2-2020

# Tobacco/HIV-1-Induced Myeloid Cell-Derived Extracellular Vesicles in HIV-1 Pathogenesis

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## Abstract

Introduction. Smoking, which is highly prevalent in people living with HIV/AIDS, has been shown to exacerbate HIV-1 replication, in part via cytochrome P450 (CYP)-induced oxidative stress. CYP enzymes metabolize cigarette smoke condensate (CSC), causing oxidative stress and cytotoxicity. Our previous studies have demonstrated that CSC and specific CSC constituents, benzo(a)pyrene and nicotine, potentially induce CYPs, resulting in higher oxidative stress and subsequent exacerbation of HIV-1 replication in monocytes and macrophages. However, the exact mechanism behind tobacco-induced, oxidative stress-mediated enhancement of HIV-1 replication is still poorly understood. Extracellular vesicles (EVs) have recently gained attention for their unique nature as intercellular messengers which can package proteins, nucleic acids, lipids etc. EVs are known to alter HIV-1 pathogenesis through intercellular communication. Until now, the role of EVs in smoking-enhanced HIV-1 pathogenesis has been mostly unknown. In this study, we investigated the effect of CSC on the characteristics and differential packaging of monocyte- and macrophage-derived EVs, and their influence on HIV-1 replication. We hypothesized that CSC- and/or HIV-1-exposed monocyte and macrophage-derived EVs and their components, especially pro-oxidant factors, are key mediators of HIV-1 replication.

Methods. Two monocytic cell lines, U937 and HIV-1-infected U1 cells, and macrophages derived from these monocytes, as well as macrophages derived from primary human monocytes were used. Cells were treated with 10 μg/ml/day CSC. After treatment, the cells were harvested, and the supernatant was collected for isolating EVs by Total Exosome Isolation kit. The isolated EVs were characterized for their biophysical properties. Next, monocyte-derived macrophages were exposed to EVs, as well as subjected to downstream analysis (p24 ELISA, LDH cytotoxicity assay, DNA damage assay, rtPCR, western blot, cytokine analysis).

Results. Initially, we demonstrated that CSC reduced total protein and antioxidant capacity in EVs derived from HIV-1-infected and uninfected monocytes. The EVs from CSC-treated uninfected cells showed a protective effect against cytotoxicity and viral replication in HIV-1-infected macrophages. However, EVs derived from HIV-1-infected cells lost their protective capacity. The results suggested that the exosomal defense is likely to be more effective during the early phase of HIV-1 infection and diminishes at the latter phase.

Next, we investigated differential packaging of specific contents in EVs subjected to CSC and HIV-1 exposure. We observed CSC-induced upregulation of catalase in EVs from uninfected cells, with a decrease in the levels of catalase and PRDX6 in EVs from HIV-1-infected cells. We also observed higher expression of CYPs (1A1, 1B1, 3A4) and lower expression of antioxidant enzymes (SOD-1, catalase) in EVs from HIV-1-infected macrophages compared to those from uninfected macrophages. Together, they are expected to increase concentrations of oxidative stress factors in EVs derived from HIV-1-infected cells. Moreover, our results show that longer exposure to CSC increased the expression of cytokines in EVs from HIV-1-infected macrophages, when compared to the shorter exposure. Importantly, pro-inflammatory cytokines, especially IL-6, were highly packaged in EVs from HIV-1-infected macrophages upon both long and short-term CSC exposures. Anti-inflammatory cytokines, particularly IL-10, had high packaging in EVs, while packaging of chemokines was mostly increased in EVs upon CSC exposure in both HIV-1-infected and uninfected macrophages.

Conclusion. Taken together, our results suggest a potential role of CSC-exposure in modulating HIV-1-infected and uninfected cell-derived EVs, thereby affecting HIV-1 replication in recipient cells. Our study also suggests the packaging of increased levels of oxidative stress-inducing and inflammatory

elements in EVs upon exposure to tobacco constituents and/or HIV-1, which would ultimately enhance HIV-1 replication in macrophages via cell-cell interactions.


## Document Type
Dissertation

## Degree Name
Doctor of Philosophy (PhD)

## Program
Pharmaceutical Sciences

## Research Advisor
Santosh Kumar, Ph.D.

## Keywords
Cytochrome P450, Cytokine, Extracellular Vesicles, HIV-1, Oxidative stress, Smoking

## Subject Categories
Diseases | Immune System Diseases | Medicine and Health Sciences | Other Pharmacy and Pharmaceutical Sciences | Pharmaceutics and Drug Design | Pharmacy and Pharmaceutical Sciences | Virus Diseases

UNIVERSITY OF TENNESSEE HEALTH SCIENCE CENTER

DOCTOR OF PHILOSOPHY DISSERTATION

# Tobacco/HIV-1-Induced, Myeloid Cell-Derived Extracellular Vesicles in HIV-1 Pathogenesis

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*A Dissertation Presented for The Graduate Studies Council of*
*The University of Tennessee Health Science Center*
*in Partial Fulfillment of the Requirements for the*
*Doctor of Philosophy degree from*
*The University of Tennessee*

*in*

*Pharmaceutical Sciences: Pharmacometrics*
*College of Graduate Health Sciences*

*May 2020*

## DEDICATION

I dedicate this dissertation to my beloved parents MD. Shamsul Hoque Mondal and Mrs. Shaheda Khatun, source of my motivation and inspiration. I am who I am today, because of my parents' unconditional support, love, and trust on me. I am lucky to have them as my parents.

## ACKNOWLEDGEMENTS

My utmost gratitude to my mentor Dr. Santosh Kumar, for giving me the opportunity to pursue this project as well as his outstanding guidance and mentorship to make it a success. I have grown as a scientist, learned the philosophy of being a doctorate, and become a better soul through his moral teachings. I am forever grateful to him for his exceptional mentorship.

I am sincerely thankful to my committee members, Dr. Theodore J. Cory, Dr. Wei Li, Dr. Francesca-Fang Liao, and Dr. Murali Yallapu for their time, valuable inputs and suggestions to complete my project and dissertation. I would also like to acknowledge Nature Publishing Group, MDPI AG, and PLOS ONE for allowing me to reprint and modify the contents from the respective manuscripts into my dissertation.

My earnest appreciation to the Department of Pharmaceutical Sciences for the administrative and financial support to conduct my graduate study and project. I would also like to thank National Institutes of Health for providing grants: DA042374, DA047178 to fund my project.

I would like to sincerely thank my wonderful lab mates, Narasimha Midde, Benjamin Patters, Namita Sinha, Sunitha Kodidela, Yuqing Gong, Arif Rahman, and Sabina Ranjit for their help and support throughout this journey. I am also thankful to my friends in the department of pharmaceutical sciences and CGHS for making my graduate life an unforgettable experience.

Last but not the least, I would like to thank my brilliant dear husband, Dr. Arif Rahman, for being my strongest support throughout this journey. He has been a helpful lab mate, an amazingly caring life partner who stayed by me through the challenging times and inspired me to be the better version of me. I am eternally grateful to my mother for believing in me, my (late) father who would have been the proudest of his daughter's achievement. I am thankful to my mother and father in-law for their kindest and warmest love and support. I am blessed to have my kid sisters Shayonti Hoque Ahona and Nilufar Yasmin and their purest love for me. I am thankful to Mrs. Rahima Sheikh Rumi, for her unbound affection throughout my life. My deepest gratitude to my lovely cousins, relatives, teachers, friends, and all the well-wishers for their support and love. I feel privileged and grateful to have this wonderful opportunity.

## ABSTRACT

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**Conclusion.** Taken together, our results suggest a potential role of CSC-exposure in modulating HIV-1-infected and uninfected cell-derived EVs, thereby affecting HIV-1 replication in recipient cells. Our study also suggests the packaging of increased levels of oxidative stress-inducing and inflammatory elements in EVs upon exposure to tobacco constituents and/or HIV-1, which would ultimately enhance HIV-1 replication in macrophages via cell-cell interactions.

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| | |
|---|---|
| AOE | Antioxidant Enzyme |
| B(a)p | Benzo(a)pyrene |
| CSC | Cigarette Smoke Condensate |
| CYP | Cytochrome P450 enzyme |
| DMSO | Dimethyl Sulfoxide |
| EV | Extracellular Vesicle |
| HAND | HIV-1-associated neurocognitive disorders |
| HIV-1 | Human Immunodeficiency Virus Type-1 |
| IL- | Interleukin- |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| MDM | Monocyte-derived Macrophage |
| MISEV | Minimal Information for Studies of Extracellular Vesicles |
| PLWHA | People living with HIV/AIDS |
| RANTES | Regulated on activation, normal T cell expressed and secreted |
| TNF-α | Tumour Necrosis Factor alpha |

# CHAPTER 1. INTRODUCTION[1]

## Smoking-Enhanced HIV-1 Pathogenesis and the Role of Oxidative Stress via the Cytochrome P450 Pathway

Once a lethal pandemic, AIDS is now considered a lethal chronic condition. As of 2018, at least 37.9 million people were living with HIV-1, with more than a million new cases each year [1]. The majority of these people living with HIV-1/AIDS (PLWHA) have a life expectancy comparable to healthy adults, which is attributed to remarkable advances in medicine, especially the introduction of combination anti-retroviral therapy (cART) [2-5]. However, a huge portion of PLWHA have a poor quality of life and suffer from high morbidity and mortality associated with drugs of abuse, including tobacco. More than 40% of PLWHA in the USA are cigarette smokers, which significantly affects their life expectancy - reducing their average life span by over 6 years [6-8]. Smoking cessation can improve life expectancy, although studies have shown that this is difficult to achieve [9]. Cigarette smoke disturbs the redox reaction balance in the body by affecting both antioxidant pathways and reactive oxygen species (ROS) levels. These alterations cause oxidative stress and inflammation, which lead to cellular toxicity and damage in various tissues [10-13]. Despite the use of cART, smoking is known to exacerbate morbidity and mortality in PLWHA [14-16]. In the PLWHA, smoking further weakens the immune system, resulting in a higher risk of virological rebound, an increased rate of immunologic failure, and a decreased response to cART [17, 18]. The progression of smoking-associated diseases is more rapid in PLWHA than in uninfected smokers [19]. Furthermore, several reports also support the hypothesis that smoking enhances HIV-1 infectivity, replication, and progression to AIDS [20-24]. In addition, approximately 50% of PLWHA demonstrate a pattern of cognitive, motor, and behavioral dysfunction, cumulatively termed HIV-1-associated neurocognitive disorders (HAND) [25-27]. In the presence of cigarette smoke, the risk of peripheral neuropathy and HAND in PLWHA increases significantly [7, 28-31]. Some reports demonstrate a conflicting impact of cigarette smoke on PLWHA in terms of neurocognitive disorders [32-34], which further indicates the necessity to study whether cigarette smoking is a causative factor for HAND.

While the exact mechanistic pathway for smoking-mediated exacerbation of HIV-1 pathogenesis is not yet fully understood, literature reports and our own studies have, in part, established the role of cellular oxidative stress in HIV-1 pathogenesis by altering the pro- and anti-inflammatory and pro- and anti-oxidant factors in monocytes and macrophages upon exposure to cigarette smoke [10, 21, 22, 35-37]. Our lab demonstrated that, benzo(a)pyrene (B(a)p), a potent component of cigarette smoke, significantly enhanced cytochrome P450 (CYP) 1A1 expression in monocytes and macrophages [10].

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[1] Modified from final submission with open access permission. Sanjana Haque, Sunitha Kodidela, Kelli Gerth, Elham Hatami, Neha Verma and Santosh Kumar. Extracellular Vesicles in Smoking-Mediated HIV Pathogenesis and their Potential Role in Biomarker Discovery and Therapeutic Interventions. Cells 2020, 9(4), 864; https://doi.org/10.3390/cells9040864 [215].

Moreover, CYP2A6, the primary nicotine-metabolizing enzyme, was shown to be highly expressed in macrophages in the presence of a stimulus like alcohol [38, 39]. Interestingly, nicotine metabolism has also been found to be notably higher in HIV-1-positive smokers compared to HIV-1-negative smokers [40]. Furthermore, our studies have suggested an altered level of cytokines (particularly IL-8 and MCP-1) in HIV-1-positive smokers [22]. In summation, tobacco smoke increases oxidative stress, which potentially aggravates HIV-1 pathogenesis. The oxidative stress is generated via the induction of CYP-mediated metabolism and activation of cigarette smoke condensate (CSC), as well as by modification of cytokine expression. However, CYP-mediated oxidative stress generation only partially explains the exacerbation of HIV-1 replication in cigarette smokers. It is likely that these highly expressed pro-oxidants are released into the plasma. This could potentially affect metabolic activity, and therefore HIV-1 replication, in neighboring or distant cells upon internalization.

One possible mechanistic pathway of tobacco smoking-induced HIV-1 pathogenesis could be the transportation of oxidative stress-related agents and inflammatory modulators via extracellular vesicles (EVs), commonly referred to as exosomes prior to 2018. EVs are biological nanoparticles and are released by almost all cells [40, 41]. They are considered both inter- and intra-cellular messengers, able to modify their cargos according to the condition or stimulus affecting the parent cells [41, 42]. Upon internalization by recipient cells, EVs can modulate the pathophysiological state in those cells [43, 44]. EVs play an important role in HIV-1 pathogenesis, either improving or deteriorating existing conditions; however, the exact role of EVs in HIV-1 pathogenesis is poorly understood [45, 46]. Currently, only a handful of studies have investigated the role of EVs in smoking-induced toxicity in the setting of HIV-1 [35, 47, 48].

Therefore, there is an apparent correlation between cigarette smoking and HIV-1 pathogenesis as demonstrated via EVs. Nevertheless, there are unresolved questions to be answered. This niche field has drawn researchers to enhance understanding of the role of EVs containing oxidative stress and inflammatory agents in smoking-enhanced HIV-1 pathogenesis. However, before elaborating on the potential roles of EVs in tobacco smoking-enhanced HIV-1 pathogenesis, and any links between the two, it is imperative to establish the current understanding available in the literature on EVs, especially with regards to their isolation and characterization, as well as their potential in biomarker discovery and therapeutic interventions.

## Extracellular Vesicles: A Brief Introduction

### Discovery of and Basic Characteristics of EVs

The discovery of EVs, especially exosomes, a particular type of EV, dates back to the 1980s. Two research groups led by Philip Stahl [49] and Rose Johnstone [50] independently described EVs that are released from cells by exocytosis [51]. Initially,

these vesicles were assumed to take part in the removal of unwanted substances from the cells. In the following decade, it was revealed that these vesicles carry functional molecules, including genetic materials, which can modify the pathophysiological conditions of recipient cells [52-55]. Subsequently, the body of literature on these vesicles has grown exponentially, and the knowledge about their physiological roles is still evolving. There are various forms of EVs, including exosomes, microvesicles, apoptotic bodies, microsomes, ectosomes, prostasomes, and oncosomes, the former three being the most heavily studied. The EVs that are generally formed in multivesicular bodies by endosomal sorting complexes required for transport (i.e. protein-based biogenesis) are exosomes [56]. These exosomes are the nano-sized vesicles (~150nm) that capture and transport functional messages from cells and are thus the primary vesicles of interest [57, 58]. Microvesicles (100-1000nm) and apoptotic bodies (50-5000nm) are generated directly from the plasma membrane, respectively by budding and blebbing during apoptosis [59]. Unfortunately, isolation and purification of exosomes from other types of EVs remain challenging. Thus, most isolated EVs are a mixed population of different sorts of vesicles. Therefore, according to the guidelines provided by the Minimal Information for Studies of Extracellular Vesicles (MISEV) in 2018, it is more appropriate to use the generic term extracellular vesicles (EVs) to designate these vesicles [60].

New insights are coming forward in terms of understanding cellular uptake of EVs. Some EVs demonstrate cell-specific uptake, some are universally internalized by all cells, and some EVs could be rejected altogether by recipient cells [56]. The exact reasons for this semi-selective targeting characteristics are still to be discovered, however some studies have identified specific receptor-binding molecules on the surfaces of EVs that might be responsible [61]. On the other hand, EVs carry very small cargo molecules from their parent cells. Therefore, the targeting properties of both the EVs and the recipient cells need to be highly specific and efficient [62]. Depending on the uptake process, EVs can release their cargo at the plasma membrane of the recipient cells or go through endocytosis to be re-released from the recipient cells, with modified characteristics. In addition to the cellular targeting and uptake process, whether the EVs will have a functional or phenotypical impact on the recipient cells largely depends on the cargo release mechanism of the EVs. Unfortunately, there is still a significant lack of information which would be necessary to fully comprehend the true import of EV-mediated intercellular communication. Nevertheless, after internalization and release of cargo, the role of EVs in the physiological modulation of recipient cells is well-established [48, 62-67].

## Isolation Method and Characterization Techniques

Due to the complex nature of EVs, successful isolation of a single variety of EVs still remains challenging. Owing to the persistent discrepancies in EV isolation procedures, the MISEV 2014 guidelines recommended choosing the isolation method according to the need of the downstream analysis [68]. Simultaneously, the source and availability of the EV-originating biofluid, reproducibility, and availability of resources should be considered. Until now, ultracentrifugation has been considered the gold

standard method for the isolation of EVs from cell culture media and biological fluids [69]. However, this method has its own limitations that include the requirement for a large volume of starting fluid and co-precipitation of unwanted materials [70]. Several isolation techniques have been developed to overcome these limitations. Sucrose gradient centrifugation, the modified sucrose gradient method using iodixanol (optiprep™), size-exclusion chromatography, polymer-based precipitation using polyethylene glycol, antibody-specific EV separation, and microfluidic techniques are among the most commonly employed separation techniques [59, 70-74]. Recently, Rong et al. have demonstrated a sequential ultrafiltration method to isolate two subtypes of EVs (exosomes and microvesicles) from cancer cells, which uses smaller pore size filters in each subsequent step. Although the sequential ultracentrifugation method allows for the separation of more purified exosomes, it requires a substantial amount of starting material [75]. Mengxi et al. have developed an integrated acoustics and microfluidics method to isolate EVs from undiluted whole blood, with high purity and yield in an automated system [76]. Furthermore, a commercial column method to isolate EVs is also currently available [77]. However, there is still potential for improvement, in terms of purity, yield, and the cost of isolation procedures [78].

After isolation of EVs, it is equally important to characterize the particles to confirm their identity as EVs. According to the updated MISEV 2018 guidelines, molecular techniques to identify at least three or more exosome-enriched proteins, such as tetraspanin membrane proteins (e.g. CD9, CD63 and CD81), heat shock proteins (HSPA8 and HSP84), membrane transporters (GTPases), and lipid-bound proteins should be presented. In addition, the vesicles should be devoid of cell-specific proteins such as β-actin and GAPDH [60]. At least 2 different biophysical techniques should be performed to characterize EVs. These techniques could be optical particle tracking to determine the size dispersion (ranging 10 nm to 2 μm) and concentration of EVs, which includes transmission electron microscopy (TEM), as well as atomic force microscopy to characterize individual EVs [79-81]. Comparable data from nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing are the basic requirements for EV characterization [68]. However, MISEV stressed that since the research field of EVs is highly diverse and continuously evolving, investigators have the flexibility to divert from the suggested guidelines with appropriate reasoning [60].

## EVs in Cellular Communication

The unique characteristics of EVs is their role in cell-to-cell communication. EVs have the capacity to package cargo from both healthy and diseased cells. The contents of the EVs are subject to alteration in the presence of an external trigger, which may include pathogenicity from viruses, bacteria, cancer, inflammation, environmental toxins, drugs of abuse etc. Once formed and released from cells, EVs act as cellular messengers to deliver the packaged materials to other cells or organs. This distinctive property allows EVs to be considered as a) convenient biomarkers for disease conditions and b) potential therapeutic carriers for drugs, nucleic acids, etc. A brief depiction of characteristics of EVs and their role is in **Figure 1-1**.

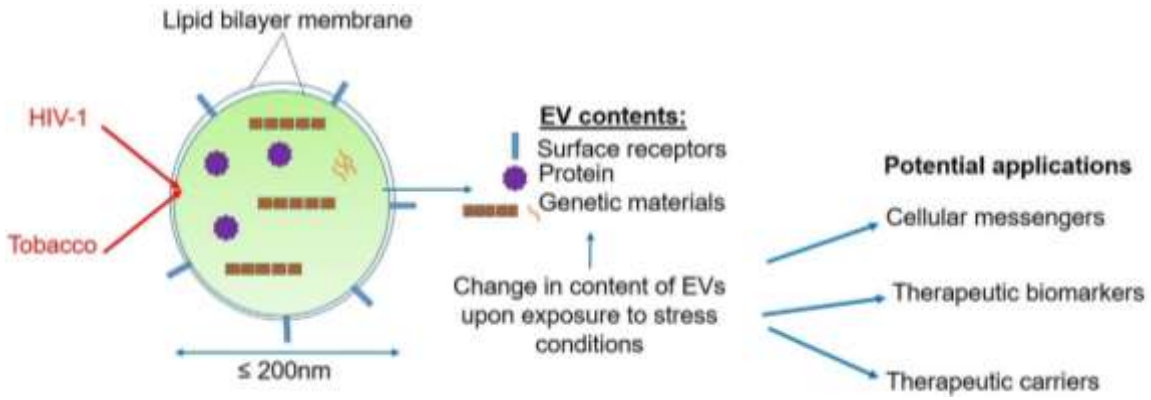


**Figure 1-1. Brief Characteristics and Potential Application of EVs.**
EVs are nanosized vesicles within the size of ≤200 nm, surrounded by a lipid bilayer membrane. EVs have surface receptors, proteins, genetic material etc., which can be modified upon exposure to stress conditions, such HIV-1 infection and tobacco. Based on this characteristic, EVs could potentially be used as cellular messengers, as well as in developing therapeutic biomarkers and carriers.

