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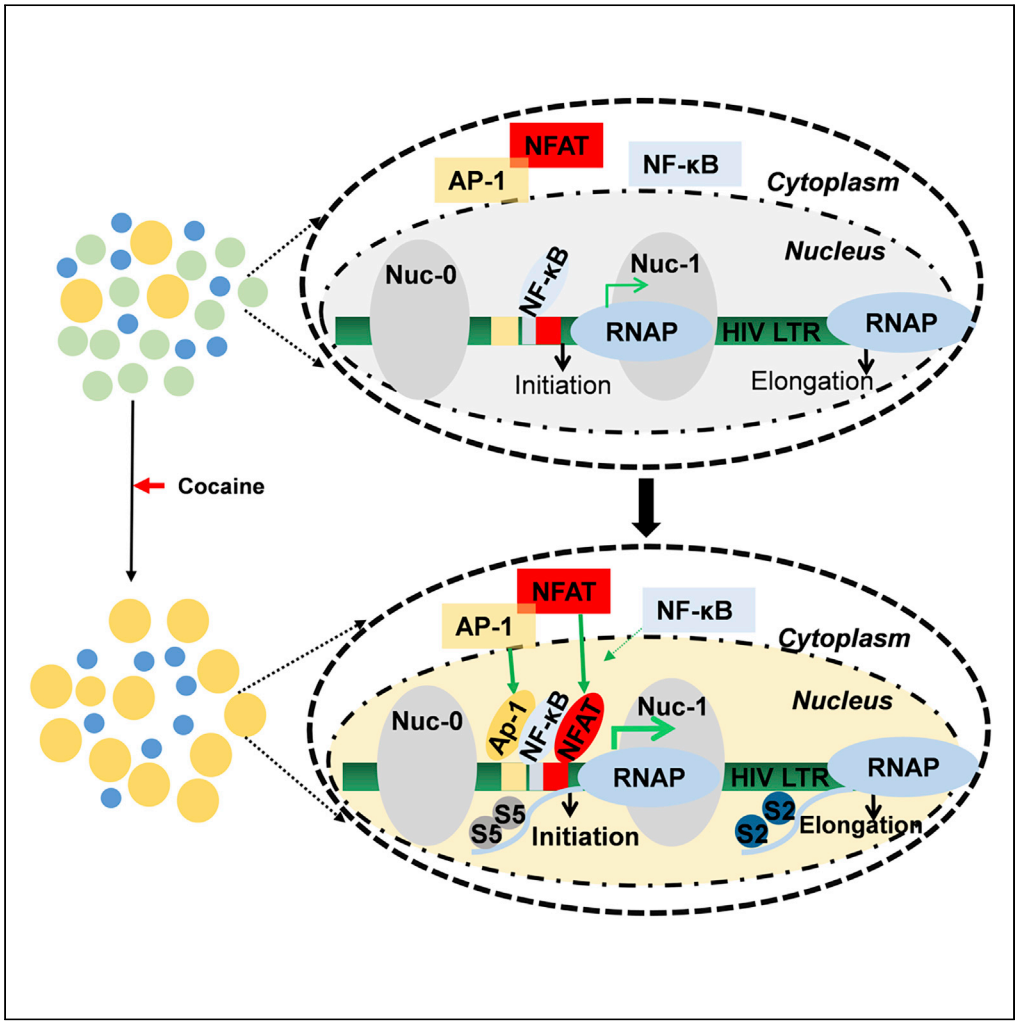

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Highlights

Cocaine upregulates the overall cell metabolism by activating the NFAT and AP-1

Cocaine sensitizes partially active CD4$^+$ T cells to support HIV infection

Cocaine-induced AP-1 works in tandem with NFAT to augment HIV transcription

Cocaine boosts HIV transcription by increasing RNAPII CTD phosphorylation at Ser 5 & 2

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Cocaine sensitizes the CD4$^+$ T cells for HIV infection by co-stimulating NFAT and AP-1

Adhikarimayum Lakhikumar Sharma,[1] Dylan Shafer,[1] Daniel Netting,[1] and Mudit Tyagi[1,2,*]

SUMMARY

The productive infection of HIV, which generates new viral progeny, depends on the activation status of the cell. In this study, we found cocaine exposure sensitizes partially active CD4$^+$ T cells and makes them poised for productive HIV infection. We discovered that cocaine treatment enhances the metabolic state of the cells by co-stimulating several transcription factors, mainly NFAT and AP-1, the two transcription factors, which specifically play a crucial role in enhancing both HIV and the overall cellular gene expression in T cells. We found that cocaine-induced AP-1 works in tandem with NFAT to boost HIV transcription. The enhanced HIV transcription upon cocaine exposure was further confirmed through higher phosphorylation of the crucial serine residues at the carboxyl-terminal domain (CTD) of RNA polymerase II. The insights gained from this study could aid in developing highly specialized therapeutics combating the deleterious effects of cocaine on the cocaine-using HIV population.

INTRODUCTION

HIV/acquired immunodeficiency syndrome (AIDS) has become one of the most devastating pandemics in recorded history. Since the inception of HIV in 1981, the global HIV/AIDS pandemic has claimed approximately 36.3 million [27.2 million–47.8 million] lives, with 37.7 million people currently living with HIV-1 infection.[1] Despite tremendous progress in HIV/AIDS research over the last four decades, a preventative vaccine has yet to be developed.[2–6] However, HIV/AIDS-related mortality has been dramatically reduced in patients who regularly take antiretroviral therapy (ART).[7,8] Unfortunately, the illicit drug use population has exacerbated the global HIV/AIDS epidemic. Despite unparalleled success in controlling HIV, the use of illicit drugs is one of the major factors for new HIV infections.[9,10] Therefore, the illicit drug-using population continues to be the main impediment in the fight against HIV/AIDS. These drugs impair individuals' judgment, which can lead to risky sexual behavior, such as sex trading for drugs and needle sharing.[11–14] These impairments under the influence of illegal drugs are also a potent cofactor that impedes timely diagnosis of HIV transmission, pathogenesis, and adherence to therapy.[13,15] Clinical studies implicate drug abuse in increased viral load, accelerated disease progression, and worsening of AIDS-related mortality even in ART-adherent patients.[16,17] Therefore, these drugs remain a major obstacle in combating the global HIV epidemic.

Cocaine is a commonly used drug among HIV-positive individuals in the United States, which significantly enhances HIV infection and transmission.[18–22] Evidence shows that cocaine use results in the accelerated decline of CD4$^+$ T cells, even among ART-adherent patients, with manifestations similar to AIDS.[23] Moreover, enhanced HIV replication in human peripheral blood mononuclear cells and higher viral load in humanized mice upon cocaine exposure is well documented.[24] However, all the underlying mechanisms through which cocaine increases HIV transcription, gene expression, and replication have yet to be defined.

The CD4$^+$ cells are a crucial component of the host immune system. CD4$^+$ T cells (helper T cells) are the most vulnerable CD4$^+$ cell population, which is specifically impacted upon HIV infection. Notably, the productive infection of HIV to CD4$^+$ T lymphocytes depends primarily on the activation state of the cell. HIV replication primarily depends on the host cell transcription and translation machinery, and only the metabolically active cells have enough ingredients required for HIV to complete all the steps of its life cycle. Therefore, metabolically active CD4$^+$ T lymphocytes are highly susceptible for productive HIV infection,[8] but CD4$^+$ T cells that are quiescent or metabolically dormant, such as resting CD4$^+$ T cells, are quite resistant to productive HIV infection or replication.[8,25] Given the fact that most of the HIV infections are not readily detected, HIV infection reached the chronic stage. The chronic phase of HIV infection is characterized by the persistent

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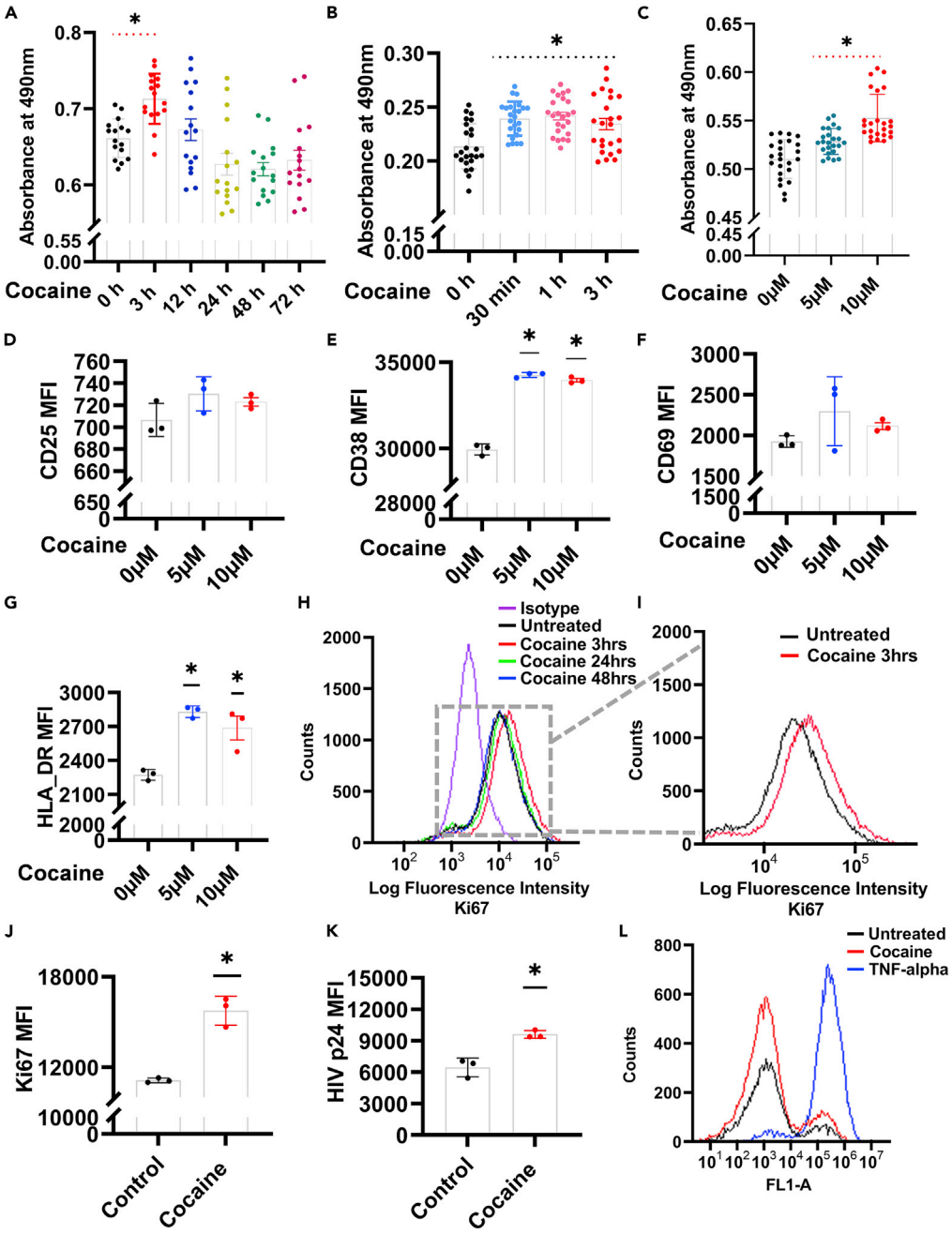

Figure 1. Cocaine sensitizes CD4⁺ T cells for HIV infection by upregulating cell metabolism

(A and B) Jurkat cells were treated in a time-dependent manner with 10 μM cocaine and a (C) dose-dependent manner. Later, the cell metabolic activity was analyzed using the MTS-PMS cell proliferation assay.

(D–G) Cells were exposed to cocaine for 3 h, harvested, and analyzed for surface markers by flow cytometry. The untreated cells were used as a control. The data were plotted graphically with mean fluorescence intensity (MFI).

(H) Cells were exposed to cocaine for 3, 24, and 48 h or untreated as control, harvested, and analyzed by flow cytometry for Ki67 intracellular marker expression changes. (H) is further represented again as (I and J) by plotting only the control and 3 h cocaine.

(K) Cells were pre-treated with cocaine for 3 h before being infected with replication-competent HIV (HIV Type 1 strain 93/TH/051) for 24 h, then analyzed for HIV p24 using flow cytometry.

(L) The impact of cocaine-mediated cell stimulation on the reactivation of latent HIV was examined. TNF-α was used as positive control. Each dot in the figures represents an independent experiment.

