EXOSOMES FROM UNINFECTED CELLS CAUSE TRANSCRIPTIONAL ACTIVATION OF LATENT HIV-1 VIRUS IN INFECTED IMMUNE CELLS

by

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Exosomes from Uninfected Cells Cause Transcriptional Activation of Latent HIV-1 Virus in Infected Immune Cells

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

By

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Bachelor of Science
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DEDICATION

I dedicate this work to my grandmother, Rose Plowman, who, though she is no longer with us, inspired me to pursue a career of science and research.

ACKNOWLEDGEMENTS

I'd like to thank my mom, dad, my brother, Aaron, and the rest of my family for believing in me and being supportive throughout the thesis process. I'd also like to thank my PI, Dr. Fatah Kashanchi, as well as fellow lab members, Dr. Sergey Iordanskiy, Dr. Gavin Sampey, and Angela Schwab, for their invaluable help throughout this process.

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LIST OF ABBREVIATIONS AND SYMBOLS

Human Immunodeficiency Virus……HIV
Acquired Immunodeficiency Syndrome……AIDS
Ribonucleic Acid……....RNA
Deoxyribonucleic Acid……...DNA
Rev Response Element……...RRE
Trans-Activating Response Element……..TAR
Messenger RNA...…….mRNA
Micro RNA…...……miRNA
Human T-cell Lymphotropic Virus……HTLV
Epstein-Barr Virus……..EBV
Toll-like Receptor…….....TLR
Carbon Dioxide……..CO_2
Degrees Centigrade……ºC
Fetal Bovine Serum……FBS
Milliliters……mL
Acetylcholine Esterase……...AchE
Combination Antiretroviral Therapy……..cART
Reverse Transcriptase……RT
Quantified Polymerase Chain Reaction……..qPCR
Nucleoside Reverse Transcriptase Inhibitor……..NRTI
Micromolar……µM
Nanometers……nm
Water……..H_2O
Chromatin Immunoprecipitation……ChIP
Exosome……Exo
Figure……Fig.
RNA Polymerase II……....Pol II

ABSTRACT

EXOSOMES FROM UNINFECTED CELLS CAUSE TRANSCRIPTIONAL ACTIVATION OF LATENT HIV-1 VIRUS IN INFECTED IMMUNE CELLS

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George Mason University, 2016

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HIV-1 infection causes AIDS, a significant problem in today's world. It can be combatted through the use of combination antiretroviral therapy, which can lower viral load to undetectable levels. However, because HIV-1 is able to integrate its genome with the host cell's, achieving latency, there is no effective way to target the viral reservoir. In previous studies, it has been shown that there is a link between HIV-1 and exosomes. Specifically, exosomes were shown to transport viral proteins and RNA from infected cells to neighboring uninfected cells. These viral products could then suppress the PKR pathway, leading to increased pathogenesis. In this study, exosomes from uninfected cells were observed to increase short and long-length viral transcripts within wild-type HIV-infected cells. This effect was again observed when infected cells were under cART. An investigation into a possible mechanism for this phenomenon revealed that the exosomes potentially cause an increase in RNA Polymerase II activity within the

infected cells. Collectively, these results imply that exosomes from uninfected cells cause an activation of HIV-1 from latency in infected cells.

CHAPTER 1: INTRODUCTION

HIV

Human Immunodeficiency Virus (HIV) is the causative agent of AIDS (Acquired Immunodeficiency Syndrome), a significant problem in today's society. According to the World Health Organization, approximately 37 million people at the end of 2014 lived with HIV/AIDS (15). There is no cure for this virus; neither is there a vaccine (13). Therefore, it is imperative that the virus, especially mechanisms related to pathogenesis, is studied so that further efforts to defeat the disease can be launched and bear fruit.

HIV has a relatively small genome, made up of nine genes: the structural genes *gag*, *pol*, and *env*, the regulatory genes *rev* and *tat*, and the accessory genes *vif, vpr, vpu*, and *nef*. Of the structural genes, the *gag* gene codes for a polyprotein made of four subunits (p6, p7, p17, and p24) which are eventually cleaved by HIV protease and take part in assembling the interior of new virions (13). The *pol* gene codes for proteins HIV needs to replicate and integrate itself into the host genome; these proteins include reverse transcriptase, integrase, and protease (this is the viral-coded protease that cleaves the *gag* precursor polyprotein) (13). The *env* gene codes for HIV's envelope proteins, gp120 and gp41 (13). Structural proteins of note are gp120, which is needed to bind to CD4, the host receptor needed to initiate HIV infection, reverse transcriptase, which converts

HIV's RNA into DNA, and integrase, which integrates the converted DNA into the host genome (13).

The two regulatory genes of HIV, *rev* and *tat*, code for Rev and Tat, respectively. The rev protein, which operates in the nucleus, is most well-known for binding to viral RNAs containing the RRE (Rev Response Element), a specific sequence found in freshly transcribed viral RNA. When bound to Rev, viral RNAs are able to be exported from the nucleus to the cytoplasm for translation (13). The Tat protein can also operate in the host cell's nucleus, where it binds to the 5' end of newly made HIV RNA transcripts at a specific double stranded stem-and-loop structure called TAR (Trans-Activation Response Element). This binding of Tat to TAR allows viral transcription to proceed from initiation to elongation by recruiting the host factor p-TEFb to the site of transcription, thus allowing full length viral transcripts to be produced (13).

The accessory genes, *vif*, *vpr*, *vpu*, and *nef*, code for Vif, Vpr, Vpu, and Nef proteins, respectively. Vif inhibits the host anti-viral protein APOBEC3G, which allows for viral replication, while Vpr plays a role in the import of HIV's reverse transcribed DNA from the cytoplasm to the nucleus (13). Vpu protein enhances viral release (13). Nef has a number of functions which contribute to pathogenesis, but one of the more important functions is the downregulation of CD4 on the surface of infected cells, thus decreasing the likelihood of a T cell response against the infected cell (13).

Interestingly, some of these viral proteins, along with viral RNAs, can be packaged into vesicles and transported to neighboring cells. For example, HIV Nef

protein and TAR RNA have previously been shown to travel from infected cells to uninfected cells thru these vesicles (2). These vesicles are termed "exosomes".

