted Towards the Megakaryocytic Lineage. Differential expression of both CXCR4 and CCR5 has been previously documented in lineage-committed HPCs obtained from peripheral blood (45, 46). These studies had indicated that the effect of Tat may be lineage specific and may be potentiated after multiple exposures. Hence, in the following experiments, we monitored CXCR4 and CCR5 expression in precommitted cells (Fig. 6). In addition, in contrast to a single exposure to Tat, as performed in the previous experiments, we monitored the effect of multiple exposures to the Tat protein in the following studies (Fig. 6). The K562 cells were stimulated with 16 n*M* PMA for 12 hrs before their exposure to increasing concentrations (1–100 ng/ml) of Tat, which was replenished every 12 hrs. Co-receptor expression was monitored by RT-PCR (Fig. 6A) and flow cytometry (Fig. 6B).

The RT-PCR assays show the co-receptor mRNA levels (Fig. 6A). The densitometric values were normalized to *GAPDH* mRNA levels in each sample and values



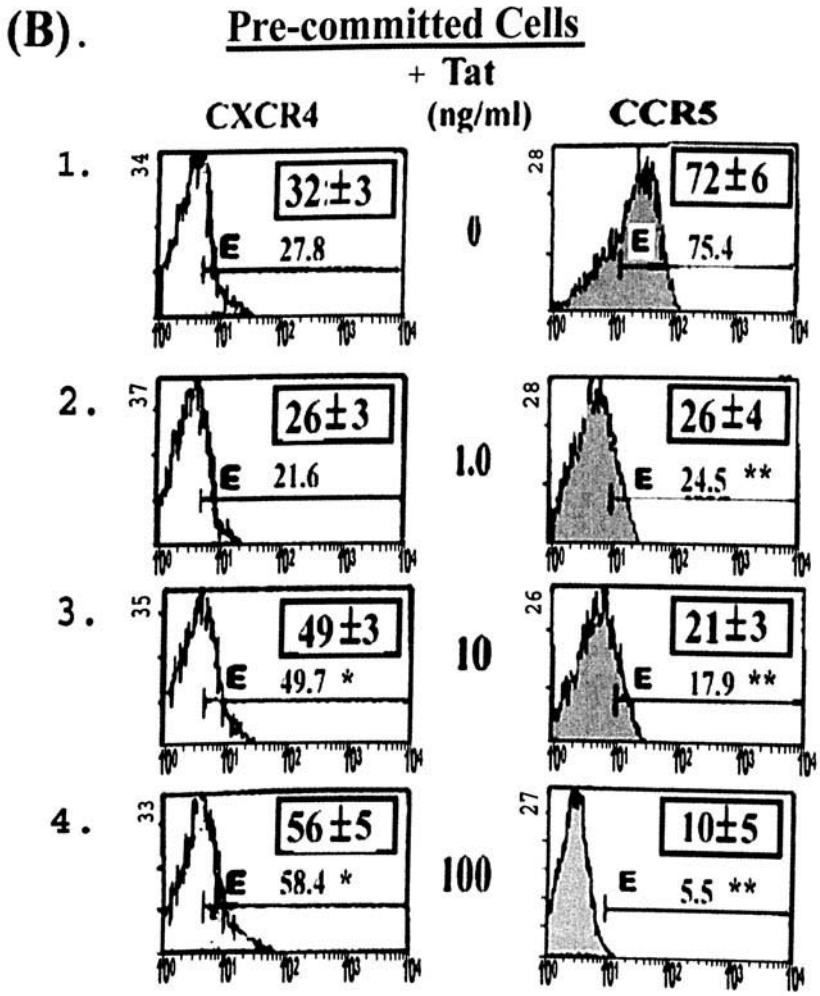


Figure 6. Effect of multiple Tat exposures of megakaryocytically precommitted cells on *CXCR4* and *CCR5* expression. K562 cells were prestimulated with 16 n*M* PMA for 12 hrs before initiation of 1–100 ng/ml of Tat treatment. Cells were harvested after 24 hrs for RT-PCR (A) and after 36 hrs for flow cytometry analysis (B). The representative RT-PCR assay in (A) shows changes in *CXCR4* and *CCR5* mRNA levels in unstimulated cells (Lane 1), in PMA-stimulated cells (Lane 2), and in cells co-stimulated with Tat and PMA (Lanes 3–5). The normalized D.U. values for RT-PCR products are provided at the bottom of the gel picture ($n=3$). Significant changes are shown as *P* values (*, $P < 0.05$). In (B), the representative FACScan analysis and the average changes in co-receptor–expressing cells are shown in each panel ($n=3$). As compared with cells prestimulated with PMA alone (Lane 1), significant changes in *CXCR4* and *CCR5* after multiple 1–100 ng/ml of Tat treatments (Lanes 2–5), are documented as *P* values (*, $P < 0.05$; **, $P < 0.01$).