Figure 1. *Continued*
The results are expressed as mean ± SD, analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test (A–G) or unpaired t test (J and K). Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated cells vs. control (untreated) cells.

high level of HIV replication and continuous loss of immune control due to virus replication. In certain subsets of T cells, persistently high-level exposure to HIV results in cell exhaustion.[26–28]

As suggested by its name, the nuclear factor of activated T cells (NFAT), a transcription factor, is crucial during T cell activation and differentiation.[29–31] The NFAT superfamily of transcription factors comprises five NFAT proteins: NFAT1 (also known as NFATc2 or NFATp),[32] NFAT2 (NFATc1 or NFATc),[32] NFAT3 (NFATc4),[33] NFAT4 (NFATc3 or NFATx),[32] and NFAT5.[34] Upon cell activation, a calcium-calmodulin-dependent phosphatase, calcineurin, dephosphorylates NFAT and exposes its nuclear localization signal, which triggers nuclear translocation of NFAT and activation of NFAT-responsive promoters. Since NFAT is key in T cell activation and tolerance, it has emerged as an important factor in deciding T cell stimulation and overall immune modulation. NFAT directs the effector arm of the immune response in the presence of activator protein 1 (AP-1) and T cell anergy/exhaustion in the absence of AP-1. AP-1 is a dimeric transcription factor composed of proteins belonging to each Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) families. In T cells, AP-1 is the main partner of NFAT during the transcription of cellular genes.[35,36] The T cell activation involves recruitment of NFAT and AP-1 at the promoters of several cytokine genes, such as IL-2, IL-4, IL-5, IL-13, IFN-γ, and GM-CSF.[35–39] Most of these genes and many more genes that are induced during T cell activation contain two adjacent binding motifs for both transcription factors.[29,36] The binding of NFAT–AP-1 enhances the expression of several cellular genes, including cytokine genes, which eventually results in the activation of T cells and their neighboring cells.[35,36] These complexes have an extensive network of protein-protein contacts, which explains their stability and cooperative nature.[40] The activation and subsequent cooperation between NFAT and AP-1 factors is usually triggered upon activation of two main signaling pathways that are induced during T cell stimulation: 1) calcium signaling, which activates NFAT proteins and 2) the RAS–MAPK pathway, which stimulates Fos and Jun subunits.[41] NFAT–AP-1 cooperation during T cell activation is responsible for a specific pattern of gene expression, which induces the functional changes characterizing activated T cells. Therefore, NFAT is a key regulator of T cell activation through its interaction with proteins of the AP-1 family of transcription factors. The expression of NFAT is cell type specific, and its role in supporting HIV gene transcription is well established, especially during the initiation phase of HIV transcription in $CD4^+$ T cells.[42–45]

In this study, we demonstrated that cocaine sensitizes $CD4^+$ T cells for HIV infection by co-stimulating NFAT and AP-1, the two transcription factors that play a vital role in HIV transcription and overall cellular gene expression.

RESULTS

Cocaine by upregulating cell metabolism of $CD4^+$ T cells sensitizes them for HIV infection

It is well recognized that the activation/metabolic state of a cell is crucial for productive HIV infection. We and others have shown the significant stimulation of HIV transcription and replication by cocaine.[22,46] However, the impact of cocaine in priming $CD4^+$ T cells for HIV infection was never defined. In this investigation, we found that cocaine exposure sensitizes the partially active T cells for productive HIV infection by upregulating their metabolic activity. The cocaine-mediated upregulation of cell metabolism renders the partially active cells suitable for HIV infection by reducing their threshold for HIV infection.

To assess the impact of cocaine on the metabolic state of T cells, Jurkat cells (a $CD4^+$ T cell line) were seeded in 96 well plates at concentrations of 2×10^4 to 2×10^5. The cells were either treated with cocaine for 3, 12, 24, 48, and 72 h or left untreated, as a control. Later, cells were analyzed for cell proliferation/metabolic activity, using Cell Titer 96® Aqueous One Solution Cell Proliferation (MTS-PMS) Assay (Promega, Madison, WI), following the manufacturer protocol. The assay is based on the presence of NAD(P)H-producing dehydrogenase enzymes, which predominantly exist in metabolically active cells. Upon cocaine treatment, we found a significantly higher absorption value, demonstrating enhanced NADPH/NADH levels and dehydrogenase activity, validating the upregulation of metabolic activity in $CD4^+$ T cells, which peaked in just 3 h of cocaine treatment but diminished with time (Figure 1A). This shows that within 3 h, cocaine significantly enhances NADPH oxidase activity, a marker of upregulated metabolic state of $CD4^+$ T cells. The results were confirmed by further evaluating the effect of cocaine on cell metabolism

for shorter time intervals: 30 min, 1 h, and 3 h (Figure 1B). Later, we examined the impact on cell metabolism of two different doses of cocaine (5 and 10 μM). Both cocaine doses enhanced the metabolic activity of cells (Figure 1C). These results demonstrate a significant upregulation to the metabolic state of T cells upon cocaine treatment, which lasts for a short duration.

To determine the impact of cocaine on cell state, we evaluated the levels of different cell-activation markers, such as CD25, CD38, CD69, and HLA-DR after short exposure to cocaine (3 h). Following cocaine treatment, we found a subtle but significant upregulation to CD38 and HLA-DR expression; however, CD25 and CD69 did not increase significantly (Figures 1D, 1E, 1F, and 1G). The stimulation of these cell activation markers shows the metabolic stimulation or activation of T cells upon cocaine exposure.

We further analyzed the cell proliferative markers to check the impact of cocaine on cell proliferation. Jurkat cells were treated with cocaine for different time intervals; 3, 24, and 48 h. Subsequently, we stained the cells to quantify nuclear antigen Ki67 using a specific antibody labeled with fluorescent dye. Ki67 protein is a nuclear protein present in cells that are metabolically active and undergoing proliferation. Like MTS-PMS assays (Figure 1A), the cocaine-treated $CD4^+$ T cell population showed higher levels of Ki67 expression, which peaked at 3 h but diminished with time (Figure 1H). Figure 1H is further represented as Figures 1I and 1J to show the higher Ki67 expression upon 3 h of cocaine exposure. Together, these validate the metabolic stimulation upon cocaine exposure.

Later, we investigated if acute cocaine treatment primes the cells for HIV infection. Jurkat cells were treated with cocaine for 3 h before being infected with HIV Type 1 strain 93/TH/051 (replication-competent HIV) for 24–48 h. The cells were harvested and stained for HIV Gag/p24 protein, a marker for HIV infection, using a specific antibody against p24 and labeled with fluorescent dye. The enhanced expression for p24 in cells that are primed with cocaine (Figure 1K) confirmed that cocaine exposure sensitizes the cells for HIV infection.

Subsequently, we examined the impact of cocaine-mediated cell stimulation on the reactivation of latent HIV. We treated a latently HIV-infected Jurkat T cell line, 2D10,[47] with cocaine. After 24–48 h, we quantified green fluorescent protein (GFP), which is expressed through HIV LTR promoter. The TNF-α, which we used as a positive control, was able to stimulate latent HIV in more than 70% of cells. However, we did not find significant upregulation of GFP in cocaine-treated latently infected 2D10 cells (Figures 1L and S1A). Notably, as expected based on our findings described above, we noticed higher mean fluorescent intensity of GFP in cocaine-treated cells (Figure 1L). These results further validate that cocaine by augmenting the metabolism of partially active cells equip them to support HIV infection. Given the fact that T cell exhaustion regulates AP-1 levels in the cell,[48] we examined the presence of PD-1, CD160, and TIM3 (Figures S1B–S1D). Together, these results confirmed that although cocaine-mediated cell stimulation is not sufficient to reactivate latent HIV provirus in completely quiescent cells, yet a certain population of partially active cells can be made suitable for HIV infection.

Cocaine enhanced both HIV gene expression and replication

We evaluated the impact of cocaine on HIV gene expression using Jurkat cells freshly infected with pHR'P-Luc (Jurkat-pHR'P-Luc).[47] These cells harbor an HIV-based lentivirus that expresses *luciferase* reporter gene under the control of the HIV LTR promoter (Figure 2A). Therefore, luciferase expression marks HIV gene expression. The cells were treated with different amounts of cocaine (5, 10, and 20 μM). After 48 h, cell extracts were prepared, and the level of reporter luciferase protein expression was determined via luciferase assays (Figure 2B). As anticipated from our previous findings,[22] we noticed significant upregulation of luciferase counts in a dose-dependent manner, indicating enhanced HIV gene expression in cocaine-treated samples. Furthermore, to ensure that the amount of cocaine used was not toxic to the cells, Jurkat-pHR'P-Luc cells were cultured with cocaine at different concentrations (5, 10, 15, 20, and 30 μM) for 48–72 h, and cell cytotoxicity was determined by trypan blue in automated counter. We did not observe any toxicity even at 30 μM of cocaine treatment (Figure S1E).

To confirm the impact of cocaine on HIV gene expression and replication, Jurkat cells were pre-treated with cocaine for 3 h before being infected with a replication-competent dual tropic HIV Type 1 strain 93/TH/051 for either 24 or 48 h. The HIV transcripts were quantified using real-time qPCR using primer sets that amplify the protease region of the HIV genome. A significant upregulation of HIV gene expression was confirmed in

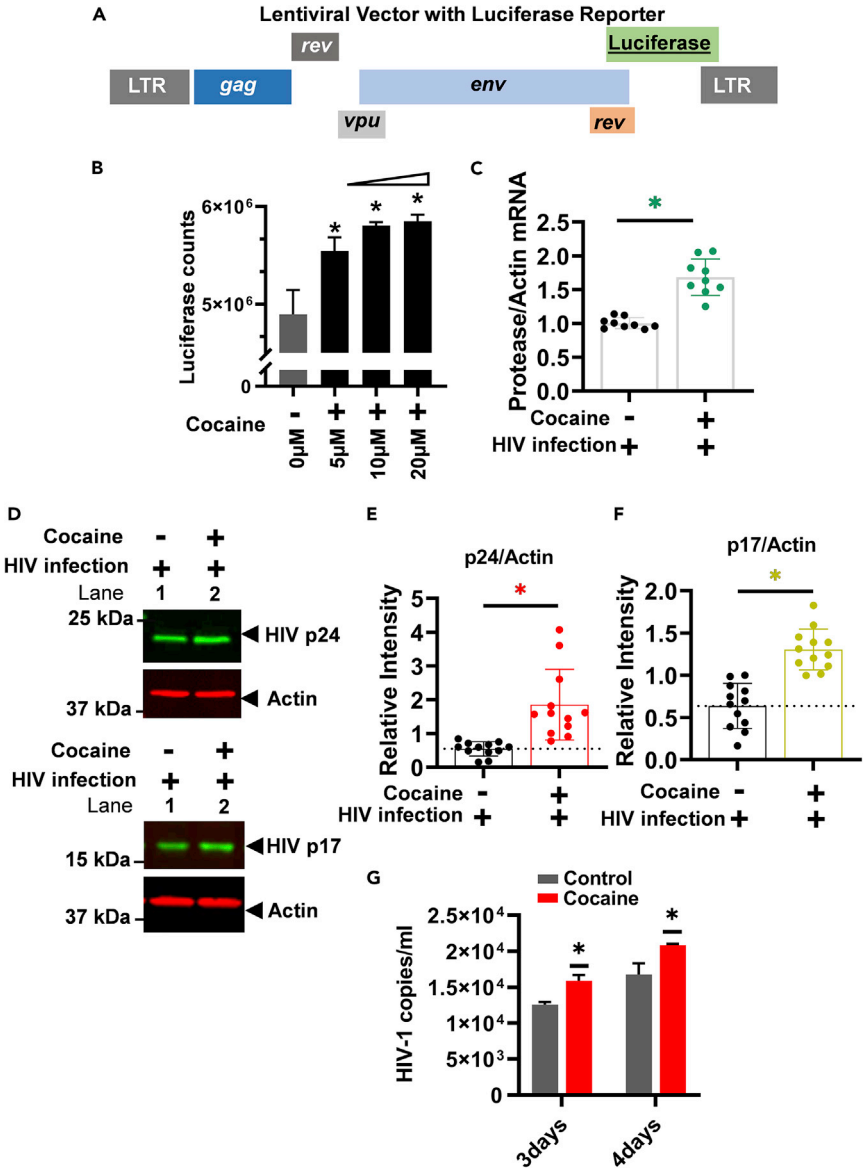

Figure 2. Cocaine enhances HIV gene expression and replication

(A and B) Jurkat cells harboring pHR'-P-Luc provirus were treated with different concentrations (doses) of cocaine (5, 10, and 20 μM). Later, cells were lysed, and the level of reporter protein expression was determined by the luciferase assay. (C–G) Cells were pre-treated with 10 μM cocaine for 3 h before being infected with a replication-competent dual tropic HIV Type 1 strain 93/TH/051 for 24–48 h. (C) The level of HIV transcripts was assessed by amplifying the protease region of HIV genome via real-time PCR. (D–F) The level of HIV Gag protein was analyzed by immunoblotting, using specific antibodies against p24 and p17. The constitutively expressed protein actin was probed as a loading control. (G) The amount of HIV virions was quantified using 200 μL of the conditioned media after 3–4 days. Each dot in the figures represents an independent experiment.