## EVs as Cellular Messengers

The unique nature of EVs is their role as cellular messengers. Speculation on this quality started with the finding that EVs are involved in indirect activation of CD4-positive T cells by carrying major histocompatibility complex and T cell costimulatory molecules, as well as promoting their exchange between cells [82, 83]. In 2007, Valadi et al. took EVs research another step forward by demonstrating that these vesicles packaged and shuttled mRNA and miRNA to recipient cells for translation to functional proteins [54, 83]. Since then, extensive studies have revealed that EVs are released from nearly all kinds of mammalian cells [41]. Some describe EVs from intestinal epithelial cells that participate in antigen presentation, or EVs from neurons and astrocytes that contribute to regulatory functions at synapses, tumor cell EVs containing tumor rejection antigens to induce anti-tumor effects, or stem cell EVs that propagate immunomodulatory activities, and much more [84-87]. EVs contain proteins, genetic materials (including DNA, miRNA, LncRNA, mRNA, mitochondrial RNA), and lipids [88, 89]. The contents within the EVs reflect the pathophysiological condition of the originating cells. Thus, the potential to use EVs in biomarker identification has rapidly grown, expanding from cancer biomarker identification to liquid biopsy in ADME (absorption, distribution, metabolism, and excretion) phenotyping of drugs [90-92]. EV production has been shown to be enhanced in the presence of triggers, which could be inflammatory conditions, various disease states, drugs of abuse, etc. [93-97]. The intricate role of EVs in disease pathogenesis is thus a matter of deep interest. The most studied is perhaps the role of EVs in cancer and tumor pathogenesis [98-104]. Additionally, EVs have been demonstrated to participate in disseminating pathogens like HIV-1, Hepatitis C virus, Epstein-Barr virus (EBV), prions etc. [105-109]. The focus of this project will be on this role of EVs in HIV-1 pathogenesis.

## EVs in Biomarker Discovery

The contents within EVs hold valuable information about the pathophysiological conditions of their source. Hence, in the last decade, the potential of EVs in biomarker discovery has been extensively explored [110]. Furthermore, EVs can be isolated from accessible biofluids such as serum, urine, saliva, seminal fluid, breast milk, and amniotic fluid collected by non-invasive methods [110]. Until now, the role of EVs as biomarkers has been mostly studied in the cancer field including pancreatic, hepatocellular, and renal cell carcinoma, melanoma, prostate, ovarian, breast, gastric, and bladder cancers [89, 111-114]. In fact, a blood-based cancer diagnostic tool that utilizes EVs has been commercially available since 2016 [115]. In addition to cancer, circulating EVs and their contents have shown promise as potential biomarkers including, but not limited to the following conditions: arthritis in psoriasis patients, functional dyspepsia, aging and cognitive decline, and acute respiratory distress syndrome [116-119]. EVs could serve as a diagnostic tool to identify viral infections as well. For example, EV contents can be used as biomarkers for HIV-1, hepatitis A, B, and C viruses, human adenovirus, human T cell lymphotropic virus, herpes simplex virus 1, EBV, cytomegalovirus, and human papilloma virus [120-123]. In addition to serum, EVs could also be isolated from

cerebrospinal fluid and potentially brain tissues, which are being studied to identify biomarkers for neurological conditions such as Alzheimer's disease, Parkinson's disease, Down syndrome, and Huntington's disease [124-130].

### EVs as Therapeutic Carriers

EVs are also gaining recognition as biological vehicles to carry various molecules, including drugs. EVs are natural nanocarriers, with low immunogenicity and semi-selective targeting capacity [61, 131-133]. Thus, EVs are capable of evading phagocytosis or engulfment by macrophages and lysosomal degradation, with a long circulating half-life [134-136]. Moreover, as EVs potentially evade first pass metabolism, they have a greater potential to reach target organs [137]. As with biomarker discovery, EV drug loading capacity and efficiency has been explored mostly in the context of anti-cancer therapy [134, 138-143]. Use of EVs as drug carriers has also shown potential to induce immune responses against tuberculosis, toxoplasmosis, and diphtheria toxoid, via delivery of small interfering RNA and much more [134, 138-143]. In addition, EVs have been used to deliver antioxidant enzymes (AOEs) through the blood-brain barrier (BBB) to combat oxidative stress-related toxicity in central nervous system (CNS) diseases [144]. Several studies have suggested that EVs obtain homing patterns from their originating cells, suggesting that it is crucial to consider this natural tropism of EVs while developing therapeutic carriers [145, 146]. Engineered EVs are being developed to more specifically bind to target cells by enrichment with specific antibodies, nanobodies, peptides, or nucleic acids, as well as by the pseudotyping method commonly used in altering viral tropism [61, 142, 147].

Undoubtedly, EVs have great potential to be developed as biomarkers and therapeutic carriers for many conditions. However, the EV field is still evolving, with newer challenges in terms of isolation and characterization techniques, as well as the comprehensive profiling of their contents. In brief, there is much that remains unknown about EVs that must be discovered before EVs can be fully employed for diagnostic purposes.

## Role of EVs in HIV-1

EVs have a vital role in HIV-1 pathogenesis. To begin with, EVs and HIV-1 share a multitude of similarities in terms of size, origination, and cellular release mechanisms [148]. EVs carry viral proteins like Negative Regulatory Factor (Nef), transactivator of transcription (Tat) encoding protein, entry receptors like CCR5, etc. [149-151]. By the same token, HIV-1 can utilize the release pathways of EVs to effect viral transmission [150]. It is well established that oxidative stress is involved in various stages of the HIV-1 life cycle, including viral replication and the inflammatory response, ultimately leading to the destruction of T cells, as well as damage to nuclear and mitochondrial DNA in brain tissue [152-157]. However, it is still not clearly understood how these oxidative and immunomodulatory factors are translocated from their cells of origin to neighboring cells

or even to distant organs. Based on the role of EVs in cell-cell communication, it is hypothesized that EVs carry these agents from parent cells to recipient cells in distant tissues, including the brain.

It is well documented that HIV-1-infected populations have elevated levels of EVs in their plasma in comparison to healthy individuals [93]. EVs that originate from HIV-1-infected cells can reveal important information about these cells, including the phase of the infection. Indeed, Madison et. al suggested that EVs may influence the stages of the viral life cycle [158]. Most importantly, they can provide information about the cell's exposure to stressors [158-160]. Chettimada et al. have shown that EV cargos provide a wealth of information about immune responses and oxidative stress. Their study not only confirmed the increased level of plasma EVs in PLWHA, but also showed that this increase directly correlated with elevation of the oxidative stress markers cystine and oxidized Cys-Gly. They also detected other such markers such as catalase, peroxiredoxins (PRDX1, PRDX2), and thioredoxin-1 (TXN), markers of EVs, immune markers, and Notch4 in plasma EVs by untargeted proteomic analysis [161]. Moreover, they showed reduction in levels of the anti-inflammatory polyunsaturated fatty acid (PUFA), which are known to be responsible for sensitizing cells to oxidative stress and eventually leading to cell death and apoptosis [161].

Recognition of specific protein and RNA contents in plasma EVs from PLWHA can be used for both diagnostic and prognostic purposes, as well as for estimation of treatment efficacy [162]. EVs isolated from a small group of HIV-1-suppressed patients demonstrated that miR-21 was downregulated in HIV-1-controlled patients, with a decreasing pattern of CD4-positive T cell count [163]. In addition, EVs obtained from an PLWHA without antiretroviral therapy are larger in size and greater in quantity compared to ART-suppressed and control subjects [93, 164]. In addition, the same group had a low amount of miRNA-155 and miRNA-223 [93, 164]. These findings suggest that EVs could potentially be used to indicate stages of infection, as well as markers of response towards treatment. Additionally, urinary EVs collected from PLWHA may provide a good source of HIV-1 biomarkers, including p24 [165]. In fact, commercial diagnostic tools using EVs are being developed to identify HIV-1 in patients [162, 166]. EVs could also indicate comorbid conditions in HIV-1-positive patients. For instance, EVs from PLWHA are enriched with miR-122 and miR-200a, which are known markers for liver disease [167]. Amyloid beta (Aβ) deposition is a well-recognized biomarker for Alzheimer's disease, and is common in PLWHA with HAND. Interestingly, neuron-derived EVs from HIV-1-infected populations show high levels of Aβ, which may serve as a possible biomarker for Alzheimer's disease in PLWHA [168, 169]. Markers of general neurodegeneration, e.g. HMGB1 and NF-L, are also found in these EVs, suggesting their potential role in biomarker identification for PLWHA with HAND as well [169, 170].

Taken together, EVs can potentially direct HIV-1 pathogenesis, and whether this is towards remission or exacerbation will require deeper investigation. Moreover, EVs shuttle various biomarkers that could prove useful in clinical applications.

## Role of EVs in Tobacco Smoking

Tobacco smoke contains over 4,500 chemical constituents, including a substantial amount of reactive oxygen species (ROS) [171]. These chemicals can induce pro-inflammatory reactions, contribute to DNA damage, and potentiate the development of various smoking-related illnesses associated with oxidative and nitrosative stress [11, 38, 172-178]. In addition to enhancing oxidative stress, Wang et al. demonstrated that B(a)p-exposed retinal pigment cells and mice exposed to chronic cigarette smoke expressed a greater number of key exosomal markers compared to control tissues [179]. It is thought that oxidative stress conditions induced by cigarette smoking trigger the release of EVs, as EVs may play a key role in inflammatory pathways [180] and in some cases may mediate protective signals during oxidative stress, reducing cell death [181]. Consequently, it is hypothesized that EVs transport oxidative stress-associated agents to either protect or cause more stress to recipient cells.

In addition to enhancing oxidative stress, tobacco smoking has been linked to the release of EVs in various cell types, as well as in the serum, saliva, and urine of smokers [182]. Recently, our lab has shown that a relatively high abundance of CYP1B1 and CYP2A6 is packaged into plasma EVs of healthy nonsmokers, likely originating from the lungs and the liver [183]. Further studies involving cytokine and proteomic profiling of plasma EVs from smokers provide evidence that tobacco smoking modulates the cargo of EVs. Cholesteryl ester transfer protein (CETP) protein levels are downregulated in plasma EVs from smokers compared to nonsmokers, a trend previously associated with smoking-related cardiovascular disease [47, 184]. It is reasonable to suggest that EVs may serve as biomarkers for smoking-related illnesses and nicotine dependence, as tobacco smoking is associated with variations in metabolic enzyme expression and lipid transport [183]. Additionally, the cytokine IL-6, involved in smoking-induced inflammation, was found to be completely packaged in plasma EVs relative to plasma from smoking subjects—suggesting that EVs may also be used to target cytokine signaling pathways [47].

Furthermore, we have demonstrated that B(a)p induced CYP1A1 mRNA expression in U937 monocytes, accompanied by enhanced oxidative stress, cytotoxicity, and AOE expression (SOD1, catalase), with similar findings in cigarette smoke condensate (CSC)-exposed monocytes [10, 21]. Our subsequent studies revealed a higher expression of CYP1A1 and AOE mRNAs in EVs from CSC-exposed monocytes and point toward a protective role of EVs against CSC-induced cytotoxicity [48]. These findings are consistent with prior theories, which have suggested that EVs may be involved in cell-to-cell protective signaling under oxidative stress conditions via nucleic acid shuttling [181].

EVs obtained from smokers could potentially be used in the diagnosis of smoking-induced diseases. Ryu et al. have summarized the potential biomarkers found within EVs in response to cigarette smoke. Their review suggested that progression towards chronic obstructive pulmonary disease, cardiovascular disease, and non-small cell lung cancer, in the presence of cigarette smoke, could be indicated by identifying

miRNAs and protein content present in EVs derived from plasma, serum, bronchoalveolar lavage, and urine [182].

## Role of EVs in HIV-1 and Tobacco Smoking Comorbidity

As discussed earlier, cigarette smoke contributes to the deterioration, in terms of viral replication of HIV-1 by various mechanisms, with one of them being the CYP-mediated oxidative stress pathway [22, 37, 185, 186]. However, other studies challenge these observations, which prompts further investigation [20, 187]. Although the exacerbation of HIV-1 pathogenesis via cigarette smoke condensate inducing oxidative stress has been frequently proposed in the literatures, the role of EVs in these interactions has scarcely been studied.

Our recent study with plasma EVs from HIV-1-positive smokers has revealed some exciting information. First, we have shown the downregulation of properdin in plasma EVs from HIV-1-positive smokers, a protein in the human complementary system that interacts with viral proteins, suggesting a relatively high risk of secondary infections [184]. We also observed an alteration in the levels of CD9, CETP, and vitronectin (VTN) in plasma EVs from the HIV-1-positive smoker population; however, further studies are required to reach to any conclusion. We have also looked at the differential cytokine packaging within the same population [47]. We identified two cytokine/chemokines, IL-6 and MCP-1, which were significantly packaged within plasma EVs from HIV-1-infected smokers [47]. These findings suggest that: a) EVs can potentially be used as a biomarker for smoking-enhanced HIV-1 pathogenesis and b) modulating EV-mediated HIV-1 pathogenesis has potential for therapeutic intervention. Several other studies are also looking at potential biomarkers to indicate the possible association between HIV-1 and tobacco smoking, as well as to predict the development of non-AIDS-related illnesses in HIV-1-positive smokers [188-190]. Il-6 stands out as a pro-inflammatory cytokine which is elevated in HIV-1-positive smokers [191]. In addition, Steel at el. have suggested that the chemokine RANTES could be one of the potential candidate biomarkers, as it was elevated in their study with HIV-1-positive smokers [188]. Our studies have also demonstrated that these agents are also highly packaged in EVs in the presence of both HIV-1 and smoking. Moreover, soluble CD14 and transforming growth factor beta (TGF-β1) are also reported to be elevated in virally suppressed HIV-1 positive smokers [189]. Soluble CD14 is a biomarker for monocyte activation, which is also associated with cardiovascular disease [192]. On the other hand, TGF-β1 has a crucial role in regulating the immune system, and increasing TGF-β1 has been correlated to defective T cell proliferation in an HIV-1-infected population [193]. Among these two, TGF-β1 is reported to be present in EVs, however confirming its potential as a biomarker for smoking-mediated HIV-1 progression warrants further investigation [194].

In recent years, more studies with regard to EVs in HAND have revealed that EVs influence the CNS, ultimately exacerbating HAND [27, 137, 169, 195-197]. For example, HIV-1-induced microRNA-7 and cathepsin B were shown to be transported by EVs to neurons, resulting in neuronal damage [27, 195]. Additionally, Pulliam et al. have

identified several other biomarkers of neurodegeneration, which include L1CAM, Aβ, NF-L, and HMGB1, in neuron-derived EVs from individuals with HAND [169]. Although cigarette smoking is postulated to be associated with exacerbated HIV-1 and HAND pathogenesis, to our knowledge, to date no biomarker has been identified that could indicate the development of HAND in HIV-1-positive smokers. A few possible markers could be- IL-6, RANTES, soluble CD14, and properdin, which have been identified within EVs isolated from the plasma of HIV-1 smokers [47, 184, 188, 189, 191-194]. However, further studies are warranted to establish their true potential as biomarkers of smoking-enhanced HIV-1 pathogenesis. Moreover, future investigations to discover biomarkers in plasma EVs that are altered only in the presence of both HIV-1 and cigarette smoking, will also be highly beneficial. A summary of the discussions above is summarized in **Table 1-1**.

## Potential Role of EVs Containing Oxidative Stress and Inflammatory Agents in Smoking-Enhanced HIV-1 Pathogenesis

The complete eradication of HIV-1 is nearly impossible due to viral latency in cellular reservoirs such as CD4 T cells, cells of the myeloid lineage (monocytes and macrophages), and dendritic cells [198-201]. Monocytes and macrophages are considered one of the most suitable cells for studying viral latency due to their long lifespan, as well as their ubiquitous presence throughout the body. The infected monocytes/macrophages cross the BBB and can infect cells of CNS such as perivascular macrophages and microglia [199, 201-203]. EVs derived from monocytes and macrophages potentially have a profound effect on recipient cells [204, 205]. Studies have shown that EVs derived from uninfected cells have protective properties, while infected cell-derived EVs influence HIV-1 infection into new host cells [158, 206]. In addition, macrophage-derived EVs can readily cross the BBB, suggesting a potential role of EVs in either disseminating or alleviating HIV-1 pathogenesis [207]. Moreover, proteomic and cytokine analyses of plasma EVs obtained from HIV-1-positive and negative smokers demonstrated a differential packaging of proteins in the EVs [47, 168].

A body of literature suggests that oxidative stress and inflammatory modulators play a crucial role in HIV-1 pathogenesis, especially in the presence of drugs of abuse. Our previous studies have demonstrated that cigarette smoke condensate (CSC), as well as prominent component chemicals of CSC, like nicotine and B(a)p, potentially exacerbate HIV-1 replication in established HIV-1 *in vitro* systems [35]. We observed a similar trend in *ex vivo* plasma samples of HIV-1-infected smokers and non-smokers. Additionally, cytokines and chemokines were shown to impact smoking-enhanced pathogenesis [208]. The potential underlying mechanism could be an alteration in the expression of CYPs, AOEs, and cytokines in monocytes and macrophages, in the presence of smoking constituents and HIV-1 exposure. However, it is still poorly understood that how these localized effects are translocated to distant cells and organs. Therefore, in this study we will address the gap in understanding the role of pro-and anti-oxidants as well as inflammatory agents in HIV-1 pathogenesis, particularly, how these are communicated with other cells via EVs.

**Table 1-1. Role of Extracellular Vesicles in HIV-1, Tobacco Smoking, and HIV-1 and Tobacco Smoking Together.**

| HIV-1-associated EV component | | Smoking-associated EV component | | HIV-1 + smoking-associated component | |
|---|---|---|---|---|---|
| **Type** | **Specific** | **Type** | **Specific** | **Type** | **Specific** |
| Viral proteins | Nef, Tat [151] | CYP enzymes | CYP-1A1 mRNA [48] | Cytokines | IL-6, MCP-1, RANTES [47, 188, 191] |
| Viral entry receptors | CCR5 [150] | Anti-oxidant enzymes | Catalase, PRDX6 [48] | Others | Properdin [184], soluble CD14, TGF-β1 [189] |
| Oxidative stress markers | Cystine, oxidized cys-gly, Catalase, PRDX1, PRDX2, and TXN [161] | Cytokines | IL-6 [47] | | |
| Anti-inflammatory marker | PUFA [161] | Other | CETP, VTN [184] | | |
| Immune activation markers | CD14, CRP, HLA-A, and HLA-B [161] | | | | |
| Neurodegeneration markers | Aβ, HMGB1 and NF-L [168-170] | | | | |

# CHAPTER 2. SCIENTIFIC PREMISE, HYPOTHESES, AND OBJECTIVES

## Scientific Premise to Study the Role of EVs in HIV-1 Pathogenesis via an Oxidative Stress-Mediated Pathway

For last several years, our lab has been investigating the role of drugs of abuse in exacerbating HIV-1 pathogenesis via oxidative stress-mediated pathways. In particular, we have demonstrated that nicotine causes oxidative stress, and that B(a)p exacerbates HIV-1 replication via cytochrome p450 (CYP)-mediated oxidative stress in cells of myeloid lineage [11, 209]. Furthermore, our studies with monocytic cells have also demonstrated that cigarette smoke condensate (CSC) induced expression of CYP1A1, leading to increased reactive oxygen species (ROS) generation, which has been suggested to correlate with HIV-1 viral load [11, 21]. In addition, we have observed increased viral load, nicotine metabolism, and CYP-mediated oxidative stress in HIV-1-infected smokers compared to uninfected smokers [22, 40]. Moreover, cytokines and chemokines play a critical role in the immune system by providing a precise control mechanism in the migration and position of immune cells [208]. Imbalances in the levels of cytokines and chemokines during HIV-1 infection vary greatly depending upon the stages of infection [210]. Our earlier studies have shown dysregulation of cytokines and chemokines in HIV-1-infected smokers and non-smokers [22, 47]. Furthermore, cytokines are shown to significantly impact the mRNA and protein expression levels of CYP enzymes in human peripheral blood mononuclear cells (PBMCs) [211]. Therefore, CYP enzymes and cytokines play a crucial part in HIV-1 pathogenesis. Cigarette smoke has been shown to be responsible for oxidative stress in myeloid cells, as well as in CNS cells, perhaps through a similar mechanistic pathway [11, 21]. Moreover, circulating CYPs, AOEs, cytokines, and chemokines might as well contribute to the enhanced expression of these agents in monocytes and macrophages. Thus, it is reasonable to postulate that a carrier is responsible for transporting them securely through the blood and delivering them in functional form to distant cells and organs.