Exosomes

Exosomes are very small, on the order of 30-100 nm in diameter, and come from late endosomal compartments which bud off the plasma membrane (6). They can carry a variety of macromolecules, including lipids, proteins, and nucleic acids (such as mRNA and miRNA), and it has been shown in previous studies that exosomes can bind to multiple target cells and elicit a response in said cells (6). Furthermore, exosomes have been shown to participate in cancer progression, viral release, host immune responses, and can even carry prions (7).

Exosomes are produced by a number of cell types throughout the body. T cells and monocytes, both immune cells, produce exosomes, as do B cells (6, 8). A 2006 study on rat cortical culture isolated L1 protein from exosomes, which is found only in neurons (7). Other studies have shown that stem cells and even cancer cells produce exosomes (5). Regardless of where they come from, all exosomes carry unique molecular markers, which allow for isolation and purification. Some examples of these markers are CD63 (a tetraspannin), Hsp90 (a heat shock protein), and alix (a protein involved in multivesicular body biogenesis) (8).

These vesicles can be isolated in cell culture. Through a combination of ultracentrifugation and passaging through a 0.22 μm filter, it is possible to separate exosomes from cells and other types of vesicles (14). Purification of exosomes can be

done through the use of an iodixanol or sucrose gradient (14). What results is two to three distinct fractions, one containing exosomes, a second containing virus (if the cells are infected), and a third containing histones (6).

Exosomes and Viruses

In previous studies, a relationship between viral infection of host cells and exosome production and packaging has been established (2, 10). Both RNA viruses and DNA viruses have been shown to participate in this relationship (2). Examples of RNA viruses are HIV and Human T-cell Lymphotropic Virus (HTLV). An example of a DNA virus is Epstein-Barr Virus (EBV).

HIV, HTLV, and EBV all have the ability to form a provirus within a host cell (13). Once this has occurred, the virus can produce viral proteins, which can then be packaged and exported out of the cell via exosomes. When cells are infected with HIV, p24, Nef, and TAR RNA have been reported in exosomes secreted from the infected cell (2). LMP1 and LMP2A, EBV viral proteins, have been found in exosomes secreted from EBV infected cells (2). Furthermore, Tax, a HTLV protein implicated in oncogenic activity, has been found in exosomes from HTLV infected cells (3). Even viruses that do not form a provirus within the host can utilize exosomes for packaging and transport of viral materials. Hepatitis C Virus has been demonstrated to transport entire virions from infected liver cells to uninfected liver cells, leading to productive infection in the uninfected cells (20).

The transport of the aforementioned viral material in exosomes causes enhanced infectivity and increased pathogenesis in target cells (2). Exosomes originating from infected HIV-1 cells can elicit neighboring infected, resting T cells to replicate HIV-1 through a combination of Nef and ADAM17 (1). Exosomes containing the EBV protein LMP1 cause immunosuppressive activity by restricting T cell proliferation (2). Tax can modulate NF-κB, a protein that enhances cytokine production, and interfere with DNA repair, leading to DNA mutation and the possibility of cancer (4). However, there can be some positive effects from viral transport of proteins and/or nucleic acid by exosomes. HCV RNA released into healthy dendritic cells was shown to enhance production of Interferon α (2). In another study, viral RNA from HIV-1 infected cells allowed for TLR activation and production of NF-κB (10).

Clinical Latency

Some viruses, such as HIV, HTLV, and EBV, can undergo a phenomenon known as clinical latency, which is defined as an infectious state in which no infectious particles are produced (9). In the case of HIV, there are several known mechanisms behind latency: sequestration of host transcription factors (such as NF-κB) in the cytoplasm, epigenetic silencing (by methylating the DNA), transcriptional interference, sequestration of p-TEFb, and antagonistic competition on p-TEFb between HIV's Tat protein and the host's BRD4 protein (12).

It is because of latency that treating HIV and other proviral infections is so difficult. Since the virus is latent, it goes undetected by the host's immune system, thus

causing a persistent reservoir that prevents the clearance of the infection (13). However, latency can be reversed by certain stimuli, which then allows for full viral production (9). This is what leads to AIDS in the case of HIV patients.

As previous studies show (1, 2, 3, 4, 10), exosomes from infected cells lead to a response in uninfected recipient cells; based on this, we hypothesized that exosomes from uninfected cells lead to a response in neighboring infected cells and, furthermore, that they are, in fact, a causative agent of viral activation from latency. To investigate this, we will isolate exosomes from uninfected cells, treat infected cells with the exosomes, and examine whether there is any change at all in viral transcription.

CHAPTER 2: MATERIALS AND METHODS

Cells

CEM cells (uninfected T cell), Jurkat cells (uninfected T cells), Jurkat E4 cells (HIV infected T cells), ACH2 cells (HIV infected T cells), U937 cells (uninfected promonocytic cells), U1 cells (HIV infected monocytes), and OM10.1 cells (HIV infected myeloid-derived cells) were grown in RPMI complete media at 37º C and 5.0% CO_2. Bovine exosomes were excluded from culture media by ultracentrifugation of fetal bovine serum (FBS) at 100,000 X *g* for seventy minutes prior to growth of cells for exosome isolation.

Reagents and Antibodies

Complete culture media consisted of RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% streptomycin/penicillin. Antibodies used for ChIP assay were IgG (sc2027), RNA Polymerase II (sc899), and p65 (ab7970). Antibodies used for Western blot were p24 (NIH AIDS Reagent, Cat. # 4121) and actin (ab49900).

Exosome Isolation

CEM, Jurkat, and U937 cells were grown in appropriate media supplemented with 10% exosome-free FBS. Exosome preparation was made with 100 mL of cells culture supernatants (produced from a culture of one million cells per mL for 5 days). Cells were pelleted by centrifugation at 300 X *g* for 10 minutes. An additional centrifugation at 2000 X *g* for 10 minutes was used to pellet dead cells. The supernatant was then filtered through a 0.22 µm filter and ultracentrifuged at 10,000 X *g* for 30 minutes to remove any cell debris. This was followed by two ultracentrifugations at 100,000 X *g* for 70 minutes to pellet the exosomes. The resulting pellet was resuspended in 100 µL of Dulbecco's Phosphate Buffered Saline (PBS). All spins were performed at 4º C. Protein levels of the exosomes were determined using AchE assay.

