Extracellular Vesicles activation of latent HIV-1 is driven by EV-associated c-Src and cellular SRC-1 via the PI3K/AKT/mTOR pathway

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Abstract

HIV-1 is the causative agent of AIDS, infecting nearly 37 million people worldwide. Currently, no cure exists, mainly due to HIV-1's ability to enact latency. Our previous work has shown that exosomes, small extracellular vesicles (EVs), from uninfected cells can activate HIV-1 in latent cells, leading to increased mostly short and some long HIV-1 RNA transcripts. This is consistent with the notion that none of the FDA-approved antiretroviral drugs used today in the clinic are transcription inhibitors. Furthermore, these HIV-1 transcripts can be packaged into EVs and released from the infected cell. In this study, we investigated the specific mechanism behind the activation of latent HIV-1 by EVs. We observed that the EV-associated kinase c-Src is able to activate latent HIV-1 in infected cells via the PI3K/AKT/mTOR pathway and SRC-1/p300-driven chromatin remodeling. We discovered that upon inhibiting each of the proteins involved in the PI3K/AKT/mTOR pathway, HIV-1 transcription, as well as levels of HIV-1 Gag p24 in the cell supernatant, was decreased. Collectively, our data suggests that the EV activation of latent HIV-1 is initially started by EV-associated c-Src being delivered into a recipient cell, where it is able to activate the PI3K/AKT/mTOR pathway, eventually leading to the activation and translocation of SRC-1 to the nucleus, promoting a pro-transcription state.

Introduction

- There is no cure for HIV-1. However, through the use of Combination Antiretroviral Therapy (cART), viral replication or active infection is inhibited. This forces the cell into a state of clinical latency where no infectious viral particles is observed in the blood.
- Current FDA-approved cART regimens lack a transcriptional inhibitor, focusing only on interfering with HIV-1 at various points in the viral life cycle.
- Recently, the lack of transcriptional latency has been called to the forefront where viral RNA has been found in various regions in the body of HIV patients under cART.
- One explanation behind this could be through the workings of EVs. A study done by our lab discovered that EVs from uninfected cells resulted in the activation of latent HIV-1 in latent infected cells under cART.
- The purpose of this study is to elucidate a mechanism by which EVs activate latent HIV-1.
- EVs are small vesicles (30-120 nm) released from numerous cells types and have been shown to contain nucleic acids, proteins, and lipids. EVs are involved in essential cellular processes such as intercellular communication and immune functions. They have also been linked to various disease pathogenesis and increased susceptibility to infection by eliciting changes in recipient cells.

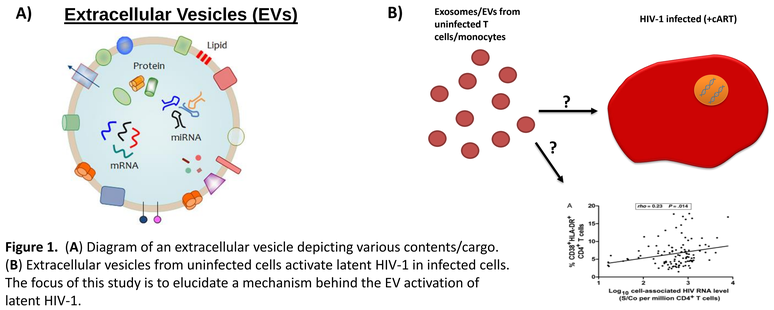


Figure 1. (**A**) Diagram of an extracellular vesicle depicting various contents/cargo. (**B**) Extracellular vesicles from uninfected cells activate latent HIV-1 in infected cells. The focus of this study is to elucidate a mechanism behind the EV activation of latent HIV-1.