Therefore, in this study we proposed that EVs impact the oxidative stress-mediated pathway of smoking-induced HIV-1 pathogenesis by acting as cellular messengers. The contents of EVs are generally more stable than their freely secreted form [212, 213]. Moreover, EVs have been shown to influence HIV-1 pathogenesis by carrying viral proteins or modulators. There are multiple sources of EVs to be considered. Circulating plasma EVs have been suggested to contain an appreciably high quantity of functional CYP enzymes [183]. This is conceivable, considering the EVs could be originating from CYP-rich organs such as liver. In the presence of triggers such as cigarette smoke and/or HIV-1, CYP expression is thought to be elevated, which could be directed towards secretion through EVs [214]. When these EVs are taken up by other cells like macrophages, they could potentially contribute to overall oxidative stress. Another source of EVs could be particular cells, such as monocytes and macrophages. As discussed earlier, upon exposure to cigarette smoke and HIV-1, oxidative stress and immunomodulatory agents are highly expressed in these cells [11, 209, 215]. These contents are secreted from the cells within EVs, which are in turn suggested to follow the

homing pattern of their parent cells. Thus, the EVs derived from myeloid cells will likely migrate towards the same cell types as the parent cells. Monocytic cells play a crucial role in HIV-1 pathogenesis by harboring the virus in latent form, produce more virus over a longer time period of time than T cells [216]. In addition, their ubiquitous presence throughout the body, including the brain, makes monocytes and macrophages the most suitable *in vitro* system to study HIV-1 as well as HAND pathogenesis.

Thus, the current project will aim at finding the missing link between cigarette smoke-enhanced HIV-1 pathogenesis and EVs transport of oxidative stress-related and immunomodulatory agents, as well as direct towards the future applications of this study.

## Hypotheses and Objectives

Based on our previous findings and literature reports, we hypothesized that under the influence of tobacco/CSC and HIV-1 infection, monocytes and monocyte-derived macrophages (MDM) would release EVs, which would contain differential amounts of oxidative stress and inflammatory pathway components that are key contributors to HIV-1 pathogenesis. **Figure 2-1** depicts a graphical representation of this central hypothesis. To test the central hypothesis, this project was divided into two broad aims:

### Aim 1: Determine the Role of Monocyte-Derived EVs upon Exposure to CSC in the Cytotoxicity and HIV-1 Replication Within Recipient MDM via Oxidative-Stress Mediated Pathways

Here, we hypothesized that the EVs, that were derived from monocytes exposed to CSC could influence cytotoxicity in MDM as well as affect viral replication in HIV-1-infected MDM, possibly by oxidative stress mediated pathways. The objective of this aim was to determine the contribution of CSC exposure-derived EVs on cytotoxicity and/or viral replication in uninfected and infected MDM.

We would perform *in vitro* studies with EVs isolated from monocytes treated with CSC in the absence or presence of HIV-1. First, we would characterize the isolated vesicles to identify EVs. Next, we would evaluate the effect of the EVs collected from these sources on recipient MDM, followed by validating the in vitro findings in primary macrophages obtained from healthy donors.

### Aim 2: Identify Differential Packaging of Oxidative Stress Modulators and Inflammatory Cytokines/Chemokines in HIV-1-Infected and Uninfected Monocytes/MDM-Derived EVs upon Exposure to CSC

We hypothesized that monocytes and MDM-derived EVs components, especially those related to pro- and anti-oxidant as well as pro- and anti-inflammatory factors

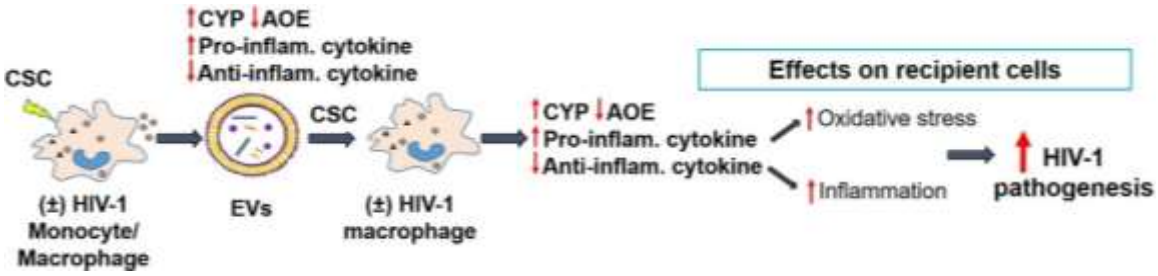


**Figure 2-1. Graphical Presentation of the Central Hypothesis.**
CSC exposure to HIV-1 uninfected and infected monocytes or MDM alters the expression of the components of oxidative stress and inflammatory pathways (CYPs, AOEs, pro- and anti-inflammatory cytokines). These differentially expressed components are packaged within EVs and released from the affected monocytes and MDM. When these modified EVs are taken up by recipient MDM, overall oxidative stress and inflammation is increased, ultimately exacerbating HIV-1 pathogenesis. CSC: Cigarette smoke condensate; EVs: Extracellular Vesicle; MDM: Monocyte Derived Macrophages; CYP: Cytochrome p450 enzyme: AOE: Anti-oxidant enzyme.

(CYPs, AOEs, cytokines, chemokines) are key mediators that influence changes in smoking-induced toxicity and HIV-1 pathogenesis. Our objective was to determine the differential packaging of specific components in CSC-treated and/or HIV-1-infected MDM-derived EVs that modulate smoking-enhanced cytotoxicity and HIV-1 pathogenesis.

We would determine the mRNA and protein levels of CYP450 enzymes in the EVs that are responsible for the metabolism of the primary CSC constituents. Next, our goal would be to observe whether CSC and/or HIV-1 exposure has any significant effect on the expression of CYPs, AOEs, cytokines, and chemokines in the monocytes and macrophage derived EVs.

The detailed experiments and subsequent findings of both aims will be discussed in the following sections.

# CHAPTER 3. MATERIALS AND METHODS[2,3]

## Cell Culture and Treatment

For *in vitro* studies, two monocytic cell lines, U937 cells from ATCC (Manassas, VA) and the HIV-1-infected U1 cells from the NIH AIDS Reagent Program (Germantown, MD) were used. U937 and U1 are model cell lines that have been used extensively by our group and many other researchers to study the effects of drug abuse, including tobacco smoking, in the context of HIV-1 infection [21, 35, 48, 217, 218]. The data obtained from these cell lines have been consistently verified and strongly correlated with primary macrophages [218, 219]. The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 media [106], supplemented with 2% sodium bicarbonate, 1% L-glutamine, and 0.1% gentamicin [10]. In order to generate MDM, the monocytes (0.8 million cells/well) were seeded in 6-well plates with RPMI media including 80 nM phorbol 12-myristate 13-acetate (PMA). After 3 days, media and non-adherent cells were removed by washing with phosphate-buffered saline (PBS). Monocytes (0.1 million/ml) and MDM were seeded in plates with 10% exosome-depleted fetal bovine serum (Exo-FBS) containing RPMI at 37°C with 5% $CO_2$ [106]. The cells were treated everyday with CSC (10 µg/ml) and dimethyl sulfoxide (DMSO) as control for 4 days. Our previous studies have shown that acute treatment of 50 µg/ml CSC, which is near the physiological concentration of nicotine and other constituents, increases the ROS level, without appreciable toxicity [21, 220]. For the current study, we chose a CSC concentration that would accumulate to 40 µg/ml after 4 days of treatment, would generate minimal toxicity, and would provide time for sufficient production of EVs for further analysis. For short-term and long-term exposure, the cells were treated with CSC for at least 4 days or 6/8 days respectively. Treating cells for 4 days allows optimum time for EVs to capture the contents of cells and be released into the media. With longer exposure, the CSC treatment is observed to be toxic to the cells after 6 and 8 days in U1 and U937 cells, respectively. Therefore, our long-term exposure was defined by 6/8 days of treatment.

For treating MDM with EVs, EVs were isolated from 1 ml supernatant from each well and were exposed to differentiated U937 and U1 MDM with 1 ml media. Our EV treatment was based on volume/volume rather than protein amount to closely reflect physiological conditions. The differentiated U937 and U1 cells were treated with DMSO or CSC, as well as with the EVs that were isolated from the same amount of media from each control and CSC-treated cells for 3-4 days.

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[2] Modified from final submission with open access permission. Sanjana Haque, Namita Sinha, Sabina Ranjit, Narasimha M. Midde, Fatah Kashanchi & Santosh Kumar. Monocyte-derived exosomes upon exposure to cigarette smoke condensate alter their characteristics and show protective effect against cytotoxicity and HIV-1 replication Sci Rep 7(1), 16120 (2017). https://doi.org/10.1038/s41598-017-16301-9. [48].

[3] Modified from final submission with open access permission. Sanjana Haque, Sunitha Kodidela, Namita Sinha, Prashant Kumar, Theodore J Cory, Santosh Kumar. Differential packaging of inflammatory cytokines/ chemokines and oxidative stress modulators in U937 and U1 macrophages-derived extracellular vesicles upon exposure to tobacco constituents. PLOS ONE. In Press [230]

## Collection of Peripheral Blood Mononuclear Cells and HIV-1 Infection

Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient fractionation from the buffy coat of blood samples collected from de-identified healthy subjects that were free from infections, particularly HIV-1, and had no reported use of drugs of abuse, especially tobacco. Blood samples were provided by Interstate Blood Bank Inc. (Memphis, TN)[21]. All procedures were performed under the guidelines of the Institutional Review Board of The University of Tennessee Health Science Center. Monocytes were separated from whole blood by RosetteSep™ Human Monocyte Enrichment Cocktail [221] layered on Ficoll and centrifuged at 1200 g for 20 minutes. The white ring of PBMCs were collected carefully, washed at least 3 times to remove Ficoll, and incubated with ammonium-chloride-potassium lysis buffer to lyse red blood cells. Following an overnight incubation with RPMI media containing human serum, the media was replaced with fresh media supplemented with macrophage colony stimulating factor (50 ng/ml) to promote differentiation into macrophages [222]. After 7-10 days, macrophages were collected and treated with Polybrene (2 μg/ml) and interleukin-2 (10 ng/μl), and then infected with the HIV-1-Ada strain at 20 ng/$10^6$. Infected and uninfected cells were seeded in 6-well plates and fresh media was added every 3-4 days. The infection level was measured using an ELISA kit to quantify the viral p24 antigen [21]. Once the infection was confirmed, the cells were cultured in fresh RPMI media containing Exo-FBS and treated with control/CSC and/or EVs as described above.

## Preparation and Isolation of EVs

Supernatant from cell culture media was used to isolate EVs using the Invitrogen Total Exosome Isolation (from cell culture media) kit (Life Technologies, NY) and using ultracentrifugation [223, 224]. We isolated EVs from both monocytes and MDM. Studies have demonstrated that although monocytes and MDM both can be infected by HIV-1, MDM are more susceptible to infection [225]. Moreover, inflammatory conditions provoke generation of MDM at the site of the stimulus [226]. For isolation of EVs, cell supernatant was centrifuged (2000 g for 30 minutes) and the total exosome isolation reagent was added to the supernatant followed by overnight incubation at 2°- 8°C. After an hour of centrifugation (10,000g, 2°- 8°C) EV pellets were obtained. For ultracentrifugation, initially the cells were centrifuged at lower speeds (300 g, then 2000 g for 10 minutes each) followed by higher speeds (10,000 g for 30 minutes and 100,000 g for 70 minutes) to remove cell debris. The pellets containing EVs were washed with PBS and centrifuged again (100,000 g for 70 minutes). The ultracentrifugation step is tedious and requires >10-times media; therefore, it was avoided for experiments that needed large amount of EVs.

For isolation of EVs from plasma, we used the Plasma Exo Kit from Applied Biosystems (Foster City, CA) [67]. Plasma was collected from 4 study groups: healthy (n=4), smoker (n=4), HIV-1 (n=4), and HIV-1-smoker (n=4), with written informed consent and with approval from the Institutional Review Board of the University of Missouri-Kansas City (Kansas City, MO) and the Institutional Ethics Committee of the

Provincial Regional Hospital of the Ministry of Public Health in Bamenda, Cameroon [47]. EVs were collected following the manufacturer's protocol as discussed in our previous studies [47, 67]. Briefly, 100 µl plasma from each study group was centrifuged at 2000 g and 10,000 g for 20 minutes each to remove debris. Next, 0.5 volumes of 1X PBS was added to the clarified plasma followed by addition of 0.2 volumes of exosome precipitation reagent. After 10 minutes of incubation period, the mixture was centrifuged at 10,000 g for 5 minutes. EV pellets were collected as a pellet on the bottom of the tube. The EVs isolated were already well-characterized in terms of size and shape by dynamic light scattering and transmission electron microscopy (TEM), exosomal biomarkers by western blot, and acetylcholinesterase activity by kit assay in our previous publications [47, 67, 183].

To determine the impurities of HIV-1 and/or viral proteins in EV samples, we measured the relative amount of a viral p24 protein in the U1-derived EVs and compared it with the U1 media. EVs carry 10-15 times less p24 than the media. The presence of detectable p24 in the EVs may be as a result of a 5-10% impurity in isolation method or packaging of p24 in EVs. This suggests that p24 present in EVs is unlikely to affect our results. This is also reflected in our findings, in which the exposure of EVs from U1/HIV-1-infected primary human macrophages (PHM) to naïve U1 or HIV-1-infected PHM showed a decrease in the viral load. EVs were further characterized using dynamic light scattering, TEM, and identification of exosomal marker proteins (CD63, CD81, and Alix).

## Electron Microscopy and Dynamic Light Scattering

EVs were analyzed by dynamic light scattering using a Zetasizer Nano-ZS from Malvern Instruments Inc. (Malvern, UK), as described previously [227]. The EVs were dissolved in 1 ml ultra-pure water and 1 ml PBS for determining size and zeta potential, respectively. To further confirm the size, shape, and quality of EVs from cultured media, we employed JEOL 2000EXII TEM (The Neuroscience Institute, University of Tennessee Health Science Center). Freshly prepared EVs were resuspended in 1X PBS and loaded on slides as described [228].

## EV Counting

The isolated EVs were evaluated by tunable resistive pulse sensing (TRPS), with the help of qNano gold (Izon Science, Christchurch, New Zealand). Initially, the instrument was calibrated with polystyrene beads (CPC100), using a final dilution of 1:500. 35µl of each sample was analyzed using a polyurethane nanopore (NP150, Izon), with a stretch of 47.01nm and blockade voltage of 0.30mV. The standard and EV samples were allowed to pass through the nanopore at 15mbar pressure. A minimum of 500 particles was counted for each run. Data processing and analysis were performed using the Izon control suite v3.3 as per the manufacturer instructions.

## EVs Labeling, Uptake, and Flow Cytometry

The uptake of EVs by U937 macrophages was monitored by using Exo-GLOW$^{TM}$ Exosome Labeling Kits from System Biosciences (Palo Alto, CA). EV pellets containing 100-500 µg protein were suspended in 500 µl of 1X PBS, with the subsequent addition of 50 µl 10X Exo-Green exosome protein fluorescent label. The EV solution was incubated at 37°C for 30 minutes followed by the addition of 100 µl ExoQuick-TC to stop the labeling reaction. The labeled pellet was exposed to U937 macrophages and their uptake at different time points was visualized by fluorescent microscopy and flow cytometry.

## DNA, RNA, and Protein Isolation

DNA and RNA were isolated from cells using DNeasy Blood & Tissue Kit and RNeasy Mini kits from QIAGEN (Germantown, MD), respectively [10]. For isolating DNA, cell samples were lysed with proteinase K in DNAeasy Mini Spin column and centrifuged. DNA was bound to the membrane. The contaminants and remaining enzyme inhibitors were washed. Isolated DNA was eluted with the preferred buffer, and the concentration was measured at 260 nm. For obtaining RNA, the samples were lysed and homogenized in guanidine-thiocyanate–containing buffer to purify intact RNA. Next, ethanol was added to the sample, followed by transferring the samples to the RNAeasy mini spin column. Total RNA was bound to the membrane while the contaminants were washed out. RNA was then eluted in water and concentration was measured at 260 nm. Exosome RNA and Protein Isolation Kits (Life Technologies, NY) were used to obtain RNA and protein from the EVs. For isolating protein and RNA, the EV samples were resuspended in the buffer provided with the kit. This solution could be directly used for protein quantification. For RNA, denaturing solution was added to the solution, followed by a brief incubation and addition of one volume of Acid-Phenol:Chloroform. The samples were mixed thoroughly by vortex and centrifuged to separate into organic and aqueous phases. RNA would be in the aqueous phase, to which ethanol was added. The lysate/ethanol mixture was applied on the filter cartridge, centrifuged, and washed with wash solution, with subsequent centrifugation. The RNA was bound to the filter cartridge. Next, RNA was eluted with elution solution. A Nanodrop 2000c Spectrophotometer from Thermo Fisher Scientific (Rockford, IL) was utilized for quantifying RNA and DNA. All procedures were performed following the suppliers' protocols.

## Quantification of Protein and Antioxidant Capacity

Protein from cells and EVs was quantified by BCA protein assay kit (Thermo Fisher Scientific). Protein from cells was lysed using RIPA buffer with protease inhibitor, and EV pellets were resuspended in exosome resuspension buffer. The required volume and dilution of protein isolated from cells/EVs was taken in a 96-well plate and mixed with BCA working reagent, at a 1:8 ratio. After 30 minutes of incubation at 37ºC, the absorbance was measured at 540-590 nm using a plate reader (Cytation 5). The protein

isolated from EVs was subjected to an antioxidant capacity assay, using the Total Antioxidant Capacity Colorimetric Assay Kit from BioVision (Milpitas, CA) as described by the manufacturer. The protein samples were diluted according to the manufacturer's suggestion and incubated with 100 µl working solution at a 1:1 ratio for 1.5 hours. The absorbance was measured at 570 nm.

## Cytotoxicity

For measuring the cytotoxicity caused by our various treatment protocols, we used the PierceTM LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) as described by the manufacturer. This assay measures extracellular lactate dehydrogenase (LDH) in the media, which is indicative of cellular damage. The supernatant was incubated with LDH reaction mixture and the optical density was measured at 490 and 680 nm.

## DNA Damage Assay

The compound, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a common marker for DNA damage [229]. The EpiQuik$^{TM}$ 8-OHdG DNA Damage Quantification Direct Kit (Colorimetric) from EpiGentek (Farmingdale, NY) was used to measure 8-OHdG levels following the manufacturer's protocol. First, DNA was isolated from cell samples as mentioned earlier, followed by DNA denaturation. DNA was digested with nuclease PI. Next, the sample was mixed with a 8-OHdG conjugate-coated plate and incubated at room temperature on a shaker. Anti-8-OHdG antibody was added to each well and incubated for an hour. Then, the wells were washed 3 times with wash buffer. DNA was bound to the wells by binding solution and incubated sequentially with capture and detector antibodies. After addition of the developer solution, the signal was measured at 450 nm [22].

## Viral Load Count

The viral load was determined in the supernatant collected from U1 and PHM (post treatment with control/CSC and/or EVs) by using the HIV-1 p24 Antigen ELISA kit from Zeptometrix Corporation (Buffalo, NY) [21]. This assay measures the level of p24, a structural protein of HIV-1. Briefly, the viral antigen was captured on the immobilized antibody, followed by a reaction with biotin-conjugated human anti-HIV-1 antibody and subsequent incubation with streptavidin-peroxidase. The reaction between the substrate and antibody was measured at 450 nm. We used a standard curve for p24 to determine the viral load from our samples.

## Quantitative Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

Quantitative RTPCR was employed to determine relative mRNA fold expression level following the protocol described in our previous studies [10, 21]. Briefly, purified RNA from cell extract (80-120 ng) and EVs (10-20 ng) was reverse transcribed to cDNA using a SimpliAmp Thermal Cycler (Applied Biosystems) and was amplified in a Step-One Plus Real-Time PCR System (Applied Biosystems) using the TaqMan Gene Expression kit (Applied Biosystems). We determined the mRNA expression level of CYPs 1A1 (Hs01054794_m1), 2A6 (Hs00868409_m1), and 2E1 (Hs00559368_m1), and the antioxidant enzymes (AOEs) superoxide dismutase (SOD1 (Hs_00533490m1) and SOD2 (Hs00167309_m1)), catalase (Hs00156308_m1), and glutathione S-transferase kappa 1 (GSTK1) (Hs00210861_m1). We tested the expression of a known exosomal marker RNA (U6snRNA) and the cellular house-keeping genes actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to analyze the relative expression of EV mRNAs compared to cells, and the effect of CSC exposure on the expression of these mRNAs. The cycle numbers of U6snRNA and actin were undetermined and very low, respectively. However, the expression of GAPDH mRNA was low (<40 cycles) but sufficient to be measured without much difference between samples. Therefore, we used GAPDH as an internal control for both cellular and EV mRNAs. As expected, the relative level of GAPDH mRNA expression in EVs was much lower than in cells. The relative expression of cellular and EVs mRNAs were calculate using the fold expression for these genes by the $2^{-\Delta\Delta Ct}$ method.