obtained with the PMA-alone group were arbitrarily designated as 1.0 D.U. The change in D.U. values are presented at the bottom of each gel picture. Results showed that, as compared with the *CXCR4* and *CCR5* gene expression in precommitted cells (Lane 2), multiple 1–100 ng/ml Tat exposure potentiates the differential effects on co-receptor mRNA levels (Lanes 3–5). In contrast to single treatment with Tat, a significantly higher increase in *CXCR4* and a more robust suppressive effect on *CCR5* was observed with multiple Tat treatment. A 2–3-fold increase in *CXCR4* was seen, even with 1–10 ng/ml of Tat, and a 4–6-fold induction was observed with the 100 ng/ml concentration of Tat. In contrast, a 75 ± 8% suppression of *CCR5* was observed with 10 ng/ml of Tat and an almost complete suppression (90 ± 5%) was seen after treatment with the 100 ng/ml of Tat concentration; even the 1 ng/ml of Tat concentration showed a 52 ± 2.5% decrease in *CCR5* mRNA levels.

Flow cytometric analysis for expression of CXCR4 and CCR5 proteins was monitored in precommitted K562 cells after exposure to increasing concentrations of Tat (1–100 ng/ml; Fig. 6B). Stimulation with PMA for 48 hrs (12 hrs + 36 hrs) showed 32 ± 5% of cells expressing CXCR4, and 67 ± 6% of cells expressing CCR5. Although a slight decrease in CXCR4 was observed with the lowest concentration of Tat, exposure to the higher concentrations (10 ng/ml and 100 ng/ml) was found to further increase PMA-stimulated CXCR4 to 49 ± 8% and 56 ± 6% of the cells, respectively. In contrast, significant suppression of CCR5 protein levels was observed at all three concentrations of Tat used. A 55 ± 5% suppression of CCR5 was observed even at 1 ng/ml. This was evident by a decrease from 67 ± 6% to 29 ± 6% of cells expressing CCR5. At 10 ng/ml of Tat, we observed further downregulation of CCR5 to 21 ± 4% and at 100 ng/ml of Tat, only 7 ± 3% of cells expressed CCR5. This demonstrated a more than 60% and an almost 90% decrease in CCR5-expressing cells after multiple Tat exposures of the megakaryocytic progenitors (Fig. 6).

Discussion

The rapidity of reservoir establishment and the persistence of HIV-1 clearly suggest that HIV-1 factors may play a crucial role in regulating the kinetics of viral reemergence. Studies have shown that the initiation of HAART, even within 10 days after the onset of symptoms of primary HIV-1 infection, cannot prevent the generation of HIV-1 reservoirs (51). Studies have suggested that the HIV-1–infected dendritic progenitors may act as long-term reservoirs for both X4 and R5 strains of HIV-1 (24, 26, 27), and viral propagation and selective co-receptor usage may occur after viral transmission to replication competent T lymphocytes and possibly in megakaryocytes (25, 28).