Results

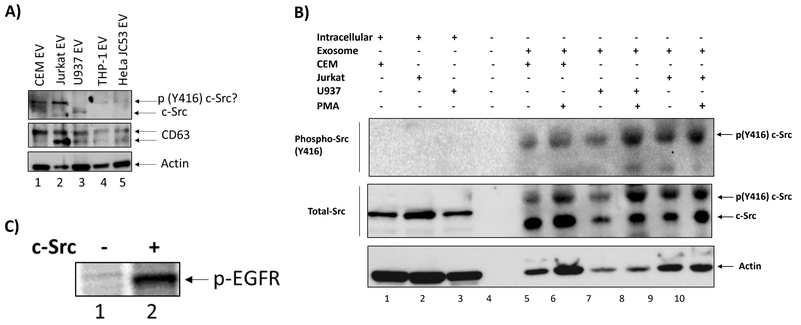


Figure 2. Activated c-Src is present in EVs. (**A**) Cell supernatant from CEM, Jurkat, U937, THP-1, and HeLa JC53 cells was collected and treated with nanotraps (NT80/82) prior to rotating for 72 hours at 4° C to concentrate EVs. EVs were then Western blotted for c-Src and CD63 (EV marker). Actin was used as a loading control. (**B**) Cells were treated with 100 nM PMA and allowed to incubate for five days at 37ºC with a second PMA treatment on Day 3. EVs were then concentrated using NT80/82 and rotated overnight at 4 °C. Samples were Western blotted for c-Src and actin. Whole-cell extracts (WCEs) were used as positive control for Western blots. (**C**) c-Src phosphorylates Epidermal Growth Factor Receptor (EGFR). ACH2 WCEs were immunoprecipitated (IP) overnight with antibody against c-Src. Complexes were then precipitated with Protein A/G agarose beads for 1 hour at 4 °C. IPs were washed twice with TNE buffer and kinase buffer prior to incubation with γ-32P ATP. The IPs were then used for *in vitro* kinase assays using EGFR as a substrate.

Results

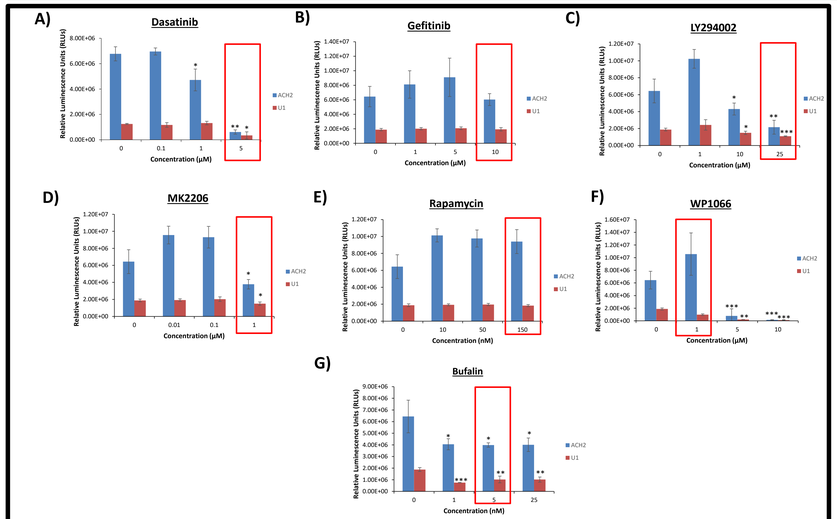


Figure 3. **Inhibitor titration of latent HIV-1-infected cells.** 5X10^4 cells were plated with different concentrations of kinase inhibitors and allowed to incubate for 48 hours prior to a Cell-Titer Glo assay. (**A**) ACH2 and U1 cells treated with dasatinib (c-Src inhibitor) at 0, 0.1, 1, or 5 μM. (**B**) ACH2 and U1 cells treated with gefitinib (EGFR inhibitor) at 0, 1, 5, or 10 μM. (**C**) ACH2 and U1 cells treated with LY294002 (PI3K inhibitor) at 0, 1, 10, or 25 μM. (**D**) ACH2 and U1 cells treated with MK2206 (AKT-1 inhibitor) at 0, 0.01, 0.1, or 1 μM. (**E**) ACH2 and U1 cells treated with rapamycin (mTOR inhibitor) at 0, 10, 50, or 150 nM. (**F**) ACH2 and U1 cells treated with WP1066 (STAT-3 inhibitor) at 0, 1, 5, or 10 μM. (**G**) ACH2 and U1 cells treated with bufalin (SRC-1 inhibitor) at 0, 1, 5, or 25 nM. Red boxes signify concentrations utilized in subsequent experiments. Student's T-test compared untreated cells with cells treated with drugs. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.D.

