Semen exosomes: intrinsic inhibitors of HIV-1 infection

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ABSTRACT

Exosomes are cell-derived vesicles that circulate in bio-fluids and enclose cell-associated cargo, producing various consequences in intercellular communication and may contribute to microbial pathogenesis. Exosomes are similar in composition to enveloped viruses, making differentiation between exosomes and enveloped viruses difficult. Yet, exosomes and enveloped viruses may vary considerably in function. Exosomes produced from infected cells may incorporate viral material as they are simultaneously produced with viruses and depending upon the cargo, contribute to disease pathogenesis. Conversely, exosomes from uninfected cells may contribute to protection from infection. Exosomes from breast milk, vaginal fluid, and semen of healthy donors protect against HIV-1. The functional dichotomy of exosomes is unknown. Here, we focus on the function and physical qualities of exosomes found in semen (SE) and how these influence HIV-1. As semen is the major body fluid involved in HIV-1 transmission, exosomes from semen that regulate HIV-1 may contribute to the low incidence of HIV-1 sexual transmission in vivo.

Previous studies indicate healthy donor derived SE but not blood exosomes (BE) inhibit HIV-1 in a donor-independent manner. The composition and function of exosomes depends on the status of the exosome-producing cell. Thus, donor characteristics that alter the condition of exosome-producing cells may alter the antiviral phenotype of SE. Illicit drug use enhances HIV-1 replication, negatively affects male fertility, and alters exosome biogenesis pathways. Thus, illicit drugs may alter SE physical, composition, and functional properties. Indeed, SE from donors with a history of illicit drugs were altered in composition which correlated with a diminished ability to inhibit HIV-1. Similarly, because exosomes derived from HIV-1 infected cell cultures promote infection, donor HIV status may contribute to a proviral phenotype of
exosomes. Infectivity studies by HIV-infected ART-naïve SE revealed that SE from healthy donors and HIV-infected donors are inhibitory, but BE are not inhibitory. Therefore, the inhibitory phenotype of SE is conserved regardless of donor HIV status. Significantly, BE and SE from HIV-infected ART-suppressed donors not only inhibited HIV-1, but contained inhibitory levels of antiretroviral (ARV) medications, indicating that body-fluid derived exosomes may act as carriers of ARV drugs.

Previous studies found that the antiviral mechanism of SE was targeted to multiple HIV-1 lifecycle steps; however, the mechanism of inhibition was unclear. Transcription specific analysis revealed that SE reduced HIV-transcription at multiple steps including association of transcription factors NF-kB and Pol II with the viral promoter, as well as transcription initiation and elongation. SE inhibited HIV-driven promoter activation and viral gene expression. Importantly, SE targeted inhibition of viral protein Tat transcriptional activities.

Overall, these findings from donor characteristics that may alter the condition of the cellular source of SE demonstrate that SE antiviral factor(s) is highly conserved. Illicit drugs alter SE-associated factors and may reduce SE inhibitory activities. However, HIV status does not affect the antiviral function of SE. Significantly, donor-ARV medications are associated with SE and BE indicating a potential role of exosomes in drug delivery in vivo. Mechanistically, SE suppress HIV-1 by targeting host and viral transcription factors that could be therapeutically exploited for novel anti-HIV strategies. These data emphasize the need for additional studies on the composition and function of SE to harness the antiviral potential of SE inhibitory factors.
Exosomes are nano-sized vesicles released from most cell types and are found in most body fluids. Healthy cells and cells infected with different viruses release exosomes that may inhibit or enhance infection. Recent research showed that exosomes found in semen of healthy human donors inhibit HIV infection, but that exosomes from blood do not. We tested whether exosomes from HIV-infected donors inhibit HIV, and found that semen exosomes from HIV-infected donors inhibit infection regardless of whether donors are on anti-HIV medications or not on medication. HIV-infected donors’ blood exosomes inhibit HIV if the donor is on anti-HIV medications, but do not inhibit if they are not on anti-HIV medications. We found that this was due to exosomes packaging the anti-HIV drugs taken by the HIV-infected donors.

Illicit drug use is associated with increased susceptibility to HIV and worsening disease, and may cause changes in both the male reproductive tract and generation of exosomes. We determined that donor illicit drug use altered semen exosomes by changing exosomes composition, and these exosomes were less able to inhibit HIV-1 infection. We investigated how semen exosomes inhibit HIV and found that they restrict the virus from copying its genetic information to make more viruses. Our results suggest that regardless of HIV status, semen exosomes are a natural protective factor against HIV. The findings of our work will likely aid mechanistic understanding of how exosomes affect viral infections, and potentially the development of novel anti-HIV therapies.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>ix</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
</tbody>
</table>

## CHAPTER I: INTRODUCTION- VEHICLES OF INTERCELLULAR COMMUNICATION: EXOSOMES AND HIV-1

- HIV/AIDS ................................................................................................................. 1
- Non-viral vesicles released by cells ................................................................. 2
- Factors influencing HIV-1 sexual transmission .................................................. 3
- Pro- and anti-HIV seminal components ................................................................. 6
- Non-semen body fluid exosomes and HIV-1 ......................................................... 9
- Semen exosomes and HIV-1 .................................................................................. 11
- Semen exosome cargo ......................................................................................... 19

## CHAPTER II: ISOLATION OF EXOSOMES FROM SEMEN FOR IN VITRO UPTAKE AND HIV-1 INFECTION ASSAYS

- Abstract .............................................................................................................. 36
- Introduction ......................................................................................................... 36
- Materials and methods ...................................................................................... 37
- Data analysis ....................................................................................................... 50
- Notes ................................................................................................................... 51
- Recipes ............................................................................................................... 54

## CHAPTER III: EFFECT OF PROLONGED FREEZING OF SEMEN ON EXOSOMES RECOVERY AND BIOLOGIC ACTIVITY

- Abstract .............................................................................................................. 62
- Introduction ......................................................................................................... 62
- Materials and methods ...................................................................................... 64
- Results ................................................................................................................ 70
Discussion ........................................................................................................................................... 76

CHAPTER IV: BODY FLUID EXOSOMES IN HIV-INFECTED SUBJECTS ENCASE ANTIRETROVIRAL DRUGS AND CONFER ANTI-HIV PHENOTYPE TO TARGET CELLS ........................................................................................................ 90

Abstract ............................................................................................................................................. 90

Introduction ......................................................................................................................................... 90

Materials and methods ....................................................................................................................... 92

Results .................................................................................................................................................. 95

Discussion .......................................................................................................................................... 99

CHAPTER V: SEMEN EXOSOMES PROMOTE TRANSCRIPTIONAL SILENCING OF HIV-1 BY DISRUPTING NF-KB/TAT CIRCUITRY ......................................................................................... 110

Abstract .............................................................................................................................................. 110

Introduction ......................................................................................................................................... 110

Materials and methods ....................................................................................................................... 112

Results .................................................................................................................................................. 118

Discussion .......................................................................................................................................... 125

CHAPTER VI: SUMMARY AND FUTURE DIRECTIONS .......................................................................... 138

Semen exosomes inhibit HIV-1 ........................................................................................................... 138

HIV-1 inhibition by semen exosome surface protein ............................................................................ 138

Identifying semen exosomes antiviral source by donor characteristics ............................................. 139

Role of exosomes derived from HIV-infected donors in HIV replication ......................................... 140

Anti-retroviral drugs are incorporated in body-fluid exosomes .......................................................... 141

Semen exosomes target viral factors .................................................................................................. 141

Future research of semen exosomes .................................................................................................. 142

APPENDIX: GLYCEROL MONOLAURATE (GML), AN ANALOGUE TO A FACTOR SECRETED BY LACTOBACILLUS, IS VIRUCIDAL AGAINST ENVELOPED VIRUSES INCLUDING HIV ....................................................................... 146
Abstract ..................................................................................................................................146
Introduction ............................................................................................................................146
Materials and methods ...........................................................................................................149
Results ....................................................................................................................................153
Discussion ..............................................................................................................................158
REFERENCES ............................................................................................................................169
LIST OF TABLES

Table 1: Classification of cell-associated vesicles .......................................................... 31
Table 2: Characteristics of donor and semen samples .................................................. 81
Table 3: Effect of length of storage and illicit drug use on semen exosome protein concentration .......................................................... 82
Table 4: HIV-infected donor clinical characteristics ................................................. 103
Table 5: HIV-infected ART-suppressed donor demographics .................................. 104
Table 6: ARV concentrations in exosome-free plasma and exosomes from HIV-infected ART-suppressed donor blood and semen ......................................................... 107
Table 7: Primer sequences ....................................................................................... 129
LIST OF FIGURES

Figure 1: Schematic representation of the HIV-1 lifecycle .......................................................... 30
Figure 2: Schematic representation of the effects of semen on the mucosal microenvironment during HIV-1 transmission ........................................................................................................... 32
Figure 3: Spectrum of the function of seminal components during HIV-1 infection ................... 33
Figure 4: Electron micrograph of semen exosomes by negative staining .................................... 34
Figure 5: Semen exosomes inhibit HIV-1 lifecycle steps............................................................. 35
Figure 6: Internalization of fluorescent exosomes by FACS........................................................ 55
Figure 7: CD63 expression on SE................................................................................................. 56
Figure 8: Acetylcholine esterase activity of SE ........................................................................... 57
Figure 9: SE size and concentration estimation ........................................................................... 58
Figure 10: Separation of SE into different fractions ........................................................................ 59
Figure 11: RNA integrity of SE .................................................................................................... 60
Figure 12: SE inhibition of HIV-1 ................................................................................................ 61
Figure 13: Physical properties of SE isolated from diverse conditions ........................................ 83
Figure 14: Protein concentration and electrophoretic patterns of SE from different donors at different freezing times ................................................................................................................. 84
Figure 15: Length of semen storage alters SE-associated AChE enzymatic activity ................. 85
Figure 16: Levels of CD63 and CD9 on the surface of SE........................................................... 86
Figure 17: Content and quantity of SE RNA from different donors and after different freezing times .............................................................................................................................................. 87
Figure 18: Effects of length of freezing and donor drug use on SE-mediated HIV-1 inhibition . 88
Figure 19: Association between CD63 expression and AChE activity with SE-mediated inhibition of HIV-1 infection ................................................................................................................. 89
Figure 20: SE from HIV-negative and HIV-infected ART-naïve donors inhibit HIV-1............ 105
Figure 21: Presence of ART in HIV-infected ART-suppressed donor body-fluids protect against HIV-1 .............................................................................................................................................. 106
Figure 22: Exosomes from HIV-infected ART-suppressed donors contain ARV drugs........... 108

Figure 23: IgG does not contribute to HIV inhibition in HIV-infected ART-suppressed donors........................................................................................................................................ 109

Figure 24: Effects of SE on viral RNA species produced within HIV-1-infected cells .......... 130

Figure 25: Semen exosome-mediated reduction in HIV RNA expression is operative in host cytoplasmic and nuclear subcellular compartments ........................................................................................................... 131

Figure 26: Semen exosomes do not alter the integrity of host RNA ........................................ 132

Figure 27: HIV-driven LTR promoter transactivation is significantly down-regulated by SE .. 133

Figure 28: SE alone do not up-or down-regulate basal LTR promoter activity ...................... 134

Figure 29: The NF-kB transcription factor pathway is implicated in SE-mediated control of HIV-1 transcription........................................................................................................................................................................ 135

Figure 30: SE reduce RNA Pol II association with HIV-1 LTR ............................................. 136

Figure 31: SE inhibition of HIV-1 transactivation is mediated by interference with Tat protein-mediated LTR activation ........................................................................................................................................................................ 137

Figure 32: Exosomes from human semen have various effects on HIV-1 replication depending on donor characteristics ........................................................................................................................................................................................................ 145

Figure A.1: GML-mediated inhibition of HIV-1 requires virus contact .................................. 161

Figure A.2: GML reduces HIV-1 binding and entry .................................................................. 162

Figure A.3: Exposure of HIV-1 to GML impairs virus activity ................................................ 163

Figure A.4: GML is virucidal against diverse enveloped viruses .............................................. 164

Figure A.5: GML is inactive against non-enveloped viruses .................................................... 165

Figure A.6: Envelope maturation reduces sensitivity to GML-mediated inactivation .......... 166

Figure A.7: Molecular structures of GML and reutericyclin .................................................. 167

Figure A.8: An analogue to GML, reutericyclin, inhibits HIV-1 ............................................. 168
Published work

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CHAPTER I: INTRODUCTION- VEHICLES OF INTERCELLULAR COMMUNICATION: EXOSOMES AND HIV-1

Cells release nanometer sized particles that enclose various cell-associated nucleic acid and protein cargo [1, 2]. The importance of cell-secreted vesicles in intercellular communication is clearly accepted. However, the terminology and distinguishing aspects for the wide variety of vesicles released from cells is much less clear. Such vesicles are commonly referred to as exosomes, extracellular vesicles, oncosomes, microvesicles, and others. Here, we will refer to them as exosomes for simplicity.

The wide-range of definitions for cell-derived vesicle subtypes results in considerable overlap with the defining features of enveloped viruses. Functionally, viruses and cell-associated vesicles mediate intercellular communication by circulating, binding to and entering cells, and delivering their cargo to target or recipient cells [3]. In addition to similarities in function, exosomes and viruses are also similar in composition potentially due to their overlapping use of biogenesis pathways. In infected cells, viruses and exosomes are simultaneously produced, resulting in the incorporation of viral material into exosomes. These features make differentiation and separation between the types of particles difficult.

Distinguishing between exosomes and viruses in biological samples is important to understand their independent and dependent contributions to disease and identify potential therapeutic interventions. It is clear that exosomes may contribute to disease protection or pathogenesis, the reason for which is often unknown or cell-condition specific. Purification techniques aim to address this issue, however, all current methods of separation contain caveats that may influence downstream applications [4]. As a consequence of the range of defining characteristics, lack of universal marker, and the ability of exosomes to contain viral material, it is often impossible to validate homogenous separation from a heterogenous population.
In this review, we compile the current understanding of similarities and differences of exosomes in general and enveloped viruses, emphasizing HIV-1, an enveloped retrovirus. Despite similar physical qualities, HIV-1 and exosomes may considerably vary in function, which may be attributed to exosome body-fluid source. Here we summarize the function of body-fluid exosomes during HIV-1 infection. We focus on the association of exosomes and HIV-1 with semen, important during sexual transmission of HIV-1. We emphasize the current understanding of semen exosomes and their role during HIV-1 infection. Because exosomes from semen regulate HIV-1 infection, we suggest this subtype of vesicle may contribute to the low risk of HIV-1 sexual transmission per exposure.

**HIV/AIDS**

More than 35 million deaths have been attributed to Acquired Immune Deficiency Syndrome (AIDS) since the disease was recognized in 1981 [5]. HIV-1, the causative agent of AIDS, is classified as a member of the Lentivirus genus in the family of Retroviridae [6]. Although primate lentiviruses were known to exist at the time AIDS was identified, it is believed that the non-human primate form (simian immunodeficiency virus or SIV) of HIV-1 was relatively contained within the simian population until a transmission event led to human infection through cutaneous or mucosa exposure [6, 7]. Subsequent viral adaptation to humans led to the emergence of HIV-1 and the resulting AIDS pandemic [7]. The isolation of HIV-1 from human semen and the ability of asymptomatic carriers to transmit the virus via cell-free and cell-associated seminal fluid has contributed to the emergence of HIV-1 [8].

**HIV-1 replication**

HIV-1 virions are ~100 nm spherical particles that contain an envelope (Env) comprised of a lipid bilayer with intermittent viral glycoproteins [6]. These glycoproteins mediate HIV-1
cellular tropism and account for primary targeting of CD4+ cells [9]. The HIV-1 capsid contains two single-stranded copies of the RNA genome encased in the nucleocapsid, along with several viral enzymes and proteins [10]. The HIV-1 lifecycle begins with a binding/attachment event of the viral Env to the CD4 receptor on the host cell surface. HIV-1 further requires a co-receptor (CCR5 or CXCR4) for entry [9]. Binding to the viral receptor/co-receptor stimulates cell membrane fusion with viral Env resulting in capsid entry, following which viral RNA is released into the cytoplasm. The HIV-1 single-stranded viral RNA is converted into double-stranded DNA (dsDNA) by the viral RNA-dependent, DNA polymerase (reverse transcriptase). The resultant dsDNA is transported to the nucleus and integrated into the host cellular (chromosomal) DNA equating to viral insertion into the host genome (proviral DNA). In activated, proliferating cells, proviral DNA replicates its genome utilizing host machinery for transcription of viral RNA and translation of viral proteins. Accumulation of viral RNA and proteins leads to assembly of new HIV-1 particles that are moved to the cell membrane. These new virions bud from the cell to result in new infectious particles (Figure 1) [11]. A hallmark of HIV-1 pathogenesis is viral-mediated regulation of the host immune response and molecular pathways during infection [12]. As such, HIV-1 virions act as important vehicles in regulating intercellular communication.

**Non-viral vesicles released by cells**

Cell-derived vesicles are important vehicles of intercellular communication which play significant roles in several pathologies, including cancer, neurodegenerative disorders, and infectious diseases, such as viral infections. There are different types of cell-derived vesicles; however, for simplicity we will refer to all cell-derived, non-viral particles as exosomes throughout this review. Nevertheless, it is important to appreciate that cells produce diverse vesicles often resulting in heterogenous vesicle composition. Cell-associated vesicles are
characterized based on their origin, mechanism of release, size, and potential markers (Table 1). The term extracellular vesicle is often used to generically describe most membranous cell-associated vesicles. However, by definition, extracellular vesicles (EVs) are a mixed population of exosomes, microvesicles, large oncosomes, and apoptotic bodies [13, 14]. Exosomes account for small membranous vesicles that range in size from 40-100 nm, although this definition may evolve as smaller non-membranous vesicles (~35 nm) have recently been described as exomeres [1, 15]. Exosomes exist as intraluminal vesicles within multi-vesicular bodies (MVBs) prior to release via fusion of MVBs with the plasma membrane [1, 16]. Microvesicles are slightly larger ranging from 100-1000 nm, while apoptotic bodies often account for 500-4000 nm vesicles. Both microvesicles and apoptotic bodies bud directly from the plasma membrane [17-19]. On opposite ends of the spectrum, large oncosomes range from 1-10 μm whereas newly described exomeres exist as <50 nm (average 35 nm) vesicles. Large oncosomes and exomeres are distinguishable by their cancer cell-association and non-membranous structure, respectively [14, 15]. These vesicles can also be classified based on originating cell type; for example, prostasomes specifically originate from the prostate epithelium and ectosomes often originate from monocytes and neutrophils [20, 21]. Although potential markers and cargo are described for each vesicle classification, there generally exists a large overlap between nucleic acid and protein content. This overlap in multiple characteristics of cell-associated vesicles has resulted in the frequent use of nonspecific terminologies.
Exosomes and enveloped viruses

By strict definition exosomes are different from viruses by their inability to replicate their contents. Unlike viruses, exosomes are metabolically inert and cannot reproduce their contents to generate progeny from producer cells [22]. However, as previously reviewed, exosomes and viruses do not conform to strict definitions [22]. Intermediate particles exist on the spectrum between virus and exosome that contain both host and viral components making it nearly impossible to classify these vesicles as either defective viruses or exosomes that contain viral components [22]. Intermediate particles are often classified as a virus or exosome derivative depending on the preference of the investigator, but once these vesicles deviate from strict definitions they may be more accurately defined as an assortment of lipid-encased particles that cannot be easily differentiated [22].

There are structural and functional similarities between exosomes and enveloped viral particles. Although heterogeneous, exosomes are similar in size compared to retroviruses (~120 nm), and the biogenesis of both exosomes and viral particles involve shared cellular pathways [22]. An important difference between exosomes and enveloped viral particles is the ability of enveloped viral particles to replicate their contents. Although exosomes may contain virus-associated nucleic acids and proteins, true exosomes do not replicate [22]. However, the recent discovery of quasi-enveloped hepatitis E and A viral particles complicates this distinction. In the case of these non-enveloped viruses, exosomes provide a pseudo-envelope that allows the particles to transmit infection [23-25]. Similarly, exosomes carrying infectious Hepatitis C virus RNA can transmit infection [26]. However, in these cases it may be argued that these virus-RNA containing exosomes are more similar to viral particles that have hijacked exosome biogenesis pathways rather than typical, cell-derived exosomes. These intermediate exosome-viral particles
have been reviewed elsewhere [22, 27]. Due to the inherent nature of exosomes to reflect the status of the producer cell at the time of biogenesis, the role of exosomes during HIV-1 infection is diverse [28]. Simply put, exosomes contribute to viral pathogenesis by regulating cellular functions that may inhibit or enhance viral infection [28]. The secretion of exosomes from nearly all cell types and their detection in a variety of biological fluids illustrates their wide-reaching influence on the cell microenvironment. Further, the localization and cellular derivation contributes to the complexity in identifying specific exosome subtypes and exosome-conveyed properties responsible for modulating host functions [29].

**Factors influencing HIV-1 sexual transmission**

Vaginal and rectal sexual intercourse accounts for >70% of HIV-1 infections worldwide [30]. During mucosal exposure, HIV-1 infection often occurs via transfer of virus from dendritic cells (DCs) to monocytes/macrophages, while direct infection of CD4+ T cells is more likely during parenteral transmission [6]. It is important to note that during mucosal infections, HIV-1 must transverse the layers of the epithelium to reach these target cells [31]. However, during HIV-1 sexual transmission intercourse may result in trauma to the epithelial cell layer leading to increased access to CD4+ cells [31]. Although sexual transmission accounts for the majority of infections worldwide, the risk of contracting HIV-1 per exposure is far greater during blood exposure than with semen exposure (risk/exposure: 9,250/10,000 from blood transfusion, 63/10,000 from needle sharing, and 8/10,000 vaginal intercourse) [32]. While blood components may influence infection, semen is the major transmission vehicle during sexual transmission [31]. High viral loads contained in semen of HIV infected individuals (up to 1.3 x 10^7 RNA copies/ml) suggest that semen may transmit HIV-1 via both seminal CD4+ cells or as cell-free virus [33, 34]. Despite semen serving as carrier of HIV-1, individual seminal components have
different roles during HIV-1 transmission and may facilitate or inhibit infection [35, 36]. Semen is highly complex, with more than 900 proteins and additional carbohydrates and lipids [35, 36]. This may explain the finding of both pro- and anti-HIV-1 factors reported in semen.

**pH regulatory and immunomodulatory role of semen**

The immunomodulatory effects of semen alter inflammatory signaling pathways, mucosal barrier integrity, and are crucial for reproductive success [37]. In addition to aiding reproduction, these functions can alter susceptibility to HIV-1 infection during sexual transmission. For example, normal vaginal pH is acidic (4.0 to 6.0), reduces sperm viability and motility, and is hostile to HIV-1 particles that are inactivated at pH < 5.0 [31, 38]. However, semen is alkaline and raises vaginal pH to the range of 6.0 to 7.0 [31], potentially favoring fertilization and HIV-1 infection [38, 39]. Aside from pH changes, some factors contained in semen promote disruption of the mucosal barrier thereby enhancing HIV-1 infection of immune cells that reside in the subepithelial layer of the reproductive tract. Typically, HIV-1 virions become trapped in the mucosal barrier and are less efficient at diffusing through intact epithelial layer [40].

**HIV-1 enhancing immune factors of semen**

In addition to supporting spermatozoa by promoting liquefaction of the coagulated fluid increasing spermatozoa motility, semen is also associated with modulation of immune responses within the reproductive microenvironment [41]. Semen, in comparison to blood, contains increased levels of inflammatory cytokines regardless of HIV infection status, although specific cytokines appear to be up-regulated during HIV-1 infection [35]. Semen induces cellular secretion of the inflammatory cytokines/chemokines IL-8, MCP-1, IL-6, GM-CSF, CCL20, and others. These factors may function in HIV-1 target cell recruitment, maturation, and activation
The enrichment of TGF-β, PGE2, and IL-7 in semen is thought to exert both pro- and anti-inflammatory responses on a variety of immune cells. This may influence cell influx and act to suppress host immunity, thus facilitating HIV-1 replication [31, 42]. It is postulated that seminal cytokines may influence inflammatory changes in order to support tolerance in pregnancy, as introduction of material into the female reproductive tract would typically elicit an immunogenic response [43]. Perhaps the pro-survival properties of semen that support spermatozoa within the female reproductive tract also promote the survival of HIV-1 virions during infection [44]. Semen CCL2 levels negatively correlate with CD4+ T cell counts, and seminal HIV-1 RNA concentration positively correlate with semen IL-6, IL-16, CCL2, CCL11, and CXCL12b [44]. This finding has been reproduced in a macaque model of SIV transmission [45], suggesting that seminal-associated cytokines promote HIV-1 infection.

**HIV-1 inhibitory factors in semen**

The cytokine/chemokine profile of semen may confer antiviral activity during infection. It has been suggested that CCR5-binding cytokines and CXCR4 ligands in semen may suppress replication of CCR5- and CXCR4-tropic strains of HIV-1, respectively [46]. Moreover, an unidentified component of semen inhibits HIV-1 infection of DCs by inhibiting viral attachment to DC-SIGN, an important mode of HIV-1 entry [36, 47, 48]. Semen clusterin was recently implicated in this inhibition, as it binds to DC-SIGN and competes for HIV-1 binding [49]. Clusterin is a glycoprotein expressed in many tissues as either a secreted or nuclear-associated form, and it is implicated in numerous functions [49]. Semen clusterin contains an abundance of fucosylated N-glycans with a high binding affinity for DC-SIGN, as shown by the inability of deglycosylated and defucosylated semen clusterin to inhibit binding of HIV-1 to DC-SIGN [49]. Although semen clusterin is potent against HIV-1, depletion of clusterin did not completely
restore HIV-1 binding to DC-SIGN [49], suggesting the presence of multiple DC-SIGN ligands within semen that may contribute to inhibition of HIV-1 attachment. Indeed, it has been shown that seminal Mucin-6 inhibits HIV-1 attachment to DCs, and subsequent DC-mediated transfer of HIV-1 to CD4+ T cells [50]. Further, semen has been shown to inhibit HIV-1 infection of CD4+ T cells [51].