Exosome Titration

Exosomes were added in a dose-dependent manner to HIV-infected cells and HIV-infected cells that were pre-treated with cART. The exosomes were added once per day for three days. Then, the cells were allowed to incubate for 48 hours, after which they were harvested, spun down into pellets, and washed in preparation for RT-qPCR.

cART Treatment

ACH2, U1, and OM10.1 cells were pre-treated with cART for three days prior to exosome addition. Indinavir (protease inhibitor), lamivudine and emtricitabine (NRTIs), and tenofovir (NtRTI), and were added together in an equal ratio to form a cocktail. The

cocktail was then added to the cells in a way that allowed for each drug to be present at 10 μM within the cells. After three days and addition of exosomes, the cells were treated again with the cocktail. Three days after that, the cells were again treated with the cocktail.

RNA Isolation and RT-qPCR

RNA was isolated from cells treated with Jurkat or U937 exosomes respectively for five days and was extracted using TRI Reagent-LS (MRC) according to the manufacturer's protocol. A cDNA library was then created with the GoScript reverse transcription system using TAR-specific reverse primer TAR+59-R (5'-CAACAGACGGGCACACACTAC-3') and 5T25 (5'-TTTTTTTTTTTTTTTTTTTTTTTTTTGAAG-3'). qPCR was then performed using an appropriate amount of RT aliquot mixes containing iQ Supermix (BioRad) with the following primers: TARfll-F (5'-GGTCTCTCTGGTTAGACC-3'), TAR+59-R, Gag 1 (5'-TCAGCCCAGAAGTAATACCCATGT-3'), and Gag 2 (5'-CACTGTGTTTAGCATGGTGTTT-3').

GFP Assay

Jurkat E4 cells treated with CEM, Jurkat, or U937-derived exosomes for five days were measured using a fluorescence assay (Promega GloMax Multidetection System) with a wavelength of 425 nm.

AchE Assay

The Amplex® Acetylcholine/Acetylcholine Esterase Activity Assay Kit (Thermo A12217) was used to quantify exosomes by following the manufacturer's instructions. Briefly, a negative control of 1x running buffer (20 mL of H_2O and 5 mL of 5x reaction buffer) and two positive controls, one consisting of acetylcholine esterase and one consisting of hydrogen peroxide, were made and plated on a 96-well plate. Exosomes were treated as per the manufacturer's instructions and plated on the 96-well plate as well. Acetylcholine esterase activity was measured every fifteen minutes for one hour to find optimal activity.

SDS-Page and Western Blot Analysis

Cell extracts were resolved by SDS-PAGE on 4-20% Tris-glycine gels (Novex). Gels were stained using Coomassie Blue stain by standard procedure. For Western blot analysis, proteins were transferred to Immobilon membranes (Millipore) at 50 mA overnight for approximately 16 hours. Membranes were blocked with Dulbecco's PBS + 0.1% Tween 20 + 5% dry milk for 30 minutes at 4º C. Primary antibodies against specified proteins were incubated with the membranes overnight at 4º C. Membranes were washed twice with PBS + 0.1% Tween 20 and incubated with HRP-conjugated secondary antibody for two hours at 4º C. Membranes were washed two times with PBS + 0.1% Tween 20 and once with PBS prior to imaging. HRP luminescence was elicited with Super Signal West Dura ExtendedDuration Substrate (Pierce) and visualized by a Molecular Imager ChemiDoc XRS system (Bio-Rad).

ChIP Assay

Cells were harvested and their DNA and protein were crosslinked following the instructions of the Imprint Chromatin Immunoprecipitation Kit (Sigma). Samples were then sonicated, again following the instructions found in the Kit. Antibodies of interest were then added, and the samples were allowed to rotate overnight at 4º C. A 50% (v/v) protein A-Sepharose/protein G-Sepharose mix was added and allowed to rotate for two hours at 4º C. The samples were washed twice with IP Wash Buffer (Sigma) before addition of Proteinase K (Sigma). After a 15 minute incubation at 65º C, reversing solution (Sigma) was added and the samples incubated at 65º C for 90 minutes. DNA was purified through elution, following the Imprint Chromatin Immunoprecipitation Kit manufacturer's instructions. PCR was performed using NF-κB1-2F (5'-TTCCGCTGGGGACTTTCC-3') and TAR+59-R.

CHAPTER 3: RESULTS

Effect of uninfected exosomes on HIV-infected cells. In a previous study, the effect of exosomes derived from HIV-infected cells on uninfected recipient cells showed an inhibition of the PKR/eIF2α pathway as well as activation of the TLRs leading to activation of the NF-κB pathway (10). Here we asked whether exosomes from uninfected cells could potentially activate HIV transcription in infected cells. Our rationale for these experiments was that HIV latent cells may still allow either short or long transcripts to be made in the presence of stimulatory molecules present in exosomes. Exosomes are abundantly present in various bodily fluids and may contain different cytokines and transcription factors as well as kinases that could potentially activate transcription (3, 19). Therefore, we isolated and purified exosomes from uninfected T cells and monocytes prior to addition on HIV-infected indicator cells. Specifically, we added CEM, Jurkat, and U937-derived exosomes to HIV-infected Jurkat E4 cells, a T cell line lacking the *gag* gene and GFP inserted into the *nef* gene. Our read out assays were for presence of TAR RNA (short double stranded transcript; ~23bp) as well as GFP (long transcript) in the cells.