The results are expressed as mean ± SD, analyzed by one-way/two-way ANOVA followed by Dunnett's multiple comparison test (B and G) or unpaired t test (C, E, and F). Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated cells vs. untreated cells.

the presence of cocaine (Figure 2C). Next, to validate the effect of cocaine on HIV protein production, the cell lysates of cocaine-treated, and untreated cells were compared by immunoblotting using antibodies against specific Gag subunits (p24 and p17) (Figures 2D, 2E, and 2F). The significant upregulation of HIV gene expression in the presence of cocaine was confirmed (Figures 2E and 2F). Together, these results

corroborate that cocaine-mediated priming of $CD4^+$ T cells, besides enhancing HIV gene expression, facilitates HIV infection and replication.

Furthermore, to investigate the effect of cocaine on the generation of new viral progeny, cells were treated for 3 h with cocaine or left untreated as a control. Subsequently, cells were infected with HIV Type 1 strain 93/TH/051. After 3 h of infection, cells were washed with PBS and supplemented with fresh media. After 3 or 4 days, 200 μL of supernatant was collected. Viral RNA was extracted using QIAamp MinElute Virus Spin Kit following the manufacturer protocol and the cDNA was synthesized using M-MLV reverse transcriptase. The cDNA was quantified through real-time PCR using a primer set amplifying a *vpu-env* region of HIV genome (Figure 2G). As anticipated, we found temporal accumulation of new viral particles in the supernatant, validating enhanced HIV replication and viral production following cocaine treatment to the T cells.

Cocaine upregulates the overall metabolism of T cells by activating nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1)

$CD4^+$ T cells are an important component of adaptive immunity. They respond to antigen stimulation by rapidly proliferating and differentiating into effector populations. Naive T cells are metabolically quiescent, with low nutrient uptake and less building blocks of biosynthesis. On the other hand, activated lymphocytes rewire their metabolic program to support the massive and rapid increase in biosynthetic needs required for clonal expansion and effector molecule production. Emerging evidence suggests that NFAT functions as a key regulator of T cell metabolism.[49,50] Studies have shown that NFAT not only regulates T cell metabolism but also senses nutrient availability and the bioenergetic status of T cells.[49] The AP-1 is another transcription factor, which is also linked to a variety of cellular events, including proliferation, differentiation, survival, metabolism, hypoxia, and angiogenesis. Therefore, we investigated whether cocaine upregulates the overall cell metabolism of $CD4^+$ T cells by stimulating NFAT and AP-1. Jurkat cells carrying a VSV-G pseudotyped replication-incompetent HIV, pHR'P-Luc, were treated with increasing cocaine doses (5, 7.5, 10, 15, and 20 μM) for 3 h. The nuclear lysates were analyzed for the expression of NFAT and AP-1 transcription factors. The results showed a clear activation of NFAT (NFATc1 and NFATc2) and AP-1 (c-Fos and c-Jun) by cocaine in $CD4^+$ T cells (Figures 3A–3E). Moreover, densitometric analyses of protein bands validated a significant dose-dependent increase to NFAT and AP-1 levels in Jurkat cells upon cocaine treatment compared to the untreated cell control (Figures 3A–3E). These results show that cocaine intake/treatment upregulates the overall metabolism of $CD4^+$ T cells by activating the NFAT and AP-1, which facilitates the priming of $CD4^+$ T cells for HIV infection. Subsequently, to examine the kinetics of these factors (NFAT and AP-1) upon cocaine treatment, we treated the cells with a fixed dose of cocaine (10 μM) for different time durations: 15 min, 30 min, 1 h, 3 h, and 6 h (Figures 4A–4E). We analyzed the nuclear lysates to assess the levels of NFAT and AP-1 transcription factors; we noted unique kinetics of NFAT and AP-1 stimulation upon cocaine exposure. As anticipated, densitometric analyses revealed a significant increase to NFAT (NFATc1 and NFATc2) and AP-1 (both c-Fos and c-Jun) following cocaine treatment in a time-dependent manner (Figures 4A–4E). Together, these results confirm that cocaine-mediated activation of NFAT and AP-1 plays a vital role in upregulating the overall metabolism of $CD4^+$ T cells.

Specific stimulation of NFAT and AP-1 by cocaine plays a crucial role in enhancing HIV gene expression

To establish the direct impact of cocaine on the activation of NFAT, we evaluated the effect of a specific NFAT inhibitor, cyclosporin A (CsA). CsA is an inhibitor of calcineurin. Calcineurin is a phosphatase, which is required to dephosphorylate NFAT, a post-translational modification that makes NFAT functionally active by promoting its nuclear translocation. Jurkat cells carrying a VSV-G pseudotyped replication-incompetent HIV, pHR'P-Luc were treated with CsA for 1 h. Cells were then washed and replenished with fresh medium. Subsequently, cells were treated with cocaine to assess its ability to rescue NFAT stimulation, which was inhibited in the presence of CsA. The nuclear lysates were evaluated for NFAT activation by immunoblotting. We observed significant inhibition of NFAT activation by CsA in the absence of cocaine (Figure 5A, Lane 3). However, in the presence of cocaine, NFAT activation was effectively rescued (Figure 5A, Lanes 4–6) (Figure S2A). Thus, results showed that cocaine was able to rescue/reverse NFAT inhibition imposed by CsA. Similarly, we evaluated the impact of cocaine in reversing the inhibitory effect of mitogen-activated protein kinase (MAPK) inhibitor PD98059, which specifically blocks stimulation of the AP-1 transcription factor. Jurkat cells carrying a VSV-G pseudotyped replication-incompetent HIV, pHR'P-Luc were pre-treated with PD98059 for 1 h, washed, and replenished with fresh medium before being exposed to 5 to 10 μM cocaine for different time intervals. Western blotting was performed to assess

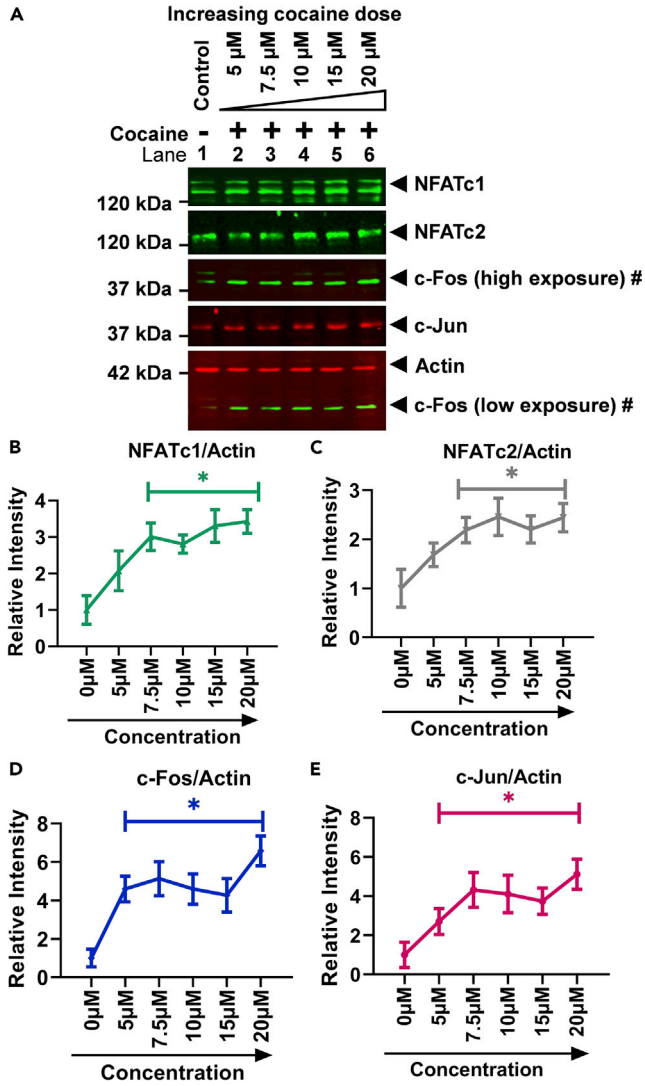

Figure 3. Cocaine activates nuclear factors of activated T cells (NFAT) and activator protein 1 (AP-1) in a dose-dependent manner

Jurkat cells harboring pHR′-P-Luc provirus were treated with different concentrations (doses) of cocaine (5, 7.5, 10, 15, and 20 μM) for 3 h (Lane 2–6).

(A) Nuclear lysates (protein samples) were analyzed by electrophoresis in SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies, as indicated. The immunoreactive protein after incubation with appropriately labeled secondary antibodies was detected with Licor. Actin was used as a loading control.

(B–E) Densitometric analysis of protein bands validated a significant dose-dependent increase to NFAT and AP-1 levels in Jurkat-pHR′-P-Luc cells upon cocaine treatment compared to untreated control cells. Immunoblots are representative of at least 3 independent experiments. # indicates the same blot but with different exposure. The results are expressed as mean ± SD for three independent experiments, analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated cells vs. untreated cells.

the impact of PD98059 and/or cocaine on AP-1 (c-Fos and c-Jun) stimulation. Both c-Jun and c-Fos were significantly inhibited in cells treated with PD98059 alone (Figure 5B, Lane 3). However, cocaine treatment significantly rescued AP-1 inhibition, which was induced by PD98059 (Figure 5B, Lanes 4–6) (Figure S2B). We noted that cocaine efficiently reversed the restriction imposed by specific inhibitors on NFAT and AP-1 stimulation, validating that cocaine is a specific and strong activator of NFAT and AP-1 in $CD4^+$ T cells. Thus, our results confirmed that cocaine-activated NFAT and AP-1 play a vital role in upregulating the overall metabolism of $CD4^+$ T cells, a requirement for productive HIV infection.

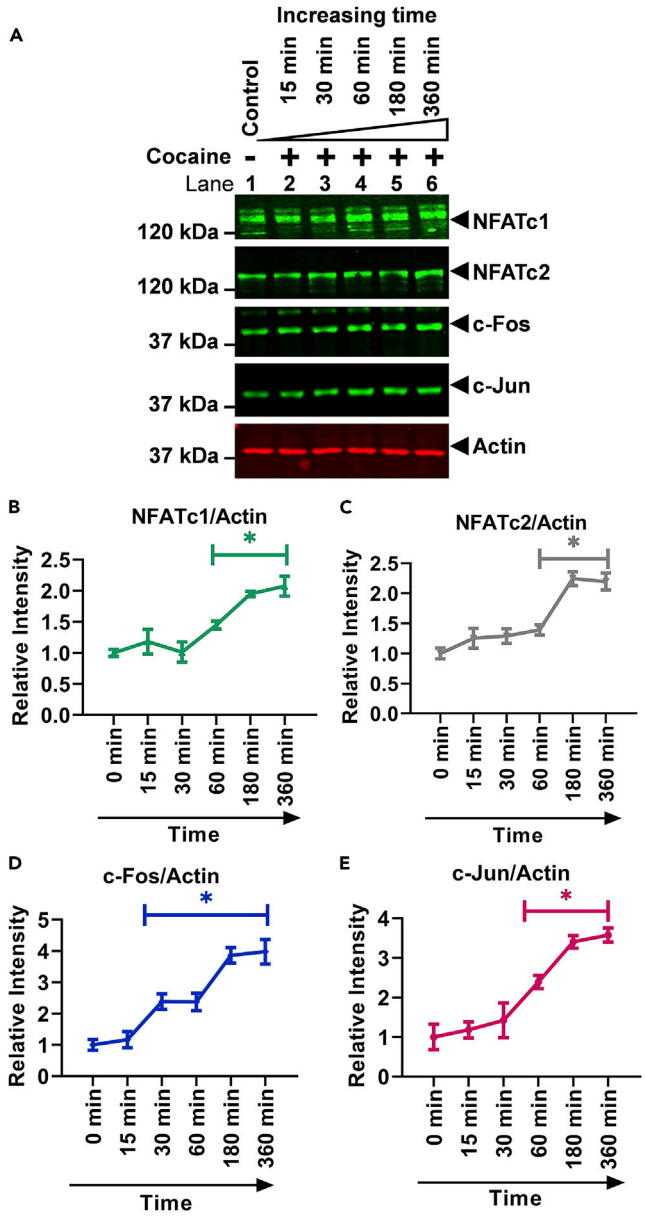

Figure 4. Cocaine activates nuclear factors of activated T cells (NFAT) and activator protein 1 (AP-1) in a time-dependent manner with unique kinetics

Jurkat cells harboring pHR′-P-Luc provirus were treated with a fixed dose of cocaine (10 μM) for different durations 15 min to 6 h (Lane 2–6).