**Pro- and anti-HIV seminal components**

Whether semen functions to promote or inhibit infection depends on multiple factors including the immunomodulatory factor content, immune or inflammatory status of the reproductive tract, HIV-1 viral load, and antiretroviral therapy (ART) [35]. It is currently unknown how exactly the factors present in semen regulate HIV-1 transmission. Semen factors may stimulate target cell recruitment, activation, and/or break-down of the mucosal barrier thereby increasing infection, or reduce susceptibility to infection by cytokine/chemokine expression or blocking viral attachment to DCs [35]. The early hypothesis that semen increased HIV-1 transmission centered on the presence of semen-derived enhancer of viral infection (SEVI) detected *in vitro* [52]. SEVI appears to be fragments of prostatic acidic phosphatase that, through the formation of amyloid fibrils, capture HIV-1 virions and increase viral transmission *in vitro* [52]. A similar attribution is assigned to semenogelin fibrils (SEM1 and SEM2) [53]. However, the relatively low frequency of heterosexual HIV-1 transmission contradicts the idea that semen greatly enhances transmission, especially given that the risk of infection per act of coitus varies between 0.0001 and 0.001 [54].

The presence of two forms of infectious HIV-1 in semen, cell-free and cell-associated virus, contributes to its role in facilitating viral transmission as both forms have advantages to overcoming mucosal barriers [40]. Predominately, cell-free virus passively diffuses through the
extracellular space to reach distal cells whereas cell-associated virus is more rapid and efficient at infecting susceptible cells [40, 55]. Spermatozoa enhances HIV-1 infection, especially of DCs, but also of macrophages and CD4+T cells [31]. Spermatozoa appears to enhance the efficiency of virus attachment to target cells when compared to cell-free virus [31]. It is unclear whether HIV-1 is able to bind to and enter spermatozoa, and this has been a source of debate despite the identification of HIV-1 nucleic acids within spermatozoa obtained from HIV-1 infected men [56]. Mannose receptors, glycolipids, and heparan sulfate on spermatozoa are proposed as potential receptors for HIV-1 binding due to their ability to interact with HIV-1 gp120 [56].

Similar to the viral capture function described for spermatozoa, the presence of soluble complement components within semen appear to opsonize HIV-1 and enhance infection of epithelial, monocyte/macrophage, T, and B cells in vitro [57]. Additionally, semen contains an inhibitor of the complement pathway in vitro, as CD59 aids escape from complement-mediated lysis [40, 58]. However, the role of complement during HIV-1 infection is controversial, making the contribution of semen to the complement system during infection unclear. In vivo, the role of semen during HIV mucosal infection is complex, and likely a summation of multiple effects [59] as depicted in Figure 2.

The presence of reactive oxygen species and cationic antimicrobial peptides are factors suggested to explain the HIV-inhibitory phenotype of semen, as these inhibit HIV-1 infection in vitro [36]. Nevertheless, the enhancing and inhibitory properties of amyloid fibrils, cationic peptides, and reactive oxygen species during HIV-1 infection are controversial [60, 61]. More recently the role of EVs, referred here as exosomes, has been investigated to understand the function of blood and semen during HIV-1 transmission. Using a variety of cell models and a murine AIDS model, exosomes derived from human semen were shown to inhibit HIV-1
infection, raising the possibility that anti-viral exosomes within semen may contribute to the low frequency of sexual HIV-1 transmission [62-64]. To summarize, semen likely contains a spectrum of components with variable functions that influence HIV-1 transmission, but the relative semen components and their activity are not clearly understood (Figure 3).

**Non-semen body fluid exosomes and HIV-1**

In recent years, the exosomes field has exploded with new exosome-attributed functions described during the progression of cancer, spread of pathogens, immune regulation, and normal cell development and differentiation. For example, *in vivo*-derived serum exosomes transmitted infectious human pegivirus (HPgV; previously GB virus C/hepatitis G virus) RNA to primary blood mononuclear cells (PBMCs) *in vitro* [65]. Cell-cultured derived exosomes from HPgV infected cells delivered HPgV protein to natural killer (NK) cells that inhibited NK cell immune functions [66]. Similarly, human serum-derived exosomes containing hepatitis C virus (HCV) RNA transferred HCV RNA to PBMCs and interfered with T cell receptor (TCR) signaling [67]. Thus, the biological impact of exosomes is widespread and significant [68]. Initially, it was not clear if exosomes represented cell debris or an artifact of the experimental systems in which exosomes were detected. Subsequent studies demonstrated the enrichment of particular cell-associated proteins regardless of the isolation strategy or cell type, confirming that exosomes are the result of distinct cellular processes giving rise to a distinct population of cellular vesicles [69]. A ubiquitous exosome-marker has yet to be formally assigned, but specific proteins such as CD9, CD63, CD81, HSP70, HSP90, MHC I and II, and acetylcholine-esterase are commonly found in exosomes, and thus may serve as useful exosome-indicators [17, 64]. The composition, cargo, and resulting function of exosomes rely on the status of the producer cell; therefore, the
ever-changing condition of the cell dictates exosome composition and biogenesis precluding the use of a specific protein to identify all types of exosomes.

**ESCRT-dependent and –independent pathways, HIV-1 and exosome biogenesis**

Endosome compartments and the endosomal sorting complex required for transport (ESCRT) pathway are essential for exosome secretion [69]. Briefly, exosomes are formed by endocytosis of plasma membrane proteins into early endosomes. Endosomes mature into MVBs where invaginations into MVBs result in the formation of individual vesicles that acquire sorted proteins, lipids, and nucleic acids [3]. Invaginations into MVBs result in incorporation of cytosolic components into individual vesicles, particularly cytosolic RNA species (mRNA, miRNA, non-coding RNA) [70]. The incorporation of RNA cargo into vesicles is highly dependent on the physiological state of the cell; therefore, the RNA cargo profile of vesicles may differ from the profile of the originating cell [3]. It is suggested that particular 3’UTR mRNA sequences may be preferentially targeted into vesicles [71]. The lipid composition of vesicles often closely resemble the composition of the originating cell, however, polyunsaturated glycerophosphoserines and phosphatidylserines seem to be particularly enriched in vesicles [3]. The vesicles’ lipid casing is derived from the lipid membrane of MVBs during the invagination step, at which time cytosolic lipids are also encased in vesicles [3, 70]. Vesicle escape into the extracellular domain from the producer cell occurs via MVB exocytosis [68]. Exosome biogenesis may occur through ESCRT-dependent and independent mechanisms, however, the ESCRT pathway is the most well understood mechanism [72]. Like exosome release, the ESCRT pathway is also required for HIV-1 budding. The HIV-1 Gag structural protein binds components of the ESCRT pathway to promote budding from the plasma membrane as the major route of viral egress [73]. For both exosomes and HIV-1 virions, utilization of the ESCRT machinery
results in the accumulation of a lipid bilayer from budding or fusion events with the plasma membrane [73].

MVBs are able to form in cells depleted of ESCRT components. During ESCRT-independent exosome secretion, ceramide formation, tetraspanins, phospholipase D2, and ADP ribosylation factor-6 mediate vesicle formation [72, 74]. ESCRT-independent pathway events may contribute to viral spread and to immune modulation during viral infections. ESCRT-independent domains alter the sorting of proteins and nucleic acids into exosomes, including the packaging of viral components into exosomes. For example, the tetraspanin CD63 mediates sorting of Epstein-Barr virus latent membrane protein 1 (LMP1) into intraluminal vesicles and packaging into exosomes [75]. The ESCRT-independent pathway may also facilitate sorting of viral components into exosomes of viruses that rely on ESCRT-components for release, possibly increasing the ability of these viruses to be released from cells. Exosome delivery of HSV-1 tegument proteins may “prime” cells for infection by immediately activating transcription upon contact with infectious virions. In addition, packaging of viral components such as HIV-1 Nef protein in exosomes enhances the ability of HIV to evade immune recognition by suppressing antiviral responses in recipient cells [76]. Exosomes and viruses exploit ESCRT-dependent and – independent pathways for biogenesis events.

**Exosomes and HIV-1 overlap in composition**

Due to utilization of the same cellular pathways, exosomes and newly synthesized HIV-1 virions incorporate similar molecules including tetraspanins [77], multi-vesicular body-associated proteins [78], and cytoskeleton proteins [79, 80]. In addition, exosomes isolated from HIV-1 infected sources contain viral materials including the viral trans-activation response element (TAR) RNA and proteins such as Nef and Gag. Transport of these viral factors to
permissive cells facilitates infection in trans [81-84]. The distinction between exosomes and HIV-1 particles becomes even more ambiguous by the existence of human endogenous retroviruses (HERVs). HERVs are evolutionarily ancient non-coding and protein coding retroviral sequences within the human genome that are unable to produce infectious virions. Specifically, HERV components including HERV-associated reverse transcriptase (RT), RNA, Gag and Env proteins are found in human exosomes [76]. HERV sequence-containing exosomes can facilitate transfer of HERV mRNA to cells in vitro [76]. These are only a few of the many constituents shared by viral particles and exosomes, and highlight the shared mechanisms of biogenesis between the two particle types.

Upon release from the producer or infected cells, exosomes and viral particles share features in how they interact with other cells via protein binding, endocytic pathway uptake, and membrane fusion [9, 85]. Following cellular entry, exosomes and HIV-1 virions act as delivery vehicles of information. Both disperse their contents into cells and influence biological processes, frequently by appropriating cellular machinery [76]. HIV-1 virions could be described as exosomes that are unique in their ability to replicate their contents. However, this is controversial based on the definition of replication, such as in the case of replication-incompetent viruses. These particles are still considered HIV-1 virions, but by definition are unable to reproduce their contents in living cells. Nevertheless, the similarities raise the question of whether or not HIV-1 virions are simply modified exosomes [22]? This question has led to the development of the Trojan exosome hypothesis [76], which reasons that exosomes and retroviruses contain extensive overlap in characteristics because retroviruses use the exosome pathway to facilitate receptor-independent infection [86]. Since the formation of retroviruses is driven by Gag protein expression, interaction of Gag with intraluminal vesicles directs
retroviruses to the exosome biogenesis pathway for the formation of infectious virions [86, 87]. While the Trojan exosome hypothesis warrants merit in understanding HIV-1 strategies outside of the classical model of receptor/co-receptor cellular infection and may offer value to understanding phenotypic similarities between exosomes and HIV-1, others argue that using the same cellular pathways does not make HIV-1 virions modified exosomes [88].

**Function of body fluid exosomes during HIV-1 infection**

It is important to again stress that exosomes may facilitate or inhibit HIV-1 infectivity, and that the effect is influenced by the producer cell of origin [28, 76]. Because biological fluid exosomes, such as those derived from blood or plasma, originate from multiple cell types, proviral or antiviral effects may be present in different fluids or from different donors [89]. For instance, blood or cell-culture derived exosomes transfer HIV-1 co-receptors, CCR5 [90] and CXCR4 [91], offering the ability to transform HIV-1 resistant cells into HIV-1 susceptible cells depending on cell expression of CD4 receptor. In addition to HIV-1 nucleic acids and protein [84, 92, 93], exosomes can also transfer cellular or viral factors that down-regulate the immune response to infection or enhance inflammatory signals [94, 95]. Exosomes from diverse biological systems contain surface-associated and encapsulated biologically active cytokines [96]; blood exosomes from HIV-1 patients contain cytokines/chemokines that increase activation levels of CD4+ and CD8+ T cells [97]. Exosomes from CD4+ T cells reactivate latent-SIV CD4+ T cells in a macaque model, potentially important for therapeutic applications targeting latent viral reservoirs [98]. Conversely, exosomes containing antiviral compounds such as APOBEC3G and interferon α/β, and exosomes from CD8+ T cells suppress HIV-1 infection *in vitro* [99-102]. Exosomes derived from CD4+T cells that contain CD4 inhibit HIV-1 infection compared to CD4-depleted exosomes [103]. In addition, exosomes derived from human breast-
milk [104], vaginal fluid [105], and semen [62-64] exhibit potent anti-HIV activity. Urine, saliva, ascites fluid exosomes have yet to be explored in HIV-1 infection, although proteomic analysis of saliva from HIV-positive heroin addicts identified that HIV infection modified the cargo of exosomes [89, 106, 107]. Remarkably, a head-to-head comparison of blood-derived exosomes with those purified from either breast-milk [104] or semen [63] showed opposing functions. Blood-derived exosomes had no effect or enhanced infection while breast-milk and semen derived particles consistently inhibited infection. The proviral and antiviral features identified in exosomes highlights that exosome composition may play an important role in cellular permissiveness and susceptibility during HIV-1 infection. However, caution should be used when comparing exosome studies, as a multitude of factors may influence observations including the exosome isolation protocol [108, 109], sample storage conditions [64, 110], and efficacy of recipient cell uptake [62, 111].

**Separating virus from exosomes**

The overlapping features of exosomes and HIV-1 particles makes purification of exosome and virus populations from the same source difficult if not impossible, complicating determination of the composition and functions of exosomes during different stages of HIV-1 infection. Popular techniques rely on velocity gradient separation such as iodixanol, since the density of HIV-1 virions and exosomes are somewhat different, although there is considerable overlap (1.13-1.21 g/l for exosomes and 1.16-1.18g/l for HIV-1) [4]. Thus, these techniques can be unreliable due to the similarity in biophysical properties and heterogenous nature of exosomes. Immuno-depletion or immuno-capture techniques have been suggested as ways to purify and concentrate exosomes from HIV-1 containing sources. Here, anti-acetylcholinesterase and/or anti-CD45 coated beads are used to capture exosomes without binding to HIV-1 [112].
This technique theoretically is a means to concentrate pure exosomes from HIV-1 particles without the addition of substances influencing down-stream HIV-1 functional assays. However, as at the time of this review, there is presently no way to remove exosomes bound to affinity beads without destroying exosome integrity, including exosome surface-associated molecules. Further, immuno-depletion or immuno-capture techniques may exclude some exosomes that are surface protein negative (or double negative) that are still capable of affecting functional studies. Exosome subpopulations contain variations in surface composition that may affect function; for example, CD63 surface protein levels from human semen-derived exosomes correlated to inhibition of HIV-1 infection where semen exosomes with reduced surface CD63 showed diminished ability to inhibit HIV-1 infection [64]. Similarly, depletion of the CD63 positive exosome population in herpes simplex virus-1 infected cells enhanced infection [113]. Thus, efficient methods of exosome and HIV-1 separation that maintain virion and vesicle integrity without complication of functional assays are needed.

**Therapeutic applications**

Although there are difficulties in separating exosomes and retroviral particles (including human and murine retroviruses and retrotransposon elements), each particle type has the ability to transfer materials to cells. This has been exploited for use as therapeutic delivery systems. Both exosome and retroviral vectors are being used to deliver immunotherapies and gene therapies because of their capacity to act as efficient transporters of bio-information [114, 115]. Both particle types provide a stable vehicle to encapsulate cargo with reduced immunogenicity [114], historically a major problem in similar delivery systems. Exosomes and lentiviruses are also proficient at interacting with multiple cell types and across tissue barriers including the blood-brain barrier [116, 117]. The surface properties of both types of particles can be modified
to allow for targeted interactions [116, 118]. Despite the similarities, exosomes and lentiviral vectors have important differences. The inherent capacity of exosomes to enclose nucleic acids, proteins, and lipids allows them to package biological and chemical agents [116]. Consequently, exosomes are employed in drug delivery systems, which is not a practical feature of lentiviral vectors. Although exosomes have been considered for use in gene therapy, particularly to carry coding and non-coding RNA including regulatory RNAs (miRNA, siRNA), lentiviral vectors have significant advantages in this therapeutic market because of their ability to confer stable integration into target cells [119, 120]. The ease of engineering and non-synthetic nature of these delivery systems offers advantages for disease targeting, and both approaches are currently being tested in human clinical trials (clinicaltrials.gov).

**Exosomes as biomarkers**

Exosomes are a “fingerprint” of the cell condition; thus, circulating exosomes are considered as potential biomarkers of disease [121]. Exosomes are considered advantageous biomarkers due to their stability, sensitivity, and specificity [122]. Cancer cell-derived exosome nucleic acid content may act as tumor markers. For example, plasma or urine-derived exosomes Survivin, PCA-3, and TMPRSS2:ERG are associated with prostate cancer [123]. Additionally, the proteomic profile of seminal plasma reveals potential markers of male infertility such as semenogelins, protein DJ-1, prostatic acid phosphatase, kallikrein 3, and prolactin-inducible protein to name a few [124-127]. Exosomes as markers of cancer and non-cancer disorders have been reviewed elsewhere [121, 128, 129]. Although studies are limited, exosomes containing viral material may be considered a marker of viral infections. Exosome-associated immune and oxidative stress markers were evaluated as indicators of HIV-1 disease progression. In addition to an increased abundance, plasma exosomes of HIV-positive ART-suppressed patients showed
increased oxidative stress markers, reduced anti-inflammatory polyunsaturated fatty acids (PUFA), and increased inflammatory response regulator, Notch4, compared to HIV-negative patients [130]. In a separate study, HIV-positive ART-naïve patients contained an increased abundance of plasma exosomes that were larger in size with enhanced miRNA levels than in HIV-positive ART-suppressed patients [131]. Exosome abundance and size inversely correlated with CD4-T cell counts and positively correlated with CD8-T cell counts [131]. These studies may indicate that exosomes may serve as indicators of oxidative stress, immune activation, and inflammation during HIV-1 progression.

**Semen exosomes and HIV-1**

During HIV-1 transmission, exosomes within human semen coexist with the virus in the male reproductive tract before widespread dissemination within the newly infected host; thus, biofluid-specific exosomes may provide novel insights into the role of exosomes in sexual transmission. Semen exosomes (SE) are a heterogeneous population (~30-200 nm in diameter) of extracellular vesicles that likely includes exomeres, generalized as exosomes for simplicity. SE are comprised of a range of morphologies and electron density (Figure 4) [63, 64]. SE abundance is estimated to range from $10^{11}$-$10^{12}$ particles per ml of semen. Since the average human ejaculate of semen is approximated at 3.7 ml, SE are highly concentrated [64, 132, 133]. In comparison, the concentration of blood exosomes is approximately $10^9$-$10^{10}$ vesicles/ml of plasma [134, 135]. Characteristic of other exosomes populations, SE contain cell-surface associated proteins shared by many exosomes including CD63, CD81, CD9, and acetylcholine esterase in addition to protein and nucleic acid cargo. Cargo may include small RNAs and mRNA capable of support or regulating gene expression [63, 64].
SE are internalized into cells by endocytosis and fusion with the cell membrane; treatment of cells with an inhibitor of macropinocytosis did not affect SE internalization, indicating that macropinocytosis is not a major route of cell entry for SE [62]. Comparison of uptake efficiency of SE and blood exosomes revealed vaginal epithelial cells internalize SE more efficiently than blood exosomes by an order of magnitude, however, blood exosomes are more efficiently internalized in monocytic (U937) and lymphocytic (SUPT1) cell lines than SE [62, 63]. This internalization efficiency may contribute to SE interference with HIV-1 infection of epithelial cells within the female reproductive tract by reducing HIV-1 transcytosis across the mucosa to reach CD4+ target cells. In both a co-culture and transwell model of HIV-1 infection, SE blocked transfer of HIV-1 from vaginal epithelial cells to monocytic (U937) and lymphocytic (SUPT1) cell lines, thus SE appear to inhibit cell-cell transmission [62]. In a more biologically relevant transwell system, SE also blocked trans infection from V428 cells to primary blood leukocytes (PBLs) [62].

The effects of SE obtained from HIV-1-negative donors reduce HIV-1 infection by more than 50% in a variety of in vitro cervical, monocytic, and lymphocytic cell models including PBLs [62, 63]. Levels of inhibition increase as concentration of SE protein increases, plateauing at ~100 μg/ml [63]. The antiviral effect is conserved across HIV-1 viral strains (R5 and X4), including lab-adapted and transmitted founder isolates, and is effective when a range of viral inocula is studied [63]. Together, these data suggest SE inhibit HIV-1 in a donor-independent and dose-dependent manner regardless of viral co-receptor tropism [63]. Most significantly, human SE-mediated reduction in HIV-1 infection is recapitulated in vivo using a murine-AIDS model of infection [62]. Infection with murine-AIDS LP-BM5 virus at the murine vaginal mucosa showed that virus incubated with human SE is reduced in viral replication and spread as
shown by reduced viral loads at the site of infection (vaginal epithelial cells) and in peripheral tissues (PBMCs, inguinal/subiliac draining lymph nodes, splenocytes), and that cell-free virus circulating in plasma is less infectious [62]. Murine vaginal epithelial cells internalized human SE, and SE cargo (human APOBEC3G mRNA) was transferred to murine vaginal epithelial cells suggesting that SE may block infection at the vaginal mucosa [62].

**Semen exosomes inhibit HIV-1 infection**

HIV-1 is notorious for its ability to circumvent control strategies and develop antiretroviral resistance. The high error rate of the HIV-1 polymerase allows the virus to select escape mutations in the presence of antiretroviral drugs that do not abrogate viral replication [136]. These mutations can occur at three main points during replication: (1) conversion of viral ssRNA to dsDNA (reverse transcription, RT), (2) copying of integrated proviral DNA, (3) transcription of viral RNA from proviral DNA [137]. HIV-1 also acquires adaptive mutations based on selection factors within different anatomical compartments. Viral populations in blood and the male genital tract differ in paired samples from chronically infected men [138], despite being identical during initial infection [138]. Therefore, combination control strategies that target different stages of the viral lifecycle have been highly successful at controlling the genetic diversity of HIV-1.

Although the exact mechanism(s) by which SE inhibit HIV-1 infection is unknown, studies show that multiple steps in the HIV-1 lifecycle are affected by SE including: (1) conversion of ssRNA to dsDNA (reverse transcription), (2) copying of integrated proviral DNA, and (3) transcription of viral RNA from proviral DNA [63, 139], as depicted in Figure 5. Since three of these replication steps are key points where HIV-1 mutations arise, SE may inhibit infection through multiple mechanisms [137]. SE reduced HIV-1 proviral DNA and viral RNA
levels in a variety of cell models across multiple HIV-1 strains [63]. These affects likely occurred post-entry as SE did not reduce intracellular HIV-1 p24 capsid protein or RT activity levels at three hours post-infection. In contrast, RT activity was reduced by twenty-four hours post-infection [63]. RT is a potential lifecycle target of SE as SE altered the ratio of RT subunits p51 and p66 by significantly reducing levels of virion-associated p66 protein [63]. The ratio of RT subunits is important for the enzymatic activity of RT [63]. Murine-AIDS virus infection implicates these same life cycle steps. Specifically, the presence of human SE reduced circulating murine-AIDS virus LP-BM5 reverse transcriptase activity (RT) in cell-free blood plasma as well as viral DNA (proviral) and RNA levels [62]. Despite no reduction in levels of intracellular RT activity in cultured naïve murine splenocytes at three hours post-infection, SE reduced RT activity by twenty-four hours post-infection indicating that there is a post-entry inhibitory effect [62]. Whether SE targets each of these lifecycle steps independently or interferes with earlier viral lifecycle stages has yet to be determined.

It has been observed that SE target viral factors and specifically blocks HIV-1 lifecycle events even when viral binding and entry steps are bypassed [139]. SE inhibit Tat-dependent transcription of a Tat expression vector and Tat exogenous protein. Specifically, SE inhibit promoter activation of de novo Tat [139]. Thus SE-mediated anti-HIV-1 activity may directly target steps in the viral life cycle as well as influence downstream replication events. SE clearly inhibit late steps of the viral lifecycle by reducing HIV-1 viral progeny RT, RNA, and infectivity levels [62, 63]. In addition, the hypothesis that SE-mediated HIV-1 inhibition may involve multiple mechanisms is supported by mimicking different cell infection conditions during in vivo sexual transmission. Although virus entry was not inhibited when SE were added to cells prior to HIV-1 infection, virus production was reduced in these cells regardless of whether SE were
added prior to, simultaneous with, or after the HIV-1 inoculum [63, 64]. SE do not alter viral entry or release as determined by intracellular and progeny p24 protein content, respectively, suggesting that SE-mediate inhibition of post-entry lifecycle steps [63]. Transcription-specific analyses show that SE diminish HIV-1 promoter activity and reduce recruitment of transcription factors to the HIV-1 promoter, implicating a regulatory mechanism [139]. Taken together, these data suggest that SE isolated from healthy donors inhibit HIV-1 at multiple stages of the viral lifecycle. It remains to be determined whether SE isolated from the semen of HIV infected individuals who are or are not on suppressive ART will control HIV infection of target cells.

It is known that HIV-1 acquires mutations to escape recognition of host immunity, yet mutations to the inhibitory effect of SE have not been identified. Thus, HIV does not appear to be passively transmitted in semen in the male genital tract [138], and the anti-HIV effect of SE may be conserved in SE isolated from HIV infected individuals, irrespective of ART. Recently, it was shown that antiretroviral drugs alter the cargo of exosomes isolated from HIV-1 infected cells cultured in the presences of drugs [140]. Similarly, chemotherapeutic drugs are packaged into exosomes, and such drugs were transferred to drug-naïve target cells [141-143]. It remains to be determined whether exosomes isolated from HIV-infected individuals on ART contain such drugs and if the drugs block HIV infection of target cells. Furthermore, it would be interesting to evaluate if blood exosomes from HIV-infected individuals on ART display ART-dependent anti-HIV phenotype not observed in blood exosomes from healthy donors [63]. Studies are underway to answer these and other pressing questions about the effect of HIV status on the antiviral phenotype of exosomes from body fluids.
Semen exosome cargo

Semen exosome nucleic acid cargo

The heterogenous nature of SE suggests that multiple cell types within the male reproductive tract contribute to the origin of these exosomes. This is supported by the finding that polyclonal antisera against SE reacts with testes, epididymis, prostate, and seminal vesicle accessory sex glands [144]. As such, SE may contain a variety of coding and non-coding nucleic acids, proteins, and lipid-associated cargo [63, 133]. SE non-coding RNAs include microRNAs, Y RNAs, and tRNAs that are postulated to act as regulatory signals within the mucosal microenvironment, and possibly contribute to the immune-regulatory profile of semen [133]. This may also contribute to immune cell trafficking during HIV-1 transmission, as SE contain an abundance of miRNA that targets immune-related mRNAs [133]. The miRNA content is estimated to be one molecule per nine SE particles, thus SE appear to be relatively abundant. In contrast, there is an estimated one miRNA copy per 47,162 exosomes derived from blood plasma [145]. miRNA signatures among individual SE donors revealed 175 miRNAs present in all SE samples analyzed. Let-7b, miR-148a, and let-7a are the most abundant [133]. Previous studies demonstrated that let-7 miRNA family members target IL-10 mRNA, and that IL-10 inhibits cytotoxic T cell responses and contributes to T cell dysregulation during HIV-1 infection [146, 147]. Targeting by miR-148a can downregulate MHC II expression, inhibit cytokine production, and reduce T cell proliferation, further contributing to immune regulation during HIV-1 infection [148]. Therefore, the non-coding content of SE may regulate immune functions influencing the proviral and antiviral milieu during infection. In addition to regulating the host immune response, the SE nucleic acids may also modulate HIV-1 replication. In relation to the HIV-1 inhibitory phenotype of SE, the protein-coding RNA element of SE may also be significant, as SE contain HIV-1 restriction factors based on gene expression analyses [62, 63]. Although there
appears to be some donor variability, SE contain an abundance of APOBEC3 genes including: A3C, A3D/E, A3F, A3G as well as BST-2/tetherin [63]. APOBEC3 and BST-2/tetherin proteins are among the most extensively studied HIV-1 restriction factors [149, 150]. These restriction factors target viral products post-integration to control viral replication [149]. Importantly, SE contains and transfers these factors to cells \textit{in vivo} [62]. Analysis of murine vaginal cells exposed to human SE showed exosome-mediated transfer of human A3G mRNA [62]. These observations may suggest that SE regulate HIV-1 infection through transfer of antiviral cargo.