## Western Blotting

To determine the relative expression of protein, approximately 20-25 µg of protein from cell extracts and 5-10 µg protein from EVs were loaded into polyacrylamide gel (4% stacking, 10% resolving gel). Upon running the gel, the proteins were transferred to a polyvinyl fluoride membrane and were blocked with 5-10 ml of Li-Cor blocking buffer from LI-COR Biosciences (Lincoln, NE) for 1 hour. The membranes were incubated overnight with primary antibodies (GAPDH, CYP1A1, CYP2A6 Rabbit Mab, Abcam, Cambridge, MA; CYP1A1 rabbit Mab, Abcam, Cambridge, MA; PRDX6 Rabbit Mab, LS Bio Seattle, WA; SOD1, Catalase, CD63, CYP3A4 Mouse Mab, CD81 Rabbit Mab, GSTK1 Goat Mab, Santa Cruz, Dallas, TX; β actin Rabbit Mab, Cell Signaling, Danvers, MA; Alix Rabbit Mab, Protein Tech, Rosemont, IL; CD9 mouse Mab, Protein Tech, Rosemont, IL) at 4°C. The antibody dilution ranged from 100 to 500 times, as appropriate. The next day, the membranes were washed and incubated with respective secondary antibodies (goat anti-Mouse Mab, goat anti-Rabbit Mab, donkey anti-Goat Mab) and the signal was detected using LI-COR (Biosciences, Lincoln, NE). To determine the fold expression of the proteins, the densitometry data was obtained using Image Studio Lite version 4.0.

## Cytokine Analysis

For cytokine analysis, we used freshly isolated EVs from 500 µl media and respective cell media. The levels of selected cytokines and chemokines, such as the pro-inflammatory cytokines IL-6, IL-8, IL-1β, and TNF-α, the anti-inflammatory cytokines IL-1ra and IL-10, and the chemokines MCP-1 and RANTES were measured using ProcartaPlex™ Multiplex Immunoassay (Invitrogen, Thermo Fisher Scientific)[230]. The EV pellets were resuspended in 50 µl Universal Assay buffer, and 50 µl of cell media was taken directly from the respective well. Following the kit protocol, the samples, standards, and magnetic beads were added to the 96-well ELISA plate and incubated (shaking at room temperature) for 1 hour. The beads were washed, followed by addition of the detection antibody, streptavidin-peroxidase, and reading buffer, and subsequently washed. The final protein concentration was measured using a Luminex 200$^{TM}$ system, and the data were analyzed by xPONENT® software.

Next, we calculated the percent of cytokines packaged in the EV. We analyzed the relative packaging of cytokines in the extracellular vesicles in comparison with the respective media cytokines. The "Percent of EV packaging" refers to the percentage of cytokine packaged in the EVs compared to the cytokines present in the media. We calculated the percentage using the following equation:
Percent of EV packaging = (Concentration of cytokines in the EVs ÷Concentration of cytokines in the media) ×100

## Statistical Analysis

Mean ± standard error of the mean (SEM) was calculated and normalized against the control group for generating data from Aim 1. Mean ± SEM was calculated and compared to the control group for generating data for Aim 2. Two-tailed t-tests and one-way ANOVA were used respectively for Aim 1 and Aim 2 to calculate the statistical significance. All the statistical calculations were performed using GraphPad Prism 7 (San Diego, CA).

# CHAPTER 4. RESULTS AND DISCUSSION

## Monocyte-Derived Extracellular Vesicles upon Exposure to Cigarette Smoke Condensate Alter Their Characteristics and Show Protective Effects Against Cytotoxicity and HIV-1 Replication[4]

Approximately 480,000 people in the United States die each year due to the hazards of smoking [231].A recent study by Mdege. et al. in 28 low- and middle-income countries has revealed a high prevalence of tobacco usage among HIV-1-infected people [232]. Cigarette smoke affects the homeostatic balance between antioxidants and ROS, leading to oxidative damage [10, 13]. The oxidative injury results in various pathological complications: respiratory (chronic obstructive pulmonary disease, asthma), brain (ischemic stroke, Alzheimer's disease, Parkinson's disease), cardiovascular (coronary heart disease, cardiac stroke), and cancers (lung, cervix, stomach, liver, kidney, and esophagus) [233-241]. In addition, the prevalence of various non-AIDS-associated diseases in HIV-1-positive smokers is significantly higher than HIV-1-positive non-smokers [19]. Several reports suggest that tobacco exacerbates HIV-1 replication as well, through the oxidative stress pathway [21, 22, 203, 242].

We hypothesized that extracellular vesicles (EVs) influence the oxidative stress mediated pathway of smoking-induced HIV-1 pathogenesis. In recent years, EVs have gained much attention due to their role in cell-to-cell communication [180, 243, 244]. The contents inside EVs may change under stress conditions such as disease and infection, suggesting their use as diagnostic biomarkers. EVs from lymphocytic and monocytic cells are shown to contain miRNA, viral transactivators, and cytokines that affect the course of HIV-1 infection [245-247]. As mentioned earlier, EVs have a dual role in HIV-1 pathogenesis, i.e. they can be either beneficial or detrimental to recipient cells, depending on their origin [158, 206]. Keeping this in mind, here we examined how EVs from monocytes communicate with uninfected and HIV-1-infected cells to influence smoking-enhanced cellular toxicity and viral replication in HIV-1-infected monocyte-derived macrophages (MDM).

Initially, we characterized the EVs derived from uninfected and HIV-1 infected monocytes treated with cigarette smoke condensate (CSC). These EVs were further exposed to uninfected and HIV-1-infected MDM. We observed that EVs derived from CSC-treated, uninfected monocytes exhibit a better protective function against cytotoxicity and HIV-1 replication than those derived from the HIV-1-infected cells. The findings in this study suggest that EVs are likely to function as a vehicle to transport protective messages from uninfected monocytes to naïve HIV-1-infected macrophages.

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[4] Modified from final submission with open access permission. Sanjana Haque, Namita Sinha, Sabina Ranjit, Narasimha M. Midde, Fatah Kashanchi & Santosh Kumar. Monocyte-derived exosomes upon exposure to cigarette smoke condensate alter their characteristics and show protective effect against cytotoxicity and HIV-1 replication Sci Rep 7, 16120 (2017). https://doi.org/10.1038/s41598-017-16301-9. [48]

We used uninfected and HIV-1-infected monocytic cells as well as HIV-1-infected and uninfected PHM in this study. These cells are model systems for HIV-1-related work. Our recent studies with these cells have also shown the effect of CSC and/or nicotine exposure on oxidative stress and HIV-1 replication [21, 242]. In this study, we extended our work to establish the role of monocytic EVs in cellular communication among HIV-1 infected and uninfected cells upon CSC exposure.

Please note that, in the initial figures, EVs are indicated by Exo because these figures were originally published in Scientific Reports, as EVs were commonly referred as exosomes at the time of publishing in 2017 [48].Since the release of the MISEV 2018 guidelines, it is recommended to refer the vesicles under the umbrella term "extracellular vesicles (EVs)". However, for consistency we have used EVs throughout the thesis and figure legends, except within these figures.

**Effect of CSC on the Physical Characteristics, Protein Content, and Antioxidant Capacity of U937 Cell-Derived EVs and Their Uptake by Recipient Cells**

Protein extracts from CSC-treated U937 cells and EVs derived from those cells were separated by SDS-PAGE and stained with Coomassie Brilliant Blue, along with EVs and lysate from untreated control cells. As shown in **Figure 4-1a**, a relatively lower total protein presence and lower quantity of individual proteins were observed in the EVs derived from the CSC-treated cells (CSC-EVs) than the EVs derived from the untreated cells (control-EVs). For the identification of EVs, we performed Western blotting using antibodies against CD63, Alix, and CD81, which are the protein biomarkers of EVs (**Figure 4-1b**). We observed weak, but detectable bands for all three proteins, which confirmed the presence of EVs in the pellet. We did not observe any band for β-actin in EV pellets, which further supports our finding. Interestingly, CSC treatment showed an increase in the expression of CD63 and Alix, and a decrease in the expression of CD81.

Furthermore, to confirm the altered level of protein in CSC-EVs (**Figure 4-1a**), the total proteins in the EVs derived from the treated and untreated cells were quantified. EVs from CSC-treated cells showed a significant reduction in protein level (**Figure 4-1c**, * $p<0.05$) compared to control. We also measured whether the altered level of protein in CSC-EVs changed total antioxidant capacity in those EVs. The results showed that EVs from CSC-treated cells showed a significant reduction in total antioxidant levels (**Figure 4-1d**, * $p<0.05$) compared to control. Thus, in the following experiments, we determined whether the decrease in EV protein levels with CSC exposure is the result of a decrease in the total number of EVs produced by these cells or a decrease in the average amount of proteins packaged per EV.

Next, the isolated EVs were characterized by transmission electron microscopy (TEM) and dynamic light scattering to observe the effects of CSC on the number and size of U937 cell-derived EVs. Negative staining of samples showed the presence of multiple membraned vesicles with a mean size of 66 ± 1.4 nm (control) and 66 ± 3.3 nm (CSC) (**Figure. 4-2a-c**). Quantitative analysis from the control-EVs (number of images = 82)

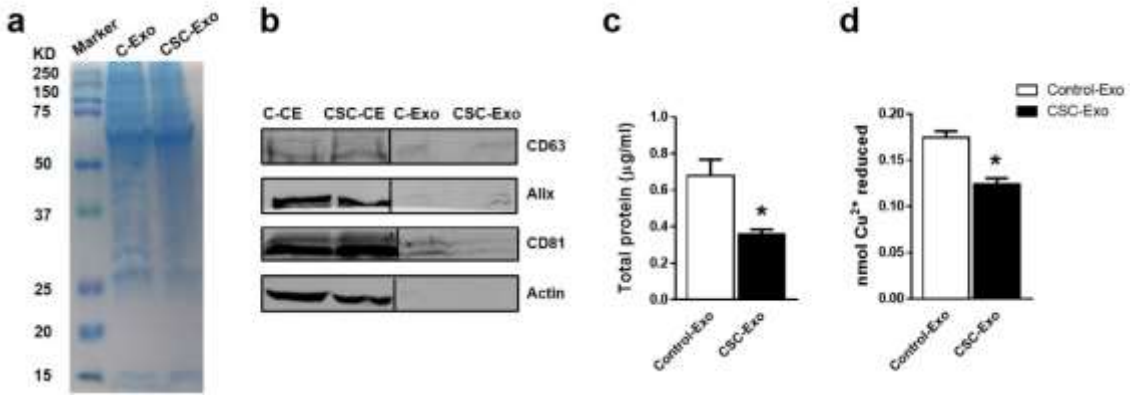


**Figure 4-1. Effect of CSC on U937 Cell-Derived EV Proteins and Antioxidant Properties.**

(a) Coomassie blue staining patterns of EVs that were derived from control or CSC-exposed U937 monocytic cells. Equal amount of protein (10 µg) was loaded in each well. (b) Western blot of EV marker proteins CD63, CD81, and Alix and cellular marker protein actin. Equal amounts of protein (10 µg) were loaded in each lane. C-CE: control-cell extract, CSC-CE: CSC-cell extract, C-Exo: Control-EV, CSC-Exo: CSC-EV. (c) Total protein levels of EVs that are quantified using the BCA protein estimation method. Bars indicate mean ± SEM values from three replicates. (d) Total antioxidant capacity of EVs measured by total antioxidant capacity assay. * indicates $p<0.05$ compared to control.

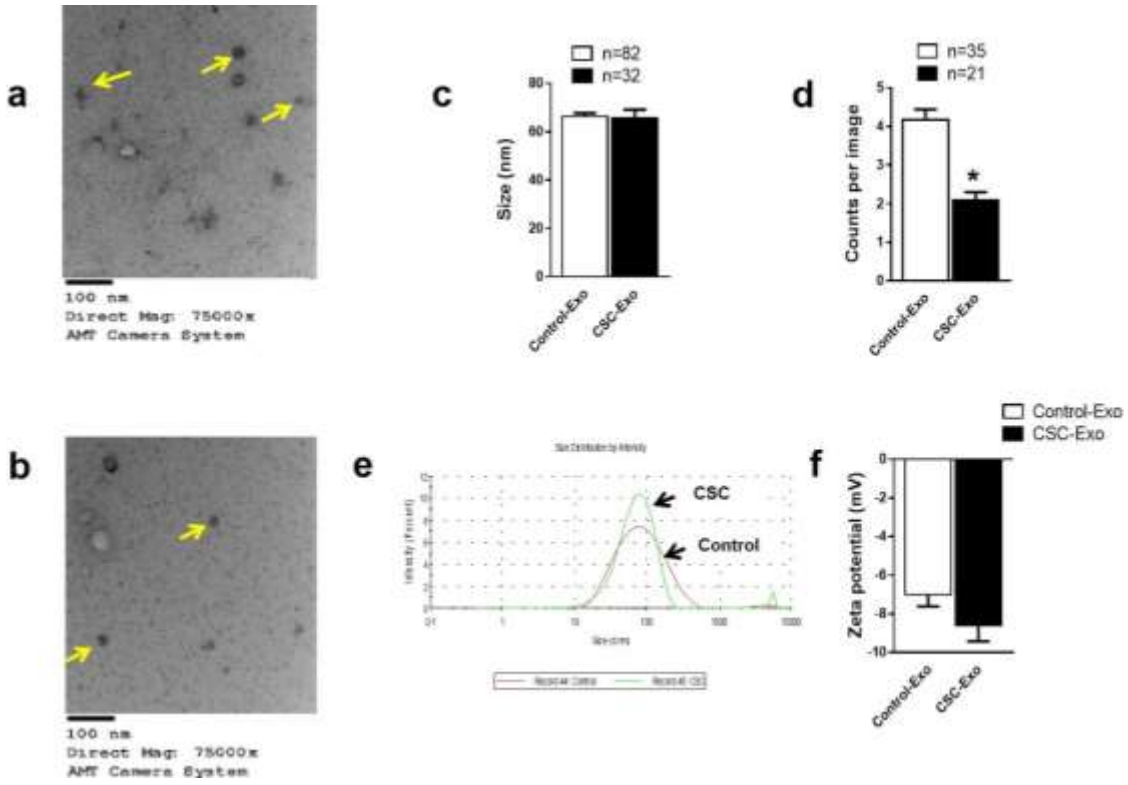


**Figure 4-2. Effect of CSC on Physical Characteristics of Monocyte-Derived EVs.** Representative TEM images of (a) control and (b) CSC treatment-derived EVs. Arrows indicate the vesicles that are considered EVs with size ~100 nm and intact membranes. Quantitative analysis of EV count and diameter from three independent experiments are shown in (c) and (d), respectively. Bars indicate mean ± SEM values; 'n' shows the number of images that were analyzed to measure EV count or size. The average hydrodynamic size and size distribution (e) and zeta potential (f) of EVs were measured using dynamic light scattering with three independent replicates. Control-exosome: Control-EV, CSC-Exosome: CSC-EV. * indicates $p<0.05$ compared to control.

and the CSC-EVs (number of images = 32) did not show any significant difference in particle size (**Figure 4-2c**). However, CSC-exposure (n=21) significantly reduced the number of EVs compared to control (n=35) (**Figure 4-2d**, * $p<0.05$). Similar to TEM size distribution, we did not observe any significant variation in the mean diameter of the EVs when hydrodynamic size was measured using dynamic light scattering (**Figure 4-2e**). However, there is a visual change in the shape of the peaks; CSC-EVs have a sharper and more concise peak than the control. Additionally, control and CSC-EVs showed mean negative zeta potentials of $-7.0 \pm 0.6$ and $-8.6 \pm 0.8$, respectively, owing to the negative charge of the phospholipid membrane. Expectedly, CSC treatment did not show any noticeable change to the zeta potential of EVs compared to the control (**Figure 4-2f**, n=3). Overall, we observed no significant difference in the size and zeta potential, but a significant decrease in the total number of EVs upon CSC exposure to U937 cells.

For determining EV uptake, EVs isolated from control and CSC-treated plasma were exposed to differentiated U937 MDM for 6 hours. The figure obtained from flow cytometry (**Figure 4-3a**) shows a clear difference in the cellular uptakes from the EVs derived from CSC-exposed cells. From the graphical representation (**Figure 4-3b**), it is apparent that the uptake of CSC- EVs is significantly lower than control- EVs (**Figure. 4.3b**, * $p<0.05$). This finding is also supported by the fluorescent microscopy images of the wells (**Figure 4-3c** and **d**). In the previous section, we demonstrated that CSC exposure causes an overall reduction in the release profile of EVs (**Figure 4-3d**), which may explain a relatively lower uptake of CSC- EVs than control- EVs.

Our detailed characterizations using size determination and zeta potential clearly suggest that the majority of EVs are within the accepted exosomal size limit of <200 nm. The TEM image, which shows the EVs to be ~100 nm in diameter, with a transparent double membrane, further validates the quality of these EVs. The uptake of EVs by U937-macrophages ensures that EVs can fuse with the recipient cells. Finally, the presence of the EV marker proteins CD63, Alix, and CD81, together with the absence or negligible level of the cellular marker protein actin, clearly suggest the purity of our EV samples.

Our analysis of EVs from CSC-exposed monocytic cells did not show altered size distribution. This result is consistent with earlier studies in which cells treated with cigarette smoke extract, cocaine, or ethanol also did not reveal any effect on EV size [21, 248, 249]. However, we observed a decrease in protein content and antioxidant capacity in the EVs derived from CSC-treated monocytic cells. Since the average size did not change upon CSC treatment, a decrease in protein content suggests a decrease in the number of EVs upon CSC exposure.

### Effect of CSC and CSC-EVs on Cytotoxicity and DNA Damage in U937 Cells

To determine the cytotoxicity caused by CSC and CSC-EVs on U937 cells, we performed lactate dehydrogenase (LDH) cytotoxicity and DNA damage assays. Differentiated U937 cells were treated with DMSO as control, with CSC, or with EVs

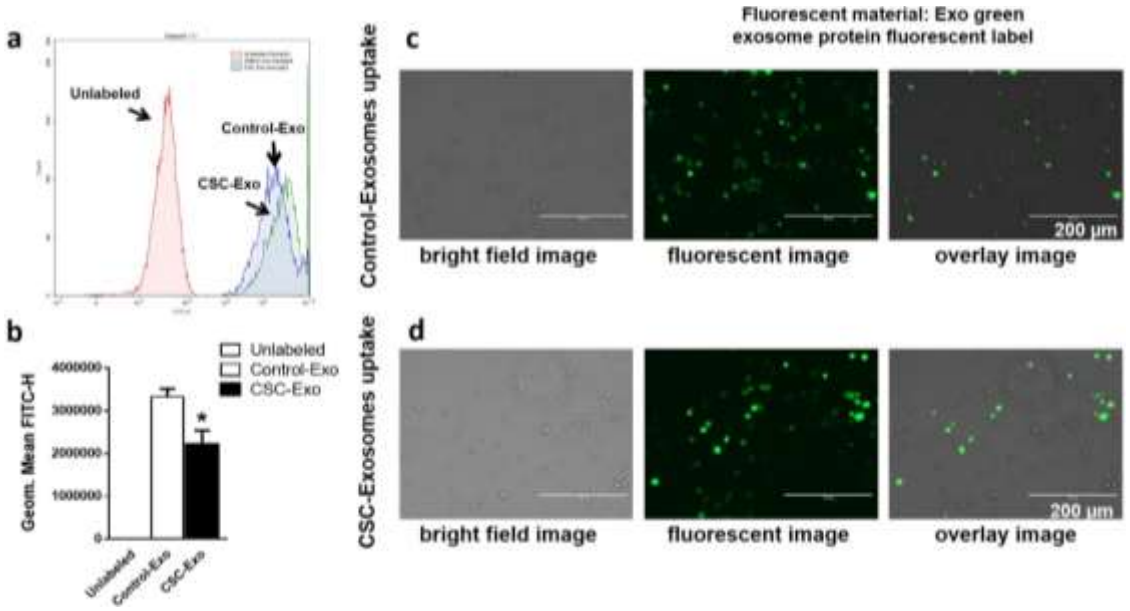


**Figure 4-3. Effect of CSC on EV Uptake into the Recipient Monocytic Cells.**
(a) Flow cytometry histogram showing the presence of labeled EVs (10X Exo-Green exosome protein fluorescent label) in the macrophages after 6 hours of exposure. (b) Fluorescence intensity in CSC- EV treated cells compared with DMSO-EV treated cells. (c and d) bright field, fluorescent, and overlay images of cells after respective treatments and 6 hours of exposure. Microscope was set to visualize particles of 200 µm diameter. Bright field and fluorescent images represent only cells and cells with EVs, respectively. Overlay image combines both of these to demonstrate presence of EVs within the cells. *indicates $p<0.05$ compared to control-EVs. Control-Exo/Control-exosome: Control-EV, CSC-Exo/CSC-Exosome: CSC-EV.

derived from control or CSC-treated U937 cells. The results obtained from the LDH assay showed that cells treated with CSC-EVs (EVs derived from CSC-treated U937 cells) suffered significantly higher toxicity in comparison with the control and CSC-treated cells (**Figure 4-4a**, ** $p<0.01$ compared to control, # $p< 0.05$ compared to CSC). CSC and control-EV treated cells also displayed significantly higher toxicity than the control (* $p<0.05$, ** $p<0.01$). Further analysis showed an additive effect of CSC- and control-EV treatment on the cytotoxicity of U937 cells.