(A) Nuclear lysates (protein samples) were analyzed by electrophoresis in SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies as indicated. The immunoreactive protein after incubation with appropriately labeled secondary antibodies was detected with Licor. Actin was used as a loading control.

(B–E) Densitometric analysis of protein bands validated a significant time-dependent increase to NFAT and AP-1 levels in Jurkat-pHR′-P-Luc cells upon cocaine treatment compared to untreated cell control. Immunoblots are representative of at least 3 independent experiments. The results are expressed as mean ± SD for three independent experiments, analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated cells vs. untreated cells.

To assess the impact of cocaine-mediated activation of NFAT and AP-1 on HIV gene expression, Jurkat cells were pre-treated with different amounts of cocaine for 3 h before being infected with dual tropic HIV Type 1 strain 93/TH/051. After 3 h, nuclear extracts were examined for NFAT and AP-1

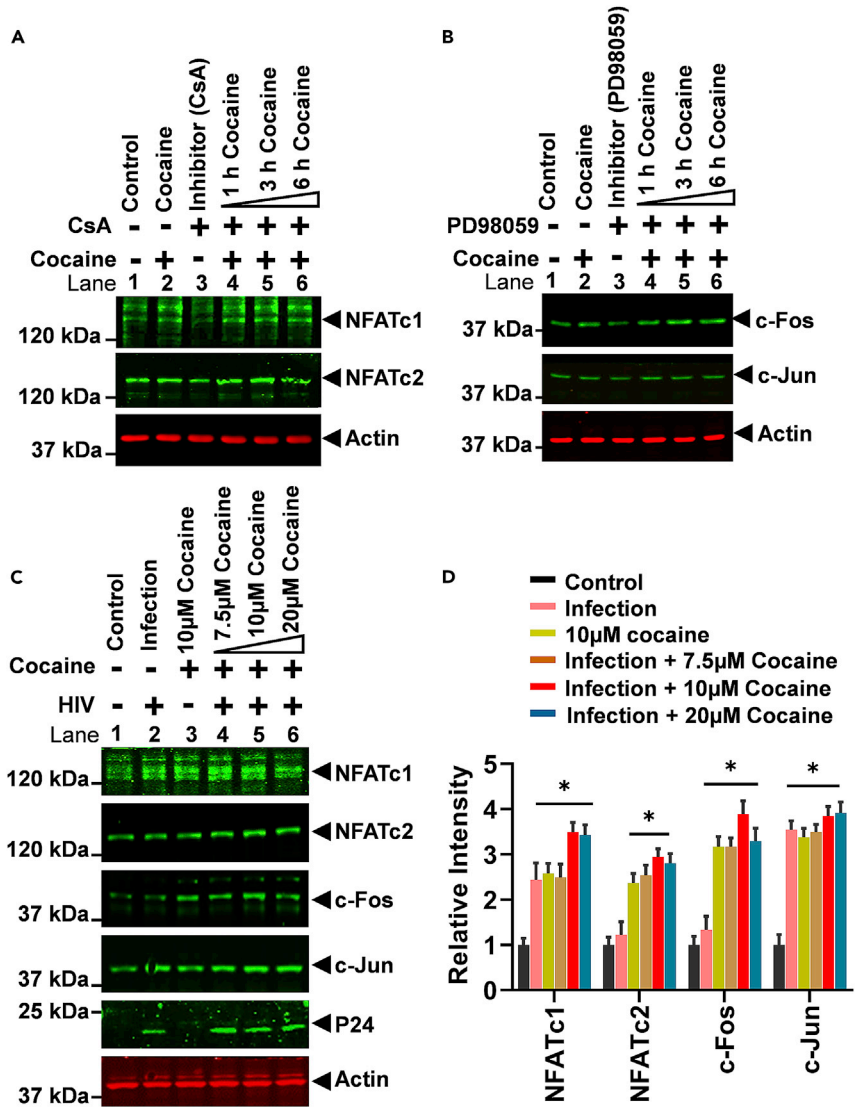

Figure 5. Stimulation of NFAT and AP-1 by cocaine plays a crucial role in enhancing HIV gene expression

(A and B) Cocaine restores NFAT and AP-1 levels, which were specifically inhibited by CsA and PD98059, respectively. Jurkat-pHR′-P-Luc cells were pre-treated with either CsA or PD98059 for 1 h, then washed and replenished with fresh medium. Later, cells were treated with cocaine for different time intervals (1 to 6 h). Nuclear lysates were evaluated via immunoblotting using specific antibodies. Actin was used as a loading control.

(C) Cocaine activates NFAT, and AP-1 was further reproduced in cells which were infected with replication-competent HIV Type 1 strain 93/TH/051. Jurkat cells were untreated and uninfected (Lane 1) or infected in the absence of cocaine (Lane 2) or treated with cocaine but uninfected (Lane 3) or infected with replication-competent HIV Type 1 strain 93/TH/051 after pre-treatment with different cocaine doses (Lane 4–6). Nuclear lysates were analyzed by immunoblotting against the specific antibodies as indicated. Actin was used as a loading control.

(D) Densitometric analysis of protein bands validated a significant increase to NFAT, and AP-1 levels compared to untreated cells (control). Immunoblots are representative of at least 3 independent experiments. The results are expressed as mean ± SD for three independent experiments, analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison against the untreated cells (controls).

stimulation. The activation of both NFAT and AP-1 upon cocaine treatment was reproduced (Figures 5C and 5D). We further validated this finding in another T cell line (MT-4 cells) (Figures S2C and S2D). For marking ongoing HIV gene expression, we evaluated the expression of p24 subunit of the Gag protein of HIV. The significant upregulation of HIV protein expression (p24) was confirmed

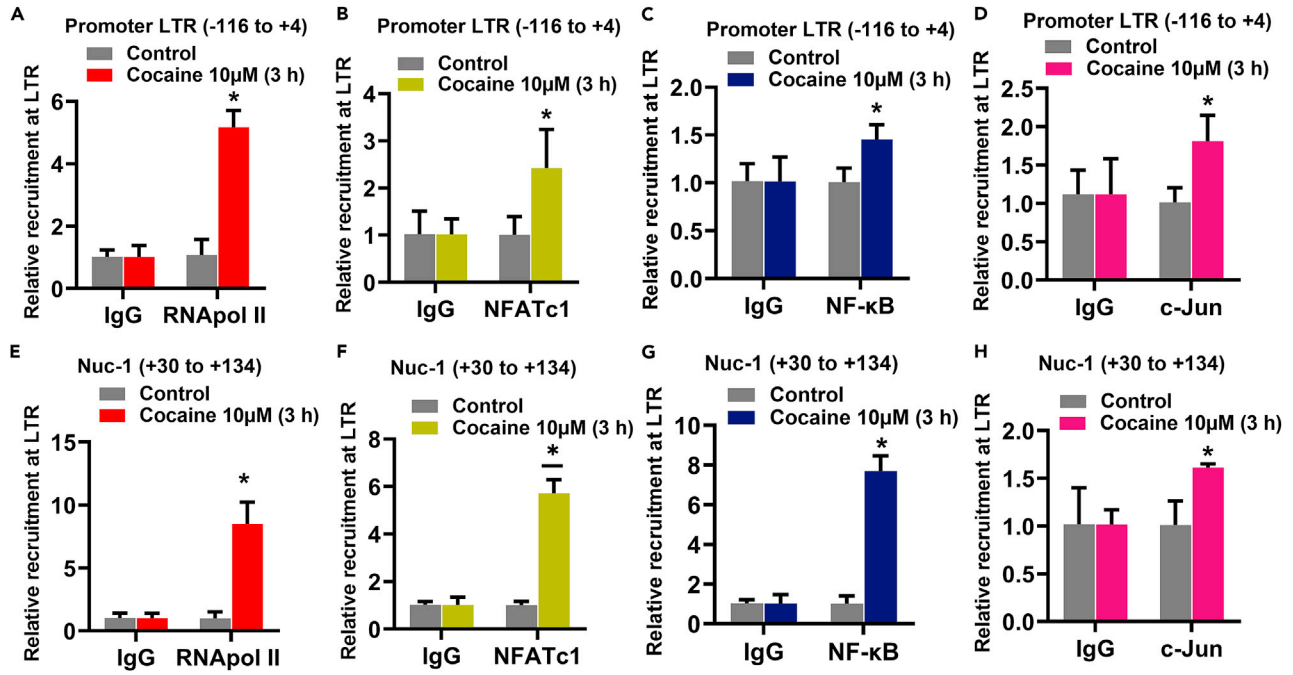

Figure 6. Cocaine enhances the recruitment of NFAT and AP-1 at HIV LTR

(A–H) Freshly infected Jurkat T cells with replication-incompetent HIV, pHR'P-Luc, were treated with 10 μM cocaine for 3 h. Chromatin immunoprecipitation (ChIP) assays were performed to examine the recruitment kinetics of RNAP II, NFAT (NFATc1), NF-κB (p65), and AP-1 (c-Jun) at the promoter (A–D) and Nucleosome-1 (E–H) regions of HIV LTR, using specific primer sets. The results are expressed as mean ± SD for three independent experiments, analyzed by two-way ANOVA. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated cells vs untreated cells.

in the presence of cocaine when compared with HIV-infected but cocaine-untreated cells (Figures 5C and 5D).

Cocaine augments HIV transcription by enhancing the recruitment of AP-1 and NFAT at HIV LTR promoter

To determine if enhanced HIV gene expression is the result of cocaine-induced higher nuclear levels of NFAT and AP-1 and corresponding enhanced recruitment to HIV LTR, we performed ChIP assays. We examined the recruitment of NFAT and AP-1 at HIV LTR in the absence and presence of cocaine, using our standardized methodology.[3,4,22,47] As controls, we evaluated the recruitment of NF-kB and RNA Polymerase II (RNAPII) at HIV LTR before and after cocaine treatment. Freshly infected Jurkat T cells with replication-incompetent HIV, pHR'P-Luc, were treated with cocaine for 3 h. The immunoprecipitated DNAs were quantified through qPCR using primer sets amplifying either the promoter region (−116 to +4) or nucleosome-1 (Nuc-1) region (+30 to +134) of LTR (Table S1). To provide a control for equal loading, the results were normalized with a housekeeping *GAPDH* gene (−145 to +21), a constitutively expressed cellular gene. We found reduced levels of NFAT and c-Jun in the absence of cocaine; however, NFAT and c-Jun significantly increased at both the promoter and Nuc-1 regions of LTR upon cocaine treatment (Figures 6A–6H). These results validate that cocaine-mediated upregulation of nuclear NFAT and AP-1 levels resulted in corresponding higher recruitment at HIV LTR, which subsequently augments HIV gene expression. This fact is also confirmed, when we examined the recruitment kinetics of RNAPII at HIV LTR following cocaine treatment, we noted more than 3-fold higher amount of RNAPII at LTR, validating enhanced ongoing HIV gene expression after cocaine treatment (Figures 6A and 6E).