\textbf{Semen exosomes protein cargo}

In addition to nucleic acid cargo, SE contain an abundance of intra-vesicle and surface-associated proteins [64]. Analysis of the SE proteome identified $>1000$ proteins, including the exosome-associated markers ALIX and HSP70 [124]. Despite variation among donors, $>300$ common proteins among two groups of pooled donors were detected in SE [124]. High physiological variation among individual donors in seminal composition may account for the relatively modest sharing of only 24% of all proteins identified among SE donors. In addition, differences in isolation strategies and experimental processing may contribute to the number and species of proteins identified. Gene ontology (GO) analysis of the SE proteome revealed a link of SE proteins to biological processes. There is a correlation between SE-associated protein levels and SE-mediated function. For example, a correlation between HIV-1 infection and acetylcholine-esterase, CD9, and CD63 exosome-surface protein levels is observed in different models of infection, and increased protein levels correlated with decreased HIV-1 infectivity [64]. Although correlation does not prove causation, these studies suggest that SE-associated proteins may play a role in the anti-HIV activity of SE. Determining the proteome of SE sub-populations may prove useful in narrowing down the cell types and tissues producing HIV
inhibitory SE within the male reproductive tract. Further, characterization of the source of the anti-HIV-1 cargo is important, as not all SE vesicles contain the same cargo [63, 64, 124]. In-depth comparative proteomics analyses of SE with other non-inhibitory exosome proteome, such as blood exosomes may aid in identifying additional SE functions and antiviral mechanisms. This is not a general antiviral effect, as SE did not inhibit herpes simplex virus-1 and -2 (HSV-1 and HSV-2, respectively) [63]. Despite the overlap between HIV-1 and HSV-1 and HSV-2 by virtue of being sexually transmitted viruses and their ability to persist quiescently in the host, HSV-1 and HSV-2 are DNA viruses with major differences in their life cycles compared to HIV-1. Although a flavivirus, the Zika virus (ZIKV) lifecycle is more comparable to HIV-1 as both are enveloped positive-sense single-stranded RNA viruses [151]. SE inhibited ZIKV infection and attachment to target cells in vitro, a potential explanation for the low frequency of ZIKV sexual transmission despite detection of high viral titers in semen [152].

**Semen exosome immunomodulatory functions**

Although further understanding of the immunomodulatory capacity of SE is needed, a few descriptions of the interaction of prostasomes with immune cells may provide some insight into SE-mediated immune regulation. Prostasomes, exosomes secreted from the prostate epithelium which are likely intermixed within SE, bind to lymphocytes, inhibit lymphoproliferation, and inhibit monocyte endocytosis in vitro [20]. This function is postulated to protect sperm during reproduction from the hostile environment of the female reproductive tract [153]. Prostasomes contain CD59, an inhibitor of the complement system, and are protected against complement-mediated cell lysis [154, 155]. Additionally, CD59 transferred from prostasomes to cells lacking CD59 in vitro maintain the ability to abrogate complement-mediated lysis [156]. In relation to viral infections, prostasomes are associated with CD46, a
receptor for measles virus and a complement system co-factor. CD46 is a co-factor during cleavage of C3b and C4b and regulates the complement cascade by inhibiting formation of the membrane attack complex. CD46 is highly enriched in seminal plasma and is precipitated with prostasomes, suggesting a prostasome association [157]. During measles virus infection, CD46 is downregulated from the cell surface, increasing the sensitivity of infected cells to complement mediated lysis [158]. During reproduction, the complement inhibitors CD59 and CD46 may protect spermatozoa from complement attack in the female reproductive tract [157]. Although these complement inhibitors may also protect viruses from complement-mediated lysis, prostasomes have been described to inhibit measles virus activity [154]. Prostasomes and seminal plasma including prostasomes inhibit measles virus infectivity, possibly through viral binding to prostasome-associated CD46 [20, 154]. It is possible that this sub-population of SE may impose an immunosuppressive effect during infection, although this has not yet been studied in the context of HIV-1 infection. Because prostasomes are speculated to temporarily reduce early immune responses, it is possible that SE-mediated immune regulation occurs early after HIV-1 infection [153].

Because the antiviral effect of SE is cell-type independent, the effect of SE during HIV-1 infection appears to interfere with the virus rather than the cell [63]. Therefore, it is likely that SE may not alter HIV-1 target cell activation during infection. Studies are underway to determine the effect of SE on lymphocyte activation and the associated cell proliferation and cytokine production. These studies will determine if SE induce viral reactivation in lymphocytes isolated from HIV-infected ART-suppressed individuals. Of note, SE had no effect on viral reactivation in an in vitro cell model of HIV-1 latency [139].
**Semen exosomes and transcription factors**

Exosomes in general are known to mediate proximal and distal cell signaling alterations including changes in cellular transcription that permit disease progression [95, 159] or mitigation [139, 160]. The details on how exosomes achieve diverse roles in cells are unclear but it is known that nucleic acid-binding proteins and transcription factor proteins are present in exosomes [161]. These proteins may therefore effect the exosome-directed phenotypic and functional changes observed. Indeed, it was recently shown that SE block HIV-1 transcription initiation, elongation, and the recruitment of transcription factors [139]. Host transcription factors, including NF-kB and Pol II as well as the viral factor Tat are specific targets of SE in in vitro HIV infection model [139]. SE reduce the DNA binding ability of NF-kB and Pol II, resulting in reduced HIV-1 LTR activation by HIV-1 or by extracellular Tat. During infection, Tat associates with Sp1 and NF-kB as well as other cellular factors to drive HIV-1 LTR elongation [162, 163]. As a multifunctional protein, Tat regulates multiple steps of viral replication including Pol II initiation of transcription, mRNA splicing, and functions of reverse transcriptase [164-170]. Tat is implicated as a probable target of SE as SE interfered in DNA binding of Pol II, reduced expression levels of HIV-1 mRNA splice variants, and altered the ratio of HIV-1 RT subunits [63, 139]. Whether the effects by SE on host and viral transcriptional regulators are distinct or related mechanisms is to be determined. In addition, these studies are not exhaustive, and other transcription factors may be affected.

Exosomes and enveloped viruses, including HIV-1, are highly similar in physical and functional characteristics. Differentiation between the two types of vesicles during HIV-1 infection is difficult, yet important to understanding their independent contributions to disease pathogenesis. Although the inhibitory or enhancing contribution of exosomes during HIV-1
infection seems to rely on bio-fluid source, the inhibitory phenotype of exosomes from human semen may account for the low risk of infection per sexual exposure. The role of semen appears considerably varied during infection, however, the nucleic acid and proteinaceous content of semen exosomes may provide useful insights into the anti-HIV function of this particular subset of exosomes. It seems likely that semen exosomes target multiple steps of the HIV-1 lifecycle, and in addition may regulate the immune response to infection to restrict viral replication. Although recent data has made important progress to understanding the interaction of these vehicles within the same biological system, many questions remain; including identification of the antiviral factors contained within semen exosomes that inhibit HIV-1, and understanding the mechanisms of SE perturbation of HIV-1 lifecycle events, as well as the immune-regulatory function of semen exosomes during infection. These questions are important to understanding the co-evolution of pathogenic (HIV-1) and protective (SE) vehicles of intercellular communication within the male reproductive tract.
Figure 1: Schematic representation of the HIV-1 lifecycle. 1. HIV-1 virions bind to cell receptor/co-receptor. 2. Fusion and entry inserts the viral core in the cell cytoplasm. 3. The viral genome is reverse transcribed from single-stranded RNA to double-stranded DNA. 4. Viral double-stranded DNA is imported into the cell nucleus. 5. Viral double-stranded DNA is integrated into host double-stranded DNA. 6. Viral RNA is transcribed from integrated DNA. Concurrently, 7A. Viral proteins are translated from viral RNA. 7B. Progeny virions are assembled with viral proteins and viral RNA. 8. New HIV-1 virions bud from the cell plasma membrane. See text for references.
<table>
<thead>
<tr>
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<th>Origin</th>
<th>Mechanism of release</th>
<th>Size</th>
<th>Potential Markers</th>
<th>Source*</th>
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<td>Mixed population of exosomes, microvesicles, apoptotic bodies, large oncosomes</td>
<td>Fusion of MVBs and direct budding from the plasma membrane</td>
<td>Variable 40 nm-10 μm</td>
<td>Varied: tetraspanins, major histocompatibility complex (MHC) molecules, cytosolic proteins</td>
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<td>Multiple cell types (endosome-associated)</td>
<td>Intraluminal vesicles exist within MVBs, upon release these vesicles are termed exosomes</td>
<td>40-100 nm</td>
<td>MHC II, tetraspanins, ubiquitinated proteins</td>
<td>[1, 16]</td>
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<tr>
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<td>Multiple cell types (commonly neutrophils or monocytes)</td>
<td>Plasma membrane budding</td>
<td>100-350 nm</td>
<td>TyA, C1q</td>
<td>[21]</td>
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<tr>
<td>Exosomes</td>
<td>Multiple cell types (endosome-associated)</td>
<td>Fusion of MVBs with plasma membrane</td>
<td>40-100 nm</td>
<td>CD9, CD63, CD81, TSG101, Alix, Hsp70</td>
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<td>Cancer cell membrane budding</td>
<td>1-10 μm</td>
<td>EGFRvIII, ARF6, Cav-1, CK18, oncogenic material</td>
<td>[14, 171-173]</td>
</tr>
<tr>
<td>Apoptotic bodies</td>
<td>Cells undergoing apoptosis</td>
<td>Blebbing and fragmentation of the plasma membrane of apoptotic cells</td>
<td>500-4000 nm</td>
<td>Phosphatidylserine, Annexin V, thrombospondin, C3b</td>
<td>[19]</td>
</tr>
<tr>
<td>Enveloped virus particles</td>
<td>Virally infected cell</td>
<td>Plasma membrane budding</td>
<td>~100 nm</td>
<td>Viral-encoded proteins</td>
<td>[22]</td>
</tr>
</tbody>
</table>

*The cited references are not an exhaustive list. We apologize to authors whose work was unintentionally omitted.

Table 1: Classification of cell-associated vesicles.
Figure 2: Schematic representation of the effects of semen on the mucosal microenvironment during HIV-1 transmission. 1. Seminal-associated cytokines/chemokines may transverse the mucosal layer and recruit cells susceptible to HIV-1 infection such as CD4+ T cells, dendritic cells, and monocytes or macrophages. 2. The alkaline properties of semen raise the acidic vaginal pH from a range of 4.0-6.0 to 6.0-7.0, disrupting the protective mucosal layer allowing more efficient viral dissemination through the epithelial cell layer. 3. Clusterin and Mucin-6 molecules in semen prevent HIV-1 attachment to DC-SIGN on dendritic cells. 4. Spermatozoa may act as carriers of HIV-1 virions to susceptible cells. See text for references.
Figure 3: Spectrum of the function of seminal components during HIV-1 infection. HIV-enhancing components (red) and HIV-inhibitory components (blue) co-exist in seminal fluid. See text for references.
Figure 4: Electron micrograph of semen exosomes by negative staining. Shown is a heterogeneous population of vesicles consisting of a range of sizes with a singular or double membrane of differing densities (translucent-light vs. translucent-dark particles). White arrows highlight a few exosome particles.
Figure 5: Semen exosomes inhibit HIV-1 lifecycle steps. Semen exosomes inhibit HIV-1 at the steps of reverse transcription, proviral integration, and viral transcription. See text for references.
CHAPTER II: ISOLATION OF EXOSOMES FROM SEMEN FOR IN VITRO UPTAKE AND HIV-1 INFECTION ASSAYS

Abstract

Exosomes are membranous extracellular nanovesicles of endocytic origin. Exosomes are known to carry host and pathogen-derived genomic, proteomic, lipidomic cargos and other extraneous molecules. Exosomes are secreted by diverse cell types into the extracellular milieu and are subsequently internalized by recipient neighboring or distal cells. Upon internalization, exosomes condition recipient cells by donating their cargos and/or activating various signal transduction pathways, consequently regulating physiological and pathophysiological processes. Exosomes facilitate intercellular communication, modulate cellular phenotype, and regulate microbial pathogenesis. We have previously shown that semen exosomes (SE) inhibit HIV-1 replication in various cell types. Here, we describe detailed protocols for characterizing SE. This protocol can be adapted or modified and used for evaluation of other extracellular vesicles of interest.

Introduction

Exosomes are membranous nanovesicles originating as a result of inward budding of endosomal membranes within the late endosomal compartment of a multitude of cell types [174]. Exosomes are released by many cell types [175] into the extracellular milieu and are found in biological fluids including blood [176], urine [177], saliva [28], and breast milk [63, 104]. Human semen contains a heterogenous population of nanovesicles [62, 63] produced by tissues of the male genital tract including prostate secretory acinar cells [178] and epididymal epithelial cells [179] as well as cells of the vasa deferentia, testes, and the vesicular glands [144, 180]. The variability in the cells that secret exosomes is reflected in the composition and function of
exosomes. Thus, exosomal cargo composition and function are regulated by many factors including the type and condition of the originating cell [1], cellular environment, and for *in vivo* derived exosomes the condition of the donor [64]. Released exosomes when taken up by target cells transfer their cargo, including proteins [175, 181], miRNA [182, 183], and mRNA [62, 63, 184] to the target cells. As a result, exosomes are known to be involved in modulation of host immune response [133, 176], and regulation of microbial pathogenesis [62, 63, 101, 104, 133, 185].

While progress has been made in the field of exosome biology, many protocols are contradictory in the most effective and efficient method of characterizing exosomes [186]. Here, we provide a detailed protocol for evaluating the function and physical properties of semen exosomes [62, 63]. This protocol lays the groundwork for evaluating other functional activities of semen exosomes, and for evaluating exosomes from other sources.

**Materials and methods**

**A. Acquisition of human semen samples**

This study utilized existing human specimens (semen) and therefore is not human subjects’ research. The samples were discarded from routine examinations and not linked to any identifiers.

1. Collect semen by dry manual stimulation and ejaculation into sterile 15 ml polypropylene conical tubes.
2. Store samples at room temperature for 30 min to promote liquefaction and then centrifuge for 10 min at 1,000 x g at 4 °C to pellet spermatozoa.
3. Remove seminal plasma from spermatozoa-containing pellets. Pellets can be discarded or stored for downstream analysis.
4. Store seminal plasma samples at -80 °C until required for exosome purification by ExoQuick or ultracentrifugation. We have found no discernable difference between the two methods of purification [62, 63].

B. ExoQuick purification of exosomes

1. Thaw seminal plasma samples before centrifuging at 2,000 x g for 15 min at 4 °C in a 50 ml conical tube. Transfer supernatant to a new tube and centrifuge again at 10,000 x g for 30 min at 4 °C in a 50 ml conical tube to pellet cellular debris.

2. Place clarified seminal plasma in a fresh 50 ml conical tube and add ExoQuick at a ratio of 4:1 (clarified seminal plasma/ExoQuick). Mix by inversion, and incubate at 4 °C overnight (12-24 h).

3. Centrifuge the clarified seminal plasma/ExoQuick mixture at 1,500 x g for 30 min at 4 °C.

4. Remove the supernatant (exosome free clarified seminal plasma and ExoQuick) and repeat centrifugation at 1,500 x g for 10 min at 4 °C without resuspension of the pellet.

5. Remove the residual supernatant.

6. Resuspend the exosome pellet in PBS to 1/10 of the original volume of seminal plasma, quantify protein concentration by Bradford assay, and aliquot [62, 63].

C. Ultracentrifugation purification of exosomes

1. Dilute clarified seminal plasma 50% in PBS, and ultracentrifuge at 100,000 x g for 2 h at 4 °C to pellet exosomes using a SW41Ti rotor.

2. Wash exosome pellets in PBS three times with ultracentrifugation at 100,000 x g for 30 min per wash (1.5 h). A volume of PBS should be used that completely fills the ultracentrifuge tubes to avoid collapse of the tubes.
3. Resuspend exosomes in PBS to 1/10 of the original volume of seminal plasma, quantify protein concentration by Bradford assay, and aliquot [187].

D. Fluorescent labeling of exosomes, liposome controls or PBS controls

1. Fluorescently label PBS control, Lipofectamine 2000 derived liposome control, or exosomes using PKH67Green or PKH26Red kits according to manufacturer’s instructions with the following modifications: Add 1 mg of purified exosomes or liposome control or an equivalent volume of PBS to 250 µl of PBS and mix with 250 µl of Diluent C in SW41Ti ultracentrifuge tubes.

2. Add 4 µl of PKH67Green or PKH26Red lipophilic dye to 500 µl of Diluent C in a separate tube at room temperature (RT) in the dark and perform all subsequent steps at RT in the dark.

3. Combine the tube containing dye and Diluent C with the tube containing exosomes or controls in PBS and Diluent C in the SW41Ti ultracentrifuge tube. Rapidly mix by manual pipetting. Incubate for 5 min in the dark at RT and vortex twice during the 5-min incubation.

4. After the 5 min of incubation, add exosome-free FBS (see Recipes) in a 1:1 ratio to the ultracentrifuge tube containing dye, exosomes or controls, and Diluent C. Incubate for 1 min to occupy unbound dye.

5. Ultracentrifuge the solution at 100,000 x g for 30 min. Discard the supernatant.

6. Resuspend the pellet containing dye-labelled exosomes or controls in PBS, transfer to another SW41Ti ultracentrifuge tube and wash three times by ultracentrifugation at 100,000 x g for 30 min per wash. After each wash, transfer the PBS resuspended pellet to a new SW41Ti ultracentrifuge tube to minimize transfer of unbound dye. A volume of
PBS should be used that completely fills the ultracentrifuge tube to avoid collapse of the tube.

7. After the final wash, remove the supernatant, and resuspend the pellet in PBS to the original volume of the exosomes. Quantify dye-labelled exosomes or controls using Bradford Protein Assay Kit, aliquot, and store in the dark at -80 °C until use [62, 63].

E. Cell culture preparation for internalization of fluorescent exosomes

1. Grow human monocytic and lymphocytic cell lines in tissue culture dishes in RPMI supplemented with 10% exosome depleted FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.3 mg/ml L-glutamine in a 5% CO2 incubator at 37 °C.

2. Grow TZM-bl vaginal epithelial cell lines in tissue culture dishes in DMEM supplemented with 10% exosome depleted FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.3 mg/ml L-glutamine in a 5% CO2 incubator at 37 °C.

3. Grow VK2 and V428 vaginal epithelial cell lines in tissue culture dishes in keratinocyte serum free media supplemented with 0.1 ng/ml prequalified human recombinant Epidermal Growth Factor 1-53, 0.05 mg/ml BPE, 100 U/ml penicillin and 100 μg/ml streptomycin.

4. Grow all cell types in 12 well tissue culture dishes. Seed cells at 1 x 10⁵ cells per well in 1 ml volume of the correct cell culture media for each cell type (see Note 1).

5. Expose cells to 25-100 μg/ml of red or green fluorescent exosomes or controls for 0, 3, 6, 9, 12 or 24 h incubation periods in a 5% CO2 incubator at 37 °C. The incubation of SE
with cells should be completed in cell culture media. Final volume of SE incubation with cells will depend on the concentration of exosomes after fluorescent labeling.

6. Wash TZM-bl, VK2 or V428 adherent cells three times in 1 ml PBS in the plate and detach the cells from the plate using 0.5 ml of 0.25% trypsin-EDTA dissociation reagent. Neutralize the trypsin with 0.5 ml 10% exosome free FBS in DMEM. Collect the cells from the wells and transfer to 5 ml polystyrene round-bottom tubes. Wash the cells three times in 1 ml PBS with 5 min centrifugations at 500 x g at 4 °C.

7. Collect monocytic and lymphocytic suspension cells in 5 ml polystyrene round-bottom tubes. Treat with trypsin as described in step E5, and neutralize with 10% exosome free FBS in RPMI. Wash the cells three times in 1 ml PBS with 5 min centrifugations at 500 x g at 4 °C.

8. Assess cellular uptake and exosome internalization kinetics utilizing FACS analysis or confocal microscopy [62, 63].
   a. For FACS analysis:
      i. Fix cells for 15 min on ice with 300 μl 2% paraformaldehyde.
      ii. Wash the cells two times in PBS with 5 min centrifugations at 500 x g in between each wash. Resuspend the cell pellets in 300 μl PBS.
      iii. Analyze fluorescence using a FACSCalibur or FACSVerse flow cytometer (BD) to detect the PKH67Green (FL-1 or FITC channel, respectively) or PKH26Red (FL-2 or PE channel, respectively) transferred from exosomes to cells during fusion and uptake.
      iv. Determine cellular frequency and fluorescence intensity using FlowJo analysis software (TreeStar) (Figure 6) [62, 63].
b. For confocal microscopy analysis of exosome internalization:

i. Coat coverslips with collagen by adding sterile, round 18 mm coverslips to the wells of a 12 well plate. Add 0.5 ml of 50 μg/ml collagen solution to the wells. Incubate the plate at 37 °C for 1 h, after which you move the plate to 4 °C for overnight incubation. After overnight incubation, remove the collagen solution and gently wash the coverslips in the wells three times with PBS. After the final wash, aspirate all the PBS and immediately proceed to step E8b.ii.

ii. Grow TZM-bl, VK2 or V428 adherent cells in 12 well tissue culture plates on top of collagen coated microscope cover slips, expose to fluorescent exosomes as described in step E4 and wash three times in PBS in the plate.

iii. Fix cells on the coverslips in the plate with 2% paraformaldehyde for 15 min with the plate on ice.

iv. Remove coverslips from the plate and add a drop of Vectashield antifade reagent to each coverslip.

v. Mount coverslips down on microscope slides.

vi. Assess fusion, uptake and internalization kinetics of fluorescent red or green exosomes using laser scanning confocal microscopy. Representative images are shown in Madison et al., 2015 [62].

F. Detection of surface exosomal markers

Here we will describe detection of human CD63 in SE, but this protocol may be modified for detection of other common exosomal surface markers in SE. Human CD63 in SE is detected per the manufacturer’s instructions using the exosome-human CD63 isolation/detection kit from Invitrogen where after acquiring SE:
1. Resuspend 25 μg of SE to a total volume of 100 μl in 0.1% BSA.

2. Resuspend anti-CD63 coated magnetic beads by vortexing for 30 sec. Transfer 20 μl of beads to a microcentrifuge tube and wash the beads by adding 200 μl of 0.1% BSA and vortexing.

3. Place the tube containing the beads on a magnet separator for 1 min. Discard the supernatant before removing the tube from the magnet.

4. Add the SE solution to the washed beads and mix by pipetting.

5. Incubate the SE/beads solution at 4 °C overnight (18-20 h) in a rotating mixer.

6. Centrifuge the SE/beads solution for 3-5 sec.

7. Wash the SE bound beads in 300 μl of 0.1% BSA and mix by pipetting before placing the tube on the magnet for 1 min and discarding the supernatant.

8. Remove the tube from the magnet. Wash the SE bound beads in 400 μl of 0.1% BSA and mix by pipetting.

9. Place the tube on the magnet for 1 min and discard the supernatant before removal from the magnet.

10. Resuspend the SE bound beads in 300 μl of 0.1% BSA.

11. Transfer 100 μl of the SE bound beads to a new microcentrifuge tube. Add 5 μl of anti-human CD63-FITC (Biolegend) and mix by pipetting.

12. Incubate the SE-bounds beads and antibody for 60 min at room temperature in the dark on an oscillating plate.

13. Wash the antibody stained SE-bound beads in 300 μl of 0.1% BSA and mix by pipetting. Place the tube on the magnet for 1 min, and discard the supernatant before removal of the tube from the magnet.
Repeat the washing step twice before resuspending the SE-bound beads in 300 µl of 0.1% BSA. Transfer the resuspended SE-bound beads to a 5 ml polystyrene round-bottom tube before analysis on FACSVerse instrument and FlowJo (Tree Star) software (Figure 7) [64]. Alternatively, common exosomal markers in SE may also be detected with the use of non-magnetic beads. Here we provide instructions for the use of latex beads to detect CD63 in SE.

a. Per manufacturer instructions, 2.5 ml of resuspended, surfactant-free, 4-µm diameter, aldehyde/sulphate, latex beads (Invitrogen, Molecular Probes, hereafter referred to as latex beads) were washed twice in 10 ml of 0.025 M, pH 6.0 2-(N-morpholino) ethanesulfonic acid (MES) buffer with centrifugation at 3,000 x g for 20 min at 4 °C.

b. Following the second and final wash, the latex beads were resuspended in 5 ml MES buffer.

c. Incubate 100 µl latex beads with 100 µl of anti MHC-II MAb or isotype control antibody prepared in MES at a concentration of 1 mg/ml at room temperature overnight with gentle agitation.

d. Sediment latex beads with conjugated antibody by centrifugation at 3,000 x g for 20 min at 4 °C.

e. Remove supernatant (unbound antibody).

f. Wash latex beads with conjugated antibody thrice in 1 ml PBS (0.1 M, pH 7.2) at 3,000 x g for 20 min at 4 °C.

g. Resuspend latex beads in 100 µl of storage buffer (see Recipes).

h. ExoQuick purified exosomes (100 µg) were incubated with *2 x 10^5 anti MHC-II or isotype control coated latex beads in a final volume of 100 µl PBS (0.1 M, pH 7.2)
first for 15 min at room temperature followed by overnight at 4 °C with gentle agitation.

*Note: Concentration of beads/ml differs by lot number.

i. The reaction was stopped by 30 min incubation with PBS (0.1 M, pH 7.2) including 0.2% glycine to saturate any vacant sites on the latex beads.

j. The exosome and antibody bound latex bead preparation was then washed thrice in FACS wash buffer (see Recipes).

k. Exosomes coated beads were then incubated with the appropriate concentration of anti-CD63 conjugated to PE (Biolegend) or isotype control antibody for 1 h at room temperature in the absence of light followed by three washes in FACS buffer.

l. The resulting immunofluorescence was analyzed by use of a FACSArria flow cytometer (BD) and FlowJo analysis software (TreeStar) (Figure 7) [63].