Since CSC and tobacco constituents are known to cause DNA damage [250], we determined the relative amount of DNA damage by using compound, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in our functional assay. The results showed that CSC-EV-treated cells had significantly higher DNA damage compared to control as well as CSC-treated and control-EV-treated cells (**Figure 4-4b**, * $p<0.05$). Similar to cytotoxicity, CSC- and control-EV-treated cells also demonstrated a pattern of higher DNA damage than control. Further, the increase in DNA damage by CSC-EV-treated cells appears to be higher than the CSC- and control-EV-treated cells.

Cigarette smoke is known to cause oxidative stress, often resulting in DNA damage and ultimately cellular toxicity [21, 251]. However, the DNA damage can be repaired depending upon the concentration and time of exposure of the toxicant [252]. In our previous study we observed a significant increase in cellular toxicity after treating monocytes and macrophages with CSC [21]. Similarly, in our current study, we observed an elevation in toxicity and significant loss of cell viability in uninfected macrophages when they were exposed to CSC. Moreover, cytotoxicity further increased with the exposure of EVs to the macrophages. EVs may carry substances that could be toxic to the cells. Lenassi et al. have shown that EVs carry an HIV-1 Nef protein, which triggers apoptosis in CD4+ T cells [253]. Another study by Emmanouilidou et al. also mentions that EVs are capable of carrying toxic proteins like α-Synuclein, which promotes neurotoxicity in Parkinson's disease [254]. With CSC and CSC-EV treatment, we observed an additive increase in cytotoxicity that is associated with an increase in DNA damage. This result suggests that cytotoxicity through CSC and EV exposure could have occurred via DNA damage pathway, which is known to be a major pathway for tobacco-induced toxicity [252, 255].

### Effect of CSC/CSC-EVs on Exosomal Protein Content, HIV-1 Replication, Cytotoxicity, and DNA Damage in U1 Cells

After observing the impact of CSC and CSC-EVs on uninfected cells, we next determined the impact of similar treatment on the HIV-1 infected U937 cell line, U1 (**Figure 4-5**).

Prior to CSC or CSC-EV treatment on U1 cells, we determined whether CSC treatment to those cells alters total EV protein and antioxidant levels. Quantification of total protein from EVs derived from control/CSC-treated U1 cells showed that CSC-EVs have a lower protein content compared to the control-EVs (**Figure 4-5a**). As observed in

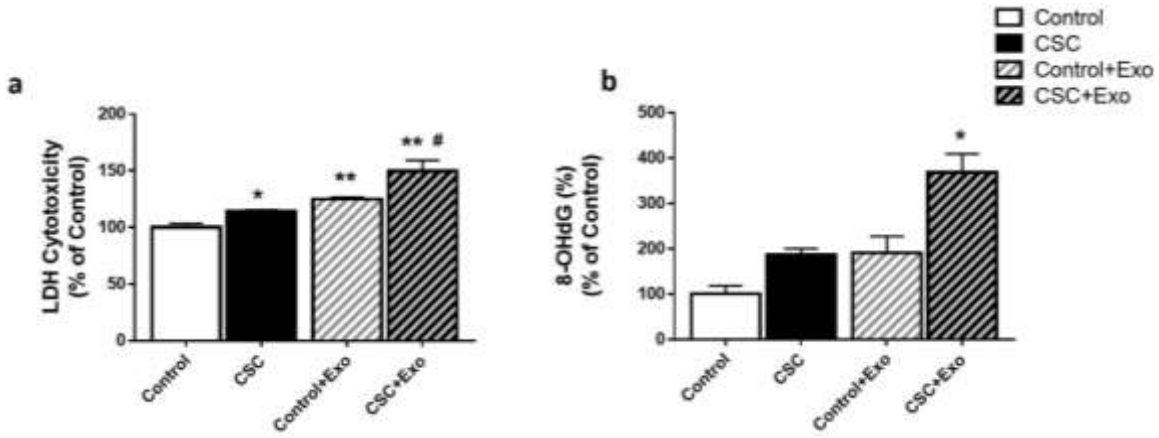


**Figure 4-4. Effect of CSC on Cytotoxicity and DNA Damage in U937 Cells.**
(a) Cytotoxicity in U937 macrophages after treating them with control, CSC, or EVs from U937 cells was determined by LDH assay. Macrophages were exposed to U937-derived EVs and 10 µg/ml CSC daily dose for 3-4 days. Bars indicate mean ± SEM values from three replicates. (b) Represents the extent of DNA damage in cells after exposure to control, CSC, or indicated EVs. We measured 8-OHdG, a major product of DNA oxidation. The amount of 8-OHdG detected is directly proportional to the extent of DNA damage due to treatment. All the bars are normalized against the control. Bars indicate mean ± SEM values from two replicates. * and ** indicate $p<0.05$ and $p<0.01$ compared to control, respectively, # indicates $p<0.05$ compared to CSC. Control+Exo: Control-EV, CSC+Exo: CSC-EV.

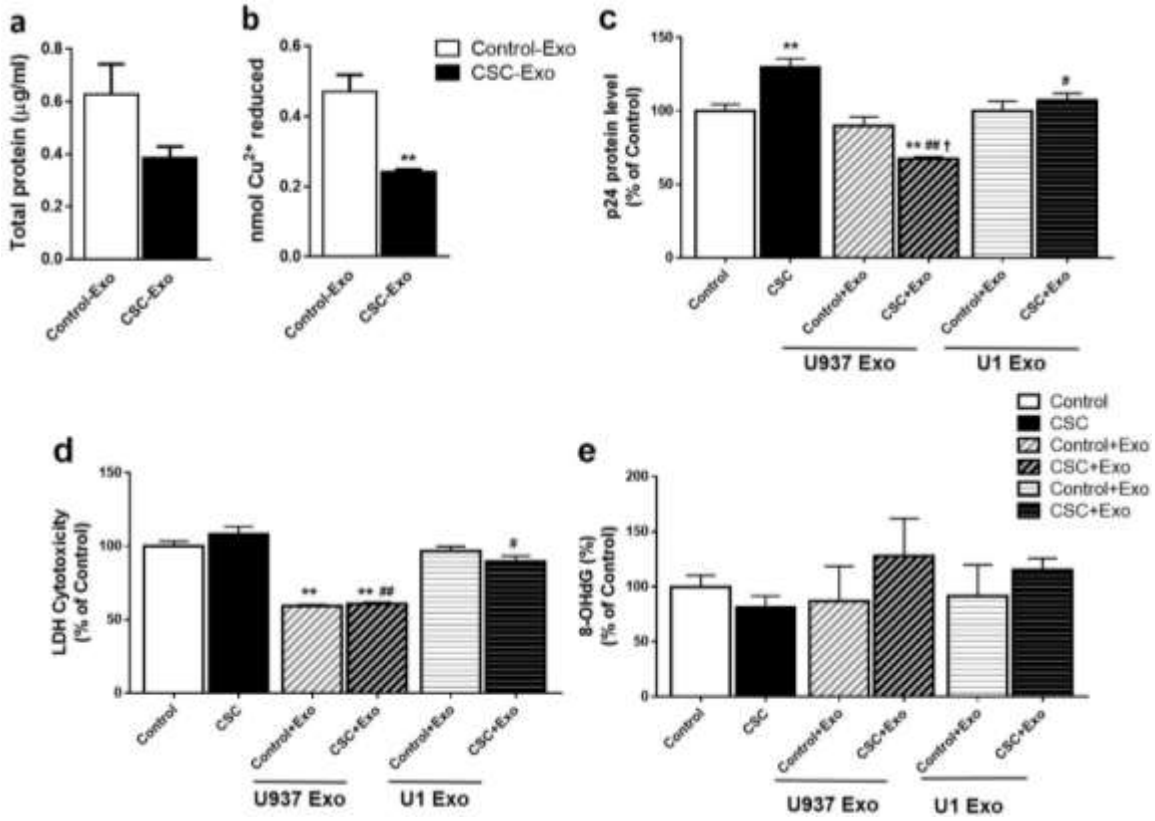


**Figure 4-5. Effect of CSC/CSC-EVs on EV Protein Content, HIV-1 Replication, Cytotoxicity, and DNA Damage in HIV-1-Infected U1 Cells.**
(a) Total protein levels of EVs from U1 cells treated with control or CSC. Protein quantification was performed by BCA protein assay kit. Bars indicate mean ± SEM values from three replicates. (b) Total antioxidant capacity of treated U1 EVs measured by Total antioxidant capacity colorimetric Assay kit. Bars indicate mean ± SEM values from two and three replicates for control and CSC EVs, respectively. Control-Exo: control-EV, CSC-Exo: CSC-EV. (c) HIV-1 p24 levels after treating HIV-1-infected U1 cells with control, CSC, or indicated EVs that were measured by ELISA kit. (d) Cytotoxicity levels in U1 cells after exposure to control, CSC, or EVs from indicated cells were determined by LDH assay. (e) DNA damage levels in terms of 8-OHdG% in cells treated with control, CSC, or indicated EVs. All bars (Fig. c-e) were normalized against controls. Bars indicate mean ± SEM values from at least three replicates. **indicates $p<0.01$ compared to control, # and ## indicate $p<0.05$ and $p<0.01$ compared to CSC, respectively, † indicates $p<0.05$ compared to control+exo. Control+Exo: Control-EV, CSC-Exo:CSC-EV.

the EVs obtained from U937 cells (**Figure 4-1d**), CSC-EVs derived from U1 cells also showed significantly lower antioxidant levels compared to the control (**Figure 4-5b**, **p<0.01). HIV-1 replication was measured in U1 cells after treating with control, CSC, and/or EVs derived from CSC-treated U937/U1 cells. The p24 level (using ELISA kit) was determined as a measure of viral replication as described earlier [21]. As expected, CSC-treated cells have a significantly higher p24 level than control (**p<0.01). The exposure of CSC-EVs from U937 and U1 cells to differentiated U1 MDM significantly reduced viral replication (**Figure 4-5c**, # p<0.05 and ## p<0.01 compared to CSC). While comparing with CSC treatment, we observed a reduction of 51 ± 3 % in p24 level by CSC-EVs from U937 (CSC-EV/U937) and a reduction of 29 ± 5 % by CSC-EVs from U1 (CSC-EV/U1). These results suggest that EVs derived from uninfected U937 cells exert more protection against viral replication than those from infected cells (p<0.037). Compared to control, CSC-U937-EV treatment reduced p24 significantly (** p<0.01). In addition, this group showed a significant reduction in the viral load compared to the control-U937-EV-treated group († p<0.05).

To investigate the association between the effect of CSC-EVs on viral load and cytotoxicity, we measured cytotoxicity in CSC-EV-treated U1 cells. LDH cytotoxicity assays performed on the same treatment groups showed that EV exposure to the cells decreased cellular toxicity compared to the control cells (**Figure 4-5d**). While CSC did not show a significant effect on cytotoxicity, the U937 EV-treated groups showed a significant reduction in cytotoxicity (** p<0.05) compared to control. When we compared the difference between the CSC treatment group and CSC-EV (U937 or U1) treatment groups, the CSC-EV/U937-treated cells showed a significant reduction in cytotoxicity compared to CSC-EV/U1-treated cells (**p<0.004).

Furthermore, we measured DNA damage in these groups and compared the results with those of cytotoxicity and HIV-1 replication. Though not statistically significant, the U1 cells treated with CSC-EV/U1 or U937 showed relatively higher DNA damage than the control or only CSC-treated cells (**Figure 4-5e**). On the other hand, the control-EV/U1 or U937 treated cells showed a pattern of a decreased DNA damage compared to the control treated cells.

Overall, we observed that viral replication and cytotoxicity in U1 cells were relatively more suppressed by EVs from U937 cells than those from U1 cells. The results also suggest a positive association between the effects of CSC-EVs on viral load and cytotoxicity. Although, we observed a protective effect from the uninfected-EV, the viral suppression is diminished when the cells were treated with CSC-EVs from HIV-1-infected cells. It could be possible that these EVs provide protection against viral replication during the early stage of HIV-1 infection. However, with the progression of the disease, the protective capacity of the EVs may be compromised. The decreased cytotoxicity level in the same cells, after the treatment of CSC-EVs from uninfected monocytes, and further increase in cytotoxicity after treatment of CSC-EVs from infected cells, also corroborates our hypothesis.

**Effect of CSC and CSC-EVs on HIV-1 Replication and Cytotoxicity in HIV-1-Infected Primary Human Macrophages**

Although U937 and U1 are authentic cell lines for uninfected and HIV-1 infected monocytic cells, respectively [256, 257], the effects observed with these cell lines may not translate fully into primary macrophages. Therefore, we further investigated the effects of CSC and CSC-EVs on viral replication and cytotoxicity in HIV-1-infected primary human macrophages (PHM) obtained from a healthy donor. As these cells are directly derived from healthy patients' blood, they are less vulnerable to stimulated oxidative stress and viral replication compared to U937 and U1 cells. Based on the previously described protocol for entry of EVs into macrophages [258] and our uptake study on U937-macrophages (**Figure. 4.3**), we performed a similar study in PHM. Our results demonstrated an overall protection on viral replication from uninfected CSC-EVs, which resembles our findings in U1 macrophages. As shown earlier by our group [21, 22], CSC-treated PHM demonstrated significantly higher p24 levels compared to the control (**Figure. 4-6a**, * $p<0.05$). When the cells were treated with CSC or CSC/control-EVs from infected and uninfected PHM, we observed an overall reduction in p24 levels in the cells treated with CSC-EVs, compared to CSC (**Figure 4-6a**). This finding is similar to that observed in U1 cells (**Figure 4-5c**). Control-EVs derived from uninfected PHM (control-EV/uninfected macrophages) group showed a significant decrease in viral load compared to the control group. However, in contrast to U1 cells, control-EVs derived from infected macrophages (control-EV/infected macrophages) showed a significant increase (*$p<0.05$, **$p<0.01$). Similar to the U1 cells, CSC-EV/infected macrophages showed a significant decrease († $p<0.05$) in p24, when compared to control-EV/infected macrophages.

Overall, our results largely validated the effect of CSC, control-EVs, and CSC-EVs in HIV-1-infected PHM. Interestingly, CSC-EVs from HIV-1-infected cells also provided protection against viral replication in infected cells. This protection may have resulted from a difference in the formation of EVs upon CSC exposure to acutely infected PHM vs. the constitutively infected U1 cells. The results suggest that although CSC increases HIV-1 replication, EVs (derived from both control and CSC-treated cells) from both uninfected and HIV-1 infected cells show a protective effect.

In conclusion, this study demonstrates that EVs secreted from monocytes upon exposure to CSC mostly retain their physical properties while altering their EV protein level and antioxidant capacity. In addition, these EVs are readily taken up by the recipient MDM. Interestingly, EVs derived from the CSC-treated uninfected monocytic cells conferred protection against HIV-1 replication in U1 cells. It has been observed in previous studies that EVs derived from cells undergoing stress can be protective to recipient cells against further cellular damage [181]. This is the first study to show a similar protective effect by EVs in HIV-1-infected cells.

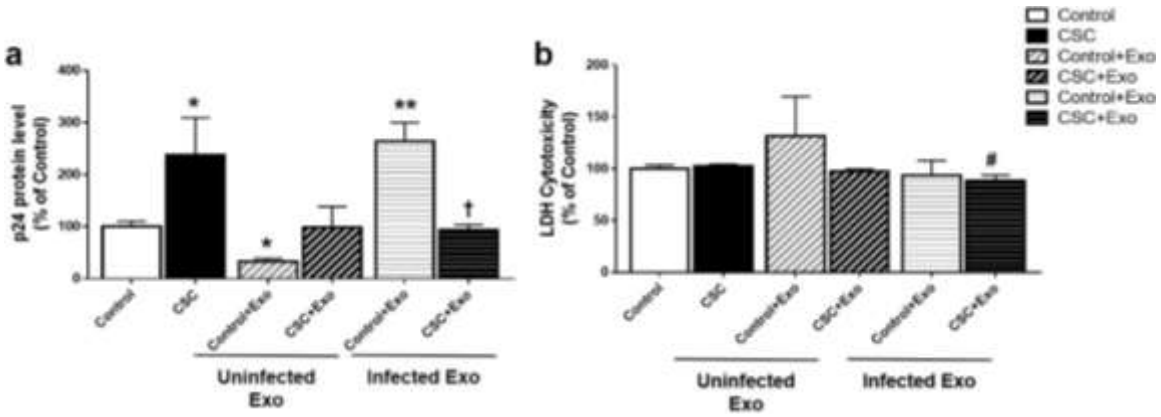


**Figure 4-6. Effect of CSC and CSC-EVs on HIV-1 Replication and Cytotoxicity in Primary Human Macrophages.**

(a) HIV-1 p24 antigen level after exposure of CSC/CSC-EVs to primary human macrophages. EVs were collected from HIV-1-infected or uninfected PHM and introduced to HIV-1-infected PHM along with 10 μg/ml of CSC. (b) LDH Assay on primary human macrophages that were treated with control, CSC, or indicated EVs. Bars indicate mean ± SEM values from two replicates. * and ** indicate $p<0.05$ and $p<0.01$ compared to control respectively, # indicates $p<0.05$ compared to CSC. † indicates $p<0.05$ compared to control+exo within the same group of treatment. Control+Exo: Control-EV, CSC+Exo: CSC-EV.

## Differential Packaging of Oxidative Stress Modulators and Inflammatory Cytokines and Chemokines in MDM-Derived EVs upon Exposure to Tobacco Constituents and HIV-1[5]

Smoking has been shown to exacerbate HIV-1 pathogenesis and its related comorbidities by dysregulating the expression of cytochromes P450 (CYPs) and antioxidant enzymes (AOEs), as well as cytokine and chemokine expression, ultimately increasing oxidative stress and inflammatory responses [11, 38, 47, 188, 259]. It has been suggested that the regulation of CYP expression by cytokines is highly variable, and could potentially have a variety of effects under different inflammatory disease conditions [260, 261]. Further, we have shown that CYP enzymes responsible for the metabolism of tobacco constituents cause oxidative stress and DNA damage, ultimately leading to the enhancement of HIV-1 replication in macrophages via an oxidative stress pathway [11, 38, 259]. Clearly, CYPs, AOEs, cytokines, and chemokines are interconnected in the case of HIV-1 pathogenesis.

We have demonstrated in the previous section that EVs can be either protective or toxic to recipient cells [48]. Our next step is to identify the components packaged in EVs that might be responsible for such effects. Therefore, in this study we aimed to investigate the packaging of oxidative stress or inflammatory modulators in EVs. We have previously reported the concentrations of cytokines and chemokines in plasma EVs collected from HIV-1-positive drug abusers. We observed differential packaging of these agents in EVs derived from HIV-1-infected and/or drugs of abuse-exposed populations [47]. In this part of the study, we aimed to measure oxidative stress-regulating elements as well as cytokines and chemokines in EVs isolated from MDM upon CSC and/or HIV-1 exposure.

We hypothesize that CSC and/or HIV-1-exposed MDM-derived EVs carry relatively high levels of pro-inflammatory and pro-oxidant cargos and/or low levels of anti-inflammatory and antioxidant cargos, which are key mediators for HIV-1 pathogenesis. EVs and the potential roles of their cargos have been vastly studied in several disease conditions; however, the understanding of their contribution to HIV-1 pathogenesis is still limited. Overall, in this study, we observed increased packaging of pro-inflammatory cytokines and oxidative stress-inducing CYP enzymes. In addition, we observed a decreased packaging of anti-inflammatory cytokines and AOEs, in EVs derived from CSC-exposed, HIV-1-infected and/or uninfected MDM. Our findings suggest that HIV-1, in the presence of CSC, can stimulate the packaging of oxidative stress and inflammatory modulators in EVs, which may potentially exacerbate smoking-enhanced HIV-1 pathogenesis. This is the first study to investigate the packaging of oxidative stress and inflammatory modulators in MDM-derived EVs, particularly upon exposure to CSC, in the absence and presence of HIV-1.