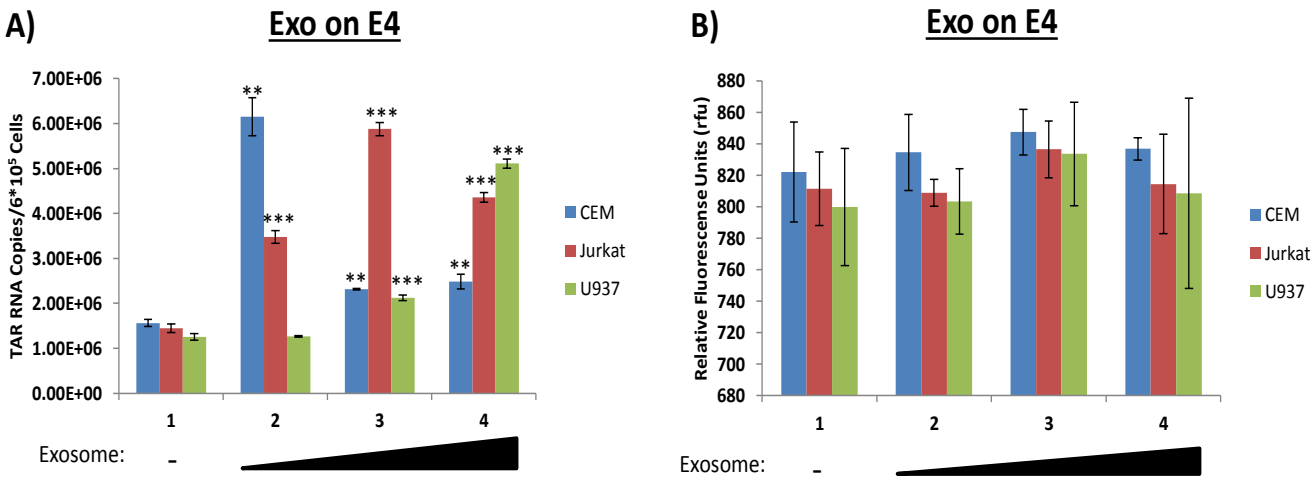

Figure 1: Uninfected exosomes on latent HIV-infected cells cause short RNA transcription but not long RNA transcription. A) Exosomes from CEM, Jurkat, and U937 cells were isolated using ultracentrifugation before being added to Jurkat E4 cells in 0 U/mL, 0.24 U/mL, 0.78 U/mL, and 2.4 U/mL increments (determined using AchE assay) once per day over the course of three days. The E4s were incubated for an additional 48 hours before they were harvested, and total RNA was isolated and subjected to RT with a TAR-specific primer. RT-qPCR was performed to quantify the total amount of TAR RNA copies. **B)** Exosomes from CEM, Jurkat, and U937 cells were isolated using ultracentrifugation before being added to Jurkat E4 cells in 0 U/mL, 0.6 U/mL, 1.2 U/mL, and 1.8 U/mL (determined using AchE assay) increments. The cells then incubated for an additional 48 hours before a GFP assay was performed. In every figure *P <0.05, ** P<0.01, ***P<0.001.

Results in **Fig. 1A** indicate that all three types of exosomes increased TAR transcription at varying levels dependent on the exosome concentration. CEM exosomes led to a 5.5-fold increase in TAR transcription at low dosage. Jurkat exosomes elicited a 4-fold increase in TAR transcription with medium dosage. U937 exosomes showed the greatest levels of TAR increase (5.5-fold) with the highest dosage used. However, there was no significant increase in GFP transcription regardless of exosome type or concentration used in these experiments (**Fig. 1B**). Collectively, these data indicate that exosomes from uninfected immune cells could potentially increase short RNA transcripts in HIV-infected recipient cells.

Effect of exosomes on wild-type HIV-infected cells. Since the Jurkat E4 cell line is infected with a virus lacking the *gag* and *nef* genes, we decided to move to different cell lines infected with wild-type viruses. We speculated that the wild-type virus could behave differently when uninfected exosomes are added to infected cells. Our rationale was that there could be a difference between short and long transcript production, as wild type virus contain both *gag* and *nef*, which are necessary for subsequent viral transcription and mRNA processing. Thus, we treated U1 (infected monocyte line), ACH2 (infected T-cell line), and OM10.1 (infected pre-myeloid line) cells with purified exosomes from uninfected T cells and monocytes. Specifically, we added Jurkat and U937-derived exosomes to the infected cells at varying concentrations and performed RT-qPCR for the presence of short and long transcripts.