G. SE acetylcholinesterase activity

1. Lyse 50 µg of SE in 2% Triton-X-100 at a 1:1 volumetric ratio.

2. Add 5 µl of SE/Triton-X reaction to a 96-well flat bottom clear plate in replicates of three.

3. Combine 1.25 mM acetylthiocholine chloride (Sigma-Aldrich) and 0.1 mM 5,5’-dithiobis-2-nitrobenzoic acid (Sigma-Aldrich) in a 1:1 ratio to reach a final volume of 100 µl per well. Substrates should be warmed to 37 °C before mixing. Add 100 µl of the mixed solutions to each of the exosome containing wells. Be sure the microplate reader has reached 37 °C before adding this solution to the wells as the reaction will start immediately after addition.
Read absorbance at 450 nm on a microplate reader for a total of 30 min in 5 min intervals at 37 °C (Figure 8) [64].

H. Dynamic Light Scattering (DLS) of SE

DLS can be used to measure the size distribution of exosomes and other small vesicles 1-500 nm in diameter.

1. Dilute 0.1 mg/ml of SE in 200 μl of PBS and analyze size by DynaPro NanoStar DLS (Wyatt Technologies) using a total volume of 150 μl in plastic cuvettes.
2. Complete data analysis using Dynamics software.
3. Use an average of ten measurements per exosome sample to determine radius, diameter, and %intensity (Figure 9) [64].

I. SE NanoSight nanoparticle tracking analysis (NTA)

NTA may be used to analyze the size, concentration, and aggregation of vesicles 10-1000 nm in size.

1. Prepare control suspension containing uniformly sized (100 or 200 nm) polystyrene particles.
2. Use the suspension to align the foci of the laser and microscope.
3. Make serial dilutions of each SE specimen in PBS to a final volume of 0.5 ml.
4. Inject 0.5 ml of diluted SE into NanoSight LM10 NTA using 1 ml disposable syringe.
5. Analyze individually the prepared serial dilutions until the raw concentration detected is within the recommended range for the instrument.
6. Using the identified dilution, record three 30 sec videos for each specimen.
7. Repeat the analysis 3 times using the same settling to ensure repeatable and accurate measurement.
8. Complete post-acquisition analysis with NTA software to determine size and concentration of SE.

9. View the results tab for i) total number of particles traced, ii) average number of particles, and iii) particle concentration.

10. Calculate total concentration of exosomes per ml of semen accounting for the dilution factor used for NanoSight analysis (Figure 9). Obtain error bars by analyzing standard deviation of the 3 measurements of each sample [64, 133].

J. Fractionation of SE into membrane and luminal contents

1. Treat 100 μg SE with 5-10x volume of 0.1 M sodium carbonate pH = 11.5. Mix by vortexing.

2. Incubate for 30 min to 1 h at 4 °C. At this point, total protein from the lysed exosomes can be measured by NanoDrop spectrophotometer at 280 nm. Complete the following steps to separate lysed SE into membrane and luminal fractions.

3. Transfer SE/sodium carbonate mix to SW60 Ti ultracentrifuge tubes. Bring up the volume with PBS to fill the tube.

4. Ultracentrifuge at 150,000 x g for 1.5-2 h at 4 °C. The pellet contains the membrane fraction while the supernatant contains the luminal fraction.

5. Remove the luminal ‘supernatant’ fraction. This can be concentrated by MW filter cutoffs before protein quantification by Bradford assay and downstream analysis or storage at -80 °C until use.

6. Resuspend the membrane fraction in PBS to the original volume of SE before lysis. Quantify protein by Bradford assay, aliquot, and store at -80 °C or use in downstream analysis (Figure 10) [102, 188, 189].
K. SE protein footprint

1. Lyse 5 μg of SE in 20 μl of PBS at a ratio of 3 μl NuPAGE lysing sample buffer (see Recipes) per 7 μl SE.

2. Heat SE in sample buffer at 90 °C for 10 min.

3. Load a maximum volume of 40 μl on a 10 well 1.5 mm NuPAGE 4-12% Bis-Tris gel.

4. Run the gel at 200 V for ~50 min in NuPAGE 20x MOPS SDS running buffer diluted to 1x.

5. Silver stain the gel following the manufacturer’s protocol (Pierce silver stain kit Thermo Scientific) [64].

L. Examination of RNA integrity and content of SE

1. Use a starting concentration of at least 12 μg of SE for RNA extraction.

2. Extract SE RNA using the Qiagen RNeasy kit per the manufacturer’s instructions and complete the optional DNase treatment using Qiagen RNase-free DNase set.

3. Determine RNA concentration by NanoDrop spectrophotometer. To evaluate RNA integrity of SE, RNA can be analyzed by an Agilent BioAnalysis run using RNA 6000 Pico chips if the RNA concentration is < 50 ng/μl. If the RNA concentration is > 50 but < 500 ng/μl, the RNA can be evaluated using RNA 6000 Nano chips (Figure 11).

4. Use equivalent concentrations of RNA for cDNA synthesis using High-Capacity cDNA Reverse Transcription Kit (ABI).

5. Use human gene specific primers to amplify CD9, CD63, and GAPDH or other genes of interest by Quantifast Sybr green technology (QIAGEN) and 7500 fast real-time machine.
6. Visualize PCR amplicons on a 2% agarose gel by ethidium bromide staining. Representative images are shown in Madison et al., 2014 [63, 64].

M. SE inhibition of HIV-1 infectivity

1. Grow TZM-bl cells in 96 well tissue culture dishes in DMEM supplemented with 10% exosome depleted FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.3 mg/ml L-glutamine in a 5% CO₂ incubator at 37 °C.

2. Preincubate 100 μg/ml SE or PBS vehicle with 8 reverse transcriptase (RT) units/ml of HIV-1 virus for 1 h at 37 °C in DMEM supplemented with 10% exosome depleted FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.3 mg/ml L-glutamine. Preincubate supplemented DMEM with PBS vehicle as uninfected control. Mix before incubation by vortexing. HIV-1 RT can be quantified by EnzChek™ Reverse Transcriptase Assay (Invitrogen) according to manufacturer’s instructions. Viral titer can also be measured by other methods such as p24 quantification, however, we recommend RT as RT more accurately quantifies infectious viral particles whereas other methods may include quantification of defective viral particles. Other viral titers may also be used; we use this titer as it typically corresponds to 100,000 luciferase units in TZM-bl reporter cells.

3. Remove DMEM media from TZM-bl cells. Add 100 μl per well in triplicate of SE/HIV-1, PBS vehicle/HIV-1, or PBS vehicle/DMEM to TZM-bl cells.

4. Incubate in a 5% CO₂ incubator at 37 °C for 24 h.

5. Access cell viability by MTT assay and HIV-1 infectivity by Steady-Glo luciferase assay (see Note 5). (Figure 12)[62-64].

   a. For MTT assay:
i. Add 20 μl 5 mg/ml of MTT reagent to each well in replicates of three. Incubate for 3.5 h in a 5% CO₂ incubator at 37 °C.

ii. Remove the media and MTT solution from the wells. Add 150 μl of MTT solvent (see Recipes) to each well and incubate for 15 min in the dark on an oscillating plate.

iii. Read absorbance at 590 nm on a microplate reader.

b. For Steady-Glo luciferase assay:

i. Remove DMEM media from TZM-bl cells. Add 100 μl of Steady-Glo luciferase reagent to each well in replicates of three, including uninfected control. Allow 5 min for cell lysis.

ii. Transfer 90 μl of lysed cells/Steady-Glo mixture to a solid white 96 well plate, avoiding the formation of bubbles.

iii. Read luciferase activity in a microplate luminometer [62-64].

Data analysis

1. Each assay should be experimentally repeated at least three times with replicates of three per experiment to verify reproducibility [62-64].

2. Average replicates of independent experiments to evaluate statistical significance. Where appropriate, compare results to vehicle control [62-64].

3. Plot data using graphing software, such as GraphPad Prism. For analyses that are not represented graphically such as Bioanalyzer, protein footprint, and FACS analysis representative images may be shown [62-64].
Notes

1. Cell culture
When growing cells, to minimize evaporation, only plate cells in the inner wells of the plate and fill the outermost wells with PBS. We recommend plating cells the day before they will be used to allow the cells to normalize. Because SE alters cell viability at differing concentrations and in a cell-type dependent manner, it is important to evaluate cell cytotoxicity in all experiments involving cell treatments with SE [62, 63]. This is also important when evaluating HIV-1 infectivity as high concentrations of virus or SE may be cytotoxic to cells and influence analysis of results.

2. Storage of exosomes
We found that repeated freeze-thawing of exosomes decreases functional activity. We recommend that after isolation, to aliquot exosomes into individual microcentrifuge tubes (< 100 μg) before freezing for storage to retain functional activity [62-64].

3. Discarding of supernatant
When discarding supernatant after pelleting or washing exosomes, we recommend removal by pipette aspiration rather than inversion to ensure complete removal. However, exosome pellets may not always be easily observed, depending on the concentration used, and caution should be used to not dislodge the pellet.

4. Sterility
As often as feasibly possible, all experimental steps should be completed under a laminar flow hood to ensure an aseptic environment. Contamination may influence downstream analyses.

5. HIV-1 infectivity
Because TZM-bl cells contain background fluorescence, we recommend plating 10,000 cells per well in a 96 well format to minimize background luciferase expression. Depending on the cell
line used, the HIV-1 isolate used may vary depending on the receptor/co-receptors expressed on that cell line. If evaluating infection in cells that are not a reporter cell line, infection may be evaluated by qRT-PCR using HIV-1 gene specific primers. Data may be confirmed by measuring intracellular or extracellular HIV-1 RT activity [62, 63]. All HIV-1 experiments must be carried out in accordance with biosafety training and laboratory environment requirements.

6. **SE-mediated inhibition of HIV-1**

SE also inhibits HIV-1 infection during a pretreatment model of infection. In this model, after growing the cells, SE is added to cells 24 h before virus infection, and remains inhibitory to infection. The HIV-1 inhibitory characteristic of SE is upheld in both the preincubation and pretreatment models with other cell lines such as: Jurkat, SUPT1, U937, PM1, THP-1, CEM, and PBLs [62, 63].

7. **Flow cytometer specifications**

Laser and filter specifications of flow cytometer systems used in this protocol are included below.

FACSCalibur flow cytometer (BD)

Lasers: Air-cooled, argon-ion, 488 nm, 15 mW

Emission detection:

- FL1 530/30
- FL2 585/42
- FL3 670 LP
- FL4 661/16
- SSC 488/10
- FSC 488/10
FACSVerse flow cytometer (BD)
Lasers: Blue laser, 488 nm, 20 mW, beam spot size 9 x 63 μm
  Red laser, 640 nm, 40 mW, beam spot size 9 x 63 μm
  Violet laser, 405 nm, 40 mW, beam spot size 9 x 63 μm
Emission detection:
  FITC 527/32
  PE 586/42
  PerCp 700/54
  APC 660/10
  SSC 488/15
  FSC 488/10

FACSAria flow cytometer (BD)
Lasers: Coherent Sapphire, solid state, 488 nm, 20mW
  JDS Uniphase HeNe, air-cooled, 633 nm, 18mW
  Point Source Violet, solid state, 405 nm, 15 mW
Emission detection:
  FITC 530/30
  PE 576/26
  PerCp 695/40
  APC 660/20
  SSC 488/10
  FSC 488/10
Recipes

1. **Exosome-depleted FBS**

   Ultracentrifuge FBS at 100,000 x g for 2 h at 4 °C in SW32Ti ultracentrifuge tubes using SW32Ti rotor. Collect supernatant and store at 4 °C for up to 1 week or at -80 °C for longer periods of time.

2. **Lysing sample buffer for protein footprint**

   Mix 250 μl NuPAGE 4x LDS sample buffer with 100 μl NuPAGE 10x reducing agent. Add mixed buffer to SE samples at 3 μl buffer per 7 μl SE.

3. **Storage buffer**

   PBS (0.1 M, pH 7.2) and 0.1% glycine.

4. **MES buffer**

   Dissolve MES in distilled water for a concentration of 0.025 M. Determine pH with a pH meter, and adjust the pH with 1 N NaOH to reach pH 6.0.

5. **FACS wash buffer**

   1% exosome-depleted FBS in PBS.

6. **MTT reagent**

   Resuspend MTT reagent in PBS for final concentration of 5 mg/ml, per manufacturer’s instructions.

7. **MTT solvent**

   0.1% NP-40 and 4 mM HCl in isopropanol.
Figure 6: Internalization of fluorescent exosomes by FACS. A. VK2 cellular uptake of PKH67 green labeled PBS vehicle or 100 μg/ml semen exosomes (SE) at 3- and 24-h post exposure. B. V428 cellular uptake of PK67 green labeled PBS vehicle or 25 or 100 μg/ml SE at 24-h post exposure. C. Jurkat, U937, and TZM-bl cellular uptake of PKH26 red or PKH67 green labeled PBS vehicle or 100 μg/ml SE at 24-h post exposure. The y-axis shows the forward scattering value (FSC) of the cell populations.
**Figure 7: CD63 expression on SE.** 25 μg SE or PBS vehicle were incubated overnight with α-CD63 coated magnetic beads to facilitate binding. Unbound SE was removed before staining of bead-bound SE with α-CD63-FITC and FACS analysis. O/N = overnight. The y-axis shows the forward scattering value (FSC) of the beads.
Figure 8: Acetylcholine esterase activity of SE. 50 μg SE or PBS vehicle were lysed in Triton-X-100. AChE activity was measured at 5 min intervals for a total of 30 min. Error bars represent standard deviation.
Figure 9: SE size and concentration estimation. A. Dynamic light scattering indicates approximate diameter of SE. 0.1 mg/ml of SE was used to measure the diameter of SE. Shown is the range of exosome diameters in the population. B. Representative image from NanoSight NTA video clip showing SE particles. C. Approximation of SE particles per ml of semen calculated from NanoSight estimation of concentration. D. Approximation of SE size by NanoSight.
Figure 10: Separation of SE into different fractions. SE can be fractionated into membrane and luminal components.
Figure 11: RNA integrity of SE. RNA was extracted from SE and analyzed by Agilent Bioanalyzer.
Figure 12: SE inhibition of HIV-1. Exosomes (100 μg/ml) or vehicle PBS were preincubated with 8 RT units/ml HIV-1 NL4.3 virus for 1 h at 37 °C before infection of TZM-bl cells for 24 h. A. Infectivity measured by luciferase units. B. Viability is determined by MTT. TZM-bl cells were pretreated with exosomes (100 μg/ml) or vehicle PBS for 24 h before infection with 8 RT units/ml HIV-1 NL4.3 virus for an additional 24 h. C. Infectivity read by luciferase units; D. Viability determined by MTT. Vehicle set as reference at 100% for infectivity and viability. Error bars are standard deviations.
CHAPTER III: EFFECT OF PROLONGED FREEZING OF SEMEN ON EXOSOMES RECOVERY AND BIOLOGIC ACTIVITY

Abstract

Exosomes are important vehicles of intercellular communication that shape host responses to physiologic, tumorigenic, and pathogenic conditions. The composition and function of exosomes are dynamic and depends on the state and condition of the cellular source. In prior work, we found that semen exosomes (SE) from healthy donors who do not use illicit drugs potently inhibit HIV-1. Following semen donation, specimens are either used immediately or frozen for use at a later time. It has been shown that short-term freezing of semen has no effect on SE-mediated HIV-1 inhibition. However, the effect of illicit drugs and prolonged freezing on SE bioactivity is unknown. Here, we show preservation of SE physical properties, (morphology, concentration, intensity/size) irrespective of illicit drug use or duration of semen freezing. Interestingly, illicit drugs and prolonged freezing decreased the levels of SE-bound CD63 and acetylcholinesterase activity respectively. Furthermore, we show differential effects of illicit drug use and prolonged freezing on SE-mediated HIV-1 inhibition. Our results highlight the importance of the source of SE and condition of semen storage on SE content and function. In-depth evaluation of donor drug-use and duration of semen storage on SE cargo and bioactivity will advance our understanding of SE composition and function.

Introduction

Exosomes are nano vesicles secreted by various cell types into the extracellular milieu, including semen. Semen exosomes (SE) enwrap various RNA (micro RNA [miRNA] and messenger RNA [mRNA]) and protein cargos [62, 63, 133, 190]. Exosomal proteins such as tetraspanins (CD9, CD63, and CD81) and acetylcholinesterase are commonly used as markers of
exosomes [22, 63, 191]. The protein and RNA content of SE have the potential to be used as intercellular messengers, biomarkers, or therapeutic tools for reproductive disorders and sexually transmitted diseases [28]. Indeed, previous studies have found that SE contain inhibitory molecules that restrict retroviral infection, including infection with MLV and HIV-1 in cultured cells [62, 63] and in a mouse model of retroviral infection [62].

SE are commonly purified from semen of donors who provide samples for reproductive purposes or for disease and drug screening. In most cases, semen samples not immediately used for isolation of spermatozoa or for other downstream analyses are placed in frozen storage, typically at -80 °C. Consequently, prolonged storage of semen in repositories or biobanks is possible. Such repositories serve as sources for semen samples that may be used in retrospective studies that address specific biological, functional, or medical questions. Moreover, conducting prospective studies involving multiple experimental time points that must be analyzed together requires freezing semen samples as they are collected. In clinical and laboratory settings, samples collected on the weekend or outside normal business hours are routinely stored frozen (possibly in -80 °C) for further processing. Thus, retrospective and prospective studies will use frozen semen samples for studies requiring SE.

While short-time freezing of semen does not affect SE function such as anti-HIV activity [62, 63], the effect of prolonged freezing on SE recovery, and biological activity is unknown. Furthermore, the effect of donor illicit drug use on SE characteristics is not known. Here, we made unexpected but novel observations on the differential effects of illicit drugs and prolonged freezing of semen on SE protein content and SE-mediated inhibition of HIV-1. The findings have identified potential markers of interest and have provided new insights regarding how
exposure to illicit drugs and environmental conditions may impact SE cargo composition and biological activity.

**Materials and methods**

**Ethics statement:** This study involves the use of existing human specimens (semen) and therefore is not human subjects' research. Dr. Amy E.T. Sparks, Director of University of Iowa In Vitro Fertilization and Reproductive Testing Laboratory provided de-identified samples of human semen from healthy donors. These samples were discarded from routine examinations and not linked to any identifiers. In addition, the Multicenter AIDS Cohort Study (MACS) provided de-identified semen samples from donors who at the time of collection reported using or not using illicit drugs. The MACS samples are also not linked to any identifiers. This study was approved by the University of Iowa Institutional Review Board.

**Semen donors:** A total of 18 donors were used for isolation of exosomes from semen in this study. Five donor semen samples were received frozen from the Multicenter AIDS Cohort Study (MACS). The MACS samples were collected approximately 30 years ago (1986) and stored in -80 °C until used. As control, 13 semen samples (one independent sample and 12 independent samples that were pooled into one sample) from the University of Iowa In Vitro Fertilization and Reproductive Testing laboratories were used. The University of Iowa samples were collected in 2014 and stored in -80 °C until used. All semen samples were thawed at room temperature. SE were isolated using a protocol previously described [62, 63] from semen of various donors who, at the time of collection, were using or not using cocaine or other illicit drugs. The rationale for inclusion of the two donor archetypes is to gain insight into how illicit drugs affect SE recovery and function. Semen samples were frozen at -80 °C since collection (~30 years ago) until thawed for this study, and will be referred to as prolonged freezing. These samples were collected and
stored by the Multicenter AIDS Cohort Study (MACS), which provided these specimens for the current study. To determine the effect of prolonged freezing of semen on the recovery of SE, we used SE isolated from semen samples obtained from the University of Iowa and stored for about two years under similar conditions, referred to as short-term freezing as control. All samples are from donors with no history of human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infection.

**Cells and viruses:** TZM-bl cells were obtained through the NIH AIDS Reagent Program and maintained in DMEM (Gibco-BRL/Life Technologies) complete with 5% exosome-depleted FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate and 0.3mg/ml L-glutamine (Invitrogen, Molecular Probes) as previously described [63]. HIV-1\textsubscript{pNL4.3} was obtained from the NIH AIDS Reagent Program and transfected into HEK293 cells using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) and as previously described [63]. HIV-1 was purified from cell culture supernatant and clarified by centrifugation and 0.45μm filtered. Viral titer was determined by EnzChek Reverse Transcriptase Assay (Life Technologies) [63].

**Isolation of semen exosomes (SE):** Semen was thawed at room temperature to allow for liquefaction. To pellet spermatozoa/cellular debris, samples were centrifuged at 7000 x g for 30 min at 4 °C. Cell-free seminal plasma was placed in a fresh conical tube and ExoQuick reagent (SBI) was added at a ratio of 4:1. Samples were re-suspended by inversion before incubation at 4 °C overnight without rotation per manufacturer’s instructions. The seminal plasma/ExoQuick mixture was centrifuged at 1500 x g for 30 min at 4 °C after which the exosome-free supernatant was removed from the exosome pellet. To remove residual ExoQuick, the exosome pellet was centrifuged at 1500 x g for 5 min. Any remaining supernatant was discarded from the exosome
pellet, and the pellet was re-suspended in 1x PBS in 1/5 of the original semen volume. This pellet is the semen exosome herein referred to as SE. SE were quantified by Bradford assay and aliquoted before use. To isolate SE from pooled donors, cell-free seminal plasma from 12 donors was combined in a conical tube before additional of ExoQuick reagent after which the protocol was followed as described above.

Transmission electron microscopy: 50 µg of SE were resuspended in 2.5% glutaraldehyde fixation solution and incubated at 4°C for 1 hr. After which, the fixed exosomes were ultracentrifuged at 100,000 x g for 2 hr at 4 °C to pellet the vesicles and remove the fixation solution. The fixed vesicle pellets were resuspended in PBS and deposited on Formvar film with carbon-coated 400-copper grids. The samples then underwent negative staining with 1% uranyl acetate for 1min and were allowed to air-dry. Images were acquired and viewed using JEOL JEM 1230 transmission electron microscope (TEM).

Quantification of SE concentration: NanoSight LM10 nanoparticle tracking analysis was used to determine concentration of SE particles per ml of semen and for estimation of particle numbers per µg of protein. Exosome preparations from each sample were diluted in PBS to a final volume of 0.5 ml and were assayed in triplicate. NanoSight NTA software was used to analyze average particle concentration from the three replicates.

Quantification of SE protein (intact and lysed) content: Intact SE protein quantification was determined by Bradford assay and absorbance at 595 nm. Total concentration of isolated SE was computed by multiplying the SE concentration in µg/µl by the volume of re-suspended SE. To quantify SE total protein which includes the luminal protein content, 5 µg of SE were lysed in 0.1M NaCO₃ for 1 hr at 4 °C before protein quantification by Bradford assay.
**Protein footprinting:** 5 μg of SE from each donor were lysed and separated on a 4-12% polyacrylamide gel before silver staining using the Pierce Silver Stain kit (Thermo Scientific) per the manufacturer’s instructions.

**Dynamic light scattering of SE:** A concentration of 0.1 mg/ml of SE in a 150 μl volume of PBS were analyzed by DynaPro NanoStar DLS (Wyatt Technologies) using disposable UVettes (Eppendorf). 10 measurements per SE sample were completed at a constant temperature of 25 °C and laser wavelength of 665nm. Analysis was determined by an average of ten measurements per SE sample. Data were analyzed using Dynamics software.

**Acetylcholinesterase (AChE) activity:** Acetylcholinesterase is an exosome specific enzyme [192] commonly used to identify exosomes and to differentiate exosomes from viruses. 50 μg of SE were lysed in a 1:1 volumetric ratio in 2% Triton X-100 in PBS. For negative control, equivalent volume of PBS was used. 5 μl of SE/Triton X fraction was added to a 96 well flat-bottom clear plate in triplicate. 1.25 mM acetylthiocholine chloride (AChE) (Sigma-Aldrich) and 0.1 mM 5,5’-Dithiobis 2-nitrobenzoic acid (DNTB) (Sigma-Aldrich) were added in a final volume of 100 μl to the exosome containing wells. Kinetic absorbance was read at 450 nm for 30 min at 37 °C at 5-minute time intervals.

**Quantification of CD63 and CD9 content of SE:** Purification of SE was further verified by expression of the exosome markers, CD63 and CD9 [63, 193]. Detection of human CD63 and human CD9 in SE using flow cytometry was completed per exosome-human CD63 isolation/detection kit following manufacturer’s instructions (Invitrogen). Briefly, 25 μg of SE re-suspended in isolation buffer (0.1% BSA in PBS) were incubated with 20 μl of 4.5 μm-diameter magnetic polystyrene beads (Dynabeads) at 4 °C overnight with rotation. The Dynabeads are pre-coated by the manufacturer with a primary monoclonal antibody for human
CD63 antigen. The SE-bound beads were then washed three times (0.1% BSA in PBS) to remove unbound exosomes, and the exosome-bound beads were stained for flow cytometry with anti-CD63-FITC (Biolegend) or anti-CD9-PE (Biolegend) for 1 hr at RT in the dark on an oscillating mixer. The SE-bound beads were then washed three times (0.1% BSA in PBS) to remove unbound antibody. CD63 or CD9 levels were analyzed through a FACSVerse instrument and mean fluorescence intensity was determined using FlowJo software (Tree Star).

**RNA evaluation:** Total SE RNA was extracted from 12 μg of SE per donor. SE RNA was purified using RNEasy kit per manufacturer’s instructions (Qiagen). RNA was subjected to treatment with DNase (Qiagen) as previously described [63]. RNA purity was assessed by the ratio of RNA absorbance (A260/A280). An A260/A280 ratio of >2.0 signifies a pure RNA preparation [194]. RNA concentration was determined by NanoDrop RNA absorbance, and equivalent concentration of RNA (1 ng/µl) was used for cDNA synthesis (ABI). Gene specific primers were used to amplify human CD9, CD63, and GAPDH (internal control) using ABI 7500 Fast real-time PCR System as previously described [63]. Thermocycler conditions were as follows: initial denaturation at 95 °C for 1 min; 45 cycles of 10 sec denaturation at 95 °C, and 30 sec annealing at 60 °C. PCR amplicons were separated and visualized on a 2% agarose gel stained with 50 µg/ml of ethidium bromide under UV light. Images were acquired with -UVP GelDock-It imaging system (UVP).