Cocaine-induced NFAT and AP-1 cooperate in stimulating HIV gene expression

The role of NFAT in supporting HIV transcription is well established.[42–45] We found that cocaine upregulates overall cell metabolism by stimulating NFAT and AP-1, the two factors that are known to cooperate in promoting transcription of several cellular genes. So, to extend further, we investigated the cooperation of cocaine-induced NFAT and AP-1 in supporting HIV transcription. In this analysis, using both the study

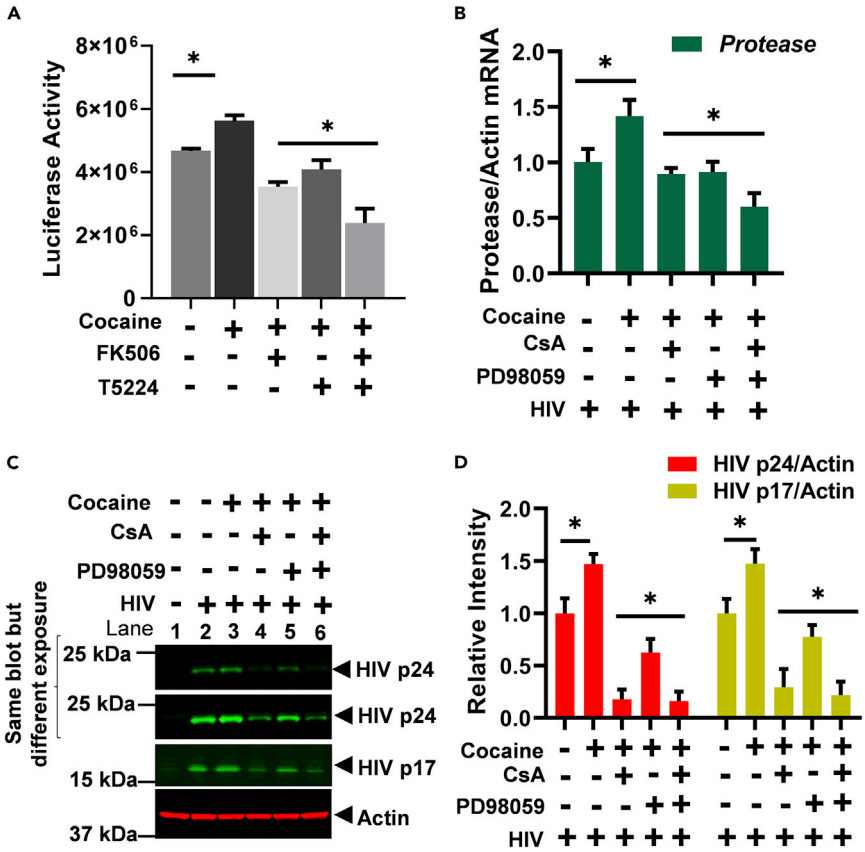

Figure 7. Cocaine enhances HIV gene expression by activating NFAT and AP-1 transcription factors

(A) Jurkat-pHR′-P-Luc cells were treated first with cocaine for 3 h, and subsequently for 48/72 h with either FK506/T5524 alone or their combination. Cells were lysed, and the level of the reporter luciferase was analyzed.

(B–D) Jurkat cells were pre-treated with cocaine for 3 h and later with inhibitors; either CsA/PD98059 alone and or their combination for 1 h before being infected with replication-competent HIV Type 1 strain 93/TH/051. (B) After 24 h, total RNA was isolated, and the HIV transcript level was analyzed by real-time qPCR (RTqPCR) amplifying the protease region of the HIV genome. (C) After 48 h, the whole-cell extract was evaluated for p24 and p17 proteins via immunoblotting, as indicated in the figure. Actin was used as the loading control. (D) Densitometric analysis of protein bands validated a significant increase to HIV p24 and HIV p17 levels in the cocaine-treated sample (Lane 3) compared to the untreated cell (control) (Lane 2). However, the presence of inhibitors (Lane 4–6) severely impairs the HIV (p24 and p17) gene expression compared to the cocaine-treated sample. Lane 1 is the negative control for this experiment. Immunoblots are representative of at least 3 independent experiments. The results are expressed as mean ± SD for three independent experiments, analyzed by one-way/two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated samples against untreated (control) and also the comparison of cocaine plus inhibitors treated against cocaine alone-treated samples.

model, cells infected with replication-incompetent (pHR′P-Luc) or replication-competent (HIV Type 1 strain 93/TH/051) HIV, we evaluated the impact of specific inhibitors, two for each target: CsA and Tacrolimus (FK506) for NFAT, and MEK inhibitor (PD98059) and T5224 c-Fos for AP-1 after being pre-treated with cocaine. Moreover, the inhibitor experiments were designed to achieve the level of ~30%–50% downregulation of NFAT and AP-1 expression as presented in Figures 5A and 5B.

Jurkat-pHR′P-Luc cells that express luciferase reporter gene under the control of HIV-1 LTR promoter were treated with cocaine for 3 h. Later, cells were treated with inhibitors; FK506, T5224, and the combination of both. After 48 h, the cells were lysed, and luciferase activity was measured. As anticipated, based on our previous findings, we found higher HIV transcription upon cocaine exposure, indicated by higher luciferase activity. However, both the NFAT inhibitor (FK506) and AP-1 inhibitor (T5224) significantly restricted HIV transcription (Figure 7A). These results again validated the specific role of cocaine-stimulated NFAT and

AP-1 in promoting HIV gene expression. However, we found a strong additive effect in restricting HIV gene expression when we treated cells together with both NFAT and AP-1 inhibitors, validating cooperation between NFAT and AP-1 in supporting HIV transcription.

To further confirm the impact of cocaine-induced NFAT and AP-1 on HIV transcription, we assessed HIV transcript levels after treating cells with NFAT and AP-1 inhibitors. Jurkat cells were treated with cocaine for 3 h. Later, cells were incubated with NFAT and AP-1 inhibitors in different combinations for 1 h. Subsequently, cells were infected with a replication-competent HIV (HIV Type 1 strain 93/TH/051) for 24 h. The RNA was extracted, and HIV transcripts were quantified via RT-qPCR using primer sets that selectively amplify a region of HIV *protease* gene. The significant upregulation of HIV gene expression upon cocaine treatment was confirmed by enhanced amplification of *protease* regions of HIV genome. Like the above result, in the presence of NFAT inhibitor CsA and/or AP-1 inhibitor PD98059, we found significantly impaired HIV gene expression (Figure 7B). These findings again validate the specific role of cocaine-induced NFAT and AP-1 in supporting HIV transcription.

To confirm our mRNA data at the protein level, the total cell lysates, detailed above (Figure 7B), were analyzed for p24 and p17 subunits of Gag protein of HIV by immunoblotting. Analogous to the above HIV transcript data (Figure 7B), we noted significant inhibition of cocaine-induced HIV protein production by NFAT and AP-1 inhibitors (Figures 7C and 7D). These data again verify the specific role of cocaine-stimulated NFAT and AP-1 in promoting HIV transcription and gene expression in $CD4^+$ T cells.

To further validate that the inhibition of NFAT and AP-1 by CsA and PD98059, respectively, restricts HIV gene expression, we employed two other specific inhibitors of NFAT and AP-1: FK-506 (Tacrolimus) and T5224, respectively. T5224 specifically inhibits the DNA binding activity of c-Fos/c-Jun. Jurkat cells were treated with cocaine for 3 h before being exposed to FK506, T5224, or their combination for 1 h. Later, cells were infected with HIV Type 1 strain 93/TH/051. The levels of p24 and p17 were evaluated after 48 h of infection by immunoblotting. The immunoblot showed a clear inhibition of cocaine-induced HIV gene expression by FK506, T5224, and combination (Figures 8A and 8B). These data again established the specific role of cocaine-stimulated NFAT and AP-1 in promoting HIV gene expression.

Subsequently, we examined whether cocaine treatment can reverse the inhibition posed by NFAT and AP-1 inhibitors on HIV gene expression. Jurkat cells were pre-treated with cocaine for 3 h before being treated with NFAT and AP-1 inhibitors for 1 h. Later, NFAT and AP-1-mediated inhibition on HIV transcription was rescued by treating those cells again with cocaine for another 3 h. Afterward, cells were infected with a replication-competent HIV (infectious virion). After 48 h of infection, the total cell lysates were analyzed for p24 levels (Figure 8C). We found restoration of p24 levels upon cocaine re-treatment (Figure 8C), again validating the crucial and specific role of cocaine-induced NFAT and AP-1 in supporting HIV transcription.

Cocaine promotes HIV transcription by enhancing the phosphorylation of RNA pol II CTD

The enhanced phosphorylation of RNAPII C-terminal domain (CTD) at Ser2 and Ser5 marks ongoing HIV transcription. The enhanced phosphorylation at Ser5 marks upregulation of the initiation phase of transcription, while higher Ser2 phosphorylation indicates productive elongation phase.[51,52] Therefore, to validate that cocaine-stimulated NFAT and AP-1 increase HIV transcription, we analyzed phosphorylation of RNA pol II at Ser2 and Ser5. Jurkat cells were untreated or treated with varying concentrations of cocaine for 3 h. Later, cells were infected with replication-competent HIV (HIV Type 1 strain 93/TH/051) for 1 h. The nuclear extract was prepared, and the levels of RNA pol II Ser2 and RNA pol II Ser5 were assessed (Figures 9A and 9B). The H5 antibody recognized the RNAP II with CTDs carrying Ser2 phosphorylation, while the H14 antibody recognized epitopes of CTD carrying Ser5 phosphorylation. A significant upregulation of Ser2 and Ser5 phosphorylation was observed following cocaine treatment. We noted a direct correlation between the extent of CTD phosphorylation and the amounts of cocaine used (Figures 9A and 9B). This suggests that cocaine enhances HIV transcription by supporting both the initiation and elongation phases of transcription, a requirement to generate full genomic transcript. We also noted that the fold increment in Ser5 phosphorylation is less pronounced compared to the fold increment in Ser2 phosphorylation following the cocaine treatment, indicating that cocaine facilitates HIV transcription predominantly by supporting the elongation phase. To further validate enhanced CTD phosphorylation upon cocaine exposure, we performed a rescue experiment using NFAT inhibitor. Jurkat cells were pre-treated with

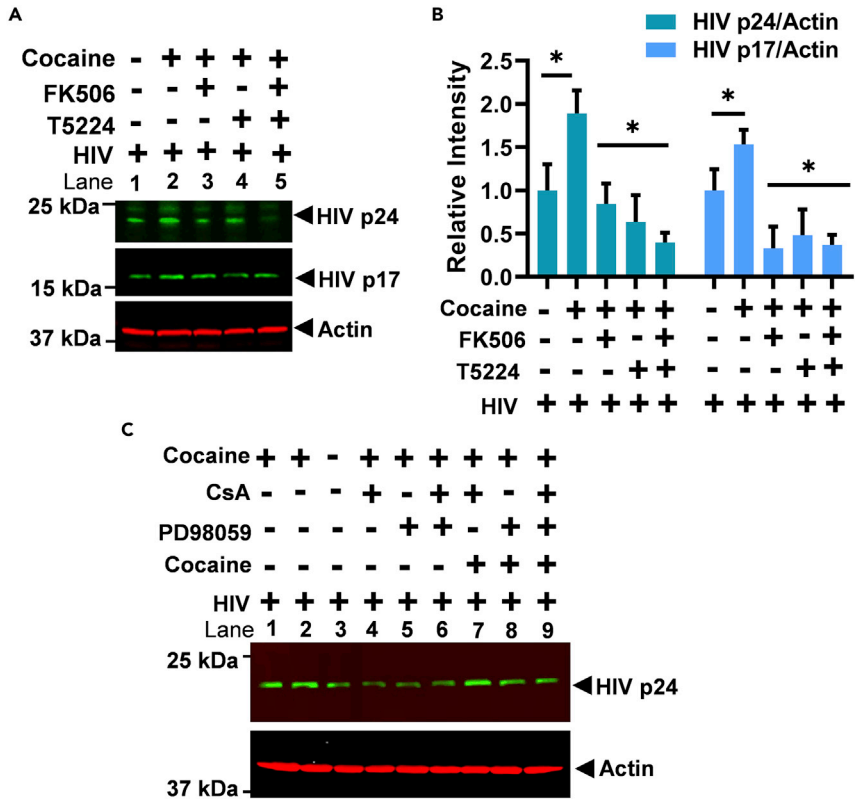

Figure 8. Cocaine restores HIV gene expression by activating NFAT and AP-1 transcription factors

(A) Jurkat cells were pre-treated with cocaine for 3 h, and later with inhibitors (FK506/T5224 or combination) for 1 h, before being infected with replication-competent HIV Type 1 strain 93/TH/051. After 48 h, the whole-cell extract was analyzed by western blotting with HIV-specific antibodies (HIV p24 and p17), as indicated in the figure. Actin was used as the loading control.

(B) Densitometric analysis of protein bands validated a significant increase to HIV p24 and HIV p17 levels in the cocaine-treated sample (Lane 2) compared to the untreated control cell (Lane 1). However, the presence of inhibitors (Lane 3–5) severely impaired the expression of p24 and p17 when compared to cells treated with cocaine alone (Lane 2). The results are expressed as mean ± SD for three independent experiments, analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated samples against untreated and also the comparison of cocaine plus inhibitors treated against cocaine alone-treated samples.