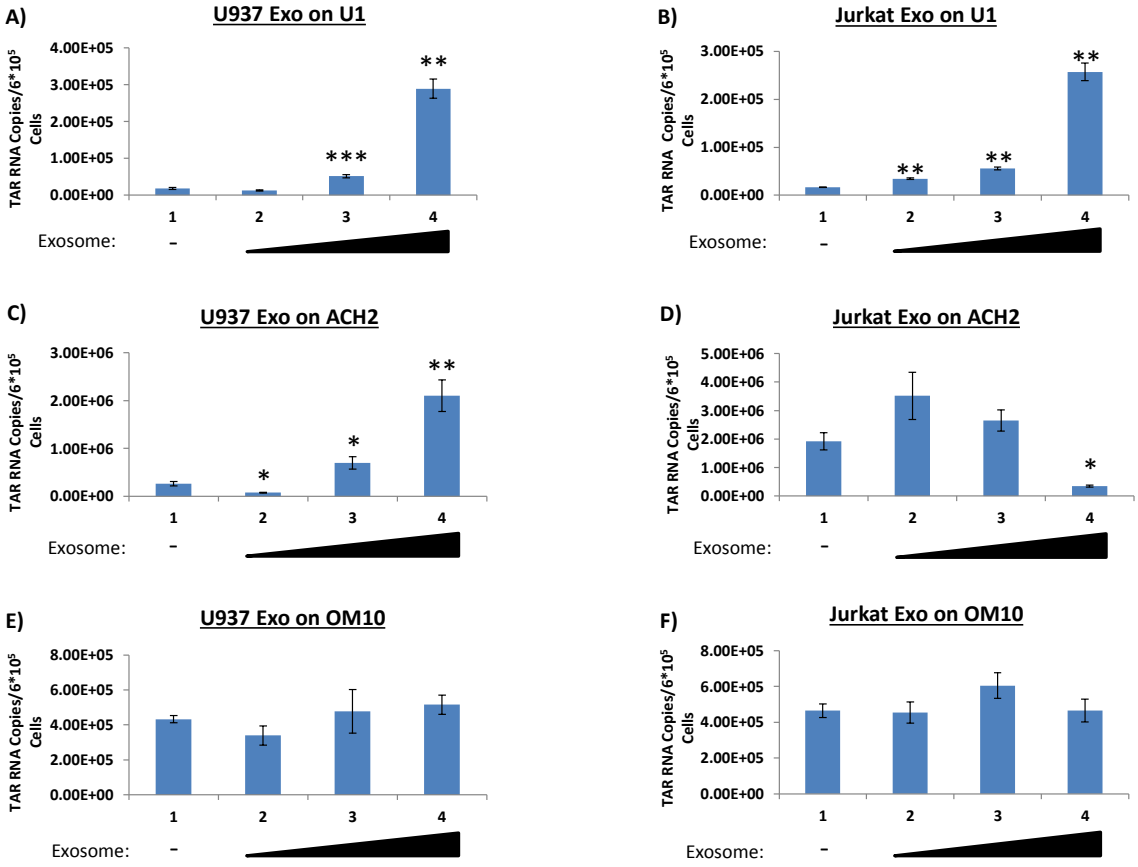

Figure 2: Short RNA transcripts observed in wild-type HIV-infected T-cells and monocytes. Exosomes from Jurkat and U937 cells were isolated using ultracentrifugation before being added to ACH2, U1, or OM10.1 cells once per day over the course of three days. The cells were then allowed to incubate for an additional 48 hours before being harvested. Total RNA was isolated, then subjected to RT with a primer specific for TAR. RT-qPCR was performed to quantify the total amount of TAR RNA copies. **A)** U1 cells were treated with 0 mU/mL, 2.79 mU/mL, 13.95 mU/mL, and 41.85 mU/mL of U937 exosomes. **B)** U1 cells were treated with 0 mU/mL, 2.90 mU/mL, 14.5 mU/mL, and 43.5 mU/mL of Jurkat exosomes. **C)** ACH2 cells were treated with 0 mU/mL, 2.42 mU/mL, 12.10 mU/mL, 36.30 mU/mL of U937 exosomes. **D)** ACH2 cells were treated with 0 mU/mL, 1.75 mU/mL, 8.75 mU/mL, and 26.25 mU/mL of Jurkat exosomes. **E)** OM10.1 cells were treated with 0 mU/mL, 2.42 mU/mL, 12.10 mU/mL, 36.30 mU/mL of U937 exosomes. **F)** OM10.1 cells were treated with 0 mU/mL, 1.75 mU/mL, 8.75 mU/mL, and 26.25 mU/mL of Jurkat exosomes. All exosome concentrations were determined by AchE assay.

Data in **Fig. 2A** and **Fig. 2B** indicate that upon addition of exosomes to U1 cells, there is a significant increase in TAR RNA transcription at the highest dosages used. Specifically, there was a 16-fold increase with the use of monocyte exosomes and approximately a 16-fold increase with the T cell exosomes. We observed a similar trend

upon addition of U937 exosomes to ACH2 cells at high dosage with approximately an 11-fold increase of TAR (**Fig. 2C**). However, when Jurkat exosomes were added to the ACH2 cells at high dosage, there was a significant decrease in the TAR levels (**Fig. 2D**). Interestingly, within the OM10.1 cells, no significant increase in TAR RNA transcription was observed regardless of exosome type or concentration used (**Fig. 2E** and **Fig. 2F**). It is important to note that OM10.1 cells are a latently HIV-infected clone of human leukemic myeloblast HL-60 as compared to U1 infected cells, which are derived from a monoblast U937 cell line.

Next, we examined whether there were any changes in the full length genomic RNA levels following treatment with uninfected exosomes. Data in **Fig. 3** indicate that upon addition of Jurkat exosomes to ACH2 cells, there was a 5-fold increase in genomic RNA while U1 cells treated with Jurkat or U937 exosomes showed an increase of about 20-fold and 31-fold of genomic RNA, respectively.

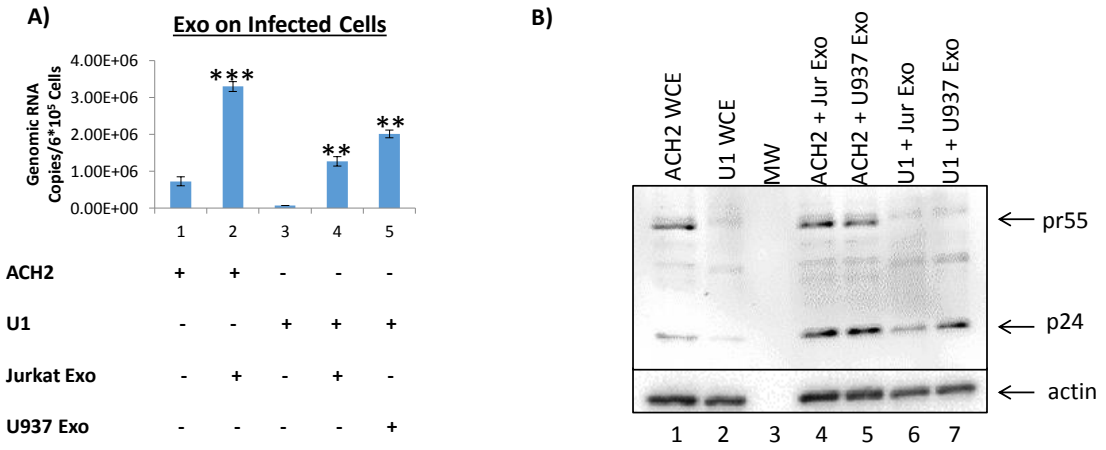

Figure 3: Uninfected exosome addition causes increase of long RNA transcripts and p24 in wild-type HIV-infected cells. A) Exosomes from Jurkat and U937 cells were isolated using ultracentrifugation before being added to ACH2 and U1 cells at concentrations of 26.25 mU/mL (Lane 2), 43.50 mU/mL (Lane 4), and 41.85 mU/mL (Lane 5) (all were determined by AchE assay). The exosomes were added once per day for three days before being incubated an additional 48 hours. The cells were then harvested; total RNA was isolated and subjected to RT with a primer specific to the 3' end of the HIV-1 genome. RT-qPCR was performed with *gag*- specific primers to quantify the levels of genomic mRNA. **B)** Exosomes from Jurkat and U937 cells were isolated using ultracentrifugation before being added to ACH2 and U1 cells at concentrations of 26.25 mU/mL (Lanes 4 and 6) and 41.85 mU/mL (Lanes 5 and 7), respectively. The exosomes were added once per day for three days before being incubated an additional 48 hours. The cells were then harvested, lysed, and the resulting lysates were run on a gel, after which a Western blot for pr55 and p24 was performed. B-actin was used as a control.