**SE inhibition assay:** The effectiveness of SE at inhibiting HIV-1 infection was accessed using two different SE inhibition (pre-incubation and pre-treatment) models as previously described [63]. In the pre-incubation model, SE was pre-incubated with HIV-1 prior to addition to target cells. This model mimics the events in HIV-1-infected men where SE is in contact with semen and SE-containing semen is discharged into the mucosa. Hence, 100 µg/ml SE or equivalent
volume of vehicle (PBS) were added to 8 RT units/ml of HIV-1\textsubscript{NL4.3}. The mixtures were incubated at 37 °C for 1 hour in complete DMEM containing 5% exosome-free FBS. After 1 hour, the SE/HIV-1 or HIV-1/PBS mixture was added to TZM-bl cells. Infectivity was determined 24 hours later by Steady-Glo luciferase substrate (Promega) emission in a luminometer. On the other hand, in pre-treatment model, SE are added to target cells to allow SE to condition the target cells prior to infection with HIV-1. This model assumes that SE elicits or endows anti-HIV-1 response in recipient cells. Thus, 100 μg/ml SE or vehicle (PBS) were added to equivalent number of TZM-bl cells and incubated for 24 hours. Then 8 RT units/ml of HIV-1\textsubscript{NL4.3} was added to cells. Infectivity was assessed 24 hours after the addition of virus by Steady-Glo emission. Experiments were repeated in duplicate with replicates of three per experiment.

**Cell viability:** The effect of SE on HIV-1 infected cells was assessed in both pre-incubation and pre-treatment SE inhibition models. Viability was determined by the MTT assay, as previously described [195] with replicates of three per experiment. Briefly, HIV-1 infected cells +/- SE or vehicle (PBS) and uninfected controls were incubated with 5 mg/ml MTT reagent for 3 hr in the dark at 37 °C. Following, MTT solvent (0.1%NP-40 and 4mM HCl in isopropanol) was added and incubated for 15 min with rocking. Absorbance was read at 590 nm using Tecan Infinite M200 Pro microplate reader.

**Statistics:** Statistical analysis was performed using the GraphPad Prism 7 software. Analysis of linear regression and the Pearson correlation coefficient (r) was used to determine correlation between variables using 95% confidence intervals and two-tailed p values. Infectivity was determined in two models of infection, referred to as preincubation and pretreatment models. Linear regression and correlation analysis was performed for each model of infectivity with CD63 or CD9 surface expression or baseline AChE activity. Prior to analysis of correlation
coefficients, normality of distribution was checked with D’Agostino and Pearson normality test. Two-tailed t test p-value (GraphPad Prism) calculations determined statistical significance; where p<.05 = *, p<.01 = **, ns = not significant. Error bars represent standard deviation (SD).

Results

Effect of prolonged freezing of semen on SE protein concentration

Donor and semen characteristics are presented in Table 2. Following isolation, we examined SE concentration by measuring the protein content of intact SE using Bradford assay [62, 63]. We found that SE concentration ranged between 5.55 µg/µl to 6.37 µg/µl. The protein content of intact SE is independent of length of storage and donor drug use. These data indicate that SE recovery was similar in the short and prolonged samples (Table 3).

Prolonged freezing of semen and SE physical properties

To analyze possible changes in physical properties of SE following prolonged freezing, SE isolated from prolonged frozen samples were examined for physical characteristics using transmission electron microscopy (TEM), NanoSight nanoparticle tracking analysis (NTA), and dynamic light scattering (DLS) measurements. Post-isolation TEM revealed similar morphology in all SE populations regardless of donor drug use or length of freezing (Figure 13A). Evaluation of SE concentration in donor semen by NTA showed that approximately 1.28 x 10^{11} - 1.78 x 10^{12} SE particles per ml were isolated from semen. This range sits within the expected particle concentration [133] and revealed that length of storage and donor drug use has no significant effect on the number of SE isolated from donor semen (Figure 13B). Additional characterization of SE intensity and size distributions were obtained using DLS. The radius of the particles was calculated using sphere approximation. Our data show that length of freezing has no effect on SE size distribution (Figure 13C-F). Similarly, drug use by semen donors has no effect on SE size
distribution (Figure 13C, G-I). These results indicate that prolonged freezing of semen has no effect on the physical properties of SE irrespective of donor drug use status.

**Effect of prolonged freezing of semen on SE protein cargo**

Although prolonged freezing of semen preserves SE physical properties, the effect of prolonged freezing on SE protein composition is unknown. Thus, we assessed the effect of prolonged freezing of semen on SE protein quality and composition. SE were lysed and total protein concentration was quantified. The data show presence of protein in all SE samples, with some donor-dependent variations (Figure 14A; see intact vs lysed bars). However, prolonged freezing of semen did not alter total SE protein, regardless of donor drug use status. Although semen exosomes are a distinctly heterogeneous population of vesicles [63, 196] (Figure 13A), it is currently unknown to what extent protein concentration varies with vesicle numbers. To ascertain how SE particle number (P) relate to protein concentration (µg), we calculated the ratio of particle to protein (P/µg). We observed subtle donor-dependent differences in SE particle to protein ratios with numbers ranging from approximately $2.2\times10^8$ to $3.0\times10^9$ particles per µg intact protein (Figure 14B). For completeness, we also computed the ratio of particle to total protein ($4.7\times10^7$ to $7.1\times10^8$) and found that particle to protein ratio diminishes with increasing protein concentration (Figure 14C). Our data suggest that SE protein content varies independent of vesicle number, donor drug use, or length of freezing.

Since prolonged freezing of semen does not decrease the protein content of SE, we sought to determine whether the protein profile will be altered following prolonged freezing. Thus, we examined SE proteome profile by performing protein foot printing. As shown in Figure 14D, random differences in band intensity were observed in the SE protein footprint. These differences are independent of freezing length and donor drug use. There was no discernible
difference in SE proteome pattern following 30 years of prolonged frozen semen storage when compared to semen stored for only 2 years. Similarly, donor illicit drug use did not change SE protein footprint (Figure 14D).

To further determine if prolonged freezing of semen alters the level of specific SE protein, we evaluated the activity of acetylcholine-esterase (AChE)—an enzyme typically associated with exosomes [192]. Analysis of AChE activity shows that all SE samples contain AChE with donor-dependent variability (Figure 15A). We found that prolonged (30 years) freezing of semen significantly decreased basal SE AChE activity (Figure 15B) while donor drug use has no significant effect on basal AChE activity (Figure 15C). As expected, measurement of the levels of SE AChE activity at different time points (0, 15, 30 minutes) show a time-dependent increase in AChE activity in all donor samples (Figure 15D). These data serve to validate that the isolated vesicles are SE. The lower basal SE AChE activity seen after prolonged frozen storage (Figure 15B) is indicative of potential AChE degradation or loss of enzymatic function following prolonged frozen semen storage.

The protein content and footprint results (Figure 14A,D) suggest that the proteome profile of SE is not markedly altered by prolonged freezing or by donor drug use. However, levels of specific proteins or enzymatic activities may be markedly altered by prolonged freezing as revealed by the results of AChE activity (Figure 15A-D). To further test this hypothesis, we assessed the effect of prolonged freezing and donor drug use on the level of the ubiquitous exosomal markers, CD63 and CD9 [63, 191].

As expected, all semen samples contain CD63 and CD9 positive SE, irrespective of length of freezing (Figure 16A-F). Strikingly, CD63 phenotyping produced an interesting distinction that separates SE based on donor drug use. SE purified from donors that used illicit
drugs have reduced surface CD63 intensity compared to SE purified from donors who did not use drugs (Figure 16A-C). SE from donors that used illicit drugs showed a similar pattern and appeared to contain CD9 with lower intensity compared to SE from donors who did not use drugs (Figure 16D). However, CD9 differences did not reach statistical significance (Figure 16E-F). These results indicate that a drug-dependent mechanism may be involved in the suppression of tetraspanin protein (CD63 and potentially CD9) expression in the cells that secrete SE or in the incorporation of CD63 and potentially CD9 into SE.

**Effect of prolonged freezing of semen on SE RNA content**

Our previous work revealed that SE isolated from semen contain substantial amounts of RNA [63]. However, whether SE RNA content change following prolonged freezing is unknown. This was assessed by isolation of total SE RNA followed by evaluation of RNA concentration by spectrophotometric analysis. We found donor-dependent variations in SE RNA content (Figure 17A). The observed donor-dependent difference in RNA content is independent of donor drug use or prolonged freezing of semen.

Given the drug-use dependent difference in CD63 and possibly CD9 protein content (Figure 16A-F) and the donor-dependent difference in total RNA content (Figure 17A), we sought to determine whether prolonged freezing and donor drug use altered the level of coding mRNA loaded into SE. Reverse transcription PCR (RT-PCR) revealed that there was no difference in the abundance of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and CD9 mRNA (Figure 17B). Interestingly, we observed a significant decrease in CD63 mRNA in SE from semen of donors that used illicit drugs (Figure 17B). These data identify CD63 as a gene that is susceptible to modulation by illicit drugs. Whether the action of illicit drugs on CD63 is directed at exosome-producing cells and/or packaging into semen exosomes is currently
unknown and being investigated. It is possible that SE originate from various parts of the male reproductive organs and that the protein and RNA content of SE could vary depending upon the cell from which the SE was secreted.

**The effect of prolonged freezing of semen on SE-mediated inhibition of HIV-1**

It is known that SE isolated from semen that had been stored for a short time, obtained from healthy donors with no history of drug use, robustly inhibit HIV-1 infection [62, 63]. The inhibitory effect of SE on HIV-1 infection is operative when cells are pre-treated with SE 24 hours before infection and when SE are pre-incubated with virus for 1 hour at 37°C before addition of the SE/virus complex to cells [63]. These two different models of SE inhibition signify that SE may use multiple mechanisms to inhibit HIV-1 infection. To assess whether prolonged (30 years) freezing of semen or donor drug use alter SE-mediated inhibition of HIV-1, we evaluated SE inhibitory characteristics in both pre-treatment and pre-incubation inhibition models.

Pre-incubation of SE with HIV-1 for 1 hour before addition of the SE/virus complex to cells reveals an impairment of SE inhibitory effect of HIV-1 infection that is mediated by prolonged freezing of semen (Figure 18A). As expected, SE isolated from semen stored for ~2 years effectively inhibited HIV-1 infection (Figure 18A), supporting previous reports [62, 63]. The loss of HIV-1 inhibition by SE following prolonged freezing was not due to cell death because cell viability as determined by MTT assay was unaffected (Figure 18B). In this SE inhibition model, donor drug use has no effect on SE-mediated HIV-1 inhibition as well as on cell viability (Figure 18A-B; compare pink bars vs blue bars).

SE endows cells with anti-HIV phenotype as shown by marked inhibition of HIV-1 infection of cells following exposure of cells to SE prior to infection [63]. To evaluate the effect
of prolonged (~30 years) freezing of semen on SE-mediated anti-HIV-1 effect on target cells in the pre-treatment condition, we incubated SE with cells for 24 hours prior to infection with HIV-1. All SE from non-drug users independent of length of frozen semen storage inhibit HIV-1 infection (Figure 18C). In contrast, 1 out of 3 SE from illicit drug users inhibit HIV-1 infection (Figure 18C, donor D3). As in the pre-incubation model (Figure 18A), the differential pattern of SE inhibition in the pre-treatment model could not be ascribed to cellular toxicity because cells under all conditions were equally viable (Figure 18D).

The AChE activity data in Figure 15B,D and SE-mediated inhibition of HIV-1 infection in Figure 18A suggest a link between decreased SE AChE activity and SE-mediated HIV inhibition when SE and the virus are pre-incubated before infection. Indeed, further correlation analysis by Pearson r coefficient and linear regression reveals a significant correlation between decreased SE-AChE activity and absence of SE-mediated HIV-1 inhibition in a pre-incubation infection model (Figure 19A). In contrast, no correlation was observed between decreased AChE activity and SE-mediated HIV-1 inhibition in a pre-treatment model (Figure 19B). These correlative analyses suggest that the AChE activity of SE or other enzymes such as butyrylcholinesterase (BChE) that is capable of hydrolyzing acetylcholine may play a role in the SE-virus inhibitory interaction in the pre-incubation model of HIV-1 infection.

In a pre-incubation SE inhibition model, no significant correlation was observed between SE-CD63 or SE-CD9 content and inhibition of HIV-1 (Figure 19C, E, respectively). However, a significant inverse correlation was observed between surface CD63 and CD9 in SE and SE-mediated inhibition of HIV-1 infection in a pre-treatment infection model, where increased SE-CD63 or SE-CD9 content is associated with decreased HIV-1 infectivity (Figure 19D, F, respectively). This observation is in agreement with our data that show that SE with significantly
decreased CD63 or patterns of reduced CD9 isolated from donors who use illicit drugs (Figure 16A-D) had no effect on HIV-1 inhibition in 2 out of 3 donors (Figure 18C). Together, these data imply that prolonged freezing rather than illicit drugs may alter components of SE that mediate a potential SE/HIV-1 interaction that occurs upon incubation of SE and HIV-1. Furthermore, our data suggest that the use of illicit drugs rather than prolonged freezing impairs SE-mediated endowment of an anti-HIV-1 state to recipient cells. This effect may depend on other donor intrinsic factors.

**Discussion**

In this study we have shown that prolonged semen freezing has no significant effect on the recovery of semen exosomes (SE). Isolated SE have similar physical properties as determined by comparative morphology, light scattering patterns (intensity and size), and particle concentrations following prolonged and short-term semen freezing. We also show that donor drug use has no significant effect on SE recovery and SE physical properties. In our study, we isolated approximately $1.28 \times 10^{11} - 1.78 \times 10^{12}$ particles per ml of semen. This concentration is in line with previously reported SE concentration ($4.7 \times 10^{11} - 3.12 \times 10^{13}$) [133]. Similar to our findings, others have reported minimal loss of urinary exosome-associated proteins from urine that was stored at -80 °C, however this study addressed the effect of temperature storage on exosome-associated protein recovery, and did not address length of storage [110].

Although SE physical properties, RNA and protein contents are stable following prolonged freezing, the levels of specific SE cargos may be altered, as exemplified by decreased AChE activity (Figure 15A-D). Such alteration may affect some biological and functional activities of SE, although this is yet to be determined. While total protein concentration (intact and lysed) and protein footprint is conserved in all SE samples, prolonged semen freezing
reduced the level of AChE activity of SE. There was also a trend towards drug-induced reduction of SE AChE activity, but this difference did not reach statistical significance. AChE is a plasma membrane protein incorporated into exosomes during exosome biogenesis, making the enzymatic activity of AChE a commonly used exosome marker [197]. AChE activity is indicative of the enzymatic degradation of acetylcholine, a compound known to dampen inflammatory response in cells [198]. Inhibition of AChE with pyridostigmine downregulates HIV-1-induced T cell activation and T cell proliferation in chronically infected HIV-1 patients, however, viral load was not assessed in this study [198]. While a link between AChE activity and HIV-1 viral load has not been made, infection with herpes simplex virus type 1 (HSV-1) results in a steady decline in the enzymatic activity of AChE [199], indicating that AChE or other acetylcholine hydrolyzing enzymes such as BChE may play a role in viral infection. Thus, decreased AChE activity in SE following prolonged storage of frozen semen may have important biological or functional effects. Indeed, the correlation (Figure 19A) between decreased AChE activity (Figure 15B) and loss of HIV-1 inhibition by SE following prolonged freezing (Figure 18A) suggest that prolonged freezing of semen may concurrently or independently alter the activities of proteins that inhibit HIV-1 infection. Future studies are needed to investigate the role of SE-bound AChE, as well as other SE-bound proteins/enzymes in modifying processes that inhibit HIV-1 infection, and SE isolated from semen stored for at least 30 years will be valuable for such studies.

In contrast to AChE levels, prolonged freezing of semen has no effect on SE CD63 and CD9 protein and mRNA. However, drug use by semen donors markedly reduced the level of CD63 protein and mRNA, and showed patterns of reduced CD9 protein. CD63 and CD9 are cellular membrane proteins found in exosomes including SE [63]. It has been shown that CD63,
CD9 and other tetraspanin proteins play multiple important roles in HIV-1 infection [200-203] and that CD63 and CD9 are incorporated into released HIV-1 particles [202, 204]. Inhibition of CD63 reduces HIV-1 infection in macrophages [201] in a CCR5 co-receptor dependent manner [201]. On the contrary, CD63 protein incorporated into released virions attenuates HIV-1 infectivity in a virion-specific manner [202], and recombinant extracellular domains of CD63 potently inhibit HIV-1 infection in a cell type-dependent manner [200]. These reports suggest that the effect of CD63 on HIV-1 may depend both on target cells and on the virions. Although the role of CD9 in HIV infection has not been extensively studied, it has been shown that overexpression of CD9 reduced HIV-1 infectivity [203]. Additionally, CD9 has been implicated in regulating virion membrane fusion events where knock-down of CD9 expression enhanced viral entry [205]. Our finding that donor drug use reduces CD63 protein and mRNA, and potentially CD9 protein (Figures 16 and 17) and that such SE containing lower levels of CD63 and CD9 were unable to inhibit HIV-1 infection in a pre-treatment SE inhibition model (Figure 18C) suggest that CD63 and/or CD9 protein in SE may be linked to SE-mediated inhibition of HIV-1 in this model. The amount of CD63 and CD9 at the surface of SE clearly correlates with the level of HIV-1 inhibition in a pre-treatment model (Figure 19D,F) and inversely correlates with donor drug use. Further studies will be needed to link SE CD63 and CD9 to HIV-1 inhibition and to define the role of illicit drugs in the process.

Similar to prolonged freezing of semen, donor drug use may alter SE cargo composition and function, such as SE-mediated inhibition of HIV-1 infection [62, 63]. It is known that addictive stimulant drugs including cocaine increase the risk of exposure to HIV-1 infection and investigation into the chronic administration of cocaine and HIV-1 infection showed significant enhancement of cell death and toxic effects [206]. Therefore, it is probable that illicit drug use
alters the composition of exosomes during biogenesis due to its cellular affects. The impact of drug use on the exosomal pathway is further exemplified by the observation that cocaine enhanced the release of extracellular vesicles in cell cultures [207]. Indeed, the effect of illicit drugs goes beyond enhancement of HIV-1 infection. Illicit drugs such as, opioid narcotics, methamphetamines, marijuana, and cocaine have been found to adversely impact male fertility [208], although their mechanisms of action are currently unknown. Thus, identifying proteomic differences in semen exosomes isolated from donors who reported illicit drug use compared to non-user and their resulting effects on HIV-1 infectivity or male fertility may be essential in identifying exosome-induced recipient cell protective effects against HIV-1 or detrimental effects on reproduction.

The fundamental roles of SE in HIV-1 inhibition are intriguing. However, the mechanisms of action and the inhibitory factors need to be identified. The observed distinctive effects of illicit drug use and prolonged freezing of semen on the different SE inhibition models (pre-incubation vs pre-treatment) support the proposition that SE may utilize different mechanisms or pathways to inhibit HIV-1 infection. Thus, SE isolated from semen of illicit drug users and semen that has been frozen at -80 °C for a prolonged time may be used in elucidating the mechanisms of SE anti-HIV-1 activity and in identifying the inhibitory factors in SE. Additionally, SE from semen of illicit drug users may serve as a useful tool for identifying predictive bio-signatures in cases of chronic drug exposure.

Finally, although the findings of this study are intriguing, our study is not free from limitations. The small sample size limits our conclusions and requires caution in interpreting the observed associations. Increased sample size is needed to overcome these constraints and to
make conclusive statements in terms of the i) link between SE AChE, CD63, and CD9 to HIV-1 inhibition and ii) causation of the phenotypes observed in SE.
<table>
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<th>Length of Storage (years)</th>
<th>Drug Use</th>
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<th>Po</th>
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Table 2: Characteristics of donor and semen samples.
Co = Cocaine; Ma = Marijuana; Po = Poppers; PCP = Phencyclidine; EC = Ecstasy; He = Heroin; Do = Downers; Et = Ethyl chloride; Al = Alcohol; + = Self-reported ever used; - = Self-reported never used; mod = Moderate; p = pooled samples from 12 donors.
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<th>Storage definition</th>
<th>Drug Use</th>
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Table 3: Effect of length of storage and illicit drug use on semen exosome protein concentration.
Figure 13: Physical properties of SE isolated from diverse conditions. (a) Representative TEM images showing SE morphology from donors who used illicit drugs (top left) or did not use illicit drugs (top right) and following prolonged (bottom left) or short (bottom right) term freezing of semen. The insets on each image show the zoomed image of a single vesicle. Yellow arrowheads indicate the vesicle in each field used for the zoomed image. Scale bars are 1 μm. (b) Exosome concentrations were quantified by NTA and averaged from three measurements. Concentrations were calculated per ml of semen. (c) Comparison of SE size in diameter (nm) by length of storage or donor illicit drug use. (d and e) Representative DLS histograms showing SE distribution intensity by radius (nm) following prolonged (n = 5) or short-term freezing (n = 13) of semen. (f) Average SE DLS size in diameter (nm) by length of storage. (g and h) Representative DLS histograms showing distribution intensity of SE from donors who used (n = 3) illicit drugs or donors who did not use illicit drugs (n = 15). (i) Average SE DLS size in diameter (nm) by donor illicit drug use. For statistical analysis, samples were grouped into “length of the freezing” and “drug use” and analyzed comparing short to prolong or drug – to drug + respectively for F and I. For B and C statistics was performed by comparing illicit drug use (n=3) and no illicit drug use (n=15). Significance was determined by student’s t test. Differences with p values of 0.05 are considered significant. Error bars are standard errors of the mean. ns = not significant.
Figure 14: Protein concentration and electrophoretic patterns of SE from different donors at different freezing times. (a) Protein concentrations as determined by Bradford analyses for intact and lysed SE. (b) Comparison of ratio of particles to intact protein across all donor samples and under different storage conditions. (c) Comparison of ratio of particles to total protein across all donor samples and under different storage conditions. (d) SE protein footprint analyzed by silver stain. Statistics was performed by comparing illicit drug use (n=3) and no illicit drug use (n=15). Significance was determined by student’s t-test. Differences with $p$ values of 0.05 or less are considered significant. ns = not significant.
Figure 15: Length of semen storage alters SE-associated AChE enzymatic activity. Acetylcholine-esterase (AChE) specific enzyme activity was measured in SE as described in “Methods” where vehicle is PBS. (a) Baseline acetylcholine-esterase activity taken at time = 0 minutes for individual donor and pooled SE. (b) Average baseline AChE enzymatic activity at time = 0 for prolonged (n = 5) and short-term freezing (n = 13). (c) Average baseline AChE enzymatic activity for illicit drug use (n = 3) and no illicit drug use (n=15). (d) Time course of AChE activity at time = 0, 15, and 30 minutes. Statistics was based on comparing baseline AChE activity to vehicle, short to prolong, and Drug - to Drug + respectively for panels a, b, and c. For panel D, statistics time values from point 15 and 30 minutes were compared to the 0 time point. Significance was determined by student’s t test. Differences with p values of 0.05 or less are considered significant *p<0.05, **p<0.001. Error bars are SEM. ns = not significant.
Figure 16: Levels of CD63 and CD9 on the surface of SE. Analysis of SE-bound CD63 and CD9 was performed as described in the “Methods” section by binding of SE to anti-CD63 coated magnetic beads. CD63-bound SE were stained with human CD63 or CD9 and analyzed by flow cytometry. (a) Staggered histogram showing changes in CD63 on the surface of SE. (b) Mean fluorescence intensity (MFI) of CD63 on the surface of SE. (c) CD63 MFI normalized to baseline AChE. (d) Staggered histogram of CD9 levels on the surface of SE. (e) CD9 MFI on SE. (f) CD9 MFI normalized to baseline AChE. Statistics was performed by comparing illicit drug use (n=3) and no illicit drug use (n=15). Significance was determined by student’s t test. Differences with $p$ values of 0.05 or less are considered significant $^*p<0.05$. ns = not significant.
Figure 17: Content and quantity of SE RNA from different donors and after different freezing times. RNA was extracted from SE as described in “Methods”. (a) Total RNA concentration from 12 μg of SE. (b) RT-PCR analysis of mRNA of selected exosomal markers and GAPDH in SE. PCR products were from cDNA generated using equivalent amounts (1 ng/µl) of total RNA. Statistics was performed by comparing illicit drug use (n=3) and no illicit drug use (n=15). Significance was determined by student’s t test. Differences with p values of 0.05 or less are considered significant. ns = not significant.
Figure 18: Effects of length of freezing and donor drug use on SE-mediated HIV-1 inhibition. (a and b) Infectivity of HIV-1 and cell viability in a pre-incubation infection model where SE (100 μg/ml) or vehicle PBS was pre-incubated with 8 RT units/ml of HIV-1 NL4.3 for 1 hour at 37 °C before infection of TZM-bl indicator cells for 24 h. (a) Infectivity and (b) Cell viability. (c and d) Infectivity of HIV-1 and cell viability in a pre-treatment infection model where SE (100 μg/ml) or vehicle PBS was added to TZM-bl indicator cells for 24 h before infecting cells with 8 RT units/ml of HIV-1 NL4.3 for 24 h. (c) Infectivity and (d) Cell viability. Vehicle is set as reference at 100% for infectivity and viability. Statistics was performed by comparing infectivity or viability values from each donor to vehicle control. Significance was determined by student’s t test. Differences with p values of 0.05 or less are considered significant *p<0.05, **p<0.001. Error bars are SD. ns = not significant.
Figure 19: Association between CD63 expression and AChE activity with SE-mediated inhibition of HIV-1 infection. Correlation between baseline SE-AChE activity and inhibition of HIV-1 infection during (a) pre-incubation (b) pre-treatment infection models. Correlation analysis between CD63 and inhibition of HIV-1 infection during (c) pre-incubation and (d) pre-treatment infection models. Correlation analysis between CD9 and inhibition of HIV-1 infection during (d) pre-incubation and (e) pretreatment infection models. Prior to correlation coefficient analyses, normality of distribution was checked with D'Agostino and Pearson normality test. Differences with $p$ values of 0.05 or less are considered significant *$p$<0.05, **$p$<0.01.
CHAPTER IV: BODY FLUID EXOSOMES IN HIV-INFECTED SUBJECTS ENCASE ANTIRETROVIRAL DRUGS AND INHIBIT HIV REPLICATION IN TARGET CELLS

Abstract

Exosomes are cell-derived vesicles that may influence HIV-1 pathogenesis. Exosomes isolated from semen but not blood obtained from HIV-negative human donors contain protective factors that endow an anti-HIV phenotype to target cells. The reason for this is not well understood. We characterized exosomes and exosome-free plasma in HIV infected donor semen and blood and their role during HIV-1 infection. Exosomes and exosome-free plasma were purified from blood and semen of HIV-negative controls, HIV-suppressed individuals on antiretroviral therapy (ART), and HIV-infected individuals not on antiretroviral (ARV) therapy (ART-naïve). Exosomes purified from semen of HIV-negative and HIV-infected ART-naïve individuals inhibited HIV-1 infection, but blood exosomes and exosome-free blood and seminal plasma did not. Exosome-free plasma and exosomes from blood and semen of HIV-suppressed individuals on ART both inhibited HIV-1 infection. ARVs and ARV metabolites were detected in exosomes and their corresponding exosome-free fractions obtained from ART-suppressed, HIV-1 infected individuals. HIV-negative blood exosomes loaded with ART confirmed exosome-associated ARVs are protective against HIV-1. Importantly, exosomes from semen inhibited HIV-1 infection regardless of donor HIV status or ART.