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[5] Modified from final submission with open access permission. Sanjana Haque, Sunitha Kodidela, Namita Sinha, Prashant Kumar, Theodore J Cory, Santosh Kumar. Differential packaging of inflammatory cytokines/ chemokines and oxidative stress modulators in U937 and U1 macrophages-derived extracellular vesicles upon exposure to tobacco constituents. PLOS ONE. In press.[230]

**Relative Expression of CYPs and AOEs in U937 Cell-Derived EV**

We investigated the potential EV components that may provide protection to the monocytic cells. We performed an initial screening of whether U937 monocytic cell-derived EVs carry CYP or AOE mRNA or protein. The roles of cellular CYPs and AOEs in tobacco-induced oxidative stress, cytotoxicity, and carcinogenesis are well-established [256]. Our previous study has also suggested a potential role of CYPs and oxidative stress pathways in smoking-enhanced HIV-1 replication [22]. We measured the relative mRNA expression of CYPs and AOEs in U937 cells and U937-derived EVs using GAPDH as an internal control for both cells and EVs. A robust 12- and 27- fold increase in CYP2A6 and CYP2E1 levels, respectively, was observed in EVs compared to cell extract (**Figure 4-7a**, *$p<0.05$). Similarly, we observed greater mRNA expression of AOEs in EVs than in the cell extract: SOD1 (14-fold), SOD2 (6-fold) (**Figure 4-7a**, *$p<0.05$). EVs also exhibited 92-, 170-, 555-fold elevation in the AOEs catalase, GSTK1, and PRDX6 levels respectively (**Figure 4-7a**, *$p<0.05$). The results clearly suggest a selective packaging of CYPs and AOEs mRNAs in EVs compared to the house keeping gene GAPDH. GAPDH is well-accepted house-keeping gene in cells. In EVs, although GAPDH is not highly expressed, we used this to establish a parameter to compare the selective packaging of the specific genes only. The expression in mRNA level was also altered in CSC-EVs compared to control-EVs. The results showed a higher mRNA expression of CYP1A1, SOD1, catalase, and GSTK-1, while a lower mRNA expression of CYP2A6, SOD-2, and PRDX6 in exosomes derived from CSC exposure to U937 cells, compared to EVs derived from control cells (**Figure 4-7b**).

Furthermore, we performed a Western blot for the proteins obtained from the EVs and their respective cell extracts. Although the relative level of protein expression in EVs compared to cell extract was low, we observed detectable levels of catalase and PRDX6 as well as very low levels of CYP2A6 and SOD1 proteins in EVs (**Figure 4-7c**). Interestingly, the relative level of PRDX6, compared to other proteins, appeared to be higher in EVs than in the cell extracts even though the same amount of protein was loaded on the gel for the Western analysis of all proteins. As expected, the cellular housekeeping proteins GAPDH and β-actin were negligible or undetectable in EVs. We then examined whether EVs derived from CSC-exposed U937 cells had altered expression of catalase and PRDX6, and the results are presented in **Figure 4-7d** and **e**. As shown in **Figure 4-7d**, CSC-treatment to U937 cells significantly elevated the level of catalase in EVs compared to control (* $p<0.05$), while slightly decreasing the level of catalase in U1 EVs (** $p<0.01$). In contrast, a significant reduction of PRDX6 protein was observed in CSC-EVs (** $p<0.01$) both in U937 and U1 cells.

In our study, EVs derived from uninfected and HIV-1 infected macrophages treated with CSC were observed to have a lower antioxidant capacity and altered levels of both pro-oxidant and antioxidant enzymes. The mRNA profile of CSC-EVs from uninfected monocytes indicated that they packaged a high amount of CYP1A1, which plays a critical role in the metabolism of tobacco constituents. In fact, CYP enzymes have been shown to be associated with oxidative stress-induced cytotoxicity, DNA damage, and viral replication in CSC-treated myeloid cell lines [21]. Additionally, our study

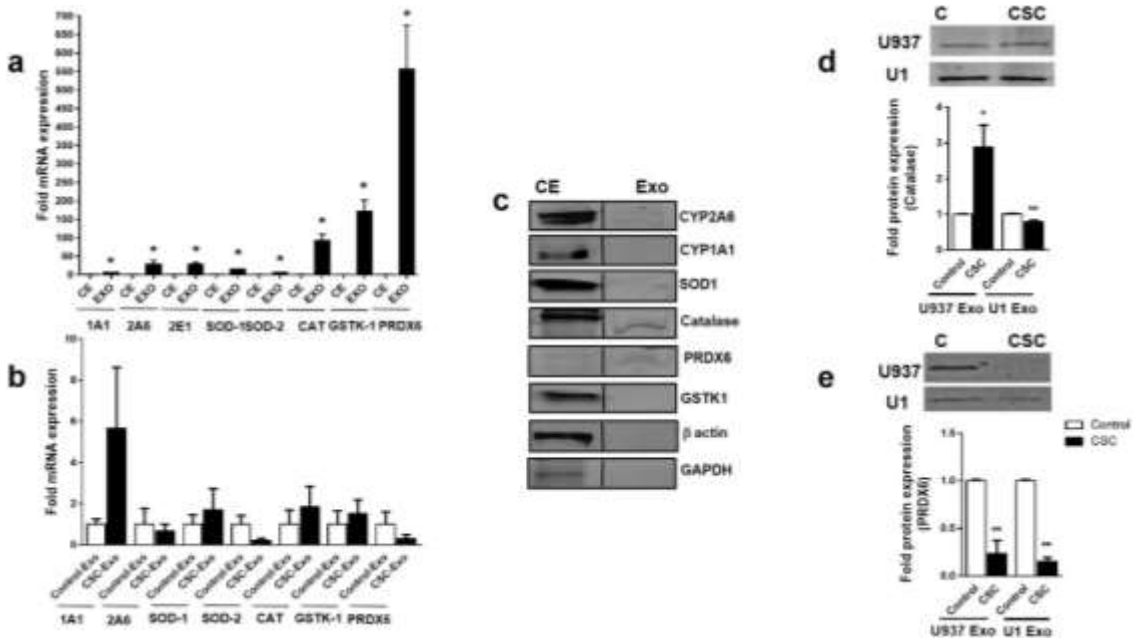


**Figure 4-7. Relative mRNA and Protein Levels of Cytochrome P450s (CYPs) and Antioxidant Enzymes (AOEs) in Monocyte-Derived EVs and Effect of CSC on EV AOE.**

(a) Relative mRNA levels of CYPs (1A1, 2A6, and 2E1) and AOEs (SOD1, SOD2, catalase, GSTK1, and PRDX6) compared with the house-keeping gene GAPDH in EVs. (b) Relative EV mRNA levels of CYP (1A1, 2A6, and 2E1) and AOE (SOD1, SOD2, catalase, GSTK1, and PRDX6) in CSC-treated cells compared to control. (c) Representative immunoblots for the expression of EV CYPs and AOEs compared to cell extracts. For cell extracts and EVs, amount of protein loaded was 30 µg and 3 µg, respectively. Effect of CSC on catalase (d) and PRDX6 (e) levels in EVs isolated from U937 and U1 cells. Equal amount of proteins (4 µg) was loaded on each lane. Bars indicate mean ± SEM values from three replicates. * and ** indicate $p<0.05$ and $p<0.01$ compared to each control respectively. CE: cell extract, EXO: EV, CAT: catalase.

demonstrated that PRDX6 expression was significantly lower in CSC-EVs, however catalase expression was observed to be high. The role of PRDX6 as an antioxidant is known to suppress lipid oxidation and therefore help in rescuing cellular toxicity from external agents [262-264]. It is possible that increased CYP1A1 expression and decreased PRDX6 level in EVs could increase oxidative stress and resultant DNA damage and cellular toxicity.

Again, the reason for higher protection against cytotoxicity provided by U937 cell-derived CSC-EVs compared to U1 cell-derived CSC-EVs can be explained by their catalase content. U937 cell-derived CSC-EVs package higher catalase protein, suggesting higher protection to recipient cells. On the contrary, U1 cell-derived EVs inherently contains lower catalase and PRDX6 protein, resulting in compromised protection against cellular toxicity and subsequently viral replication. These observations further provide an association between the two phenomena, likely mediated by altered levels of antioxidant enzymes.

CYP enzymes have been known to be involved in the metabolism of numerous xenobiotics, including polycyclic aromatic hydrocarbons, which are largely found in cigarette smoke [10, 265]. In our previous studies, we observed elevated ROS production and resulting oxidative stress followed by apoptotic cell death in monocytic cells after treatment with CSC [21]. CYP enzymes are expressed in these cells, but the underlying mechanistic pathway through which EV CYPs may affect neighboring cells is unknown. The current study revealed that EVs from naïve cells express relatively high levels of CYP1A1, CYP2A6, and CYP2E1 compared with the housekeeping genes GAPDH or actin. This may explain the increased cytotoxicity that we observed after introducing CSC-EVs in U937 cells. In addition to the CYPs, the EVs also expressed higher levels of AOEs such as SOD1, SOD2, catalase, GSTK1, and PRDX6 compared to the housekeeping gene. There was an increase in the expression of catalase in EVs derived from CSC-exposed U937 cells, however the expression of PRDX6 was lowered. The modulation of these antioxidants in EVs upon CSC exposure could have an impact on overall oxidative stress and subsequent downstream cytotoxicity and viral replication. However, other EV contents such as miRNAs and inflammatory cytokines are also possible factors for triggering oxidative stress in the recipient cells [266, 267].
We next expanded our treatment paradigm. Our goal was to observe the differential packaging capacity of EVs under the influence of short- and long-term CSC and/or HIV-1 exposure. As mentioned earlier, we have defined 4 days as short-term and 6-8 days as long-term exposure, considering optimal treatment efficacy and cell viability.

**Physical Characterization of EVs**

Physical characteristics of MDM-derived EVs in terms of size, number of EVs, zeta potential, and protein concentration, were estimated (**Figure 4-8**). The average size of EVs from uninfected U937 and HIV-infected U1 cells was similar, in the presence or absence of CSC (**Figure. 4.7a-c**). Although not statistically significant, the number of EVs per ml trended upward in presence of such stressors as CSC and HIV (**Figure. 4.8d**).

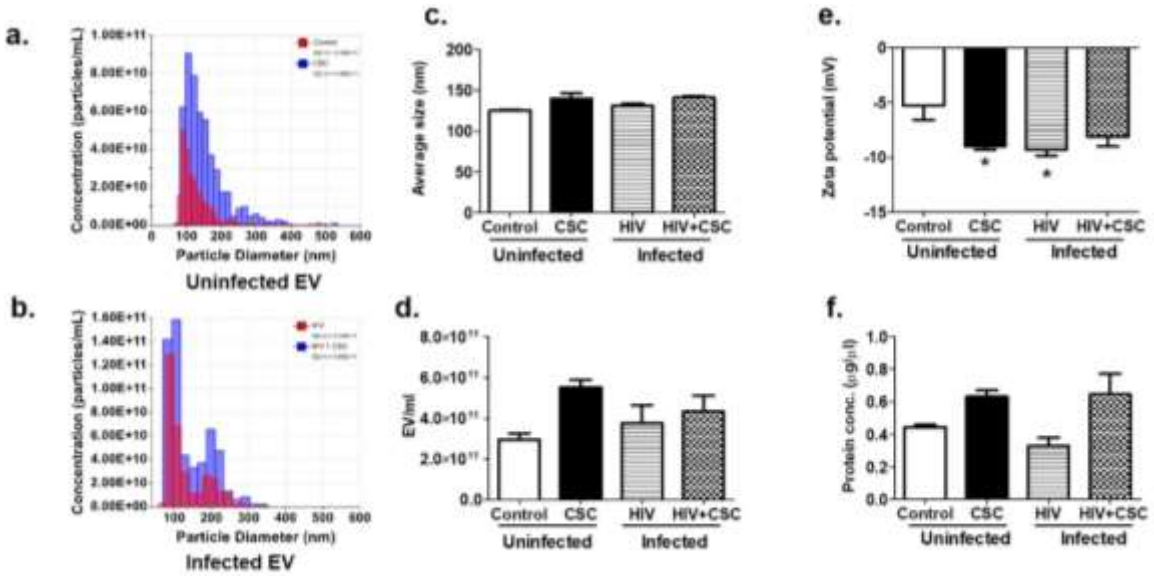


**Figure 4-8. Physical Characteristics of Extracellular Vesicles (EV) Collected from U937 (Uninfected) and U1 (HIV-1-Infected) Macrophages Treated with CSC.**
(a) Representative diagram of number of EV/ml for uninfected EV. (b) Representative diagram of number of EVs/ml for infected EV. c. Average size of EV. d. Number of EV/ml. e. Zeta potential of EV. Bars represent data from n=3 replicates. *$p<0.05$ compared to control. f. Total protein concentration of EV. Bars represent data from n=3 replicates (except the HIV group, which is n=2).

Zeta potentials ranged between -5mV to -10mV, which is optimum for EVs (**Figure 4-8e**). Next, we measured the total protein concentration in EVs (**Figure 4-8f**). We observed that protein concentration gradually increased from the uninfected control group to the uninfected CSC-treated group, with a similar trend noted in the HIV-infected groups (**Figure 4-8f**).

**Effect of CSC Exposure on Packaging of CYPs and AOEs in the EVs**

CYP enzymes, which are responsible for the metabolism of smoking constituents and subsequent induction of oxidative stress, as well as AOEs, were measured in EVs derived from HIV-1-infected U1 and uninfected U937 MDM exposed to CSC for short- and long-term durations by Western blot (**Figure 4-9a**). We observed a slight increase in the expression of exosomal marker proteins, namely CD63 and CD81, in the HIV-1-infected U1 MDM-derived EVs, compared to the EVs derived from uninfected U937 cells. CD9 also showed a variation in expression in EVs derived from infected cells. This is not surprising, as these marker proteins are not traditionally considered to be housekeeping proteins, and they are shown to be altered in EVs depending upon cellular origin and the condition of the parent cells [60]. We measured the expression levels of CYP enzymes 1A1, 1B1, 3A4 (polyaryl hydrocarbon metabolizer and major drug metabolizing enzymes [21, 259, 268-270]) in uninfected U937 and HIV-1-infected U1 MDM-derived EVs with or without CSC exposure. There appears to be a slight increase in the expression of CYP1B1 in HIV-1-infected U1 MDM-derived EVs. However, CYPs 1A1 and 3A4 were undetectable in EVs derived from uninfected U937 cells but present in EVs derived from HIV-1-infected cells. Finally, we also measured common AOEs such as SOD-1 and catalase. There was a clear decrease in the expression of both enzymes in EVs isolated from HIV-1-infected cells compared to EVs derived from uninfected cells. Our data suggest that EVs derived from infected cells demonstrate higher CYP expression and lower AOE expression. Together this indicates an overall increase in oxidative stress-inducing elements in EVs derived from HIV-1-infected cells. However, in all cases, CSC did not alter the expression level of either CYPs or AOEs in EVs from uninfected or HIV-1-infected cells.

We also checked the presence and impact of smoking on plasma-derived EVs from healthy, HIV-1-positive, smoker, and HIV-1-smoker subjects (**Figure 4-9b**). We observed a consistent band for CD63, weaker bands for CD81 for all the groups, and stronger band for CD9 in the HIV-1-positive group. We also observed the presence of CYPs and AOEs, however, they did not demonstrate any notable differences among group. A larger cohort of samples would likely be more informative in this regard.

Tobacco constituents are known to induce oxidative stress via the NFκB pathway [35, 271]. Literature reports have also established that CSC increases oxidative stress via inducing CYPs, especially CYP2A6 and 1A1, which in turn metabolize smoking constituents in macrophages [10, 38, 272, 273]. However, the lack of increased CYP packaging in EVs upon CSC exposure suggests that despite CSC-induced enhanced cellular expression of CYPs, their packaging into EVs is limited. On the other hand, a

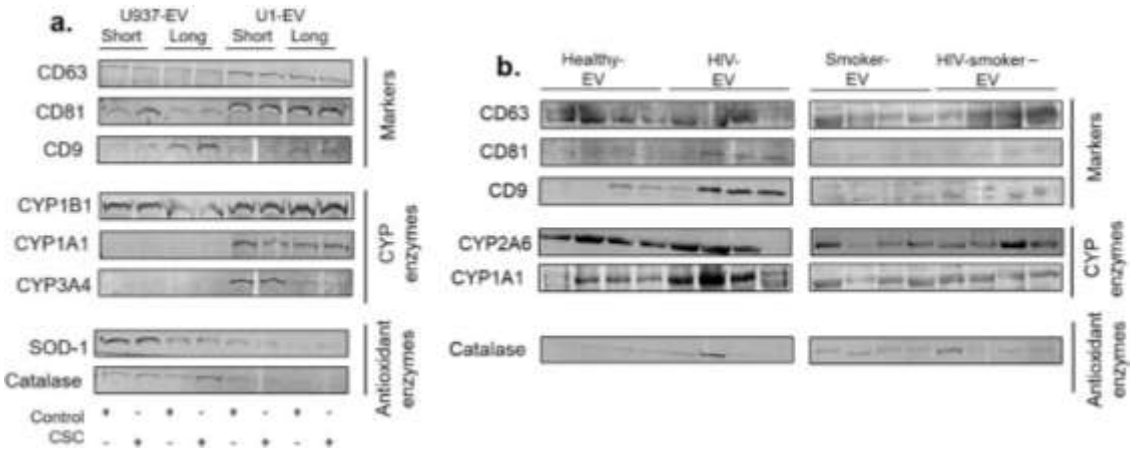


**Figure 4-9. Cytochrome P450 Enzymes (CYPs) and Antioxidant Enzymes (AOEs) in EVs Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC for Short-Term and Long-Term Durations.**
(a) Markers for extracellular vesicles, namely CD63, CD81, and CD9, were present in EVs. Protein expression levels of CYP enzymes such as CYP1B1, CYP1A1, and CYP3A4, and of AOEs such as SOD-1 and catalase were checked. This is a representative blot of at least two experiments. Equal amounts of protein were loaded in each well. (b) EVs were isolated from 100 µl of plasma collected from healthy, HIV-positive, smoker, and HIV-positive smoker patient groups.

higher packaging of CYPs 2A6 and 1A1, concurrent with a lower packaging of SOD1 and catalase in EVs upon HIV-1 exposure, suggests an overall increase in the packaging of oxidative stress factors in EVs from HIV-1-infected cells. The finding is somewhat consistent with our own previous *ex vivo* finding that there is a relatively higher cellular expression of CYPs in monocytes from HIV-1-infected subjects than those of uninfected individuals [21]. Overall, this finding suggests a role for EVs in exacerbating HIV-1 pathogenesis via oxidative stress pathways.

## Effects of Short-Term CSC Exposure on Cytokines and Chemokines

### Pro-inflammatory Cytokines

Levels of pro-inflammatory cytokines such as IL-6, IL-1β, IL-8, and TNF-α were measured in uninfected U937 and HIV-1-infected U1 MDM-derived EVs treated with CSC (**Figure 4-10a-d**). In general, cytokine levels (except IL-6) increased after CSC exposure, both in the media and EVs from uninfected cells, compared to their respective controls. In the case of HIV-1-infected U1 MDM-derived EVs, IL-6 showed an upward trend. Inversely, IL-1β, IL-8, and TNF-α showed a downward trend, both in media and EVs, after CSC treatment. The percentage of cytokines packaged in EVs was low, only 0.05%-6.5% of the total cytokines present in media. Further, the IL-6 level in the media of HIV-1-infected cells was significantly higher than in the media of uninfected cells. However, in the presence of CSC, IL-6 concentration decreased significantly in the media of HIV-1-infected U1 cells compared to the media of infected cells without CSC treatment. EVs packaging might be the contributor to this change, as it showed a significant rise in cytokine concentration within EV (**Figure 4-10a**). About 3% of total IL-6 was packaged in HIV-1-infected U1 MDM-derived EVs upon exposure to CSC, which is higher than in control EVs (**Figure 4-10a**). IL-1β also demonstrated higher EVs packaging after exposure to CSC, both in the absence and presence of HIV-1 infection (**Figure 4-10b**).

### Anti-inflammatory Cytokines

We measured the concentration of two anti-inflammatory cytokines, IL-1ra and IL-10, in the same experimental setting (**Figure 4-11a, b**). CSC mostly reduced anti-inflammatory cytokines in the media and EVs from uninfected U937 and HIV-1-infected U1 cells (except IL-1ra in CSC-treated media and EVs from uninfected cells). However, there was a slight increase in the EVs packaging of IL-1ra and IL-10 upon exposure to CSC and in the presence of HIV-1-infection, compared to the proportion packaged in EVs without CSC treatment (**Figure 4-11a**). Moreover, the packaging of IL-10 in EVs was significantly higher (**Figure 4-11b**) with both CSC-treatment and HIV-1-infection cells when compared to HIV-1 infection alone.