We finally asked whether an increase in genomic RNA could also result in increased pr55 or p24 levels. Data in **Fig. 3B** indicate that p24 levels were slightly increased when either of the infected cells were treated with exosomes derived from uninfected cells. However, the amount of RNA increase did not correlate to a similar increase in protein levels (in ACH2, they increased by ~10.5-11-fold while in U1, they increased by ~6-10.5-fold), indicating that most of the exosomal effects may be at the transcriptional activation stage. Collectively, these data suggest that exosomes from uninfected immune cells can activate transcription of short and long RNA transcripts in latent T-cells and monocytes and that these transcripts can be potentially translated into viral proteins.

Effect of exosomes on wild-type HIV-infected cells under cART treatment

Patients infected with HIV are often under cART therapy, treatment where HIV titers drop dramatically in blood (10). We then decided to ask whether short or long RNA transcript levels increase in recipient cells when treated with cART. Our rationale for the experiment was that patients infected with HIV are often under cART, and we asked whether exosomes could regulate transcription under such conditions. Therefore, we again treated infected cells, including U1, ACH2, and OM10.1 with purified exosomes from uninfected T-cells and monocytes. Specifically, we added Jurkat and U937-derived exosomes to the infected cells at varying concentrations and performed RT-qPCR for the presence of short and long transcripts.

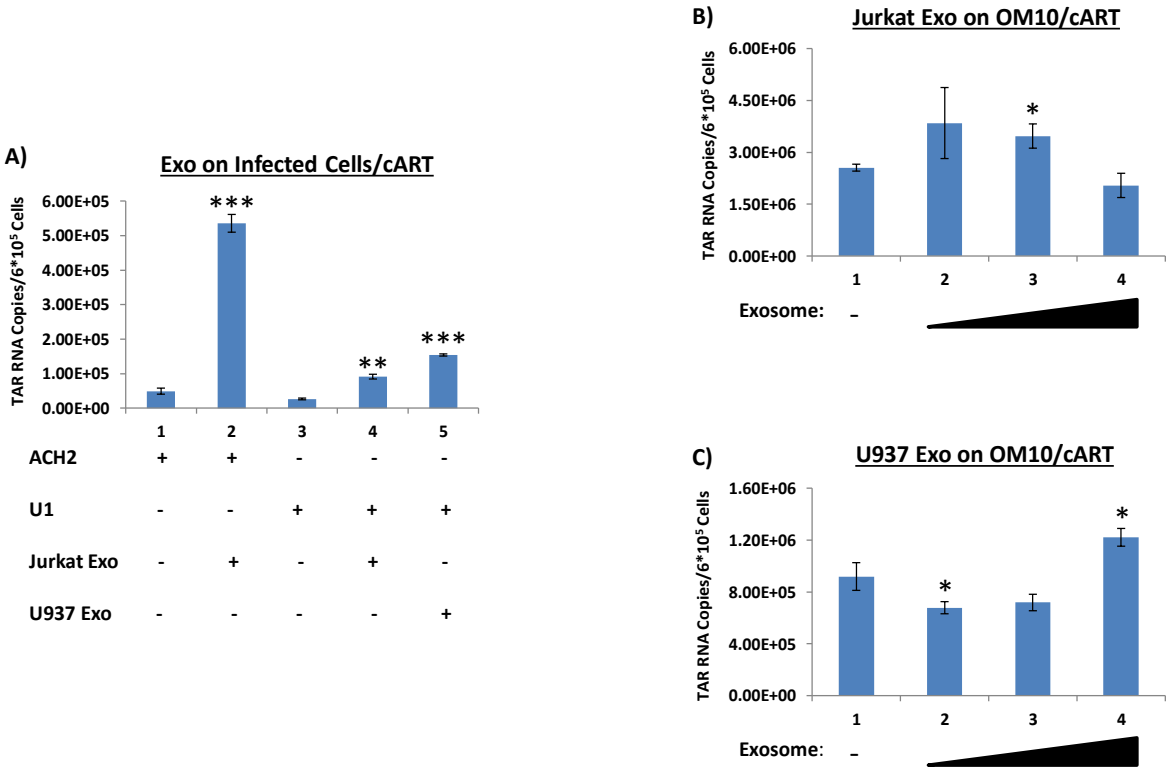

Figure 4: Short RNA transcripts observed in wild-type HIV-infected T cells and monocytes under cART conditions. Exosomes from Jurkat and U937 cells were isolated using ultracentrifugation. The aforementioned exosomes were added to either ACH2 cells or U1 cells under cART treatment. cART consisted of an equal parts cocktail of indinavir, lamivudine, tenofovir, and emtricitabine at 10 µM. **A)** The exosomes were added at concentrations of 1.60 mU/mL (Lanes 2 and 4) and 1.88 mU/mL (Lane 5) once per day over the course of three days. The cells were allowed to incubate an additional 48 hours before they were harvested and their total RNA was isolated then subjected to RT with a TAR-specific primer. RT-qPCR was performed in order to quantify the total amount of TAR RNA copies. **B)** OM10.1 cells were treated with exosomes derived from Jurkat cells. The concentration of exosomes added to each sample is as follows: 0 mU/mL (Lane 1), 0.107 mU/mL (Lane 2), 0.535 mU/mL (Lane 3), and 1.60 mU/mL (Lane 4). **C)** OM10.1 cells were treated with exosomes derived from U937 cells. The concentration of exosomes added to each sample is as follows: 0 mU/mL (Lane 1), 0.125 mU/mL (Lane 2), 0.626 mU/mL (Lane 3), and 1.88 mU/mL (Lane 4). All exosome concentrations were determined by AchE assay.