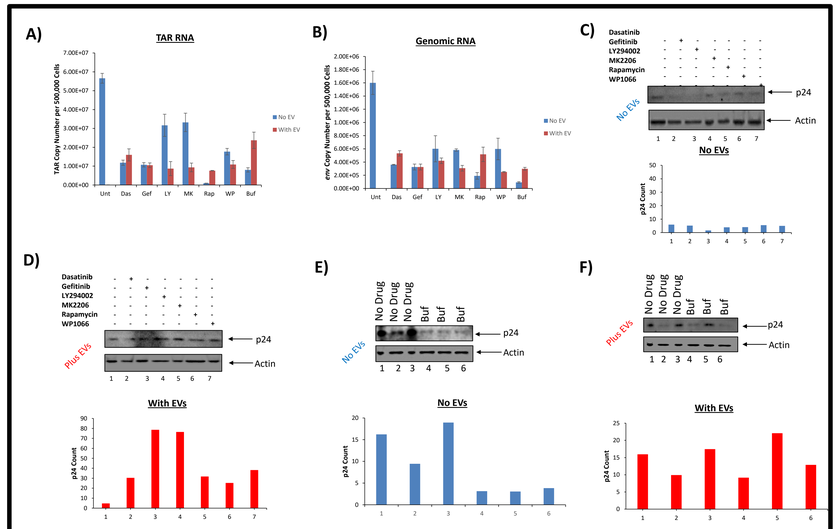


Figure 4: Effect of EVs on the activation of HIV-1 in the presence of various kinase inhibitors. U1 cell were plated and treated with 5 μM dasatinib, 10 μM gefitinib, 10 μM LY294002, 1 μM MK2206-2 HCl, 150 nM rapamycin, or 1 μM WP-1066 and allowed to incubate for 48 hours. A second drug treatment was then performed followed by a 2 hour incubation and a CEM EV treatment (1 cell:10,000 EVs). A second EV treatment was performed after a 24 hour incubation period. Cells were then harvested and the cell supernatant collected and rotated overnight at 4ºC with NT86. Total RNA was isolated and subjected to RT-qPCR for HIV-1 TAR RNA (**A**) and genomic RNA (**B**). (**C-D**) NT86-treated samples were Western blotted for HIV-1 Gag p24. (**E-F**) U1 cells were plated and treated with 5 nM bufalin and allowed to incubate for 48 hours, followed by a second drug treatment. CEM EVs were added once a day for 2 days. Samples were then Western blotted for HIV-1 Gag p24. For all figures, EV untreated samples were used as a negative control.

Results

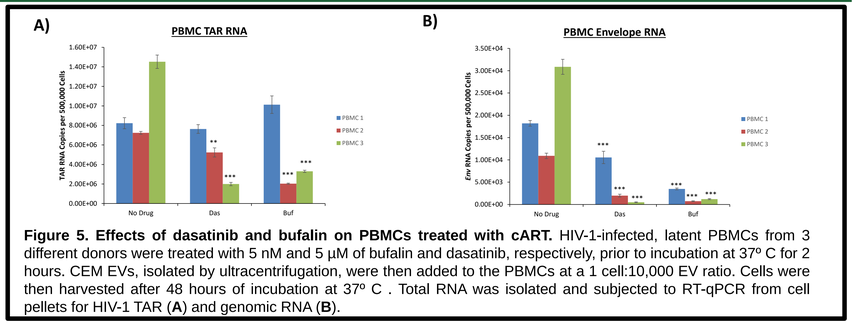


Figure 5. Effects of dasatinib and bufalin on PBMCs treated with cART. HIV-1-infected, latent PBMCs from 3 different donors were treated with 5 nM and 5 μM of bufalin and dasatinib, respectively, prior to incubation at 37º C for 2 hours. CEM EVs, isolated by ultracentrifugation, were then added to the PBMCs at a 1 cell:10,000 EV ratio. Cells were then harvested after 48 hours of incubation at 37º C . Total RNA was isolated and subjected to RT-qPCR from cell pellets for HIV-1 TAR (**A**) and genomic RNA (**B**).