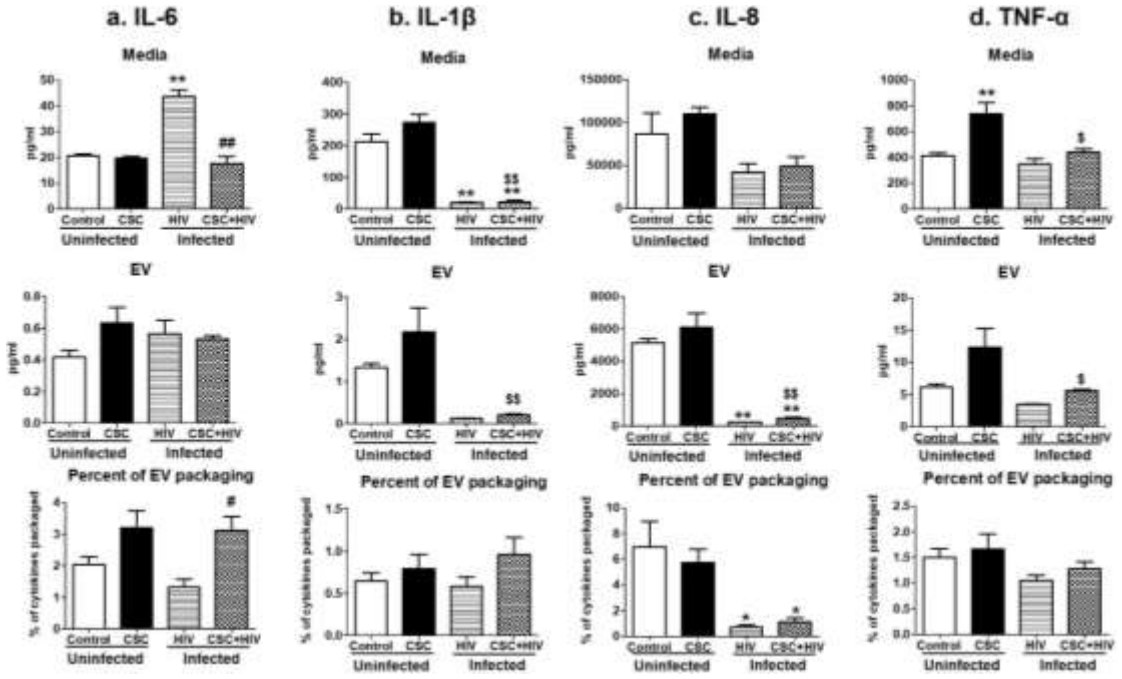


**Figure 4-10. Pro-inflammatory Cytokines in Media and EVs Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC Short-Term.** Concentration of cytokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) IL-6, (b) IL-1β, (c) IL-8, and (d) TNF-α. *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV; $p<0.05 and $$p<0.001 compared to CSC. Bars represent data from n=3 replicates. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

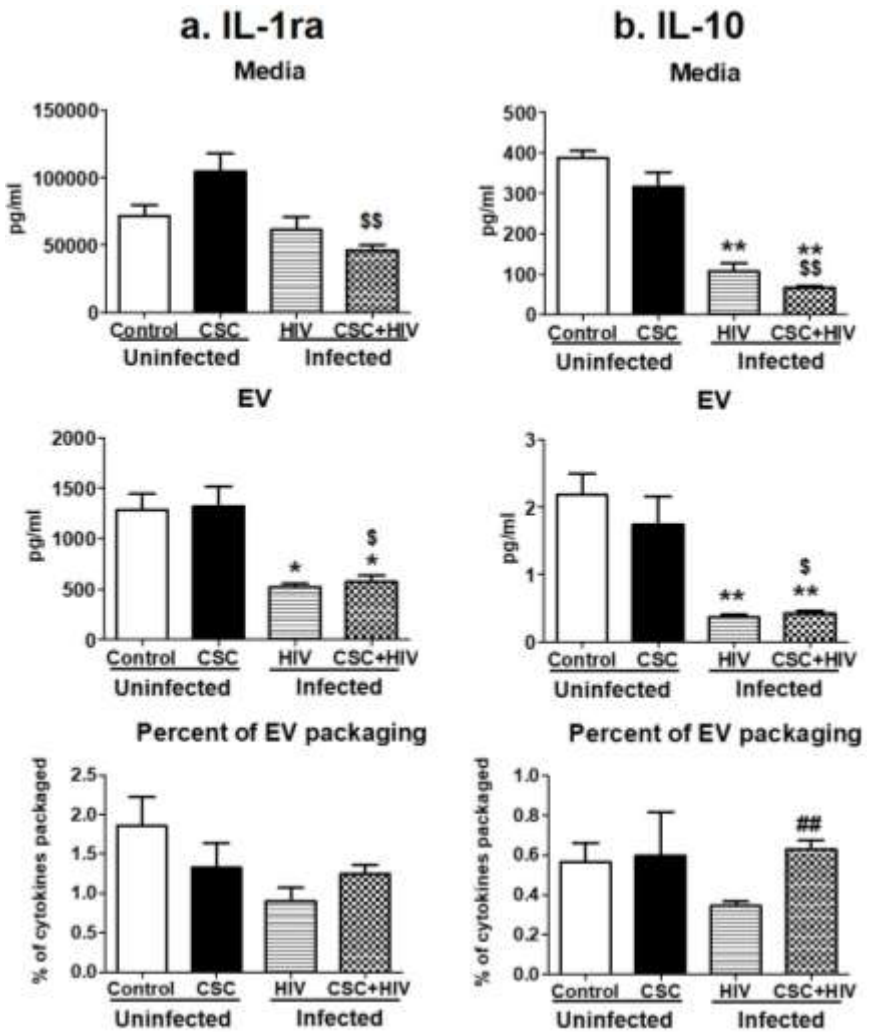


**Figure 4-11. Anti-inflammatory Cytokines in Media and Extracellular Vesicles (EV) Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) Macrophages Treated with CSC Short-Term.**

Concentration of cytokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) IL-1ra and (b) IL-10. *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV. $p<0.05 and $$p<0.001 compared to CSC. Bars represent data from n=3 replicates. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

### Chemokines

MCP-1 and RANTES were the two chemokines measured in this study. The amount of these cytokines varied among all groups, especially MCP-1 (**Figure 4-12a**). The amount of RANTES in media was similar among the groups, except after CSC treatment to uninfected U937 cells (**Figure 4-12b**). In EVs, the concentration of RANTES increased with CSC treatment, HIV-1-infection, and with both conditions together, compared to control, which was statistically significant in all groups (**Figure 4-12b**). This effect was also reflected in the proportion of EVs packaging. Especially after CSC and CSC + HIV-1 exposure, EVs packaging of RANTES was found to be around 15% and 12%, respectively, which was higher than control (**Figure 4-12b**).

Cigarette smoking is strongly associated with inflammatory responses, demonstrated by alterations in cytokine expression, which can vary widely among population groups based on age, and other associated conditions [274]. We have previously demonstrated the differential packaging of cytokines in plasma-derived EVs from HIV-1-infected and uninfected smokers [47]. Consistent with this past study, the current study also demonstrates alterations in the cytokine and chemokine levels of MDM-derived EVs under the influence of CSC and HIV-1. Moreover, CSC primarily induced the expression of pro-inflammatory cytokines, both in the media and in EVs derived from MDM. IL-6, in particular, displayed a noticeably high percentage of EVs packaging (**Figure 4-10a**). Our finding is also supported by previous studies indicating that IL-6 is increased in smokers [275]. Further, the mixed response we observed regarding anti-inflammatory cytokines, both in the media and in EVs, is also supported in the literature. In fact, nicotine is known to have conflicting impacts on cytokine production [276-278].

## Effects of Long-Term CSC Exposure on Cytokines and Chemokines

### Pro-inflammatory Cytokines

The concentration of pro-inflammatory cytokines remained mostly unaltered in media and MDM-derived EVs from uninfected U937 cells (**Figure 4-13a-c**). IL-1β was significantly lower in the media of uninfected U937 cells after long-term CSC treatment compared to control (**Figure 4-13b**). Interestingly, in the presence of both CSC and HIV-1, IL-6 and IL-1β showed more packaging into EVs from infected cells than those from uninfected cells (**Figure 4-13a, b**). Specifically, IL-6 was shown to be significantly high in media and in EVs from HIV-1-infected cells with or without CSC treatment, compared to control and CSC treatment to uninfected cells. Approximately 18% and 23% of IL-6 was packaged in EVs from untreated HIV-1-infected U1 cells and CSC-treated U1 cells, respectively (**Figure 4-13a**). In the case of IL-1β, media from HIV-1-infected U1 MDM had a lower cytokine concentration compared to media from uninfected cells, whereas EVs from infected cells showed a significantly higher IL-1β concentration (**Figure 4-13b**). These results suggest that the cytokines are driven towards being packaged in EVs upon HIV-1 ± CSC exposure. In fact, approximately 67% and 28% of IL-1β was

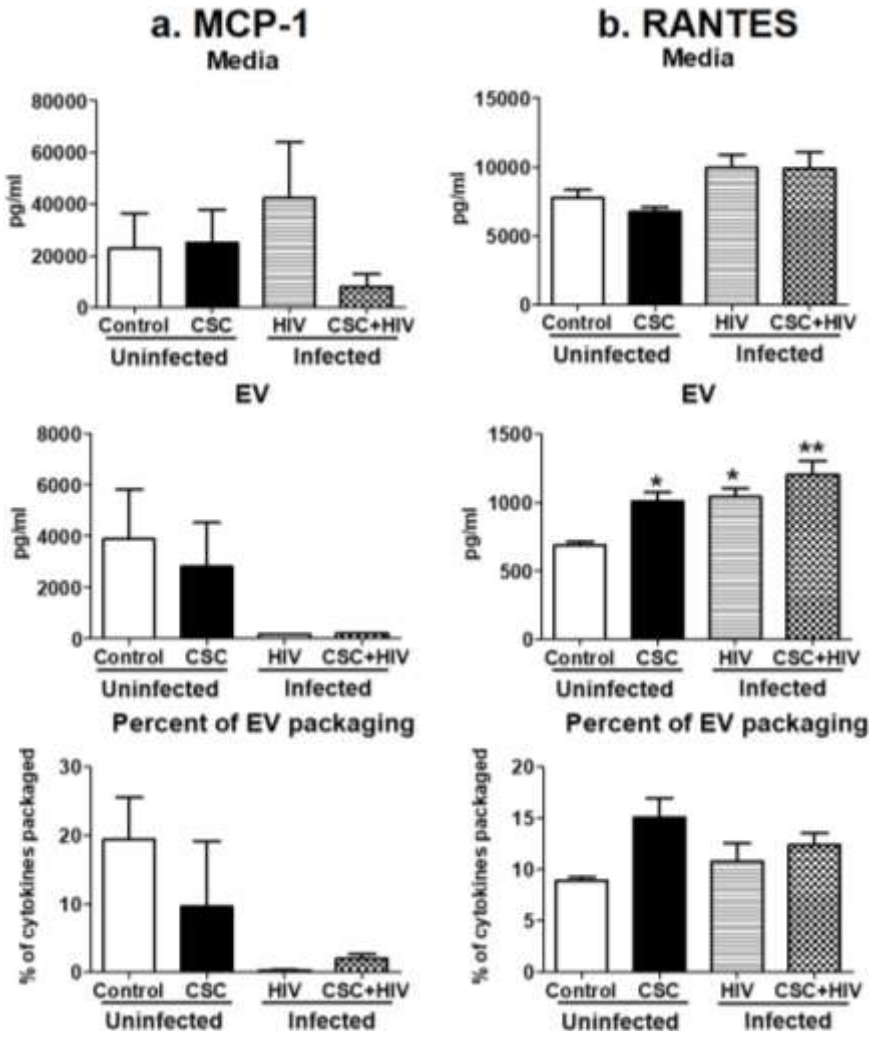


**Figure 4-12. Chemokines in Media and Extracellular Vesicles (EV) Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC Short-Term.** Concentration of chemokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) MCP-1 and (b) RANTES *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV. Bars represent data from n=2 replicates for MCP-1. Due to lack of sensitivity, MCP-1 could be measured only from two replicates. Therefore, we did not provide any significance value and reported only the change in the trend. Bars represent data from n=3 replicates. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

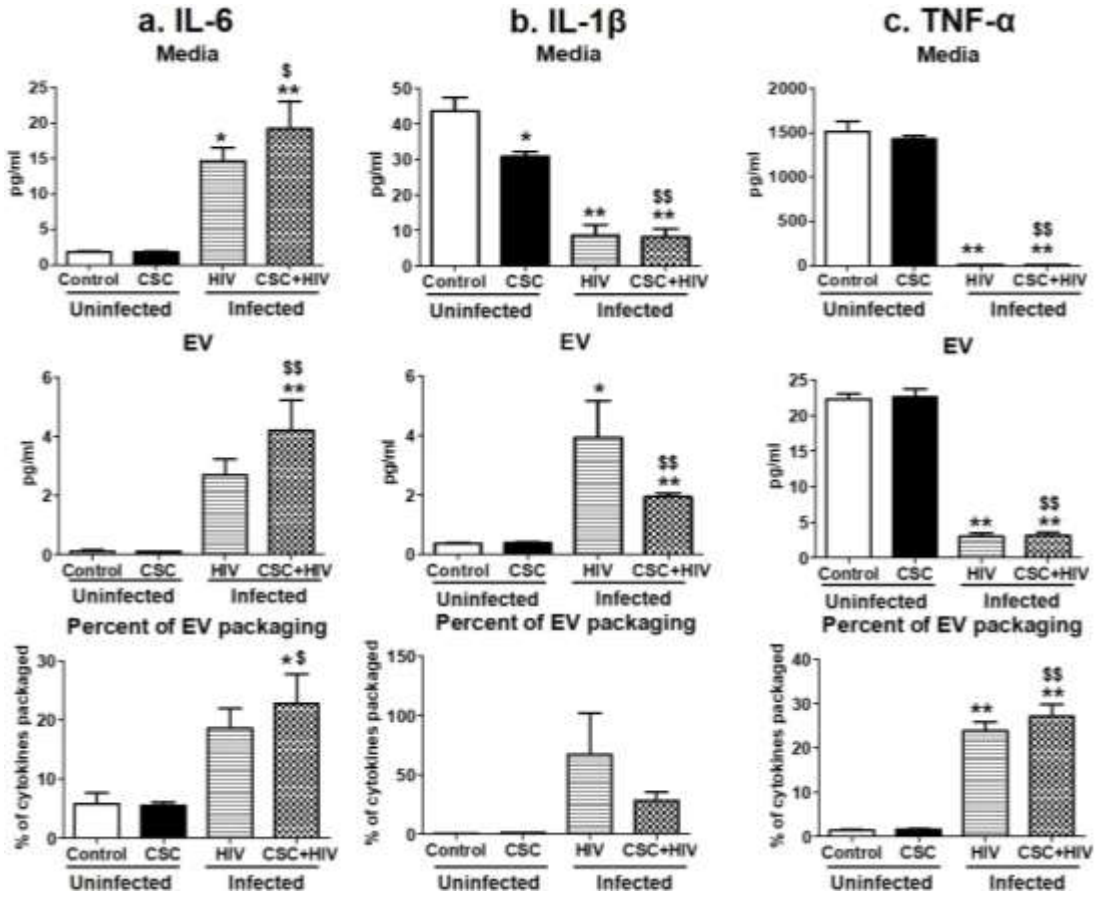


**Figure 4-13. Pro-inflammatory Cytokines in Media and Extracellular Vesicles (EV) Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC Long-Term.**

Concentration of cytokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) IL-6, (b) IL-1β, and (c) TNF-α. *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV; $p<0.05 and $$p<0.001 compared to CSC. Bars represent data from n=2 replicates. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

packaged in EVs from HIV-1-infected U1 MDM and HIV-1-infected U1 MDM upon CSC exposure, respectively (**Figure 4-13b**). Finally, the amount of TNF-α was higher in the media and in EVs from uninfected U937 cells compared to the media and EVs of HIV-1-infected U1 cells in the absence or presence of CSC. In terms of EVs packaging, 24% and 27% of total TNF-α was packaged within EVs derived from HIV-1-infected cells that went untreated and received CSC treatment, respectively (**Figure 4-13c**).

**Anti-inflammatory Cytokines**

In media, IL-1ra and IL-10 demonstrated a pattern of decreasing cytokine concentration from HIV-1-infected cells upon CSC exposure, compared to control (**Figure 4-14a, b**). CSC treatment reduced the IL-1ra levels in EVs from HIV-1-infected U1 cells with and without CSC exposure, compared to control. Interestingly, EVs packaging of IL-1ra in HIV-1-infected U1 cells in the absence and presence of CSC exposure was significantly high at 80% and 50%, respectively, explaining the reduction of free cytokines in the media from these groups (**Figure 4-14a**). EVs packaging of IL-10 was greater in HIV-1-infected U1 cells in the absence and presence of CSC exposure compared to EVs from uninfected U937 cells. This was evident by the amounts of cytokines packaged in EVs from HIV-1-infected U1 cells with and without CSC exposure, which was 16% and 27%, respectively (**Figure 4-14b**).

**Chemokines**

The chemokines MCP-1 and RANTES mostly decreased significantly after long-term CSC exposure in HIV-1-infected U1 MDM-derived EVs (**Figure 4-15a, b**). In terms of packaging, MCP-1 was highly driven towards EVs from HIV-1-infected U1 cells (**Figure 4-15a**), whereas RANTES showed high EVs packaging from HIV-1-infected U1 cells upon CSC treatment (**Figure 4-15b**).

HIV-1 is known to be associated with a significant increase in IL-6 in the presence of cigarette smoking and other comorbid conditions, even in patients who are on cART [191, 279-282]. EVs derived from HIV-1-infected macrophages are shown to exacerbate cytotoxicity and HIV-1 replication in recipient cells [48]. Moreover, EV contents vary greatly according to the stages of viral infection [197, 283]. Consistent with previous studies, the current study shows a general upward trend in the packaging of cytokines, especially the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, as well as the chemokine RANTES, upon HIV-1 exposure. RANTES, along with other chemokines, is known to potentially enhance HIV-1 spread among macrophages by recruiting target cells [284]. Thus, our findings suggest that RANTES, produced within cells, is transported to other cells via EVs, further contributing to HIV-1 replication. Both MCP-1 and RANTES demonstrated opposite trends in packaging after short- and long-term exposures. This suggests that the duration of smoking constituent exposure may impact chemokine levels. A decrease in the secretion of anti-inflammatory cytokines, especially IL-10, in the media following CSC and HIV-1 exposures, conforms to the role of IL-10 as an HIV-immunosuppressive agent [285]. In contrast, we observed an increase in the EVs packaging of anti-inflammatory cytokines, including IL-10, upon exposures to CSC

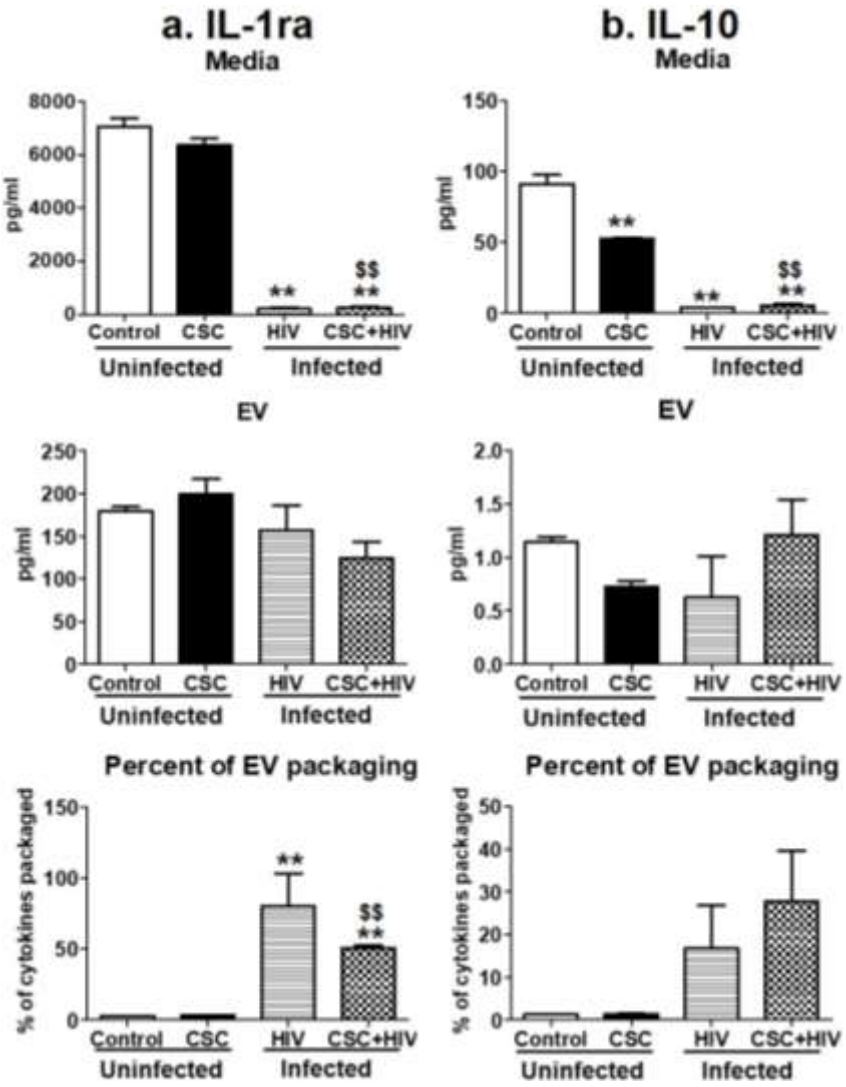


**Figure 4-14. Anti-inflammatory Cytokines in Media and Extracellular Vesicles (EV) Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC Long-Term.**

Concentration of cytokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) IL-1ra and (b) IL-10. *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV; $p<0.05 and $$p<0.001 compared to CSC. Bars represent data from n=3 replicates. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