Our current findings implicate a novel role for the HIV-1 Tat protein in regulating both lineage-specific differentiation, and co-receptor expression in the K562 cells. Tat co-exposure suppressed BA-induced erythroid differentiation and stimulated PMA-induced megakaryocytic differentiation. Interestingly, the differential effect of Tat on CXCR4 and CCR5 expression was observed only in the PMA-stimulated K562 cells. In the megakaryocytic cells, Tat selectively upregulated CXCR4 and downregulated CCR5 expression, and this differential effect was potentiated when Tat was added to the precommitted cells. Differentiation-specific functions (Fig. 1), as well as both gene expression (Fig. 2) and protein levels (Fig. 3) for lineage-specific molecular markers, were shown to be altered by Tat co-treatment. Interestingly, exposure to Tat alone did not increase serotonin uptake or CD61 expression; however, Tat was able to suppress the percent of benzidine-positive cells and glycophorin-A expression in unstimulated control cells. We believe that this may be because the erythroleukemic K562 cells are precommitted toward the erythroid lineage, as evident from high basal levels of benzidine-stained cells and glycophorin-A expression. A pronounced suppressive effect after Tat treatment alone may be evident in these erythroid cells that may not be observed in the absence of a PMA-mediated switch toward a megakaryocytic lineage. Indeed, a similar facilitative effect of Tat alone was observed in megakaryocytic differentiation when cells were precommitted toward this lineage via prestimulation with PMA before their Tat treatment (Fig. 6). These observations suggested that the Tat protein affects differentiation of K562 cells, which may play a direct role in HIV-1–induced hematologic dysfunctions in AIDS. Indeed, greater than 70% of asymptomatic patients develop anemia at some time during their disease course (18), and thrombocytopenia occurs in 25–50% of patients (28). Calenda *et al.* (53, 54) and Zauli *et al.* (37) had previously documented that Tat exposure of HPCs can suppress hematopoietic differentiation via the erythroid lineage. In another study, Calenda and Cherman (52) had also documented an inductive effect of Tat on granulomonocytic differentiation. Similar effects of infectious HIV-1 clones were also observed on both erythroid and granulocytic differentiation of HPCs (18, 53, 54) suggesting a pivotal role played by the Tat protein in regulating these effects of HIV-1. Our findings corroborate with these previous studies and show a direct role for Tat in dysregulation of the lineage commitment, possibly via altering the signal transduction pathway being used. As suggested from our findings, the HIV-1–induced anemia may partially be caused by the Tat-mediated suppression of erythroid differentiation in HPCs. In contrast, our findings showed that Tat may enhance early commitment of progenitor cells toward the megakaryocytic pathway, which may not directly correlate with the findings of HIV-1–induced thrombocytopenia. However, the primary mechanism for HIV-1–induced thrombocytopenia has been suggested to be an increase in peripheral platelet destruction rather than the suppression of megakaryocytopoiesis (55). A possible explanation of decreased platelet count may result from an increase in differentiation-induced apoptosis and/or

abrogation of function in megakaryocytes growing under an HIV-1–infected microenvironment.

Both fully differentiated megakaryocytes and megakaryoblasts can be productively infected by R5 and X4 strains of HIV-1 (28). The effects of Tat on CXCR4 and CCR5 expression has been documented in different cell types; however, the dramatically opposite effect we have observed in the megakaryocytic lineage-committed cells, has not been reported earlier, as far as we know. Previous studies have shown that increased secretion of C-C chemokines by HIV-1–exposed HPCs can selectively limit the entry of R5-tropic viruses (56). Furthermore, a Tat-mediated increase in C-X-C chemokine SDF-1α production in lymphocytes has been implicated in decreased infectivity of HPCs via the X4-tropic strains (42, 43). A number of recent studies have also documented both inductive and suppressive effects of Tat on CXCR4 and CCR5 expression in different hematopoietic cells (41–45). Inductive effects of Tat on both co-receptors were observed in lymphocytes and monocytes and macrophages (41). Tat treatment has also been shown to upregulate CXCR4 expression in both resting CD4+ T cells (44) and in HPC committed toward the erythroid cells (45). Similarly, our observations show increase in CXCR4 expression in K562 cells stimulated with BA (Figs. 4 and 5).

Interestingly, in PMA-stimulated cells, the *CXCR4* mRNA levels (Fig. 4) did not correlate with their protein levels (Fig. 5). Tat increased *CXCR4* gene expression; however, a decreased cell-surface CXCR4 protein expression was observed. This may be interpreted from data obtained in previous findings, which showed that extracellular Tat may decrease cell surface CXCR4 levels (42, 43). This Tat-mediated antagonism of CXCR4 has been shown to be caused by a direct binding and internalization of cell-surface CXCR4 at higher concentrations of Tat (100 ng/ml) used. However, in contrast to the effect of a single exposure to Tat and PMA, the effects of multiple exposures to Tat in K562 cells that were prestimulated with PMA for 12 hrs showed a direct correlation between both the *CXCR4* mRNA and protein levels (Fig. 6). In fact, a more pronounced increase in both *CXCR4* gene expression and cell-surface protein levels were seen in these studies. In addition, we also observed a more profound suppression of CCR5 at both gene expression and cell surface protein levels. The lower concentrations of Tat (1–10 ng/ml) were also found to be far more potent in these PMA prestimulated cells, where significant changes in both CXCR4 and CCR5 were seen even at these lower concentrations of Tat. Hence, our findings, shown in Figure 6, suggested that chronic exposure of megakaryocytic lineage committed cells to the HIV-1 Tat protein may severely alter co-receptor gene expression. Both CXCR4 and CCR5 are expressed early during megakaryocytic lineage commitment (25, 57), however, the preferential reemergence of CXCR4 tropic viruses seen in long-term seroconverters (30, 31) raises the possibility that viral factors may specifically dictate the transmission of the X4 strains and suppress that of the R5 strains. Similar suppression in *CCR5* gene expression has been documented in monocyte-derived macrophages after stimulation with bacterial lipopolysaccharide (58), and in macrophages grown under conditions of hypoxia (59). It has also been documented that the bone marrow–derived megakaryoblasts secrete MIP-1α and RANTES, and high levels of these ligands may suppress CCR5 usage by HIV-1 (29). These previous observations may elucidate a possible molecular mechanism via which Tat can differentially effect co-receptor expression in the K562 cells.