(C) Cocaine treatment can reverse/restores the inhibition posed by NFAT and AP-1 inhibitors on HIV gene expression validating the crucial and specific role of cocaine-induced NFAT and AP-1 in supporting HIV transcription. Jurkat cells were first treated with cocaine for 3 h and later with inhibitors (CsA, PD98059 or combination) for 1 h, then cells were washed, replenished with fresh media, and again treated with cocaine for another 3 h, before being infected with HIV Type 1 strain 93/TH/051. After 48 h, the whole-cell extract was evaluated by western blotting with specific antibodies, as indicated in the figure. Actin was used as the loading control. Immunoblots are representative of at least 3 independent experiments.

CsA for 1 h, washed, replenished with fresh medium, and later treated with cocaine (10 μM) for 1, 3, and 6 h. The nuclear extract was analyzed for RNA pol II CTD phosphorylation at Ser2 and Ser5. We noted a significant loss of CTD phosphorylation at both Ser2 and Ser5 in CsA-treated samples (Figures 9C and 9D, Lane 2) when compared to untreated samples (Figures 9C and 9D, Lane 1). However, we found significant upregulation/restoration of CTD phosphorylation at both Ser2 and Ser5 in CsA cocaine-treated samples (Figures 9C and 9D, Lane 3–5) when compared to CsA alone-treated sample (Figures 9C and 9D, Lane 2). Thus, we noted that cocaine was able to rescue the CsA-induced loss of CTD phosphorylation (Figures 9C and 9D). These results demonstrate that cocaine promotes both the initiation and elongation phases of HIV transcription by enhancing RNAP II CTD phosphorylation at both Ser5 and Ser2. Completion of both phases is essential to generate complete/full HIV genomic transcripts, a requirement to produce new HIV progeny.

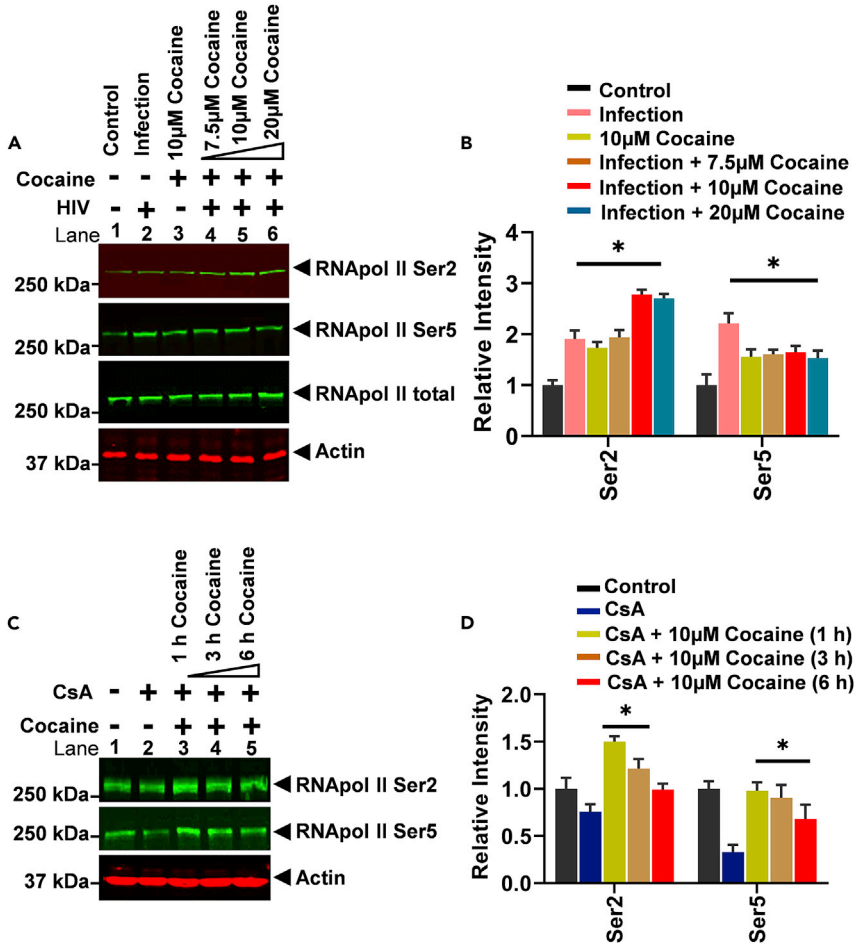

Figure 9. Cocaine promotes HIV transcription by enhancing the phosphorylation of RNA pol II CTD

(A) Jurkat cells pre-treated with different doses of cocaine (7.5–20 μM) were infected with replication-competent HIV Type 1 strain 93/TH/051 (Lane 4–6). The cells were harvested, and nuclear extracts were assessed for the phosphorylation state of the CTD of RNAP II through a western blot using antibodies that are specific for RNAP II and its phosphorylated forms (Ser2 and Ser5). Actin was used as a loading control.

(B) Densitometric analysis of protein bands validated a significant increase to RNA Pol II Ser2 and Ser5 in cocaine-treated samples or in infected samples and infection in presence of cocaine compared to untreated cells (control). The results are expressed as Mean ± SD for three independent experiments, analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. *$p < 0.05$ is for the comparison of cocaine-treated samples, infection, and cocaine-treated plus infection against untreated samples (control).

(C) Cocaine rescued CsA-induced restriction on RNA Pol II Ser2 and Ser5 phosphorylation. Jurkat cells were treated with CsA (Lane 2–5), then washed, and then replenished with fresh medium. Further cells were treated/untreated with cocaine in a time-dependent manner (1 to 6 h) (Lane 3–5). The cells were harvested, and nuclear extracts were evaluated through a western blot for the phosphorylation state of RNAP II CTD using specific antibodies. Actin was used as a loading control.

(D) Densitometric analysis of protein bands validated restoration of RNA Pol II Ser2 and Ser5 in cocaine-treated samples (Lanes 3–5) compared to CsA-treated samples (Lane 2). Densitometry analyses were performed on western blot bands using ImageJ software. The results represent the Mean ± SD of three independent experiments, analyzed by two-way ANOVA followed by Dunnett's multiple comparisons test. Asterisks over the bar indicate significant differences. Statistical significance is set as $p < 0.05$ (*), compared to CsA alone-treated cells.

DISCUSSION

ART is currently the only available effective methodology to control HIV.[53] ART regimens usually consist of three anti-HIV drugs targeting HIV at different steps of its life cycle. ART has been highly effective in preventing HIV replication and consequently viral load in the bloodstream.[2,54,55] Hence, adherence to ART is the prerequisite to maintaining a functional immune system and HIV levels below toxic limits. Unfortunately,

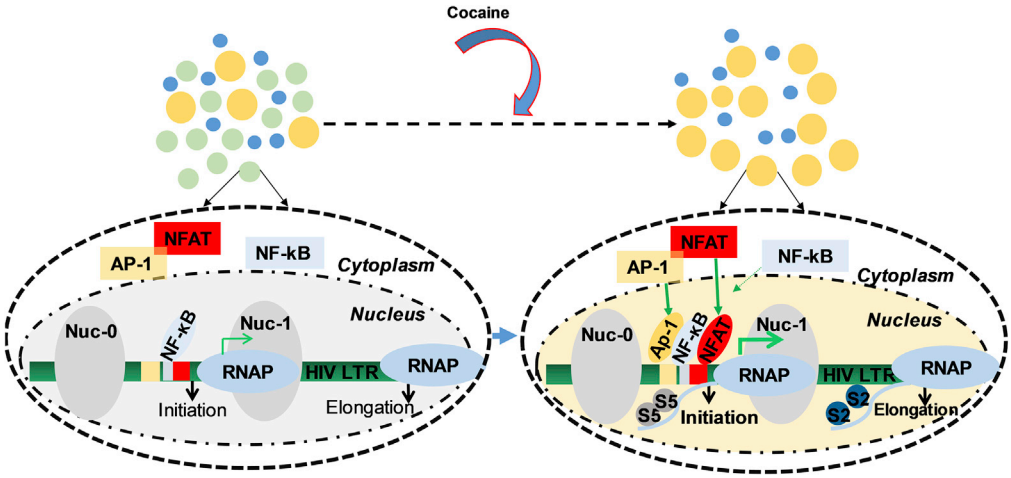

Figure 10. A proposed model demonstrating the activation of partially active T cells following cocaine exposure due to stimulation of major transcription factors, NFAT and AP-1, and ultimately enhancing the HIV transcription and gene expression

The metabolic state of the cell plays a crucial role in supporting HIV transcription and replication. Cocaine is unable to activate the quiescent cells. Our finding suggests that cocaine exposure is efficient enough to sensitize partially active $CD4^+$ T cells and make them poised for productive HIV infection. In this model, we suggest that cocaine exposure enhances the metabolic state of the cells by co-stimulating several transcription factors, mainly NFAT and AP-1, the two transcription factors, which specifically play a crucial role in enhancing both HIV and the overall cellular gene expression in T cells. Cocaine enhances HIV transcription by supporting both the initiation and elongation phases of transcription. The small blue circles indicate $CD4^+$ naive T cells, green circles indicate partially active T cells, and yellow circles indicate activated T cells.

illicit drug use by patients with HIV greatly accelerates the deterioration of both immune and nervous systems. Consequently, even shorter interruptions of ART result in severe damage to brain and immune functions in patients with HIV using illicit drugs compared to non-drug-using HIV-infected individuals.[17,56–59] Cocaine, one of the most abused drugs in the world, is an important cofactor in spreading HIV by enhancing both HIV transmission and replication, in addition to making cells suitable to support HIV infection.[22,57,58] Notably, in ART naive patients, use of illicit drugs accelerates AIDS progression.[60] It is noted that despite effective ART, cocaine-using patients with HIV have lower $CD4^+$ T cell counts, mainly due to higher rates of $CD4^+$ T cell depletion/killing.[19,61] Cocaine has been shown as a cofactor for HIV replication; however, the underlying molecular mechanisms by which cocaine enhances HIV transcription and replication remain unclear. In this investigation, we explored some of the key factors/pathways through which cocaine enhances HIV transcription. HIV transcription depends primarily on the metabolic state of the host cell, as metabolically active cells have the availability of all ingredients/building blocks required to complete HIV life cycle and generate new viral progeny. We studied whether cocaine could modulate cell metabolism and transcriptional machinery. Our findings suggest that cocaine is not a strong activator, as we observed subtle changes in cell metabolism and T cell activation markers. Nevertheless, this stimulation is efficient in priming/sensitizing partially active cells to the level where they could support HIV life cycle (Figure 10). We found that cocaine enhances cell metabolism and sensitizes partially active T cells for HIV infection by stimulating two crucial transcription factors, NFAT and AP-1. We discovered that cocaine-induced signaling pathways lead to the activation of NFAT and AP-1, which besides increasing HIV gene expression, enhances the expression of several cellular genes linked to cell cycle; thus, it augments the metabolic state of the cells sufficient enough to support the HIV life cycle.

Many studies, including ours,[22,57,58,62,63] have shown the stimulation of NF-kB by cocaine and the vital role of NF-kB in promoting HIV transcription. In this investigation, we defined the specific role of cocaine-induced NFAT in upregulating T cells metabolism and its effect on HIV gene expression. Subsequently, we investigated the impact of cocaine-induced AP-1 and its cooperation with NFAT in supporting HIV gene expression. Thus, in the present work, we report the specific role of cocaine-induced NFAT and AP-1 in augmenting HIV gene expression in lymphoid cells (Figures 2, 3, 4 and 5). We demonstrated that cocaine intake/exposure enhances HIV replication by priming/sensitizing a group of partially active

T cells. We confirmed our findings by reproducing our results using replication-competent HIV (Figures 7 and 8). To validate the specific role of cocaine-induced NFAT and AP-1 on HIV gene expression and replication, we utilized two specific inhibitors each for NFAT and AP-1 in our assays: CsA and FK506 for NFAT and PD98059 and T5224 for AP-1. We measured the inhibition of HIV transcription and gene expression by these inhibitors by quantifying luciferase reporter, HIV transcript, and protein levels. We confirmed the inhibition of HIV replication by these inhibitors using replication-competent HIV (Figures 7 and 8). The results were confirmed through various independent, but complementary methodologies and experiments. The impact of specific inhibition of NFAT and/or AP-1 on HIV transcription and replication was further validated via rescue experiments (Figure 8C). The rescue experiments demonstrated that the severely impaired HIV p24 levels in the presence of inhibitors were restored by the re-treatment of cocaine (Figure 8C, Lane 7–9). The results showed that cocaine can rescue the severely impaired HIV gene expression (p24) under the influence of NFAT and AP-1 inhibitors. These results demonstrate the role of cocaine-induced NFAT and AP-1 in promoting HIV transcription and replication. Overall, our findings substantiate that cocaine-stimulated NFAT and AP-1 besides promoting suitable environment by inducing cell metabolism, also directly promote HIV transcription along with cocaine-induced NF-kB.