Introduction

HIV is disproportionately acquired from seminal transmission (~88% of new infections in the USA) [209]. However, rates of sexual transmission are infrequent (1/200-1/1000 sexual exposures) [209]. Transmission rates per sexual encounter depend on the level of virus in semen, the condition of infected cells in the male reproductive tract, and susceptibility status of the
recipient mucosal environment [209]. Low rates of sexual transmission can be attributed to antiretroviral therapy (ART) suppression of virus production in blood and seminal compartments in HIV-suppressed antiretroviral (ARV) adherent individuals [210, 211]. (Lines of evidence show) Exosomes present in semen from HIV-negative donors protect against HIV-1 infection in vitro, thus semen exosomes (SE) may contribute to the low rate of HIV-1 seminal transmission in the absence of ART [62-64, 139].

Exosomes are important mediators of disease, including viral infections. Although similar in physical characteristics to viruses, exosomes may facilitate or suppress viral infection [22, 28]. The role of exosomes during viral infection is mediated by exosome cargo or membrane components acquired during biogenesis [22]. Exosomes may enhance susceptibility to HIV-1 by delivering HIV-1 co-receptors or viral components to resistant cells, or may suppress infection by transferring antiviral components or initiate immune cell activation [22, 28]. Body-fluid exosomes, including semen and blood derived particles, are released from a variety of cells in different physiological states. Therefore, exosomes isolated from diverse body-fluids may facilitate or suppress viral infections depending on the status of the exosome-originating cell [22, 28]. Exosomes from body-fluids such as semen, breast milk, and vaginal fluid of HIV-negative donors inhibit HIV-1 infection [63, 104, 105]. Although the mechanism(s) of inhibition is often unreported, HIV-negative SE interfere with HIV-1 lifecycle steps required for replication including reverse transcription, proviral DNA integration, and RNA transcription [62, 63, 139]. SE block HIV-1 replication events, at least in part, by targeting an early viral protein, Tat, and its regulatory mechanisms including host transcription factor recruitment and transcription initiation and elongation [139].
The balance between antiviral or proviral effects of exosomes is variable, and the cellular activation state, HIV infection, and ART treatment all influence the overall effect of exosomes on HIV-1 replication. However, the effect of donor HIV status and ART treatment on the function of body-fluid exosomes is currently unknown. ARV drugs are detectable in blood plasma and seminal fluids [211], and the presence of ARV drugs modifies cell-culture derived exosomes [140]. Thus, ARV drugs may alter the composition and function of body-fluid derived exosomes toward an antiviral phenotype. Conversely, exosomes from HIV-1 infected cell cultures may lead to a proviral phenotype [83, 212]. The effect of semen and blood exosomes in HIV-1 infected subjects with active HIV-1 viremia or suppressed with ARVs has not been systematically examined. Thus, we investigated the effect of SE and BE from HIV-infected ART-naïve and ART-suppressed individuals on HIV-1 infection. We examined the effect of donor HIV infection and ART status on the effect of body-fluid exosomes on HIV-1 infection.

**Materials and methods**

**Ethics:** This study was approved by The University of Iowa and Stony Brook University Institutional Review Board (IRB), and all experiments were completed according to approved University regulations. All participants provided written informed consent, and all samples were studied with laboratory personnel blinded to clinical data. Demographics and clinical characteristics were subsequently obtained through review of medical records (Table 4 and 5). Plasma HIV-1 RNA viral load was measured using the Roche Cobas method, and CD4+ T-cell counts were determined using flow cytometry by the University of Iowa Hospitals and Clinics clinical laboratory as previously described (Table 4 and 5) [213]. HIV-negative donors had no history of human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus.
(HCV) infection. HIV-infected donors were classified as ART-suppressed or ART-naïve based on viral load and therapy adherence at the time of collection (suppressed = <50 copies/ml).

**Purification of exosomes and exosome-free plasma:** Semen and whole blood samples from subjects were processed for exosome isolation within 4 hours. Peripheral blood mononuclear cells (PBMCs) were purified from blood using Vacutainer® cellular preparation tubes (CPT) according to manufacturer’s instructions (BD Biosciences). Exosomes were purified from blood plasma and semen as previously described [62-64, 111]. Briefly, cell-free blood and seminal plasma were mixed with ExoQuick reagent (SBI), according to manufacturer’s instructions and exosomes pelleted by centrifugation. Supernatant was removed and saved as exosome-free plasma. The exosome pellet was re-suspended in PBS and is referred to as blood (BE) or semen exosomes (SE) depending upon sample source. Exosome and exosome-free plasma protein quantification was determined by NanoDrop absorbance at 280nm.

**Cells and viruses:** TZM-bl cells were obtained through the NIH AIDS Reagent Program. 293T cells were purchased from ATCC. 293T and TZM-bl cells were maintained in complete DMEM (Gibco-BRL/Life Technologies) containing 5% exosome-depleted FBS (Gibco) as previously described [111]. HIV-1 pNL4.3 plasmid was provided by NIH AIDS Reagent Program, and HIV-1 NL4.3 was produced by transfected 293T cells with pNL4.3 plasmid as previously described [63, 64, 139]. Virus titers were determined by TZM-bl renilla luciferase units (RLU) and EnzChek Reverse Transcriptase Assay (Life Technologies).

**HIV-1 inhibition:** 100 μg/ml exosomes or exosome-free plasma (blood and semen) were simultaneously added with HIV-1 NL4.3 virus (100,000 RLU/100 μl) to TZM-bl indicator cells in complete DMEM containing 5% exosome-free FBS. Equivalent volume of PBS was used as
vehicle control. Infectivity was determined after 24 hours by Steady-Glo (Promega) read-out of RLU. Experiments were completed with replicates of three per donor [64, 111].

**Cell viability:** Viability was determined by MTT assay with replicates of three per donor at the time of infection read-out, as previously described [64, 111].

**Antiviral drug concentration measurement:** ARV drug concentrations were detected by LC-MS/MS using a Shimadzu Nexera liquid chromatography system and AB Sciex 6500 triplequadrupole mass spectrometer [214].

**ARV-loaded blood exosome formulation and characterization:** Two approaches were used to incorporate ARV into HIV-negative BE. Emtricitabine (50 μg FTC) (NIH AIDS Reagent Program) was Cy3-labeled with Cy3 Fast Conjugation kit (Abcam) according to manufacturer’s instructions. Modifier Reagent (1 μl per 10 μl FTC) was mixed with Cy3 Conjugate. Cy3 Quencher reagent (1 μl per 10 μl FTC) was used to stop the reaction. First, HIV-negative BE (200 μg) were combined with 50 μg FTC-Cy3 or buffer control for 90 minutes at 37°C before ultracentrifugation at 100,000 x g for 70 minutes. Pellets containing FTC-Cy3 loaded or unlabeled FTC control exosomes were resuspended in PBS to original volume and protein quantified by Nanodrop absorbance before Cy3 detection and HIV inhibition studies [215]. Alternatively, 200 μg HIV-negative BE in 50 μl volume were incubated with 50 μg FTC-Cy3 in 20μl, 10 μl ExoFect reagent (SBI), and 70 μl PBS. The reaction was incubated for 10 minutes at 37°C and stopped by addition of 30 μl ExoQuick reagent. The transfection/ExoQuick solution was placed on ice for 30 minutes prior to centrifugation at 13,000 rpm for 3 minutes. Pelleted exosomes were resuspended in the original volume of PBS before quantification, Cy3 detection, and HIV inhibition studies [216]. Both approaches were completed with three independent donors. 100 μg/ml exosomes were used for HIV inhibition studies.
**IgG purification**: IgG was depleted from HIV-infected ART-suppressed exosomes and plasma by incubation with Protein G-coated magnetic beads (Dynabeads® Protein G, Life Technologies) for 2 hours at room temperature with rotation (1 μg protein/1 μl beads). Beads were exposed to magnet to remove IgG from the supernatant. Binding and incubation with IgG-depleted supernatant fluids were repeated three times with new protein-G beads each time. IgG bound beads were exposed to 20 μl 0.2 M glycine pH 2.0 and incubated at room temperature for 5 minutes to facilitate IgG elution, and beads were removed from IgG magnetically. The eluted IgG was neutralized with 10% volume 1 M phosphate buffer pH 7.5 before IgG quantitation and HIV inhibition studies. IgG quantitation was determined by human IgG antigen ELISA according to manufacturer’s instructions (Molecular Innovations). IgG studies were repeated with four independent donors.

**Statistics**: Data are reported as the mean and standard deviation (SD). Paired two-tailed student’s t test p-value determined statistical significance P<.05=*, P<.01=**, P<.001=***, ns=not significant (GraphPad Prism).

**Results**

**Semen exosomes from HIV-negative and HIV-infected ART-naïve donors inhibit HIV-1**

Exosomes isolated from human, HIV-1 uninfected semen inhibit HIV-1 infection whereas blood exosomes do not [62-64, 111, 139]. Because semen is the major vector for HIV-1 sexual transmission and the function of exosomes is dependent on the cell condition during exosome biogenesis, we sought to expand these studies in HIV-infected ART-naïve donors. To determine if these findings relate to exosome-specific effects, the effects of exosome-free plasma (blood and semen) on HIV-1 replication were also evaluated. Exosomes and exosome-free plasma were isolated from blood and semen obtained from HIV-negative and HIV-infected
ART-naïve donors (Table 4). Hereafter these specimens are referred to as blood exosomes (BE), exosome-free blood plasma (EFBP), semen exosomes (SE), and exosome-free seminal plasma (EFSP). TZM-bl reporter cells containing HIV-1 LTR that expresses luciferase when infected showed that in a direct comparison, SE inhibit HIV-1 while BE do not (Figure 20A). Comparison of HIV-negative EFBP and EFSP showed that EFSP have no effect on infection whereas EFBP enhance infection (Figure 20A). Interestingly, these findings were recapitulated in HIV-infected ART-naïve fractions; SE inhibited and EFBP enhanced infection while BE and EFSP had no effect (Figure 20B). These effects were independent of cell viability (Figure 20C-D). Clinically-determined viral loads confirmed plasma viral RNA in HIV-infected, ART-naïve donors (Table 4). Thus, HIV-1 inhibition by HIV-infected, ART-naïve SE could not be attributed to suppressed donor infection. These results suggest HIV status does not affect the antiviral phenotype of SE, or the enhancing function of EFBP.

**Exosomes and exosome-free plasma from HIV-infected ARV-suppressed donors inhibit HIV-1**

A recent report shows ARV drugs alters the content of cell-culture exosomes released from HIV-1 infected cells [140]. The effect of donor-ART on exosomes and exosome-free plasma was evaluated in HIV-infected ART-suppressed donors (Table 4) with a median duration of ART >5 years (Table 5). HIV-1 infectivity assays showed that HIV-infected ART-suppressed BE, EFBP, SE, and EFSP robustly inhibit infection (Figure 21A) without affecting cell viability (Figure 21B). Because we expect the cell environment to differ between HIV-infected ART-suppressed donors and HIV-negative donors, affecting exosome biogenesis and incorporation of host factors, we hypothesized that levels of SE-mediated inhibition may vary. Direct comparison of SE from HIV-negative and HIV-infected ART-suppressed donors revealed no significant
difference in HIV-1 inhibitory potential (Figure 21C). Taken together with the result that the inhibitory phenotype of SE is conserved in HIV-infected ART-naïve donors (Figure 20B), these data strongly suggest that the inhibitory activity of SE is irrespective of ART.

**Exosome-associated ARV drugs protect against HIV-1**

Because BE only inhibited infection in the cohort of ART-suppressed donors, we evaluated whether ARVs could be incorporated into BE. Two independent methods for loading fluorescently labeled ARVs into HIV-negative BE demonstrated uptake of fluorescent-labeled FTC in the exosome fraction (Figure 21D). Challenge with HIV-1 showed that FTC-loaded HIV-negative BE inhibited infection while unloaded HIV-negative BE or HIV-negative BE loaded with only fluorescent marker had no effect on infection (Figure 21E) or cell viability (Figure 21F). These results indicate that donor ART may contribute to HIV-1 inhibition by BE and exosome-free plasma.

**Inhibitory levels of ARV drugs are detectable in exosomes and exosome-free plasma from HIV-infected ART-suppressed donors**

ARV drug concentrations are reported for semen, testicular tissues, and blood in HIV-infected donors; it is currently unreported whether ARVs are detectable in body-fluid exosomes [211, 217-219]. As expected, detectable concentrations of ARVs were found in EFBP and EFSP (Table 6). Interestingly, and novel to the field, exosome-associated ARVs were detected in BE and SE preparations (Table 6, Figure 22A-D). Tenofovir (TFV) and emtricitabine (FTC), included in the regimen of all donors analyzed, were detected in all fractions of all donors (Table 6, Figure 22A-B). The activate metabolites tenofovir diphosphate (TFVDP) and emtricitabine triphosphate (FTCTP) were also detected in EFSP (Table 6). Detection of cell-free TFVDP and FTCTP is significant as intracellular phosphorylation traps these metabolites in the cell lipid
membrane, making them susceptible to degradation by phosphatases in the extracellular environment [220]. In subjects receiving efavirenz (EFV) or dolutegravir (DTG), these ARVs were detected in all fractions (Table 6, Figure 22C-D).

Quantification results showed ARV drug levels in BE and SE reach FDA-half maximal inhibitory concentrations (IC$_{50}$) for HIV-1 (DTG=0.02-2.14 nM, FTC=0.0013-.64 μM, EFV=1.7-25 nM)(Table 6, Figure 22A-E) [221-223]. IC$_{50}$ levels for TFV (0.04-8.5 μM) [224] were found in SE from all donors but only in BE from one donor (Table 6, Figure 22A-E). Although limited by sample size, possible trends of compartmentalization of drug incorporation in exosomes are suggested. TFV and FTC may be more concentrated in SE rather than BE whereas EFV and DTG appear to be more concentrated in BE than SE (Table 6, Figure 22A-E). These results are the first to show that ARVs are body-fluid exosome-associated, and strongly suggest that the HIV-inhibitory phenotype of HIV-infected ART-suppressed BE, EFBP, and EFSP may be due to the presence of ARVs. In addition, these results indicate that ARVs may be selectively compartmentalized into different body-fluid fractions. Further investigation is needed to better characterize observation.

**IgG does not contribute to inhibition by HIV-infected ART-suppressed body-fluid fractions**

Although controversial, some studies suggest that HIV-1 specific IgG antibodies may limit infection [225]. To confirm that the inhibition of HIV-1 by SE and other fractions is due to the presence of ARVs, we evaluated the role of IgG on HIV-1 inhibition. By reducing IgG levels in four donors’ blood and seminal fractions (Figure 23A), IgG was detected in BE, EFBP, SE, and EFSP (Figure 23A-B). The purified IgG from BE, EFBP, SE, and EFSP did not alter HIV-1 infection or reduce cell viability (Figure 23C-D). Because ARV drugs remain in the IgG depleted fractions, infectivity by IgG depleted fractions could not be uncoupled from inhibition.
by ARV drugs. It should be noted that IgG neutralizing antibodies from human specimens are specific to the HIV-1 clinical strain of the donor; thus, human-derived IgG antibodies may not neutralize the lab-adapted strain used in these studies. Nevertheless, these data support the conclusion that donor-ART contributes to the inhibitory phenotype of blood exosomes and blood and semen exosome-free plasma, and indicate that IgG does not contribute to HIV-1 inhibition.

**Discussion**

Although, new HIV-1 infections are declining globally [226], new infections are rising in selective populations. In these populations, sexual transmission is the major mode of transmission and semen the main vector [31, 227]. Understanding the contribution of semen to infection is essential for preventative interventions. Importantly, semen contains intrinsic inhibitors to infection. Exosomes purified from human semen of HIV-negative donors significantly reduce HIV-1 replication at multiple steps in the HIV-1 lifecycle [62-64, 139]. However, previous studies were limited to HIV-negative donors, and the role of HIV status or ART on SE function was not addressed. Here, we investigated how proviral (HIV infection) and antiviral (ART treatment) cell conditions affect the function of exosomes from blood and semen. These studies are important to understanding how body-fluid exosomes affect HIV-1 transmission, and expand the HIV-inhibitory understanding of SE needed to identify and harness SE protective antiviral factor(s) for therapeutic development.

We show that donor HIV status does not affect the function of blood and semen exosomes, or exosome-free plasma. BE and EFSP from HIV-negative and HIV-infected ART-naïve donors had no effect on HIV-1 infection while EFBP enhanced infection. Interestingly, SE from HIV-negative and HIV-infected ART-naïve donors inhibited infection. These results indicate that the inhibitory phenotype of SE is conserved regardless of HIV status, suggesting
that the antiviral factor in SE is stable in different exosome-producing cell environments. These data further support the theory that in vivo SE may contribute to low levels of HIV-1 sexual transmission.

Here, we report the novel finding that exosomes isolated from blood and semen of HIV-infected ART-suppressed donors contain ARV drugs. This is the first report to show the association of body-fluid exosomes with ARVs. However, it is currently unknown whether exosome-associated ARVs are surface-associated or enwrapped as luminal cargo. We provide evidence that ARV-containing exosomes and exosome-free plasma from blood and semen protect against HIV-1 infection in vitro. Protection was attributed to the presence of donor-ARV drugs as BE and exosome-free plasma from HIV-infected ART-naïve donors did not inhibit HIV-1. In vitro loading of ARV drugs into HIV-negative BE support this hypothesis as ARV-loaded BE inhibited infection while HIV-negative BE without ARVs did not. Furthermore, inhibition was not due to virus neutralization by IgG antibodies as IgG purified from HIV-infected ART-suppressed fractions did not affect infection. ARV quantification showed BE and SE levels of DTG, FTC, and EFV reach IC₅₀ values for HIV-1 (DTG=0.02-2.14 nM, FTC=0.0013-64 μM, EFV= 1.7-25 nM) [221-223]. SE from all donors while BE from only one donor reach IC₅₀ values for TFV (0.04-8.5 μM) [224]. Taken together, these data provide strong evidence that exosome and plasma associated-ARV drugs from diverse body-fluids may mediate protection from infection.

These results may indicate that ARV drugs are preferentially compartmentalized in diverse biologic systems. We show that while present in both semen and blood, TFV was more abundant in seminal fractions and FTC followed the same general trend. Although at a lower abundance, the active metabolite TFVDP was detected in the exosome-free seminal plasma of
three of four donors, but was below quantitation in the exosome-free blood plasma. Similarly, the active metabolite of FTC, FTCTP, was only detected in the exosome-free blood plasma of one donor, but was detected in the exosome-free seminal plasma of three donors. Further studies are warranted to determine if TFV and FTC preferentially accumulate in donors’ reproductive tract. Conversely, DTG, an integrase inhibitor, and EFV, a non-nucleoside reverse transcriptase inhibitor (NNRTI) were more abundant in blood fractions suggesting that these drugs are not concentrated in the genitourinary system. Perhaps the pharmacodynamics of ARVs mediates drug distribution or biological half-life in different body-fluids.

ARV compartmentalization may occur as EFV (NNRTI) metabolites were found to accumulate in blood plasma over seminal plasma, potentially due to differential protein binding [228, 229]. Differential accumulation of ARVs in semen and blood plasma was previously hypothesized from the observation that the NRTIs zidovudine and lamivudine, not analyzed here, were more accumulated in semen over blood plasma [230]. In addition, ARV drug-induced toxicities can be physiological compartment- and cell type-specific, including in semen [231-234]. Relevant to our findings, the NRTI tenofovir disoproxil fumarate (TDF) showed much higher concentrations in seminal plasma versus blood plasma [235]. Finally, our data are in line with a report that shows NRTIs are highly concentrated in the female genital tract (FGT) whereas NNRTIs are poorly concentrated [236]. This is also supported by the observation that the NNRTI, nevirapine, showed lower to equal accumulation in semen as blood plasma [230]. Although previous findings support our hypothesis, these speculations of modern era ARVs need to be validated by increased sample size with an increased range of ARV drugs.

The role of ARV-associated exosomes in vivo has yet to be determined. Nanoparticle encapsulated ARVs are currently being studied to expand drug half-life and lower drug-induced
cytotoxicity [237]. Perhaps, body-fluid exosome-associated ARVs prolong intracellular drug retention and maintain levels of protection in biological systems distal to the site of ingestion, such as the reproductive tract. In summary, our results validate the presence of a potential intrinsic inhibitor in SE that influences HIV-1 infection that may provide an opportunity for future interventions aimed at prevention of sexual transmission.
<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Isolation Vol. (ml) Blood Plasma</th>
<th>Isolation Vol. (ml) Semen</th>
<th>Viral load (copies/ml)</th>
<th>ARV Therapy</th>
<th>CD4 count (cells/mm$^3$)</th>
<th>Years on ART</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td>1.0</td>
<td>120,000</td>
<td>Therapy-naïve</td>
<td>39</td>
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<td>1.0</td>
<td>1,318,000</td>
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<tr>
<td>5</td>
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<td>1.2</td>
<td>ND</td>
<td>Abacavir, Lamivudine, Efavirenz</td>
<td>1,037</td>
<td>&gt; 5 yrs</td>
</tr>
<tr>
<td>6</td>
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<td>1.2</td>
<td>ND</td>
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<td>545</td>
<td>&gt; 5 yrs</td>
</tr>
<tr>
<td>7</td>
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<td>ND</td>
<td>Tenofovir (TAF), Emtricitabine, Dolutegravir</td>
<td>944</td>
<td>&gt; 5 yrs</td>
</tr>
<tr>
<td>8</td>
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<td>ND</td>
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<td>597</td>
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<td>9</td>
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<td>0.4</td>
<td>ND</td>
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<td>568</td>
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<td>564</td>
<td>&gt; 5 yrs</td>
</tr>
<tr>
<td>11</td>
<td>3.0</td>
<td>0.6</td>
<td>32</td>
<td>Tenofovir (TAF), Emtricitabine, Dolutegravir</td>
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<td>1 yr</td>
</tr>
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<td>12</td>
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<td>Tenofovir (TAF), Emtricitabine, Dolutegravir</td>
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<tr>
<td>13</td>
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<td>Tenofovir (TDF), Emtricitabine, Efavirenz</td>
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<td>14</td>
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<td>Tenofovir (TAF), Emtricitabine, Ritonavir, Darunavir</td>
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<td>Tenofovir (TAF), Emtricitabine, Dolutegravir</td>
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<td>1.0</td>
<td>ND</td>
<td>Tenofovir (TDF), Emtricitabine, Efavirenz</td>
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</tr>
<tr>
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<td>1.0</td>
<td>ND</td>
<td>Abacavir, Lamivudine, Dolutegravir</td>
<td>452</td>
<td>8 mo</td>
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</table>

ND= Non-detectable
N/A= Not applicable

Table 4: HIV-infected donor clinical characteristics.
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<th>Variable</th>
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<td>Demographic characteristics</td>
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<td>Age, years (range)</td>
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<tr>
<td>Weight, kg (range)</td>
<td>67.4 (60.4–76.8)</td>
</tr>
<tr>
<td>BMI (range)</td>
<td>24.1 (21.6–26.9)</td>
</tr>
<tr>
<td>Time on ART, years (range)</td>
<td>&gt;5 (0.17–&gt;5)</td>
</tr>
<tr>
<td>CD4 count, cells/mm³</td>
<td>583 (361–1303)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<td>White</td>
<td>7</td>
</tr>
<tr>
<td>African American</td>
<td>4</td>
</tr>
<tr>
<td>Asian American</td>
<td>2</td>
</tr>
<tr>
<td>American Indian</td>
<td>0</td>
</tr>
<tr>
<td>More than one race</td>
<td>0</td>
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</table>

Table 5: HIV-infected ART-suppressed donor demographics.
Figure 20: SE from HIV-negative and HIV-infected ART-naïve donors inhibit HIV-1. (A and B) Infectivity of HIV-1 where BE, SE, EFBP, and EFSP (100 μg/ml) or vehicle PBS was added simultaneously with 100,000RLU of HIV-1 NL4.3 to TZM-bl cells for 24 h. (C and D) Viability of (A) and (B). (A and C) = HIV negative donors. (B and D)= HIV-infected ART-naïve donors. Vehicle treated cells are set as reference at 100% for infectivity and viability. Statistics was determined by comparing infectivity or viability values from all donors to vehicle control for each treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of biological replicates with triplicate values. ns= not significant.
Figure 21: Presence of ART in HIV-infected ART-suppressed donor body-fluids protect against HIV-1. (A) Infectivity of HIV-1 where HIV-infected ART-suppressed donors’ BE, SE, EFBP, and EFSP (100 μg/ml) or vehicle PBS was added simultaneously with 100,000 RLU of HIV-1 NL4.3 to TZM-bl cells for 24 h. (B) Viability of (A). Vehicle treated cells are set as reference at 100% for infectivity and viability. Statistics was determined by comparing infectivity or viability values from all donors to vehicle control for each treatment. (C) Infectivity of HIV-1 where 100 μg/ml SE from HIV-negative and HIV-infected ART-suppressed donors or vehicle PBS was added simultaneously with 100,000 RLU of HIV-1 NL4.3 to TZM-bl cells for 24 h. Vehicle treated cells are set as reference at 100%. Statistics was determined by comparing infectivity from HIV-negative SE to HIV-infected ART-suppressed SE. (D) 200 μg BE from HIV-negative donors were loaded with 50 μg Cy3 labeled emtricitabine (FTC) by two methods (ExoFect vs. Ultra). Cy3 was detected in the exosome pellet and supernatant to determine loading efficiency. Supernatant Cy3 was set at 100% for each donor and loading method. (E) Infectivity of 100,000RLU HIV-1NL4.3 in the presence of 100 μg/ml FTC-loaded BE from (D) or BE-loading or vehicle PBS controls for 24 h. (F) Viability of (E). Vehicle treated cells are set as reference at 100% for infectivity and viability. Statistics was determined by comparing FTC-loaded BE to relevant loading controls. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of biological replicates with triplicate values. ns= not significant. significant.
Table 6: ARV concentrations in exosome-free plasma and exosomes from HIV-infected ART-suppressed donor blood and semen. TFV=tenofovir; TAF=tenofovir alafenamide; TDF=tenofovir disoproxil fumarate; TFVDP=tenofovir diphosphate; FTC=emtricitabine; FTCTP=emtricitabine triphosphate; DTG=dolutegravir; EFV=efavirenz. BLQ=below quantitation. LIP=lost in processing.
Figure 22: Exosomes from HIV-infected ART-suppressed donors contain ARV drugs. Exosomes from HIV-infected ART-suppressed donors’ blood and semen were isolated as described in Methods. Drug concentrations were measured by LC-MS/MS from 4 donors with similar drug regimen. ARVs are denoted as (A) TFV (tenofovir), (B) FTC (emtricitabine), (C) DTG (dolutegravir), (D) EFV (efavirenz). Solid lines indicate FDA-half maximal inhibitory concentrations (IC_{50}) for HIV-1. (E) Molecular structures of TFV, FTC, DTG, and EFV. Structures provided by the open database PubChem https://pubchem.ncbi.nlm.nih.gov.
Figure 23: IgG does not contribute to HIV inhibition in HIV-infected ART-suppressed donors. (A) A series of immuno-capture techniques purified IgG from HIV-infected ART-suppressed exosome-free plasma and exosomes (n=4). (B) Concentration of purified IgG was measured by IgG-specific ELISA. IgG purification from HIV-infected ART-suppressed unfractionated serum was used as controls. (C) HIV-1 infectivity was measured by incubation of 100,000RLU HIV-1 NL4.3 with purified IgG fractions or controls or vehicle PBS on TZM-bl cells for 24 h. (D) Viability of (C). Vehicle treated cells are set as reference at 100% for infectivity and viability. Statistics was determined by comparing infectivity or viability values from all donors to vehicle control for each IgG fraction. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of biological replicates with triplicate values. ns= not significant.
CHAPTER V: SEMEN EXOSOMES PROMOTE TRANSCRIPTIONAL SILENCING OF HIV-1 BY DISRUPTING NF-KB/TAT CIRCUITRY

Abstract

Exosomes play various roles in host responses to cancer and infective agents, and semen exosomes (SE) inhibit HIV-1 infection and transmission, although the mechanism(s) by which this occurs is unclear. Here, we show that SE block HIV-1 proviral transcription at multiple transcriptional check points including transcription factor recruitment to the LTR, and transcription initiation and elongation. Functional studies show that SE inhibit HIV-1 long terminal repeat (HIV-LTR)-driven viral gene expression and virus replication. Through partitioning of the HIV-1 RNA, we found that SE reduced the optimal expression of various viral RNA species. CHIP-RT-qPCR analysis of infected cells identified human transcription factors NF-kB, as well as RNA Pol II as targets of SE. Of interest, SE inhibited HIV-1 LTR activation mediated by HIV-1 or Tat, but not by the mitogen PMA or TNFα. SE inhibited the DNA binding activity of NF-kB and blocked the recruitment of this transcription factor and Pol II to the HIV LTR promoter. Importantly, SE blocked Tat, NF-kB, and Pol II binding to the LTR, suggesting that SE-mediated inhibition of the functional complex—NF-kB-Pol II-Tat, may be a novel mechanism of proviral transcription repression. These data provide a novel molecular basis for SE-mediated inhibition of HIV-1 and identify Tat as a potential target of SE.