Our findings with the high concentrations of Tat (1–100 ng/ml) may not be of physiologic significance. In HIV-1 infected sera, concentrations of Tat protein has been shown to be approximately 1–10 ng/ml (42). Although we have used 10-fold higher amounts of Tat to show the most significant effects, it has been suggested that such high concentrations of Tat may exist in HIV-1–infected microenvironments. In addition, our data showing that the action of Tat is potentiated in K562 cells that were prestimulated with PMA (Fig. 6), suggesting that lower concentrations of Tat may also play a role in manifesting both hematopoietic dysfunctions and co-receptor expression.

The K562 cells have been used by a number of previous studies as a comparative model to evaluate erythroid and megakaryocytic differentiation-specific signaling in CD34+ HPCs (60, 61). The Ras/extracellular signal–regulated kinase (ERK)–dependent pathway has been implicated in both erythroid (60) and megakaryocytic differentiation of K562 cells (61). Similar signaling cascades, e.g., ERK and p38, have also been shown to be involved in thrombopoietin-induced megakaryocytic, and erythropoietin-induced erythroid differentiation of primary HPCs (62). Lineage commitment involves cross talk between various second messengers and transcription factors activated via the protein kinase C (PKC) and the mitogen-activated protein/ERK kinase (MEK) activation cascades (63–65). Zauli *et al.* (1998) also documented that an increase in cAMP response element (CRE)–binding protein (CREB) transcription factor phosphorylation at the Serine-133 residue is an early event during megakaryocytic differentiation of HPCs (66). Interestingly, in both Jurkat T cells and in PBMCs, extracellular HIV-1 Tat protein has been shown to induce a rapid Ser-133 phosphorylation and activation of CREB (67). These previous findings suggest a possible mechanism via which Tat may increase megakaryocytic commitment in K562 cells by increasing CREB function. The transcriptional co-activators, CREB-binding protein (CBP)/p300, have been shown to regulate the function of hematopoietic transcription factors, such as CREB and GATA-1 (68). Functional CRE-elements that are found upstream of *CXCR4* (69) and the GATA-1 element upstream of *CCR5* also play important roles in macrophages (70). Furthermore, SDF-1α co-stimulation enhances thrombopoietin-mediated megakaryocytic differentiation of HPCs (71), and has been shown to cause a selective inhibition of

erythroid differentiation via the upregulation of Fas/CD95 ligand (72). Tat can activate the transcriptional repressor inducible cAMP early repressor (ICER) in PBMCs (73), suggesting that ICER may be involved in Tat-mediated downregulation of glycophorin-A and CCR5 expression, which we have observed in our current studies. Indeed, Tat protein has been shown to interact with CBP and p300 (36) and our studies (unpublished observations) have also demonstrated that Tat treatment of K562 cells can increase CREB phosphorylation as well as its co-activation by CBP.

Our novel findings that Tat may affect co-receptor preference in megakaryocytic HPCs suggest that Tat may dictate infectivity, co-receptor usage, and pathogenesis in HIV-1 reservoirs. Although viral transmission from dendritic cells to T cells may occur efficiently in lymph nodes, an intermediate cell type, such as the megakaryocytic progenitors, may be involved in viral carriage from more sequestered sites, such as bone marrow. Hence, our findings may implicate that Tat can increase this infectable cell pool by increasing megakaryocytic differentiation and may play a crucial role in dictating the switch in co-receptor preference after viral reactivation from these reservoirs. Our observations demonstrating the opposing effects of Tat on differentiation and co-receptor expression may involve cross talk between different signaling cascades, interactions between lineage-specification transcription factors, and sequestration by specific sets of co-activators or co-suppressors. However, the exact mechanisms for the pleiotropic actions of Tat is not known, further studies may provide a thorough molecular understanding of the role of Tat in HPCs and may provide some physiologic significance for our findings in regulating infectivity in progenitor cell reservoirs.

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