Results from **Fig. 4A** indicate that addition of Jurkat or U937 exosomes to U1 cells increased TAR RNA transcription by 4-fold and 9-fold, respectively, while addition of Jurkat exosomes to ACH2 cells increased TAR RNA transcription by 11-fold. However, addition of U937 exosomes to ACH2 cells did not result in increased short RNA transcript levels. Interestingly, data in **Fig. 4B** and **Fig. 4C** indicate that addition of

Jurkat or U937 exosomes to OM10.1 cells did not result in a dramatic change of transcription of short RNA transcripts.

We next assayed for presence of full length genomic RNA in cART-treated infected cells. Data in **Fig. 5A** indicate that upon addition of Jurkat and U937 exosomes to ACH2 cells, there was a 2-fold and 4.5-fold increase of genomic RNA, respectively. Similarly, data in **Fig. 5B** show approximately a 4-fold increase and a 5-fold increase in genomic RNA within U1 cells after addition of Jurkat and U937 exosomes, respectively.

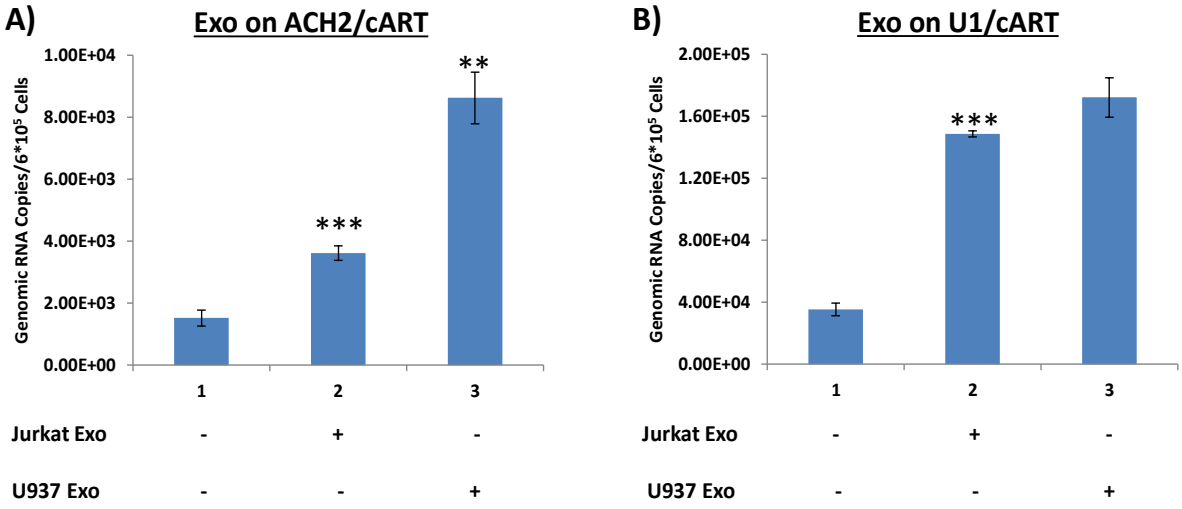

Figure 5: Uninfected exosome addition causes increase of long RNA transcripts in wild-type HIV-infected cells under cART conditions. Exosomes from Jurkat and U937 cells were isolated using ultracentrifugation before being added to ACH2 (**A**) and U1 cells (**B**); cells were under cART treatment, which consisted of an equal parts cocktail of indinavir, lamivudine, tenofovir, and emtricitabine at 10 μM. Concentrations of exosomes used were 1.60 mU/mL (Lane 2), and 1.88 mU/mL (Lane 3). Exosomes were added once per day for three days. Cells were allowed to incubate for an additional 48 hours. The cells were then harvested; total RNA was isolated and subjected to RT with a primer specific to the 3' end of the HIV-1 genome. RT-qPCR was performed to quantify the levels of genomic RNA. U937 exosome-treated U1 cells were run on a different plate than U1 cells and Jurkat exosome-treated U1 cells so the background of the U937-treated U1 cells was subtracted from the total RNA.

Collectively, these data further imply that uninfected T cell and monocyte-derived exosomes can cause significant changes in the amounts of short and long RNA transcripts in infected cells treated with antiretroviral drugs.

Mechanism for increased transcription in cells

We next investigated a possible mechanism for how transcription could increase using a ChIP assay. In this experiment, we isolated and purified exosomes from U937 cell culture prior to addition to U1 cells under cART as greatest increases in transcription involved monocyte-derived exosomes being added to monocytes. A ChIP assay was performed utilizing antibodies specific for RNA Polymerase II large subunit (Pol II), p65 (NF-κB component), and IgG (as control). Following cross linking, the immunoprecipitated DNA was isolated and quantified by qPCR.