In our prior study, we also confirmed that cocaine treatment promotes the recruitment of the positive transcription elongation factor b (P-TEFb) to the HIV-1 LTR.[22] The kinase subunit of P-TEFb, CDK9, is the main kinase that catalyzes the phosphorylation of RNAPII CTD at the Ser2 position. The hyper-phosphorylation of Ser2 of RNAPII CTD indicates the enhanced elongation phase of transcription. To expand on those findings, we found that in addition to enhancing the Ser2 phosphorylation, cocaine augments the Ser5 phosphorylation of RNAPII CTD. Ser5 hyperphosphorylation is the indicator of the augmented initiation phase of HIV transcription. However, the fold increment to Ser5 phosphorylation upon cocaine treatment was less pronounced than Ser2 phosphorylation, suggesting that cocaine facilitates HIV transcription predominantly by supporting the elongation phase. Overall, these results suggest that cocaine enhances HIV transcription by supporting both the initiation and elongation phases of transcription (Figure 9). These findings reveal yet another breakthrough in the role of cocaine in enhancing HIV transcription and gene expression. Future studies should use a combination of biochemical, genetic, and genomics approaches for a detailed investigation of this mechanism.

Understanding the molecular mechanisms that control HIV transcription and gene regulation is critical in the search for an HIV cure. Although cocaine has been shown to modulate HIV infection, the underlying molecular mechanism remains unknown. The current study provides evidence for cocaine's critical role in promoting HIV transcription and viral gene expression. Our findings unraveled the molecular mechanisms utilized by cocaine to enhance HIV transcription and replication (Figure 10). In this study, we found that cocaine upregulates the overall cell metabolism by activating the NFAT and AP-1. To the best of our knowledge, this is the first study to show that cocaine promotes HIV-1 transcription and gene expression by increasing the functional activity of the NFAT and AP-1 transcription factors. We found that cocaine-induced AP-1 works in tandem with NFAT to boost HIV transcription and replication. Our study also revealed that cocaine supports transcriptional initiation by catalyzing the phosphorylation of CTD at Ser5 and enhances the HIV transcriptional elongation by catalyzing the phosphorylation of CTD at Ser2 and promoting P-TEFb factors. Overall, these findings shed light on the molecular mechanisms through which cocaine sensitizes the partially active lymphocytes suitable for the HIV life cycle, whereby enhancing their metabolism. These findings elucidate cocaine-induced changes in $CD4^+$ T cell biology, which helps determine the underlying mechanisms by which cocaine-using patients with HIV have accelerated HIV pathogenesis and AIDS-related death.

Limitations of the study

Our study had some limitations. First, the amount of replication-competent virus used to infect cells was not always precisely quantified. We did, however, use an equal number of viruses to infect the test and control (mock) samples. Second, because our study used potent and selective inhibitors of NFAT and AP-1 that have little to no reported effect on the activities of other mitogen-activated protein kinase family members, we cannot rule out the possibility that prolonged infusion at pharmacologic doses impacted the activity of other kinase members and contributed to our *in vitro* results. Nonetheless, the findings of this study suggested that the effect of cocaine on HIV-1 transcription and gene expression is dependent on the transcription factors of NFAT and AP-1.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105651.

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AUTHOR CONTRIBUTIONS

A.L.S. and M.T. conceived the project. A.L.S. and M.T. designed the experiments. A.L.S., D.S., and D.N. performed the experiments. A.L.S. and M.T. acquired, analyzed, and interpreted the data. A.L.S. and M.T. drafted and revised the manuscript. M.T. acquired the funding. M.T. supervised this project. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HIV1 p55 + p24 + p17 antibody	Abcam (Cambridge, UK)	Cat# ab63917; RRID: AB_1139524
HIV p24 mouse monoclonal antibody	Abcam (Cambridge, UK)	Cat# ab8246; RRID: AB_306392
HIV-1 p24 gag monoclonal	NIH-ARP	#6521
HIV-1 p17	Santa Cruz (Dallas, TX, USA)	Cat# sc-69725; RRID: AB_1125168
NFATc2	Santa Cruz (Dallas, TX, USA)	Cat# sc-136206; RRID: AB_2236055
c-Fos	Santa Cruz (Dallas, TX, USA)	Cat# sc-166940; RRID: AB_10609634
NFATc1	Santa Cruz (Dallas, TX, USA)	Cat# sc-7294; RRID: AB_2152503
Pol II (CTD4H8)	Santa Cruz (Dallas, TX, USA)	Cat# sc-47701; RRID: AB_677353
c-Jun (G-4)	Santa Cruz (Dallas, TX, USA)	Cat# sc-74543; RRID: AB_1121646
RNA Pol II Ser2	Bethyl Lab (Montgomery, TX, USA)	Cat# A300-654A-M; RRID: AB_2779370
RNA Pol II Ser5	Bethyl Lab (Montgomery, TX, USA)	Cat# A304-408A-M; RRID: AB_2781885
Actin (c4)	Santa Cruz (Dallas, TX, USA)	Cat# sc-47778; RRID: AB_626632
IgG (3E8)	Santa Cruz (Dallas, TX, USA)	Cat# sc-69786; RRID: AB_1124809
IRDye 680RD	Li-cor (Lincoln, NE, USA)	Cat# 926-68071; RRID: AB_10956166
IRDye 680LT	Li-cor (Lincoln, NE, USA)	Cat# 926-68022; RRID: AB_10715072
IRDye 800CW	Li-cor (Lincoln, NE, USA)	Cat# 926-32211; RRID: AB_621843
PE anti-human Ki67	Biolegend (San Diego, CA, USA)	Cat# 350504; RRID: AB_10660752
Anti-HIV-1 p24 (APC)	Novus Biologicals (Littleton, CO, USA)	NB500-473APC
APC anti-human CD25	Biolegend (San Diego, CA, USA)	Cat# 302610; RRID: AB_314280
PE anti-human CD38	Biolegend (San Diego, CA, USA)	Cat# 356604; RRID: AB_2561900
FITC anti-human CD69	Biolegend (San Diego, CA, USA)	Cat# 310904; RRID: AB_314839
FITC anti-human HLA-DR	Biolegend (San Diego, CA, USA)	Cat# 307604; RRID: AB_314682
APC anti-human CD366 (Tim3)	Biolegend (San Diego, CA, USA)	Cat# 345012; RRID: AB_2561718
PE anti-human CD160	Biolegend (San Diego, CA, USA)	Cat# 341206; RRID: AB_2561433
FITC anti-human CD279 (PD-1)	Biolegend (San Diego, CA, USA)	Cat# 621612; RRID: AB_2832832
Virus strains		
HIV replication-competent virus (HIV-1 strain 93/TH/051; R5- and X4-tropic virus isolated from a seropositive individual in Thailand)	NIH AIDS reagent	ARP-2165
Chemicals, peptides, and recombinant protein		
DMSO	Sigma-Aldrich (Burlington, MA, USA)	Cat# D2650
BSA	Sigma-Aldrich (Burlington, MA, USA)	Cat# A9647
Triton X-100	Sigma-Aldrich (Burlington, MA, USA)	Cat# T928
RPMI1640	Invitrogen (Waltham, MA, USA)	Cat# 11835–030
DMEM	Lonza (Walkersville, MD, USA)	Cat# 12–604F
Opti-MEM (1X) +GlutaMAX™	Gibco (Waltham, MA, USA)	Cat# 51985–034
HEPES Buffer	Corning, New York, U.S.	Cat# 25-060-CI
Fetal Bovine serum	Gibco (Waltham, MA, USA)	Cat# 10082147
Pen strep	Gibco (Waltham, MA, USA)	Cat# 15140–122
Cocaine	NIDA	N/A
PD98059	Cell Signaling Technology (Danvers, MA, USA)	Cat# 9900

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cyclosporin A, 99+%	Alfa Aesar (Haverhill, MA, USA)	Cat# J63191
Tacrolimus (FK506)	Cayman Chemical Company (Ann Arbor, MI, USA)	Cat# 10007965
T5224	ApexBio (Boston, MA, USA)	Cat# B4664
PageRuler™ Prestained Protein Ladder	Thermo Scientific (Waltham, MA, USA)	Cat# 26617
Protein A-Sepharose 4B Conjugate	Thermo Scientific (Waltham, MA, USA)	Cat# 101041
Passive Lysis Buffer, 5X	Promega (Madison, WI, USA)	Cat# E194A
Trypan Blue stain	Gibco (Waltham, MA, USA)	Cat# 15250–061
Lipofectamine 2000	Thermo Scientific (Waltham, MA, USA)	Cat# 11668–019
Critical commercial assays		
RNeasy mini kit	Qiagen (Hilden, Germany)	Cat# 74104
Qiagen MinElute Virus Spin kit	Qiagen (Hilden, Germany)	Cat# 57704
BCA Protein Assay Kit	Thermo Scientific (Waltham, MA, USA)	Cat# 23225
Luciferase Assay System	Promega (Madison, WI, USA)	Cat# E1501
CellTiter 96® Aqueous One Solution Reagent	Promega (Madison, WI, USA)	Cat# G5421
M-MLV Reverse Transcriptase	Thermo Scientific (Waltham, MA, USA)	Cat# 28025013
Taq™ Universal SYBR® Green Supermix	Biorad (Hercules, CA, USA)	Cat# 1725125
Experimental models: Cell lines		
Jurkat cells line	ATCC	TIB-152
Jurkat-pHR′-P-Luc	In-house generated model	N/A
2D10 cells line	In-house generated model	N/A
MT-4 cell line	NIH AIDS reagent	ARP-120
Software and algorithms		
Prism 9	Graphpad	Ver 9.1.2 (226)
Odyssey infrared imaging software	Li-cor	Ver 3.0.30
ImageJ software	NIH	1.53e

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mudit Tyagi (Mudit.tyagi@jefferson.edu).

Materials availability

Plasmids generated in this study are available from the lead contact upon reasonable request. In-house generated materials are also available from the lead contact upon request with the completed Material Transfer Agreement.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report the original code. Any additional information required to analyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines

Jurkat and its derivatives cell lines, such as Jurkat-pHR′-P-Luc, and 2D10 were maintained in complete RPMI 1640 medium (RPMI supplemented with fetal bovine serum, penicillin, and streptavidin). MT-4 cells were

also cultured in complete RPMI while HEK293 T cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM). All the cell lines were cultured in an incubator maintaining 37°C and 5% CO_2.

METHOD DETAILS

Plasmid constructs, transfection, and VSV-G pseudotyped virus generation

pHR'P-d2EGFP was derived by inserting the *EcoRI* and *XhoI* fragment of HIV-1 pNL4-3 into the pHR' plasmid.[47,64] The short-lived version of green fluorescent protein (d2EGFP) replaces nef position at the *MluI* and *XhoI* sites. We have previously explained the generation of pHR'P-Luc.[47,65] Human Embryonic Kidney (HEK) 293 T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 g/L Glucose & L-Glutamine (Lonza, Walkersville, MD, USA); 10% fetal bovine serum (Gibco, Waltham, MA, USA); and 1 U/mL penicillin/streptavidin (Gibco, Waltham, MA, USA). Cells were grown to 70–80% confluency and washed with Opti-MEMs GlutaMAX reduced serum media (Gibco, Waltham, MA, USA) before transfection. Transfection with Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) was conducted according to the manufacturer's protocol. Briefly, 35μL of Lipofectamine 2000 reagent was diluted in 500μL Opti-MEM. In a separate tube, 18 μg of plasmid DNA mixture (4 μg pMD.G, 3 μg pCMVΔ8.9.1, 3 μg pMDL-g/p-RRE, 1 μg pRSV-Rev, and 7 μg of pHR-P-Luc/pHR'P-d2EGFP) was diluted in 500μL Opti-MEM to generate pHR-P-Luc/pHR'P-d2EGFP pseudotyped virus. The two separated dilutions were mixed and incubated at room temperature (RT) for 10 min to form the lipid-DNA complex. The complex was then added to the cells.[22] Five hours after adding the transfection cocktail, a fresh DMEM culture medium was added to the cells. The virus-containing cell supernatant was collected at 48 and 72 h post-transfection. We confirmed the successful generation of replication-incompetent pseudotyped virus expressing the Luc/GFP reporter gene under the control of the HIV-1 LTR by fluorescent microscopy or FACS analysis.