Introduction

The HIV-1 long terminal repeat (LTR) is responsible for transcriptional regulation of proviral DNA. The LTR is divided into four functional regions, namely the; modulatory element, enhancer, promoter, and Tat-activating region (TAR). TAR forms a stable RNA stem-loop that binds and recruits the HIV-encoded transactivator Tat, which activates HIV-1 transcription
through increasing host Pol II processivity via interactions with the cellular target positive transcription elongation factor b (P-TEFb) [238, 239]. Although HIV-1 proviral transcription is initiated by host Pol II, in the absence of Tat, Pol II is unable to transcribe the complete viral template, and only short viral transcripts are generated [240, 241]. Tat transactivates the HIV LTR promoter, and plays a role in HIV-1 reverse transcription [166, 168].

Tat is located in the nucleus and cytoplasm [242]. Nuclear Tat is required for transactivation of the HIV-1 LTR promoter resulting in transcription of high levels of viral mRNA by Pol II [243]. Nuclear Tat binds the HIV-1 TAR [244], promoting transcriptional initiation and elongation through interaction with cellular transacting factors and cofactors, including TFIID [245, 246], C/EBPb [247], cyclin T1/CDK9 [248, 249], E2F-2 [250], histone acetyltransferases p300/CBP and P/CAF [251], Sp1 [252, 253], and NF-kB [254]. The LTR contains key binding sites for cellular transcription factors including Sp1 and NF-kB that are involved in HIV-1 transcription. Through the induction of IKK activity and proteasomal degradation of IkB-a, Tat increases NF-kB p65 transcriptional activity [255]. NF-kB is bound to IkB proteins in the cytoplasm. Following release from IkB, NF-kB translocates to the nucleus where it associates with the HIV-1 LTR [256]. NF-kB interacts with two NF-kB binding sites that cooperatively interact with three proximal Sp1 binding sites to stimulate LTR transcriptional activity [257, 258]. Binding of these factors results in cooperation with TATA binding protein and associated factors to drive transcription. Further, these factors recruit P-TEFb and are involved in chromatin remodeling to regulate transcription [256]. Thus, NF-kB is critical for HIV promoter activity. The p65 subunit is primarily responsible for LTR transactivation, and induction of NF-kB results in NF-kB dependent HIV-1 gene expression. However, binding of NF-kB alone without Sp1 is insufficient to induce HIV-1 gene expression [241, 254, 258-260].
Factors that impair or block Tat-mediated processes reduce provirus transcription and possibly inhibit viral replication. We previously demonstrated that semen exosomes (SE), a subset of extracellular vesicles, inhibit HIV-1 infection of various cell types and inhibit HIV-1 transmission [62-64]. In addition to the protection provided by the female mucosa, SE may contribute to the low risk of HIV-1 infection per heterosexual sexual act (0.04-0.08%) by interfering with HIV replication [261]. SE inhibit HIV-1 infection and transmission in part due to their ability to impair viral reverse transcriptase function and to repress viral RNA gene expression [62-64]. Since reverse transcription and viral gene expression require Tat activity, we hypothesized that SE interfere with Tat-dependent transcriptional events. Furthermore, we hypothesized that Tat protein is targeted by SE.

We examined potential mechanisms by which SE inhibit HIV-1 provirus transcription, and characterized the binding of HIV-1 Tat, cellular transcription factors, and the recruitment of Pol II to the HIV-1 LTR in the presence and absence of SE. Our results indicate that, in addition to damping NF-kB activation, SE inhibit NF-kB binding/recruitment and interaction with HIV-1, impair Pol II recruitment to the HIV-1 promoter, and specifically block Tat protein-mediated LTR activation. These findings provide new insights into how SE control HIV-1 transcription and provirus replication. The findings also reveal a novel function for exosomes: interaction with host transcription factors.

**Materials and methods**

**Ethical approvals:** The University of Iowa Institutional Review Board (IRB) approved the use of human specimens. HIV-1 -negative subjects consented to participate in this study via written informed consent. All samples were received unlinked to any identifiers. All experiments were performed in accordance with the approved University guidelines and regulations.
**Purification of exosomes from human semen:** Semen samples received from the University of Iowa In Vitro Fertilization and Reproductive Testing laboratories were collected and stored at -80°C until isolation. All donors have no history of human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infection. Semen exosomes were isolated using previously described protocol [62-64, 111] where prior to isolation, 12 individual semen samples were thawed at room temperature, and centrifuged (10,000 x g, 30 min, 4°C) to pellet cellular debris. After which the supernatant, or seminal plasma, from individual donors was pooled and placed in a new tube and mixed by inversion with ExoQuick reagent (SBI) at a ratio of 4:1 (seminal plasma: ExoQuick), and incubated 4 °C overnight per manufacturer’s instructions. To pellet the exosomes, the mixture was centrifuged at 1500 x g for 30 min at 4 °C and the ExoQuick/exosome-free supernatant was removed. Residual ExoQuick was removed by centrifuging the exosome pellet at 1500 x g for 5 min and discarding the supernatant. The exosome pellet was re-suspended in PBS in 1/10 of the original volume of semen and is hereafter referred to as SE. 100 μg/ml SE were used for all cell treatments. The lack of effect by residual ExoQuick reagent on infection results was verified via ExoQuick extraction of PBS (vehicle) (Figure 28J).

**Cells and plasmid:** TZM-bl, SUPT1, HIV-1 LAV infected Jurkat E6 (J1.1), and Jurkat derived J-Lat Full Length (JLat 10.6) that contain a latent HIV provirus where Nef is replaced with GFP cells were obtained through the NIH AIDS Reagent Program. 293T cells were purchased from ATCC. TZM-bl and 293T were maintained in DMEM (Gibco-BRL/Life Technologies) and SUPT1, JLat 10.6 and Jurkat E6 (J1.1) cells in RPMI (Gibco-BRL/Life Technologies), containing 5% exosome-depleted FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate and 0.3 mg/ml L-glutamine (Invitrogen, Molecular Probes) as previously
described [63]. pNL4.3 plasmid was obtained from the NIH AIDS Reagent Program and pCMV Tat plasmid (p-Tat) was a kind gift from Dr. Francesca Di Nunzio (Institut Pasteur, France).

**HIV-1 Production:** HIV-1 was produced either by purification of supernatants from HIV-1 latently infected Jurkat or by 293T transfection with pNL4-3. Briefly, cell culture supernatant of J1.1 cells was clarified by centrifugation at 2000 x g for 10 min, and virus was concentrated by ultracentrifugation at 100,000 x g for 2 hours at 4 °C and resuspended in RPMI. 293T cells were seeded in 6-well tissue-culture treated plate with 2 ml antibiotic-free media 24 hour before transfection. 10 µl of Lipofectamine 2000 (Invitrogen) was combined with 150 µl Opti-MEM for 5 min at room temp. 4 µg/well pNL4.3 plasmid was diluted in 150 µl Opti-MEM. DNA and lipofectamine mix was combined for 20 min at room temperature and 250 µl of DNA-lipofectamine complex were added to cells. 24 hours post-transfection, input plasmid was removed by replacing transfection media with antibiotic-free DMEM. 48-72 hours post-transfection, supernatants were collected and clarified of cell debris by centrifugation at 2000 x g for 10 min before determination of viral titer. Viral titer was determined by EnzChek Reverse Transcriptase Assay (Life Technologies) [63].

**LTR promoter activation assay:** Vehicle (PBS), HIV-1 NL4.3 virus (8 reverse transcriptase units [RTU]/ml), 100 µg/ml SE, or 10 ng/ml PMA (Sigma-Aldrich) was added to TZM-bl indicator cells. Where indicated, treatments were added to cells for 16 or 24 hours before removal, washing, and treatment or culturing for an additional 24 hours after which cells were lysed and measured for luciferase reporter activity by Steady-Glo (Promega). Vehicle (PBS), 100 µg/ml SE, 10 ng/ml PMA (Sigma-Aldrich), or 10 ng/ml TNFα (Sigma-Aldrich) was added to JLat 10.6 indicator cells. Where indicated, treatments were added to cells for 24 hours before
removal, washing, and treatment or culturing for an additional 24 hours after which cells were measured for GFP activity by flow cytometry analysis and lysed for RNA evaluation.

**Western blot analysis:** Whole cell lysates or cells fractionated into cytoplasmic and nuclear compartments via NE-PER nuclear and cytoplasmic extraction reagents per the manufacturer’s protocol (ThermoFisher/Life Technologies) were probed with the following primary antibodies: anti-GAPDH, anti-NF-kB p65, anti-Sp1, and anti-PCNA. The appropriate secondary IRDye antibodies were used for imaging by Odyssey Infrared Imaging (LI-COR Biosciences) [262-264]. Image J was used to quantify blots.

**ChIP assay:** SUPT1 cells were treated with vehicle or infected with HIV in the presence and absence of SE for 24 hours. For NF-kB p65: SupT1 cells were treated with vehicle or infected with HIV-1 in the presence and absence of SE (100 µg/ml) for 24 hours. ChIP assay was completed using Pierce Chip Assay kit (ThermoFisher/Life Technologies). Immunoprecipitations were completed with lysates from 1x10^6 cells per reaction with 2 µg mock anti-IgG antibody, 5 µg anti-Pol II antibody (ThermoFisher/Life Technologies), or 5 µg anti-NF-kB p65 (ThermoFisher/Life Technologies). Extracted DNA was subjected to PCR with SYBR Green master mix using LTR and GAPDH primers (Table 7). The products were resolved on 3% agarose gel. Data were normalized via fold enrichment quantification [Step 1: (CT IP)-(CT mock) Step 2: (2^-∆∆CT)] (ThermoFisher/Life Technologies) where IgG serves as mock CT and was subtracted from each IP CT value.

**p24 quantification:** Progeny virion p24 released from infected cells was quantified by p24 ELISA (XpressBio) per manufacturer’s instructions. Intracellular p24 was evaluated by flow cytometry analysis via cell permeabilization and addition of primary antibody p24 (4121 NIH
AIDS Reagent Repository) before incubation with secondary antibody Alexa Fluor-488 (Jackson ImmunoResearch).

**RNA evaluation:** Cells were fractionated into cytoplasmic and nuclear compartments via NE-PER nuclear and cytoplasmic extraction reagents per the manufacturer’s protocol (ThermoFisher/Life Technologies). RNA was extracted from cells or nuclear and cytoplasmic fractions using RNeasy kit per manufacturer’s instructions (Qiagen). RNA was subjected to treatment with DNase (Qiagen) as previously described [63]. Viral RNA was extracted from virions using viral RNA isolation kit (Zymogen). RNA integrity was determined by Agilent Bioanalyzer. RNA concentration was determined by NanoDrop RNA absorbance, and equivalent concentration of RNA (1 ng/μl) was used for cDNA synthesis (ABI). Gene specific primers were used to amplify GAPDH as previously described [63] and Pre-GAPDH as well as HIV-1 LTR, Gag-Pol, U5-Gag, Vif, Vpr, Tat-Rev, NF-kB p65, and Sp1 (Table 7). Gag-pol specific primers were used for gRNA quantification (Table 7). qRT-PCR were analyzed by relative quantification for all transcripts where each transcript was first normalized to corresponding GAPDH value, and second, where indicated, either HIV-infected vehicle treated cells or uninfected vehicle treated cells set as reference value at 1. PCR amplicons were visualized where indicated by 2% agarose gel and ethidium bromide staining. PCR analysis was completed using 7500 Fast qRT-PCR (ABI) and droplet digital PCR (ddPCR) (Bio-Rad). For ddPCR, RNA extraction and cDNA synthesis were completed as described above. The ddPCR reaction mix was prepared using 100 ng cDNA, QX200 EvaGreen Supermix (Bio-rad), and primers described above according to the manufacturers protocol. Bio-rad QX200 droplet generator formed each PCR reaction into droplets. The PCR reaction was subsequently carried out by C1000 Touch thermal cycler (Bio-rad) with cycling conditions as suggested by the manufacturer. The PCR reaction was quantified
using Bio-rad QX200 droplet reader. Data were analyzed with QuantaSoft software (Bio-rad) where reactions with less than 10,000 droplets were excluded. For qRT-PCR and ddPCR, fold change values were used for statistical analysis.

**SE inhibition of HIV-1:** 100 μg/ml SE or equivalent volume of vehicle (PBS) were pre-incubated with 8RT units/ml HIV-1 at 37°C for 1 hour prior to addition to target cells. The effect of SE on HIV-1 were determined 24 hours later. Progeny virion assays were completed by infection in the presence and absence of SE for 4 hours before removal, washing, and subsequent culture in media without treatments until 24 hours post-infection. Progeny virion released from infected cells was determined by incubation with naïve TZM-bl cells for 24 hours before measurement of infectivity by Steady-Glo (Promega) luminescence.

**SE inhibition of Tat:** TZM-bl cells were transfected with p-Tat plasmid using EndoFectin™ Max Transfection Reagent (GeneCopoeia™) at a ratio of 2:1 (μl EndoFectin to μg plasmid) in reduced serum media (Opti-MEM™). 3 hours post-transfection, the medium was replaced with complete DMEM containing 100 μg/ml SE or equivalent volume of vehicle (PBS). For recombinant Tat, the protein (10 μg/ml) was pre-incubated with 100 μg/ml SE or equivalent volume of vehicle (PBS) at 37°C for 1 hour prior to addition to target cells. The effects of SE on Tat were determined 16 hours later or at the indicated times by measuring promoter activity using the Steady-Glo® (Promega) luminescence assay or the β-galactosidase Assay Reagent (Pierce, ThermoFisher).

**Cell viability:** Viability was determined by MTT assay as previously described [195].

**Statistics:** Two-tailed, paired, student’s t test p-value (GraphPad Prism) calculations determined statistical significance where P<.05=*, P<.01=**, P<.001=***, ns=not significant. Error bars represent standard error of the mean (SEM) across independent experiments unless denoted.
Results

Three classes of HIV-1 transcripts are susceptible to inhibition by SE

The effect of SE on cell-associated HIV RNA expression was determined by infecting SUPT1 cells in the presence and absence of SE prior to RNA extraction, DNase treatment, reverse transcription, and real-time quantitative (RT-qPCR) with HIV gene-specific primers (Figure 24A, Table 7). SE inhibited the accumulation of multiply spliced—Tat-Rev (Figure 24B), singly spliced—Vif, Vpr (Figures 24C-D), and unspliced U5-Gag, Gag-Pol (Figures 24E-F) HIV-1 RNAs. The extent of SE-mediated inhibition observed in SUPT1 cells was similar in a different cell line—TZM-bl cells (Figures 24G-K), indicating that the inhibition is cell type independent. Since the presence of SE produced drastic changes in steady state level of the different RNA species, we examined the levels of particle-associated RNA. Using a similar technique for assaying cell associated RNA expression, we found that SE inhibit particle-associated viral RNA (Figure 24L). Copy number analysis of particle-associated viral RNA confirmed reduction in RNA levels in the presence of SE (Figure 24M). Given the reductions in all classes of viral RNA by SE, we evaluated the effect of SE on the levels of viral protein. Analysis of intracellular p24 protein revealed no significant alteration (Figures 24N-O). Similarly, progeny p24 levels were unchanged in the presence of SE (Figure 24P). Previously, a similar disconnect between RNA expression and HIV-1 protein levels was described in which pr55\(^{\text{Gag}}\) (precursor to p24) protein synthesis can be rescued in the presence of HIV-1 RNA defects, and de novo RNA synthesis is dispensable for synthesis and processing of HIV-1 Gag [265, 266]. Normalizing for p24 protein, viral particles produced by cells incubated in SE contained reduced RNA (Figure 24L-M-P). Infectivity of these viral particles was significantly reduced (Figure 24Q), suggesting that nascent HIV-1 particles produced in the presence of SE are less infectious. Thus alteration in RNA levels may be a contributing mechanism for SE-
related inhibition of HIV-1 infection, and changes in HIV-1 RNA levels do not have to be accompanied by reduced protein levels.

SE do not alter the intracellular distribution of viral transcripts

The intracellular distribution of HIV-1 RNA in infected cells was examined using cellular fractionation (Figure 25A). Efficiency of cell fractionation was verified by measuring cytoplasmic GAPDH and nuclear PCNA (Figure 25B). Quantification of total viral genomic RNA (gRNA), as well as cytoplasmic and nuclear gRNA revealed a significant but similar decrease in viral gRNA in the different cellular compartments (Figure 25C) that could not be attributed to reduced cell viability (Figure 25D). RNA encapsidation efficiency was evaluated as the ratio of progeny virion-associated and cytoplasmic gRNA levels [267]. Incubation of cells with SE did not reduce the relative amount of packaged viral gRNA (Figure 25E). There was no preferential localization of viral RNA in either of the compartments; indicating that SE-mediated reduction in total viral RNA was not due to impairment in nucleocytoplasmic shuttling or in the encapsidation machinery.

SE have minimal off-target effects on human RNA

The potential for SE to affect the integrity of host cell total RNA and/or the expression of host mRNAs was evaluated in cells infected in the presence and absence of SE. Although SE decreased viral gRNA in SUPT1 cells (Figure 26A), SE did not affect the level of host GAPDH mRNA (Figure 26B). SE also did not affect host GAPDH mRNA levels in TZM-bl cells (Figure 26B). In order to determine if SE induced more subtle effects on host RNA, cellular genes that have more variable expression during HIV-1 infection than GAPDH were analyzed in the presence or absence of SE with and without HIV-1 infection [268]. SE did not significantly alter mRNA levels of the transcriptional regulators ELK1 and MAZ in infected and uninfected
SUPT1 and TZM-bl cells (Figures 26C-D). SUPT1 RNA integrity was assessed using the Agilent bioanalyzer. All cells analyzed (uninfected, Vehicle [HIV-1 alone], SE [HIVSE]) contained similar RNA concentrations (ranging from an average of 88 ng/µl to 127 ng/µl) and RNA integrity (ranging from an average of 5.97 to 6.23) (Figures 26E-F). Similar results were observed when we examined the levels of 28S and 18S rRNA using non-denaturing agarose gel electrophoresis in TZM-bl cells (Figure 26G). The ratios of host 28S and 18S were 2.7, 2.5 and 2.4 for uninfected, vehicle (HIV alone), and SE (HIVSE) respectively (Figure 26H), indicating that SE-mediated down-regulation of HIV-1 mRNA expression is not due to reduction in host RNA integrity. The results also suggest that SE can inhibit HIV-1 expression with minimal regulatory effects on human mRNA production, in general, but it is possible that SE may affect specific host genes that are yet to be discovered.

**SE reduces HIV-1-dependent but not mitogen-dependent LTR activity**

Results presented above suggest that SE inhibit viral RNA expression without altering production of viral RNA species (Figure 24), reducing nucleocytoplastic shuttling (Figure 25), or diminishing RNA integrity (Figure 26). Since HIV-1 LTR promoter activity is involved in viral transcription, we performed LTR-luciferase assays in cells that contain integrated HIV-1 LTR fused to beta-galactosidase (β-gal) and luciferase as reporters (TZM-bl cells) [269]. Cells were exposed to HIV, SE, vehicle (PBS, negative control) or PMA (positive control) using SE post-treatment or pre-treatment as previously described. Briefly, SE were added to cells 24 hours before HIV or PMA (pre-treatment) or SE were added to cells 24 hours after HIV or PMA (post-treatment)[64]. As expected, both HIV and PMA increased HIV-1 promoter activity in TZM-bl cells (increased relative light units) (Figures 27A-B). However, SE specifically lowered HIV-1-driven luciferase expression without decreasing PMA-induced luciferase expression
(Figures 27A-B, compare open bars in unshaded and shaded areas); regardless of the concentration and timing of PMA addition (Figures 27C-D). The inability of SE to regulate PMA-driven promoter activity was confirmed in JLat 10.6 cells, which harbor an HIV provirus containing the Green Fluorescent Protein (GFP) ORF in place of nef and a frameshift mutation in env [270], and thus GFP expression is under the control of the HIV LTR. In addition, TNFα was included as a more physiologically relevant positive control. Similar to the LTR-luciferase results, SE did not decrease PMA or TNFα-driven GFP expression in JLat 10.6 cells, regardless of the time of SE addition (Figures 27E-H, compare solid-line versus broken-line histograms and filled versus open bars). The difference in PMA/TNFα-driven GFP expression in JLat 10.6 cells between pre- and post-PMA/TNFα conditions reflects the timing of addition and removal of stimulus prior to determination of GFP expression. Cells are actively stimulated in the pre-PMA/TNFα conditions (Figures 27E and 27G), while cells have reverted to a latent state in the post-PMA/TNFα condition (Figures 27F and 27H). It is noteworthy that SE did not alter cell viability in the presence or absence of HIV-1, PMA, or TNFα pre or post treatment (Figures 27I-L). Together, these results suggest that SE specifically inhibit HIV-1-driven LTR promoter activation.

**SE do not alter basal activity of the HIV-1 promoter**

Given that SE inhibit HIV-1-driven LTR promoter activation, we examined the effect of SE on the basal activity of the HIV-1 promoter in the absence of HIV or other LTR activating agents (PMA or TNFα). TZM-bl cells were treated with 100 µg of SE alone or with HIV or PMA as controls, and examined for luciferase expression 24 h later. While HIV and PMA increased LTR activity in TZM-bl cells, basal activity of the HIV-1 promoter was not altered by the addition of SE (Figure 28A). This was recapitulated using JLat 10.6 cells. As expected, PMA
and TNFα significantly activated the LTR promoter as measured by increased GFP expression (Figure 28B, see arrows). The increase in PMA and TNFα mediated LTR promoter activation was ~25% and ~48%, respectively (Figure 28C). In the JLat system, increased promoter activity (GFP expression) results in transcription of viral RNA as observed in the PMA and TNFα-treated cells (Figure 28D). In contrast, SE had no effect on GFP expression (Figures 28B-C) and did not induce viral transcription relative to the vehicle control (Figure 28D). To further assess the effect of SE on basal promoter activity, TZM-bl cells treated with increasing concentrations of SE for 24 h did not demonstrate altered promoter activity as measured by luciferase levels (Figure 28E). Further, addition of SE to JLat 10.6 cells did not change basal GFP expression or cell viability (Figures 28F-I). Thus, SE specifically repressed viral-driven LTR activation and subsequent viral transcription.

**SE inhibit binding and recruitment of host transcription factor components (NF-kB) to the HIV-1 promoter in a cell-based assay**

The ability of SE to inhibit HIV transcription provides an experimental system in which transcriptional targets that repress HIV-1 provirus transcription can be identified. Initially, we explored whether SE affected the levels of transcription factors Sp1 and NF-kB in infected cells. SUPT1 cells infected with HIV-1 in the presence and absence of SE were examined via RT-qPCR and Western blot. SE had no effect on Sp1 and NF-kB p65 mRNA in HIV-1 infected cells (Figure 29A-B). Similarly, Sp1 protein levels were unchanged in the presence and absence of SE (Figures 29C-D). However, SE significantly decreased NF-kB p65 protein in HIV-1 infected SUPT1 cells (Figures 29C, E). A similar trend was observed in HIV-1 infected TZM-bl cells, where SE did not reduce Sp1 and NF-kB p65 mRNA levels (Figures 29F-G), but reduced the level of NF-kB protein (Figures 29H, J) without altering Sp1 protein levels (Figure 29H, I).
Further, SE reduced cytoplasmic levels of NF-kB p65 in HIV-1 infected SUPT1 cells (Figures 29K, M) and TZM-bl cells (Figures 29L, M); although nuclear NF-kB p65 levels were more variable (Figures 29K-M).