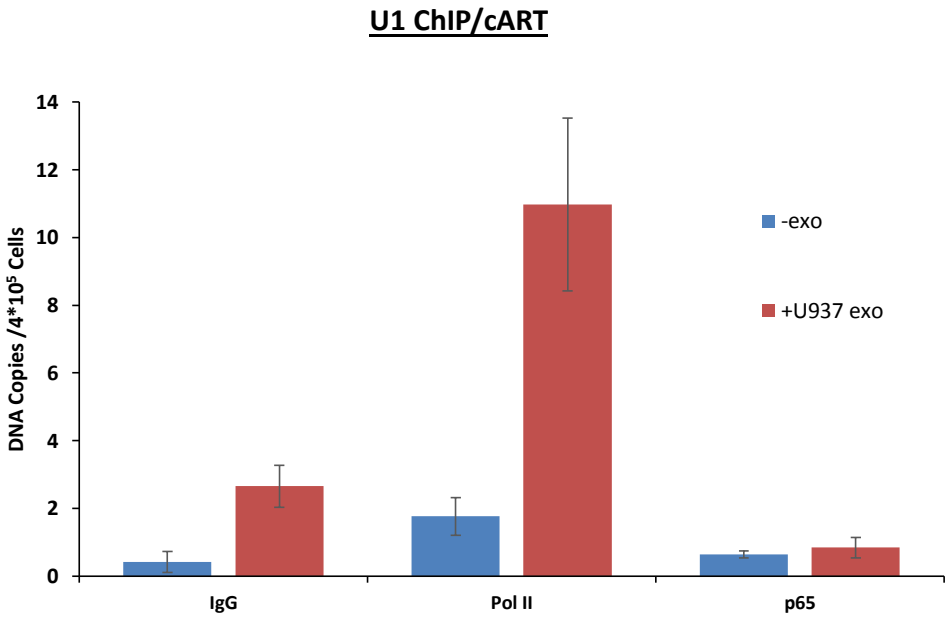

Figure 6: Mechanism for exosome-induced transcription. Exosomes from U937 cells were isolated by ultracentrifugation and added to U1 cells under cART treatment, which consisted of an equal parts cocktail of indinavir, lamivudine, tenofovir, and emtricitabine at 10 μM. The exosomes were added at a concentration of 0.403 mU/mL once per day for three days. Cells were allowed to incubate for an additional 48 hours. The cells then underwent a ChIP assay utilizing antibodies for RNA Polymerase II, p65, and IgG. DNA was then quantified using qPCR. The primers for the qPCR were NF-κB1-2F and TAR+59-R.

Results in **Fig. 6** indicate that in untreated cells, Pol II bound twice as much DNA compared to the IgG control. Upon addition of exosomes, Pol II loading onto the DNA increased by 4-fold over the control. Interestingly, the amount of DNA bound by NF-κB p65 did not change between treated and untreated cells, indicating that the effect of the exosomes on transcription may be at either late initiation or early elongation. These data indicate that exosomes derived from uninfected monocytes may cause increased transcription within HIV-infected monocytes through activation of RNA Polymerase II.

CHAPTER 4: DISCUSSION

Exosomes are known to carry proteins and RNAs from one cell (the donor) to another (the recipient) (8, 11). Previous studies have shown that virally-infected cells can spread viral proteins and/or RNA via exosomes to uninfected cells, contributing to either disease pathogenesis or increase susceptibility (2, 10). New data generated in this study shows that exosomes derived from uninfected cells can have an effect on infected recipient cells.

The focus of this study was to examine whether exosomes from uninfected donor cells could elicit a response in infected recipient cells. In Jurkat E4 cells, an indicator cell line for HIV infected with a mutant virus, it was seen that upon addition of exosomes from uninfected cells (CEM (T cell), Jurkat (T cell), and U937 (monocyte)), there was a significant increase in TAR RNA (short RNA transcript) levels but no significant increase in genomic RNA (long RNA transcript) levels (**Fig. 1**). However, the data also shows that exosomes derived from Jurkat cells and U937 cells cause increased levels of both short and long RNA transcripts, and p24, within wild-type HIV-infected recipient cells (**Fig. 2** and **Fig. 3**). Specifically, this is seen in ACH2 (T cell) and U1 (monocyte) cells. The difference between the datasets could be because E4s have a mutant virus containing GFP but lacking the *gag* and *nef* genes, which might be necessary for proper RNA transcription and splicing. Furthermore, under cART conditions, which more

accurately mimic what happens in patients infected with HIV, a similar increase in short and long RNA transcription levels upon exosome addition can be seen (**Fig. 4** and **Fig. 5**). Of note is that the data seem to indicate a more potent increase in transcription occurs in monocytes treated with monocyte-derived exosomes. This could be due to greater specificity between the exosome and the cell. Interestingly, neither Jurkat-derived nor U937-derived exosomes caused any notable increase in TAR RNA transcription within OM10.1 cells (**Fig. 2** and **Fig. 4**). This could be because OM10.1 cells are a myeloid-derived cell and thus have cellular equipment not suited to reacting with T cell-derived or monocyte-derived exosomes.

A second aspect of this study was to find a possible mechanism to explain the increases in TAR RNA and genomic RNA levels observed within infected recipient cells. Data from **Fig. 6** indicate that upon addition of U937-derived exosomes to U1 cells, there is an increase in the amount of RNA Polymerase II binding to the cellular DNA, but p65, a component of the NF-κB transcription factor, does not exhibit such an increase. This could be explained by a kinase from the donor cell being packaged into the exosome that, upon entry into the recipient cell, translocates to the nucleus and phosphorylates RNA Polymerase II. Candidates for this kinase could be AKT1, MAP2K, MTOR, DNAPK, and SRC, which have all been previously shown to be packaged into exosomes (17). In order to test this hypothesis, we will perform a future experiment where we treat U1 cells with exosomes from U937 cells and inhibitors for each of the kinases. Our assay for this experiment will be qPCR for TAR RNA. If TAR is lower in one of the inhibitor-treated

samples versus the control, this would mean that the inhibited kinase in that sample is potentially responsible for phosphorylating RNA Polymerase II.

Overall, we have shown that exosomes from uninfected donor cells cause increased transcription of both short and long RNA transcripts within infected recipient cells. However, there are still two things that should be done in the future to build upon these results. First, the kinase that drives increased RNA Polymerase II activity should be identified. This could be done by treating U1 cells with exosomes and inhibitors specific for each kinase, allowing them to incubate for five days, and then assaying for TAR RNA levels. Second, this data should be reproduced in primary cells so that it reflects more accurately what occurs *in vivo*. If the data seen in this study can be reproduced in primary cells, then it means something significant.

Exosomes are found in a variety of human fluids, such as blood serum and CSF, and constantly travel between donor cells and recipient cells (18). If exosomes from uninfected cells can elicit increased transcription upon addition to infected cells, then it means that true latency of HIV does not occur because the cells are constantly taking up exosomes, leading to constant production of viral RNA transcripts. Clinical latency, however, may still occur as that is defined as a lack of disease symptoms (9), and there may not be a correlation between RNA production and presentation of symptoms of infection.

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BIOGRAPHY

Robert Alexander Barclay graduated from George Mason University with his BS Chemistry (concentration in biochemistry) in May 2014. During his tenure as a MS student, he was a contributing author to "Presence of Viral RNA and Proteins in Exosomes from Cellular Clones Resistant to Rift Valley Fever Virus Infection" and "Exosomes from HIV-1-infected Cells Stimulate Production of Pro-Inflammatory Cytokines through Trans-Activating Response (TAR) RNA". Robert plans to continue his studies at George Mason University as a PhD student come August 2016.