Generation of latently infected Jurkat T cell clones

Isolation of Clone (WT LTR and H13L Tat) cells infected with lentiviral vectors were previously described by Pearson et al.[66] Briefly, triple transfection of 293 T cells produced Vesicular Stomatitis Virus Protein G (VSV-G)-pseudotyped HIV particles using Lipofectamine 2000 reagent (Invitrogen, Waltham, MA, USA). As we have previously explained,[55] Virus titers were determined by infecting 2×10^6 Jurkat T-cells with serial dilution of concentrated virus preparation. Six hours post-infection, cells were washed with PBS, and the fresh DMEM medium was replaced. Expression of d2EGFP was further assessed by fluorescently activated cell sorting analysis (FACS Calibur) 72 h post-infection, and d2EGFP expression was analyzed every week until cells were fully shut down without detectable d2EGFP expression before reactivation experiments.

Cell culture

Jurkat, Jurkat-pHR'-P-Luc, and clone Jurkat cells (2D10, latently HIV infected Jurkat cells) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL), streptavidin (100 IU/mL), and 25 mM HEPES at 37°C in 5% CO_2. Fresh medium was supplemented to cells every 2–3 days. The cell density was determined and maintained at 2×10^6 cells/mL.

HIV replication-competent virus

HIV Type 1 strain 93/TH/051 was procured from the NIH AIDS reagent program. Propagation of primary HIV-1 Isolates was done following the instruction provided with the datasheet. Jurkat cells were infected with HIV for 5 days, then aspirated with 25–30 mL of cultured supernatant from each T-75, leaving 10–15 mL of cell suspension in each flask. We clarified the virus by centrifugation twice, initially at 1400 rpm (370 x g) for 10 min at 4°C, then transferred virus stock into another 50 mL centrifuge tube. The second centrifugation was done at 2400 rpm (1100 x g) for 10 min at 4°C. During clarification, we labeled and aliquoted the "cell-free" virus stock at 1.0 mL/vial.

Cocaine treatment

We obtained cocaine hydrochloride through the Drug Supply Program of the National Institute on Drug Abuse (NIDA). In this study, we used cocaine at concentrations ranging from 5μM to 30μM, with a maximum of ≤30μM. All the cocaine treatment were done at 10μM cocaine unless specifically mentioned. This was much lower than human plasma levels of drug users, which typically range between 0.4 and 1.6 mM for intranasal administration.

Infection of Jurkat cells with replication-competent virus

Jurkat cells (5 × 10^6 cells) were treated with cocaine for 3 h and then infected with replication-competent HIV (HIV Type 1 strain 93/TH/051) (500μL) for 1 h, 24 h, and 48 h to analyze the HIV infection and gene expression. Treatment with inhibitors was done 1 h prior to HIV infection. All the doses of inhibitors used in this study were cyclosporine A (CsA): 50–100μM, PD98059: ≤50μM, Tacrolimus (FK506): ≤12μM and T-5224: 50–100μM.

Western blot analysis for HIV gene expression from total cell lysate

Jurkat cells (5 × 10^6 cells) were treated with cocaine for 3 h and infected with replication-competent HIV (HIV Type 1 strain 93/TH/051) for 24 and 48 h. Treatment with inhibitors was done 1 h prior to HIV infection. Thereafter, samples were collected and washed with 1 mL of ice-cold PBS, and we added 100–200μL of RIPA buffer (50 mM Tris pH 8.0, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, and 150 mM NaCl). Cells were resuspended with RIPA and incubated on ice for 30 min. During the 30 min incubation time, cells were vortexed 3 times after every 10 min to ensure complete lysis. The cell lysate was centrifuged at the highest speed for 10 min, and the supernatant was analyzed for the protein concentration using Pierce™ BCA Protein Assay Kit. Protein concentration was normalized, and an equal amount of protein was mixed with 5X Laemmle Sample buffer, heated to 100°C for 10 min. An equal amount of protein was resolved by SDS-PAGE on 12% gel at 120 volts until the dye reached the bottom and was then transferred to nitrocellulose membrane. The membranes were blocked with 3% BSA for 1 h, incubated with primary antibodies at 4°C overnight, and then incubated with Li-cor secondary antibodies (1:15000 dilution) for 1 h at RT. After three rounds of washing with 1X TBST, the blot was detected with Odyssey infrared imaging system application software version 3.0 (Li-cor Bioscience).

Western blot analysis for nuclear protein/transcription factors

Jurkat/Jurkat-pHR′-P-Luc cells (5 × 10^6 cells) were treated with cocaine (at varying doses and time points); thereafter, samples were collected, and cells were washed with 500μL of ice-cold PBS, allowed to swell in 500μL of cytoplasmic extract (CE) buffer (1 mM Hepes-KOH pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 1 mM PMSF), and vortexed for lysis. Nuclei were pelleted at 4000 r.p.m for 5 min. Cytoplasmic lysates were transferred to new eppendorf tubes. Nuclei were washed two times with 1 mL of CE buffer and pelleted at 13,000 r.p.m for 1 min. Nuclei were resuspended in 80–100μL of nuclear extract (NE) buffer (250 mM Tris pH 7.8, 1 mM EDTA, 60 mM HCl, 1 mM DTT, 1 mM PMSF) and lysed by 8 freeze-thaw cycles in liquid nitrogen-water bath. The nuclear lysate was collected by centrifugation at 13,000 r.p.m for 1 min, and supernatant was transferred into a new microfuge tube. The total nuclear protein concentration was normalized using a standard BCA assay. An equal amount of total nuclear samples was loaded and resolved by 10% SDS-PAGE gel for electrophoresis. Proteins on gels were transferred onto nitrocellulose membranes; blocked the membrane with 3% BSA for 1 h; incubated with primary antibodies overnight, and then with Li-cor secondary antibodies for 1 h at RT. After three rounds of washing with 1X TBST, the blot was detected with Odyssey infrared imaging system application software version 3.0 (Li-cor Bioscience).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were done using our established protocol.[3,8,22,47] Briefly, Jurkat-pHR′-P-Luc cells (5 × 10^7 cells) were fixed after the treatment with or without cocaine for 3 h using 0.5% formaldehyde for 10 min at RT. Cells were washed twice with 20 mL of ice-cold PBS and then allowed to swell in 5 mL of CE buffer. Nuclei were pelleted at 2000 r.p.m for 10 min at 4°C and resuspended in 1 mL of SDS-lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris-HCl pH 8.1, 1 μg/mL aprotinin, 1 mM PMSF, 1 μg/mL pepstatin A). Using our standardized conditions,[55] genomic DNS was shredded to lengths less than 800 bp by sonication (Misonex 3000) under the following sonication conditions: output 2.5 for 20s, 8 times. For each sample, 200μL of sonicated samples were added to 900μL of ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA, 1.1% Triton X-100, 16.7 mM Tris-HCl pH 8.1, 150 mM NaCl). Samples were then incubated with specific antibodies at +4°C overnight. One hundred microlitres of 25% Protein A-Sepharose were used in DNA-protein immunoprecipitation. Antibody-DNA-protein complexes were washed with 1 mL of each wash buffer in the following order: Low salt wash buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1); High salt wash buffer (0.1% SDS, 2 mM EDTA, 1% Triton X-100, 500 mM NaCl, 20 mM Tris-HCl pH 8.1); RIPA buffer (20 mM Tris-HCl pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 0.5% Na-deoxycholate, 0.1% SDS, 5 mM EDTA); and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) twice. As described in our previous publication,[55] protein DNA complexes were eluted from protein A-sepharose twice using 250μL of freshly

prepared elution buffer (1% SDS, 0.1 mM $NaHCo_3$). Twenty microlitres of 5M NaCl were added to the total eluate, and Protein-DNA complexes were reversed-cross-linked at 65°C overnight. Ten microliters of 0.5 M EDTA, 10μL of 2M Tris-HCl pH 6.5, and 2μL of 10 mg/mL proteinase K were added, and samples were further incubated at 50°C for 2 h followed by phenol extraction and ethanol precipitation. ChIP was performed using the following antibodies: NFATc1 (Santa Cruz, Dallas, TX, USA); C-Jun (Santa Cruz, Dallas, TX, USA); NF-κB p65 (Santa Cruz, Dallas, TX, USA); and Pol II (CTD4H8) (Santa Cruz, Dallas, TX, USA).

Precipitated DNA samples were dissolved in 100μL of distilled water, and 2μL of the sample was used in real-time PCR amplification using SYBR green PCR master mix (Bio-Rad). As described earlier,[55] No-antibody controls were subtracted from each sample value to remove the non-specific background signal. The promoter and Nuc-1 HIV-1 primer sets were used in real-time PCR amplification (Table S1).

RNA extraction and real-time qPCR

Total cellular RNA from the cells and viral RNA from cultured supernatant were extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) respectively, following the manufacturer protocol. Using isolated RNA as a template, cDNA was synthesized using the M-MLV Reverse Transcriptase (Thermo Scientific, Waltham, MA). cDNA was subjected to real-time PCR using the Real-Time PCR system 7500TH (Life Technologies, Carlsbad, CA, USA). For all samples from cells, Actin/GAPDH was measured as internal control and used for data normalization.

Luciferase assay

5×10^5 Jurkat-pHR′-P-Luc cells were plated in 12-well plates with RPMI supplemented with 10% FBS, penicillin, and streptavidin. The cells were incubated with 10μM cocaine and then the inhibitors for 48 h before being harvested and washed twice with PBS. Luciferase levels in the cells were assessed using a Luciferase Assay System kit (Promega, Madison, WI, USA) as described earlier.[22] Briefly, the cells were collected and lysed for 30 min at RT with 1X passive lysis buffer. 10μL of each sample was added to individual wells, followed by 50μL of luciferase substrate. Each sample was tested in triplicate. Luminescence was read in a Veritas Microplate Luminometer (Turner Biosystems) with atleast three biological repeats.

MTS-PMS cell proliferation assay

Cells were seeded at a concentration of 2×10^4 or 2×10^5 cells per well in 96 well plates with a total media volume of 100μL. Cells were untreated/treated with cocaine for 3 h, 12 h, 24 h, 48 h, and 72 h. Later, 20μL per well of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added and incubated for 4 h in a humidified, 5% CO2 atmosphere on the plate at 37°C. Absorbance was recorded at 490 nm using a 96-well plate reader.

Cell surface marker staining with CD25, CD38, CD69, and HLA-DR

Cells were treated with 5 and 10μM cocaine for 3 h and untreated as a control. Cells were harvested at 300 g for 6 min and washed with FACS buffer (1XPBS, 1% FBS, NaN3 0.04%, and 10 mM HEPES) twice. Cells were incubated with CD25, CD38, CD69, and HLA-DR antibodies (1: 100 dilution each) for 40 min. The unbound antibodies were then removed by washing twice with FACS buffer. Similar protocol was also followed to study the T cell exhaustion markers. The analyses were done with a FACS Calibur (BD Biosciences), using FlowJo software (Treestar Inc.).

Intracellular staining with Ki67 and HIV p24

The cell was treated with cocaine in a time-dependent manner for 3 h, 24 h, and 48 h and untreated as a control. For p24 staining, cells were infected for 24–48 h after 3 h of exposure to cocaine. Cells were harvested at 300 g for 6 min, and we fixed the cells with 4% paraformaldehyde for 15 min. The cell was then washed with FACS buffer (1XPBS, 1% FBS, NaN_3 0.04%, and 10 mM HEPES) twice and permeabilized for 1 h with cell permeability buffer (1XPBS, 1% BSA, and 0.5% Triton X-100). After washing twice with cell permeability buffer, the cell was incubated with Ki67 antibody for 40 min. The unbound antibodies were then removed by washing. The analyses were then done with a FACS Calibur (BD Biosciences), using FlowJo software (Treestar Inc.).

Flow cytometry (FACs) analysis

FACS analyses were performed on 2D10 cells (Jurkat cells which were infected with VSV-G pseudotyped HIV-1 virus carrying the GFP gene under the control of the HIV-1 LTR promoter) to determine the impact of cocaine on the reactivation of latent HIV. The analyses were done with a FACS Calibur (BD Biosciences), using FlowJo software (Treestar Inc.).

QUANTIFICATION AND STATISTICAL ANALYSIS

Data are expressed as the mean ± SEM and mean ± SD. Comparisons between two groups were performed by unpaired Student's *t* test. Comparisons among more than two groups were carried out using one-way or two-way ANOVA followed by Dunnett's multiple comparisons test. If the p value obtained from unpaired t test or ANOVA was under 0.05 ($p < 0.05$), then it was considered statistically significant. Asterisks over the bar indicate significant differences. *$p < 0.05$. All statistics were calculated using a GraphPad prism (Ver 9.4.0). All statistical details of the experiments can be found in the figure legends.