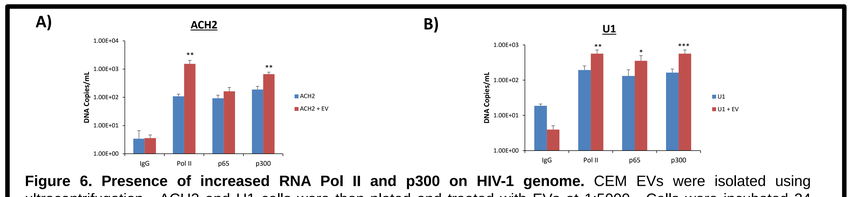
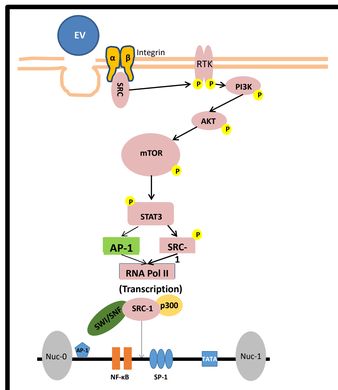


Figure 6. Presence of increased RNA Pol II and p300 on HIV-1 genome. CEM EVs were isolated using ultracentrifugation. ACH2 and U1 cells were then plated and treated with EVs at 1:5000. Cells were incubated 24 hours and again treated with CEM EVs at 1:5000. Following a second 24 hour incubation, cells were harvested and crosslinked prior to ChIP assay using antibodies against IgG, Pol II, p65, and p300. DNA was then quantified using PCR using NF-κB1-2F and TAR +59-R. IgG was used as a background control. Student T-test was used to compare untreated control and EV-treated samples. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.D.

Conclusion



- **EVs from uninfected cells contain activated c-Src (phosphorylated at tyrosine-416) that can activate a signaling cascade involving EGFR, the PI3K/AKT-1/mTOR pathway, Stat3, and SRC-1**
- **SRC-1 can then translocate to the nucleus, recruiting p300 to the HIV-1 promoter, which can then drive chromatin remodeling**
- **This would enable a more open chromatin state, promoting increased loading of RNA Pol II onto the HIV-1 promoter and driving activation of latent HIV-1**
- **EV-associated c-Src is important in driving EV activation of latent HIV-1. Dasatinib (c-Src inhibitor) and WP1066 (Stat3 inhibitor) can specifically target infected cells and could help mitigate this process**

Figure 7. Proposed model for EV activation of latent HIV-1. Uninfected EVs are taken up by HIV-1 infected cells, leading to the phosphorylation and activation of RTK by c-Src. RTK then activates PI3K, which in turn activates AKT-1. Following activation, AKT-1 phosphorylates mTOR resulting in the phosphorylation and activation of the transcription inducer, STAT3. STAT3 then recruits the cofactor SRC-1 before translocating to the nucleus and promoting HIV-1 transcription by recruiting p300. NF-κB and RNA Pol II increase loading onto the HIV-1 promoter.

References

Barclay, R. A. et al. Exosomes from uninfected cells activate transcription of latent HIV-1. J. Biol. Chem. 292, 14764 (2017).
Hatano, H. et al. Cell-based measures of viral persistence are associated with immune activation and programmed cell death protein 1 (PD-1)-expressing CD4+ T cells. J. Infect. Dis. 208, 50–56 (2013).
Kumar, A., Abbas, W. & Herbein, G. HIV-1 Latency in Monocytes/Macrophages. Viruses 6, 1837–1860 (2014).
Sampey, G. C. et al. Exosomes from HIV-1-infected Cells Stimulate Production of Pro-inflammatory Cytokines through Trans-activating Response (TAR) RNA. J. Biol. Chem. 291, 1251–1266 (2016).
Narayanan, A. et al. Exosomes derived from HIV-1-infected cells contain trans-activation response element RNA. J. Biol. Chem. 288, 20014–20033 (2013).

Acknowledgements

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