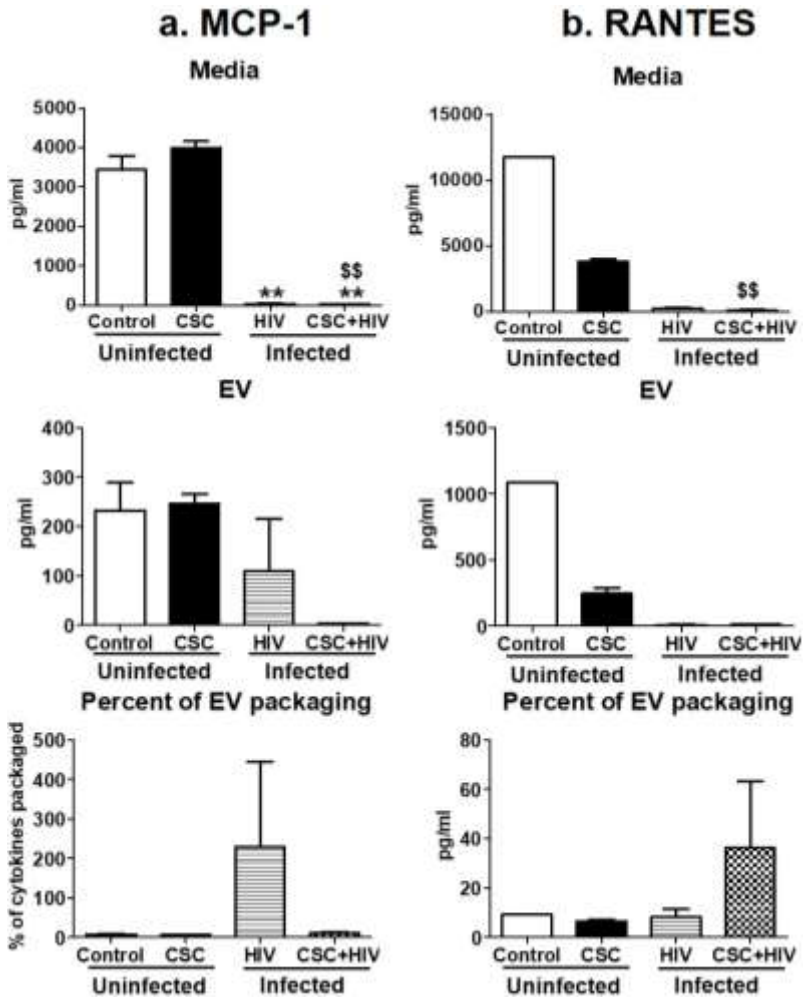


**Figure 4-15. Chemokines in Media and Extracellular Vesicles (EV) Isolated from U937 (Uninfected) and U1 (HIV-1-Infected) MDM Treated with CSC Long-Term.** Concentration of chemokines in media, concentration in EVs, and percentage of cytokine packaged in EVs for (a) MCP-1 and (b) RANTES. *p<0.05, **p<0.001 compared to control; #p<0.05, ##p<0.001 compared to HIV. Bars represent data from n=2 replicates. Due to lack of sensitivity, MCP-1 could be measured only from two replicates. Therefore, we did not provide any significance value and reported only the change in the trend. Bars represent data from at least two n=3 replicates, except control for RANTES. Statistical analysis performed: One-way ANOVA with Tukey's Multiple comparison test.

and HIV-1 together (**Table 4-1**). This trend is similar to a previously observed trend in plasma-derived EVs from HIV-1-infected smokers, where IL-10 was completely packaged in EVs [47]. This finding suggests a potential role of EVs in transmitting protective elements to recipient cells. In fact, our previous study also demonstrated that EVs from monocytes could be protective against cytotoxicity and HIV-1 replication in recipient macrophages [48]. However, the protective influence of EVs is mostly limited to the earlier stages of HIV-1 infection.

Induction of cytokines and chemokines is critical during the early and later stages of HIV-1 infection [286, 287]. Our current study illustrates a higher packaging of cytokines and chemokines within EVs following long-term exposure to CSC than short-term exposure, both in the presence and absence of HIV-1. Moreover, IL-6 packaging demonstrated an additive effect after long-term CSC exposure in the presence of HIV-1-infection. Another study has shown that latent HIV-1 can be activated by TNF-α derived from HIV-1-infected cells, which is dependent on pro-TNF-α-processing ADAM17 enzymes in exosomes [288]. Our study supports this finding. In addition, our study suggests that smoking constituents further enhance TNF-α in EVs derived from HIV-1-infected cells, which may potentially contribute to the exacerbation of HIV replication. Further, the EV packaging of the anti-inflammatory cytokines, IL-1ra and IL-10, after long-term CSC exposure in the presence of HIV-1, was noticeably high when compared to the control, as well as when compared to short-term exposures under similar conditions (**Figures 4-11b** and **4-14b**). This suggests that long-term exposures of CSC to HIV-1-infected MDM trigger relatively higher packaging of cytokine in EVs, regardless of whether the cytokines are pro- or anti-inflammatory in nature. Consistent with previous reports, the elevated level of MCP-1 in both media and EVs upon long-term CSC exposure suggests increased recruitment of monocytes and macrophages to the site of active inflammation [289]. Further, an increase in RANTES EV packaging with concurrent CSC and HIV-1 exposure is consistent with a similar trend observed *ex vivo*, in which RANTES levels are elevated in HIV-1-infected smokers, irrespective of ART use [188]. Moreover, our previous *ex vivo* study demonstrated a similar downward trend for IL-1ra and MCP-1 in the presence of cigarette smoke and HIV-1 (**Table 4-2**) [47]. It should be mentioned here that the length of smoking behavior was not clearly identified in these human samples, thus we are demonstrating a general comparison with both short-term and long-term exposure of CSC.

Overall, EVs derived from monocytes and MDM alter their contents upon exposure to CSC and/or HIV-1. CYP enzymes are generally highly expressed in EVs derived from HIV-1-infected MDM. With longer exposure to CSC and/or HIV-1, more cytokines and chemokines are packaged into EVs. Pro-inflammatory cytokines are mostly increased after short-term and long-term CSC and/or HIV-1 exposure. Interestingly, anti-inflammatory cytokines also demonstrated a trend to be packaged in EVs. Finally, our results suggest packaging of increased levels of pro-oxidant and inflammatory elements into EVs upon exposure to CSC and/or HIV-1, which would potentially contribute to enhanced HIV-1 replication in recipient MDM via cell-to-cell transmission.

**Table 4-1. Comparison Between the Short-Term and Long-Term Treatment of CSC on HIV-1-Uninfected and Infected MDM-Derived EVs.**

| | CSC-exposed | | | HIV-infected | | | HIV-infected+CSC | | |
|---|---|---|---|---|---|---|---|---|---|
| **Cytokines** | **Media** | **EV** | **% Pack-aging** | **Media** | **EV** | **% Pack-aging** | **Media** | **EV** | **% Pack-aging** |
| **Pro-inflammatory** | | | | | | | | | |
| **IL-6** | ~ | | | | | | | | |
| **IL-1β** | | | | | | | | | |
| **IL-8** | > | > | NA | > | > | NA | NA | NA | NA |
| **TNF-α** | | | | | | | | | |
| **Anti-inflammatory** | | | | | | | | | |
| **IL-1ra** | | | | | | | | | |
| **IL-10** | | | | | | | | | |
| **Chemokines** | | | | | | | | | |
| **MCP-1** | | | | | | | | | |
| **RANTES** | | | | | | | | | |

Note: ⇅=Denotes short-term exposure. ⇅=Denotes long-term exposure. >=Denotes above upper limit of quantification. ~=Denotes no change. NA=Denotes not applicable.

**Table 4-2. Comparison Between the Percentage of Cytokines Packaged in EVs after Short-Term and Long-Term Treatment of CSC in Uninfected and HIV-1-Infected MDM-Derived EVs and Plasma-EVs Derived from Smokers, HIV-1-Positive Non-Smoker Patients, and HIV-Positive Smokers.**

| Cytokine | CSC-exposed | | | HIV-infected | | | HIV-infected+CSC | | |
|---|---|---|---|---|---|---|---|---|---|
| | Short-term | Long-term | Ex-vivo[1] | Short-term | Long-term | Ex-vivo | Short-term | Long-term | Ex-vivo |
| **Pro-inflammatory** | | | | | | | | | |
| **IL-8** | ↓ | > | ↑ | ↓ | > | ND | ↓ | NA | ↑ |
| **TNF-α** | ↑ | ↑ | ND | ↓ | ↑ | ND | ↓ | ↓ | ND |
| **Anti-inflammatory** | | | | | | | | | |
| **IL-1ra** | ↓ | ↑ | ↓ | ↓ | ↑ | ↓ | ↓ | ↓ | ↓ |
| **Chemokines** | | | | | | | | | |
| **MCP-1** | ↓ | ↓ | ↑ | ↓ | ↓ | ND | ↓ | ↓ | ↓ |
| **RANTES** | ↑ | ↓ | ↓ | ↑ | ↓ | ↓ | ↑ | ↑ | ↑ |

Note: ↑↓=Denotes short-term exposure. ↑↓=Denotes long-term exposure. ↑↓=Denotes ex vivo. >=Denotes above upper limit of quantification. ND=Not detectable.

[1] Data Source: Kodidela, S., et al., Cytokine profiling of exosomes derived from the plasma of HIV-infected alcohol drinkers and cigarette smokers. PLOS ONE, 2018. 13(7): p. e0201144.

# CHAPTER 5. CONCLUSION AND FUTURE DIRECTION

## Conclusion[6,7]

Our earlier studies have largely demonstrated how cellular CYP contributes to mitigation of HIV-1 replication. We have demonstrated that in the presence of CSC, or individual constituents of cigarette smoke such as B(a)P/nicotine, myeloid cells may enhance the cellular CYP expression, resulting in oxidative stress, which ultimately increases HIV-1 replication[11, 21, 209]. Our cellular studies were largely validated by clinical samples as well [22, 40]. Previous studies have suggested involvement of oxidative stress and inflammatory modulators in tobacco-enhanced HIV-1 pathogenesis, however, how they are communicated between cells is mostly unknown. We proposed a novel mechanistic pathway involving extracellular vesicles (EVs) that carry oxidative stress and inflammatory agents to ultimately modulate tobacco-exacerbated HIV-1 pathogenesis in macrophages.

In Aim 1, for the first time, we characterized EVs from uninfected and HIV-1-infected monocytes. We demonstrated that their protein content and antioxidant capacity are altered in the presence of CSC. We observed that total protein and antioxidant capacity of the EVs, from both uninfected and infected sources, is decreased after CSC exposure. Interestingly, the average size of EVs remained the same, but the average count per TEM image of EVs reduced after CSC treatment. This suggests that the alteration in the total protein content and antioxidant capacity is likely due to an alteration in the number of EVs released. The EVs demonstrated an optimum zeta potential, which allowed them to be readily internalized by recipient cells. Importantly, we observed that EVs derived from monocytic cells exposed to cigarette smoke condensate (CSC), especially uninfected monocytes, had protective effects against toxicity and viral replication. However, EVs derived from HIV-1-infected monocytes lacked this property, which suggested that EV-mediated protection against cytotoxicity and viral replication is possibly limited to the earlier stages of HIV-1 infection.

In Aim 2, we isolated EVs from uninfected and HIV-1-infected macrophages and characterized them in terms of size, number, and total protein concentration. In contrast to the monocyte-derived EVs, we observed an increasing trend in the size and number of EVs upon exposure to CSC. This trend corroborates total protein concentration observed in the EVs, i.e., EVs obtained from CSC-exposed macrophages demonstrated higher

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[6] Modified from final submission with open access permission. Sanjana Haque, Namita Sinha, Sabina Ranjit, Narasimha M. Midde, Fatah Kashanchi & Santosh Kumar. Monocyte-derived exosomes upon exposure to cigarette smoke condensate alter their characteristics and show protective effect against cytotoxicity and HIV-1 replication. Sci Rep 7, 16120 (2017). https://doi.org/10.1038/s41598-017-16301-9 [48].

[7] Modified from final submission with open access permission. Sanjana Haque, Sunitha Kodidela, Namita Sinha, Prashant Kumar, Theodore J Cory, Santosh Kumar. Differential packaging of inflammatory cytokines/ chemokines and oxidative stress modulators in U937 and U1 macrophages-derived extracellular vesicles upon exposure to tobacco constituents. PLOS ONE. In press [230].

protein content. Next, we demonstrated that HIV-1 and CSC exposure to monocytes and MDM altered the packaging of oxidative stress and inflammatory modulators into EVs. Particularly, uninfected monocyte-derived EVs packaged higher amounts of the AOE catalase, which potentially conferred protection to CSC-exposed and HIV-1-infected recipient cells from further oxidative stress. However, catalase, as well as PRDX6, had lower expression in HIV-1-infected monocyte-derived EVs, which could explain their incapability to protect recipient cells from oxidative stress. Moreover, CYP (1A1) were present in a notably higher concentration, with a lower expression of AOEs (SOD-1 and catalase) in HIV-1-infected MDM-derived EVs, compared to EVs from uninfected cells. With longer exposure of a stimulus like CSC on HIV-1-infected MDM, more cytokines and chemokines were packaged and released within EVs, particularly the pro-inflammatory cytokines IL-6, Il-1β, and TNF-α, as well as the chemokine RANTES. We also observed a mixed response for the anti-inflammatory cytokines in the EVs.

Overall, it is reasonable to suggest that in the presence of CSC, packaging of pro-oxidant and pro-inflammatory contents into EVs is increased. This differential packaging in EVs potentially contributes towards the generation of oxidative stress, leading to exacerbation of HIV-1 in MDM. Thus, we present EVs as novel cellular messengers that likely transport CYPs, AOEs, cytokines, and chemokines from monocytic cells, altering tobacco-induced oxidative stress and enhancing viral replication in HIV-1 infection. The current study adds novel findings to the field of EVs in smoking-enhanced HIV-1 pathogenesis. EVs could be considered a major contributor to smoking-associated HIV-1 progression. It is yet to be determined exactly how and to what extent EVs carry pro-oxidants and inflammatory agents to modulate HIV-1 pathogenesis, especially in the context of tobacco smoking. Researchers have demonstrated the potential of EVs in carrying AOEs to negate oxidative stress in the treatment of Parkinson's disease [290]. This is promising in the development of EVs as therapeutic carriers for HIV-1 viral suppression or targeting latency reservoirs through the oxidative stress pathway. Similarly, EVs have a high potential as tools for diagnosis and prognosis of HIV-1 progression, as well as in biomarker discovery for smoking-affected HIV-1 pathogenesis. Future studies should be directed towards establishing a full comprehension of the mechanistic pathways involving EVs in smoking-enhanced HIV-1 pathogenesis, so that they may be employed in developing the clinical and therapeutic potentials of EVs.

The overall findings from the current study as well as a proposed mechanistic pathway is summarized in **Figure 5-1**.

## Future Direction

There are ample opportunities to further this current study. First, in order to validate our *in vitro* findings and attain a deeper understanding of the role of EVs in smoking-enhanced HIV-1 pathogenesis, future studies could be directed towards studying EVs from *ex vivo* human plasma. Plasma-derived EVs have been shown to contain considerable alcohol-inducible CYP2E1 as well as tobacco constituents-inducible CYP1A1, CYP1B1, and CYP2A6 [67] [183]. In the current project, we also observed the

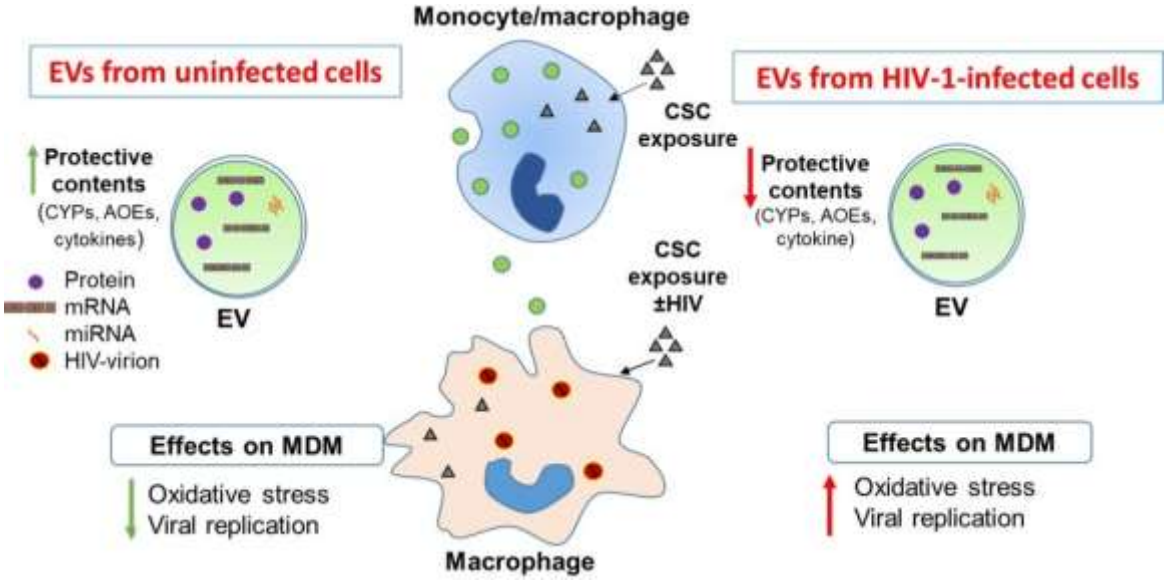


**Figure 5-1. Proposed Mechanistic Pathway for the Effect of Differential Packaging of Oxidative Stress and Inflammatory Modulators in Monocyte and MDM-Derived EVs in Exacerbating HIV-1 Pathogenesis.**
Monocytes and MDM release EVs with modified components upon exposure to CSC and/or HIV-1. EVs from HIV-1-infected cells in general package higher concentrations of pro-oxidant and pro-inflammatory elements (CYPs, cytokines) compared to the uninfected cell-derived EVs. Consequently, overall oxidative stress and inflammation in the recipient MDM are increased, leading to enhanced HIV-1 replication. On the contrary, EVs from uninfected cells impart protection by packaging higher amount of anti-oxidant and anti-inflammatory elements (AOEs, cytokines), reducing overall cytotoxicity and HIV-1 replication.

trend of higher CYP expression in the HIV-1-positive population, however further investigations to confirm this observation would be highly desirable. Perhaps a larger cohort of samples comprising healthy, HIV-1-positive nonsmoker, uninfected smoker, and HIV-1-positive smoker populations will be useful in this regard. Moreover, the use of plasma EVs isolated from smokers, HIV-1-infected nonsmokers, and HIV-1-positive smokers for *in vitro* as well as *in vivo* models will reveal useful information on their role in cytotoxicity and effects on HIV-1 pathogenesis.

Macrophage-derived EVs have the advantage of penetrating the highly selective, semipermeable BBB, allowing them a unique exposure to the resident cells of the CNS [207]. EVs from uninfected and HIV-1-infected MDM, in the absence and/or presence of tobacco exposure, can potentially deliver oxidative (e.g. CYPs) and inflammatory elements (cytokines/chemokines) to the brain and exacerbate HIV-1 pathogenesis and HAND. Therefore, future studies to investigate the role of EVs in the CNS cells would be interesting. As a preliminary study for continuing this current project, we investigated the effect of HIV-1-uninfected MDM-derived EVs on microglia cells, which demonstrated a similar protective effect from the EVs (data not shown). Thus, further investigations to identify the specific role of EVs containing cytokines and chemokines (especially IL-6), as well as CYPs, AOEs, other proteins, mRNA, and miRNA on HIV-1 and HIV-1-associated neuropathogenesis, are highly desirable.

Furthermore, the potentials of EVs are already being investigated in biomarker identification, diagnosis and prognosis of various conditions, and as therapeutic carriers of drugs in periphery as well as in the CNS. Our studies and others have postulated the prospect of IL-6, RANTES, soluble CD14, and properdin being potential biomarkers for tobacco-enhanced HIV-1-pathogenesis [47, 184, 188, 189, 191-194]. Whether they could be used in actual therapeutic settings will require extensive further study. Knowledge about EVs can help us in devising novel therapeutic agents for treating tobacco-affected HIV-1 and HIV-1-associated pathologies more efficiently.

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# VITA

Sanjana Haque, daughter of late MD. Shamsul Hoque Mondal and Mrs. Shaheda Khatun, was born in 1990 in Dhaka, the capital city of Bangladesh. She went to Viqarunnisa Noon School and College for her elementary, middle, and high school education. She attended University of Dhaka to earn her Bachelor's in Pharmacy degree. After completion, she joined Eskayef Pharmaceuticals Ltd. as a Product Executive, and continued to study for her Master's in Pharmacy degree in Clinical Pharmacy and Pharmacology from University of Dhaka. She also worked with Incepta Pharmaceuticals Ltd. as a brand executive for few months, before moving on to a teaching position at Northern University Bangladesh in the department of Pharmacy. Her keen interest in Pharmacology and toxicology research inspired her to apply to the University of Tennessee Health Science Center. Upon acceptance to the program, she moved to the United States in 2016. She joined Dr. Santosh Kumar's lab to pursue her doctoral degree in Pharmaceutical Sciences. She will be graduating in May 2020.