Direct binding of NF-kB p65 to the HIV-1 enhancer in the presence of SE was analyzed by ChIP-RT-qPCR. Chromatin was prepared from HIV-infected SUPT1 cells in the presence and absence of SE. Because we did not select cells for HIV expression, these experiments utilized a heterogeneous population of HIV-1 infected cells with a spectrum of viral gene expression. ChIP was performed using antibodies against NF-kB p65, followed by RT-qPCR for HIV-1 LTR and host GAPDH. NF-kB p65 bound to the LTR in the absence of SE (Figure 29N). However, in the presence of SE, the association of NF-kB p65 with the LTR was reduced by 43%, suggesting that SE may inhibit HIV-1 transcription in part through decreasing NF-kB p65 recruitment and binding to the HIV-1 LTR. We previously showed that cell-associated HIV-1 DNA is reduced in the presence of SE, thus these data may indicate that there is less LTR available for NF-kB binding [63]. Because there is less NF-kB protein in HIV-1 infected cells in the presence of SE (Figure 29A-M), it is also plausible that there is a lower abundance of NF-kB protein available for binding.

**Binding of RNA polymerase II (Pol II) to the HIV LTR is impaired by SE**

Because SE-mediated inhibition of NF-kB recruitment to the viral LTR was incomplete, we explored additional mechanisms by which SE might repress transcription. Productive HIV-1 transcription requires recruitment of the Pol II complex to the promoter region of the HIV-1-LTR. To explore the possibility that SE decrease Pol II activity, we assessed the presence of Pol II at the HIV-1 LTR by ChIP-RT-qPCR. While Pol II is efficiently recruited to the viral LTR in HIV-1 infected SUPT1, SE decreased the amount of Pol II associated with the LTR by 58%
(Figure 30A). Reduced Pol II was associated with decreased viral mRNA (Figure 30B). Since Pol II association with HIV-1 sequences was inhibited by SE, it is plausible that SE may inhibit HIV-1 transcription by suppressing Pol II processivity. In addition, because we previously showed less intracellular HIV-1 DNA in the presence of SE, it is also possible that there is less LTR available for binding by Pol II leading to less HIV-1 mRNA production [63].

**SE inhibits Tat-dependent HIV-1 LTR activity**

It is known that Tat participates in a positive feedback mechanism that maintains high levels of proviral transcription in HIV-1 infected cells. To examine the effect of SE on Tat-dependent HIV-1 activation, TZM-bl cells were transfected with a Tat expression vector (pCMV Tat, hereafter referred to as p-Tat) [271] followed by treatment with vehicle (PBS) or SE (100 µg/ml). SE inhibited Tat-dependent transcription by ~54%, in the luciferase assay (Figure 31A) and by ~55% in the β-gal assay (Figure 31B). SE reduced the effect of exogenous Tat protein (r-Tat) by 66%, confirming the effect of SE on Tat-driven HIV-1 LTR activation (Figure 31C). The kinetics of SE-mediated inhibition of HIV-1 promoter activity was evaluated by time-lapse analysis of HIV-1 LTR activity in cells infected with HIV-1 (Figure 31D), transfected with p-Tat (Figure 31E), or treated with r-Tat (Figure 31F). In all three scenarios, SE inhibited HIV-1 LTR activity and at 16 h, HIV-1, p-Tat, and r-Tat-driven LTR activities were inhibited by ~46%, ~71%, and ~64% (Figures 31D-F) respectively. The effect of SE on LTR activity was still observed (~58% inhibition) 24 h after exposure to HIV-1 (Figure 31D), p-Tat at ~50% (Figure 31E), and r-Tat at ~47% (Figure 31F). The activity of SE on HIV-1 and Tat were independent of cell viability as measured by MTT assay (Figures 31D-F, bottom bars). These time lapse experiments indicate that the inhibitory effect of SE occurred early and may persist up to 24 h.
The use of p-Tat that enables steady synthesis of Tat revealed that SE are capable of inhibiting LTR activation of de novo Tat (Figure 31E).

**Discussion**

The HIV-1 provirus transcribes its genes in actively infected cells, but proviral transcription is halted and suppressed in latent infection. It is known that HIV-1 transcription is regulated by disparate positive and negative regulators whose activities control the viral replication in host cells [240]. Since HIV-1 transcription is regulated by the relative levels of positive and negative regulators, any factor capable of inhibiting positive regulators of transcriotion may lead to the suppression of proviral transcription.

Possible links between SE and HIV-1 transcription have previously been inferred. In 2014 and 2015, Madison et al, [62, 63] observed that among other functions, SE induced a decrease in HIV-1 RNA expression, though it was not determined if SE per se repressed viral transcription. Here we provide evidence that SE repress viral transcription by blocking human and viral transcription factor access to the HIV-1 promoter. We hypothesize that this is accomplished through either steric hindrance or epigenetic modifications. Transcription of the HIV-1 provirus is regulated at different levels including recruitment of cellular transcription factors, chromatin organization, transcription initiation, and transcription elongation. Our data strongly suggest that SE potently induce a transcription-repressed state in the HIV-1 promoter leading to inefficient viral RNA expression. The exact effect of SE on the viral promoter is still unknown, but given that SE impair NF-kB and Pol II recruitment to the HIV-1 promoter, it is evident that SE negatively regulate HIV-1 transcription.

SE reduced interactions between cellular and viral transcription factors and the HIV-1 promoter. These observations suggest that SE may be involved in HIV-1 transcription at the
level of initiation. Previous studies linked NF-κB to the initiation of HIV-1 transcription [272]. NF-κB is required for HIV-1 transcription initiation and chromatin remodeling and plays both positive and negative roles in HIV-1 transcription and latency. Induction of NF-κB p65 and recruitment of other host factors, such as histone acetyltransferases are central for reversing proviral transcription in latency [273]. We observed that SE modestly reduced NF-κB p65 protein levels but significantly inhibited NF-κB p65 binding to the HIV-1 LTR.

Using SE to repress HIV-1 transcription allowed us to identify transcription elongation as another target of SE in provirus transcription. To our knowledge, this is the first report to suggest that Pol II recruitment/binding is targeted by exosomes. In the presence of SE, ChIP analysis demonstrated reduced Pol II enrichment at the HIV-LTR. In the same cells, we observed decreased HIV-1 RNA, suggesting an association between inhibition of Pol II recruitment and reduced RNA expression.

Following transcription initiation, the maintenance of highly efficient viral gene expression and viral replication is directed by Tat [241]. Although a viral protein, Tat associates with Sp1, NF-κB, positive transcription elongation factor (P-TEFb), and the trans-activator-responsive element TAR at the HIV-1 LTR [162, 163]. SE blocked Tat-LTR interactions. Thus, in addition to reducing transcription initiation, SE also serve as a negative regulator of HIV-1 transcription elongation via inhibition of Tat. In our system, SE reduced Tat-induced Luc and β-gal gene expression under HIV-1 LTR control. In addition to the role of Tat as the master regulator of HIV-1 gene expression and latency, Tat regulates Pol II-mediated activation of HIV-1 transcription, controls alternative mechanisms of viral replication by regulating chromatin remodeling [164], mRNA splicing [165], Rev function [274], microRNA biogenesis [275], and reverse transcriptase (RT) function [166-170]. Interestingly, we previously found that SE
regulate the ratio of HIV-1 RT subunits [63]. Whether or not the effect of SE on RT and Tat utilize distinct mechanisms of inhibition is yet to be determined. However, it is clear that two important HIV-1 proteins, RT and Tat, are susceptible to the anti-viral effects of SE, and there may be multiple SE-driven mechanisms that negatively regulate HIV-1 infection and replication.

Because we are yet to identify the factor(s) contributing to our observations, we are cautious to conclusively link these effects solely to exosomes, and recognize that these effects may be due to a host factor that is associated with exosomes during purification. However, we believe this is unlikely given that purification of semen exosomes by different methods has resulted in the same phenotype [63, 111]. Characterization of the protein and nucleic acid content of SE is needed to identify the component(s) mediating the inhibitory activity on the HIV-1 LTR.

Our study extends understanding of the role of exosomes in microbial pathogenesis. In the context of HIV-1 infection, the source of exosomes dictates their role in HIV-1 infection [28]. While exosomes produced from some HIV-1 infected cell lines may enhance viral infection, exosomes from body fluids, including breast milk, vaginal lavage, and semen inhibit HIV-1 infection [62, 63, 104, 105]. These diverse functions highlight the complexity associated with exosomes and their functions. Because of the significant role of exosomes in intercellular communication, the therapeutic potential of exosomes continues to be increasingly investigated and developed. Novel therapies against HIV-1 infection are needed to combat drug resistance adoptions by the virus and interaction with illicit substances. Exosomes with anti-viral function offer the unique opportunity to harness a naturally-evolved resistance to HIV-1 infection that has thus far not been overcome during HIV-1 evolution.

On the basis of our findings, we suggest that SE may fit this critical need. SE contain known antiviral factors such as the APOBEC family, preferentially inhibit HIV-1 infection, and
alter the balance of HIV-1 reverse transcriptase subunits [63]. Here, we show that SE interfere with the critical complex Tat-NF-kB-Pol II circuitry resulting in impaired proviral transcription and replication. Thus, the translational implication of our findings is the potential use of SE or its cargo to inhibit Tat function. The HIV-1 Tat protein is expressed early; therefore, its inhibition will result in early termination of viral replication. Although secreted, Tat inhibition may result in little or no cytotoxicity since it has no cellular homologs. Blockade of Tat is expected to prevent Tat-mediated feedback loops that enhance viral transcription and the production of viral progeny, and blocking viral reactivation to promote prolonged silencing of latent viruses. Indeed, the ability of the antiviral factor within SE to inhibit the activities of two different classes of transcription factors (host and viral) involved in HIV-1 gene expression explains why SE is such a powerful negative regulator of HIV-1 replication.
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Table 7: Primer sequences.
Figure 24: Effects of SE on viral RNA species produced within HIV-1-infected cells. Vehicle PBS or semen exosomes (100 μg/ml) were incubated with HIV-1 NL4.3 virus (8 RTU/ml) at 37°C for 1 hour before infection. qRT-PCR analysis of HIV-1 RNA species’ transcript levels in cells 24 hours after SE or vehicle treatment and HIV-1 infection. A) Schematic of HIV-1 genome. Small arrows represent primer pairs used for qRT-PCR analysis. US, unspliced; SS, singly spliced; and MS, multiply spliced. B-F) HIV-1 RNA from SUPT1 cells representing Multiply spliced Tat-Rev mRNA, singly spliced Vif and Vpr mRNA, and unspliced U5-Gag and Gag-Pol mRNA expression, respectively. qRT-PCR analysis of HIV-1 RNA species’ transcript levels in TZM-bl cells 24 hours after SE or vehicle treatment and HIV-1 infection. G-K) HIV-1 RNA from TZM-bl cells representing multiply spliced Tat-Rev mRNA, singly spliced Vif and Vpr mRNA, and unspliced U5-Gag and Gag-Pol mRNA expression, respectively. L) Progeny viral RNA was extracted from infected SUPT1 supernatants and qRT-PCR analysis was used to analyze Gag-pol mRNA expression. M) Droplet digital PCR analysis was used to measure progeny Gag-pol RNA copy numbers. Copy numbers were normalized to GAPDH. Vehicle was set as reference at 1. N) Cell-associated Gag protein was measured 24 hours after infection by cell permeabilization and flow cytometry analysis of anti-p24 or isotype control staining. Shown is one representative experiment. O) Median fluorescence intensity (MFI) was quantified from flow cytometry analysis of intracellular p24. Vehicle was set as reference at 1. P) Progeny virus produced in the presence and absence of SE were analyzed for p24 content by ELISA. Q) Naive TZM-bl indicator cells were infected with supernatants from infected SUPT1 cells for 24 hours where infectivity was measured by reporter expression and presented as Relative light units (RLU). Vehicle was set as reference at 100%. PCR analysis was normalized to GAPDH and vehicle was set as reference at 1 for all. Significance determined by student’s t test where P<.05 (*), P<.01 (**), P<.001 (***) Error bars are standard error of the mean of triplicate experiments. ns = not significant.
Figure 25: Semen exosome-mediated reduction in HIV RNA expression is operative in host cytoplasmic and nuclear subcellular compartments. Vehicle PBS or semen exosomes (100 μg/ml) were incubated with HIV-1 NL4.3 virus (8 RTU/ml) at 37°C for 1 hour before infection of SUPT1 cells. A-B) Total cellular RNA and RNA from cells fractionated into cytoplasmic and nuclear compartments 24 hours post-infection. Fractionation was evaluated by western blot analysis of cytoplasmic GAPDH and nuclear PCNA. Shown is one representative experiment. C) Total, nuclear, and cytoplasmic RNA were analyzed by qRT-PCR for viral Gag-pol mRNA expression. GAPDH and Pre-GAPDH were used for normalization of total/cytoplasmic and nucleus RNA, respectively. Vehicle was set as reference for RNA analysis from each cellular compartment. D) Cell viability was measured by MTT assay. Vehicle was used for normalization and set at 100%. E) Cellular cytoplasmic RNA and progeny viral RNA were analyzed by qRT-PCR for Gag-pol expression. GAPDH was used for normalization. Vehicle was set as reference. Encapsidation efficiency was mathematically determined as the ratio of progeny and cytoplasmic Gag-pol expression. Vehicle was used for normalization and set at 100%. Significance was taken at P<0.05 (*). Error bars are standard error of the mean of triplicate experiments. ns = not significant.
Figure 26: Semen exosomes do not alter the integrity of host RNA. Vehicle PBS or semen exosomes (100 μg/ml) were incubated with HIV-1 NL4.3 virus (8 RTU/ml) at 37°C for 1 hour before infection. Analysis of cellular and HIV-1 RNA in cells 24 hours after SE or vehicle treatment and HIV-1 infection. A) Cellular viral RNA in SUPT1 cells was analyzed by qRT-PCR for Gag-pol mRNA expression. PCR products were visualized with agarose gel ethidium bromide staining. PCR analysis was normalized to GAPDH and vehicle was set as reference at 1. B) qRT-PCR analysis of SUPT1 and TZM-bl GAPDH mRNA levels. C) qRT-PCR analysis of SUPT1 and TZM-bl ELK1 mRNA expression. PCR analysis was normalized to GAPDH and uninfected cells were set as reference at 1. D) qRT-PCR analysis of SUPT1 and TZM-bl MAZ mRNA expression. PCR analysis was normalized to GAPDH and uninfected cells were set as reference at 1. E) Agilent Bioanalyzer analysis of SUPT1 cellular RNA in the presence and absence of SE. Shown is one representative profile out of three. F) SUPT1 cellular RNA concentration and RNA integrity number of Agilent Bioanalyzer analysis from three independent experiments. G) TZM-bl cellular RNA integrity were visualized with agarose gel. Shown is one representative gel profile out of three. H) TZM-bl cellular RNA 28S/18S ratios from three independent gel profiles quantified by ImageJ. Significance was taken at P<0.01 (**). Error bars are standard error of the mean of triplicate experiments. Values in E) are presented as an average of triplicate experiments +/- standard error of the mean. ns = not significant.
Figure 27: HIV-driven LTR promoter transactivation is significantly down-regulated by SE. A) Vehicle or 100 μg/ml SE were added to TZM-bl cells for 24 hours then washed before infection with HIV-1 NL4.3 virus (8 RTU/ml) or treatment with 10 ng/ml PMA (shaded) for an additional 24 hours (i.e. cells were pre-treated with vehicle or SE). B) TZM-bl cells were infected with HIV-1 NL4.3 virus (8 RTU/ml) or treated with 10 ng/ml PMA (shaded) for 24 hours then washed before treatment with vehicle or 100 μg/ml SE for an additional 24 hours (i.e. cells were post-treated with vehicle or SE). Naïve TZM-bl indicator cells were treated with vehicle PBS or 100 μg/ml SE C) pre- or D) post- treatment with increasing concentrations of PMA. Each PMA concentration was normalized to vehicle treatment where vehicle was set at 100%. RLU was assessed via SteadyGlo. E) JLat 10.6 cells were treated with vehicle or 100 μg/ml SE then washed at 24 hours followed by addition of 10 ng/ml PMA for 24 hours. GFP expression was analyzed by flow cytometry analysis. Shown is one representative experiment. F) JLat 10.6 cells were treated with 10 ng/ml PMA then washed at 24 hours followed by addition of vehicle or 100 μg/ml SE for 24 hours. GFP expression was analyzed by flow cytometry. Shown is one representative experiment. E) and F) Flow cytometry analysis of GFP expression of three independent experiments (bars). G) JLat 10.6 cells were treated as in E) except PMA was replaced with 10 ng/ml TNFα. GFP expression was analyzed by flow cytometry analysis. Shown is one representative experiment. H) JLat 10.6 cells were treated as in F) except PMA was replaced with 10 ng/ml TNFα. GFP expression was analyzed by flow cytometry analysis. Shown is one representative experiment. G) and H) Flow cytometry analysis of GFP expression of three independent experiments (bars). Cell viability was assessed by MTT assay in I) naïve TZM-bl indicator cells that were treated with vehicle PBS, HIV-1 NL4.3 virus (8 RTU/ml), 10 ng/ml PMA, or 100 μg/ml SE. J) JLat 10.6 latently infected GFP-expressing cells were treated with vehicle PBS, 10 ng/ml PMA, 10 ng/ml TNFα, or 100 μg/ml SE. K) JLat 10.6 cells were treated with vehicle or 100 μg/ml SE before treatment with 10 ng/ml PMA or 10 ng/ml TNFα (shaded). L) JLat 10.6 cells were treated with 10 ng/ml PMA or 10 ng/ml TNFα (shaded) before treatment with vehicle or 100 μg/ml SE. For all, vehicle treatment was used for normalization and set at 100%. Where indicated, cells were treated for 24 hours before washing and subsequent treatment for an additional 24 hours. FlowJo software was used for all flow cytometry analysis. Significance was taken at P<0.01 (**). Error bars are standard error of the mean of triplicate experiments. ns = not significant.
Figure 28: SE alone do not up-or down-regulate basal LTR promoter activity. A) Naïve TZM-bl indicator cells were treated with vehicle PBS, HIV-1 NL4.3 virus (8 RTU/ml), 10 ng/ml PMA, or 100 μg/ml SE before assessment of promoter activity. B) JLat 10.6 latently infected GFP-expressing cells were treated with vehicle PBS, 10 ng/ml PMA, 10 ng/ml TNFα, or 100 μg/ml SE. Activation was assessed by GFP flow cytometry analysis. Shown is one representative experiment. C) Quantification of JLat 10.6 GFP expression from three independent experiments. D) JLat 10.6 cellular RNA was analyzed by qRT-PCR analysis for HIV-1 Gag-pol mRNA expression. GAPDH was used for normalization. Vehicle was set as reference. For all experiments, cells were treated for 24 hours before washing and culturing without treatments for an additional 24 hours before read-out. E) TZM-bl indicator cells were treated with vehicle PBS or increasing concentrations of SE for 24 hours before read-out of promoter activity. F) GFP expression was quantified by FlowJo analysis in JLat 10.6 latently infected GFP-expressing cells that were treated with vehicle PBS or 100 μg/ml SE before treatment with vehicle PBS. Shown is an average of three independent experiments. G) Viability of the JLat 10.6 cells was measured by MTT assay. H) GFP expression was quantified in JLat 10.6 latently infected GFP-expressing cells that were treated with vehicle PBS before subsequent treatment with vehicle PBS or 100 μg/ml SE. Shown is one representative experiment. I) Viability of the JLat 10.6 cells was measured by MTT assay. For all, vehicle treatment was used for normalization and set at 100%. Where indicated, cells were treated for 24 hours before washing and subsequent treatment for an additional 24 hours. J) PBS vehicle control or SE were extracted with ExoQuick reagent. Vehicle or 100 μg/ml SE were added simultaneously with HIV-1 NL4.3 virus to TZM-bl cells followed by assessment of promoter activity 24 hours later. For A) and E), TZM-bl promoter activity in relative light units was read by Steady-Glo (Promega). FlowJo software was used for all flow cytometry analysis. Significance was taken at P<0.05 (*), P<0.01 (**). Error bars are standard error of the mean of triplicate experiments. E) is plotted as median and bars are value range (min to max). ns = not significant.
Figure 29: The NF-kB transcription factor pathway is implicated in SE-mediated control of HIV-1 transcription. Vehicle PBS or semen exosomes (100 μg/ml) were incubated with HIV-1 NL4.3 virus (8 RTU/ml) at 37°C for 1 hour before infection. SUPT1 mRNA for A) Sp1 and B) NF-kB p65 by qRT-PCR. C) Whole cell lysates of SUPT1 cells were probed for anti-NF-kB p65, anti-Sp1, and anti-GAPDH by western blot. Gels shown are for one representative experiment. Relative band intensities of D) Sp1 and E) NF-kB p65 bands by ImageJ quantification. F-G) TZM-bl cells were analyzed by qRT-PCR for F) Sp1 and G) NF-kB p65 mRNA expression. GAPDH was used for normalization. Vehicle was set as reference. H) TZM-bl cells were probed for anti-NF-kB p65, anti-Sp1, and anti-GAPDH by western blot. Shown is one representative experiment. I-J) TZM-bl blot band intensity for Sp1 and NF-kB p65 were measured from three independent experiments by ImageJ quantification. Cytoplasmic and nuclear extracts of K) SUPT1 and L) TZM-bl cells probed by western blot for anti-NF-kB p65. Anti-GAPDH and anti-PCNA were used as cytoplasmic and nuclear markers, respectively. M) Relative band intensities of K) and L) by ImageJ quantification from three independent experiments. N) ChIP assay with anti-IgG or anti-NFkB p65 for qRT-PCR analysis of HIV-1 LTR. GAPDH was used for input control and HIV was set as reference at 1. ChIP products were quantified by fold enrichment method; shown is an average of three experiments. Products were visualized by agarose gel and ethidium bromide staining. Significance was taken at P<0.05 (*), P<.001 (**). Error bars are standard error of the mean of triplicate experiments. ns = not significant.
Figure 30: SE reduce RNA Pol II association with HIV-1 LTR. Vehicle PBS or semen exosomes (100 μg/ml) were incubated with HIV-1 NL4.3 virus (8 RTU/ml) at 37°C for 1 hour before infection of SUPT1 cells. A) ChIP assay with anti-IgG or anti-Pol II for qRT-PCR analysis of HIV-1 LTR. GAPDH was used for input control and HIV was set as reference at 1. ChIP products were quantified by fold enrichment method; shown is an average of three experiments. B) SUPT1 mRNA was evaluated for HIV-1 LTR gene expression by qRT-PCR. Products were visualized by agarose gel and ethidium bromide staining. Significance was taken at P<0.05 (*), P<.01 (**), P<.001 (***) and ns=not significant. Error bars are standard error of the mean of triplicate experiments.
Figure 31: SE inhibition of HIV-1 transactivation is mediated by interference with Tat protein-mediated LTR activation. TZM-bl cells were transfected with p-Tat for three hours before removal of input plasmid and treatment with SE (100 µg/ml) and incubation for 16 hours. Promoter activity was measured by A) the luciferase assay and B) the β-gal assay. C) TZM-bl cells were treated with recombinant Tat (r-Tat, 10 µg/ml) that was pre-incubated with PBS (vehicle) or SE (100 µg/ml) for 1 hour at 37 °C prior to addition to cells. Promoter activity was assessed by the luciferase assay at 16 hours post-treatment. D-F) Kinetics of SE inhibition of HIV-1 LTR promoter activity and MTT viability at time 24 h. D) HIV-1 (8 RT/ml) pre-incubated for 1 hour at 37 °C with PBS or SE (100 µg/ml); E) Vehicle, SE alone, and p-Tat transfection 3 hours before addition of SE or vehicle PBS; and F) r-Tat (10 µg/ml) pre-incubated for 1 hour at 37 °C with PBS or SE (100 µg/ml). D-F) (RLU Fold Δ) are plotted as median and bars are value range (min to max). Significance was taken at P<0.05 (*), P<0.01 (**). ns = not significant.
CHAPTER VI: SUMMARY AND FUTURE DIRECTIONS

Semen exosomes inhibit HIV-1

Previous studies showed that human semen contains an abundance of exosomes, and that semen-derived exosomes from healthy donors inhibit HIV-1 in a donor-independent manner [63, 133]. SE blocked transmitted founder and lab-adapted HIV-1 strains, in vitro and in vivo in a murine AIDS model of infection [62, 63]. Importantly, we identified the functional implication of donor characteristics including illicit drug use [64] and HIV status on the inhibitory phenotype conserved in SE, and reported a viral replication event targeted by SE that is independent of other lifecycle events [139]. Although semen contains multiple factors that may enhance or inhibit HIV-1, SE reside on the virus-impeding end of the seminal spectrum as potent inhibitory molecules (Chapter I).

HIV-1 inhibition by semen exosome surface protein

We showed that SE from illicit drug users are less effective against HIV-1 than SE from non-drug users. Correlation analysis revealed a correlation between SE CD63 surface levels and inhibitory activity; illicit drug users had reduced CD63 that associated with less potent inhibitory activity (Chapter III, [64]). These correlative results suggested that CD63 may play a functional role in the inhibitory activity of SE. Unpublished data from our group indicate that SE are a mixed population of CD63 containing vesicles where the majority of the total concentration is devoid of CD63 surface protein. Immuno-capture techniques effectively separate total SE into CD63 high and low containing fractions; although CD63 could not be completely depleted from the low containing fraction. HIV inhibition studies show that both fractions inhibit; however, the CD63 high containing fraction has significantly greater inhibition than the CD63 low containing fraction. Similar results in a HSV-1 model of infection showed depletion of the CD63 containing
exosome population enhanced infection [113]. These data indicate that CD63 may contribute to SE-mediated inhibition of HIV-1 and other viruses, but it is not the only inhibitory molecule contained in SE.

**Identifying semen exosomes antiviral source by donor characteristics**

The cellular source of SE inhibitory factor(s) have not yet been identified; however, SE likely originate from multiple cell types within the male reproductive tract as polyclonal antisera against SE reacted with testes, epididymis, prostate, seminal vesicles, and bulbourethral glands [133]. Donor characteristics that affect the heterogenous makeup of SE may aid in discovering the cell source that mediates protection. Our unpublished data show that SE from vasectomized donors vary in inhibitory function despite having no difference in particle concentration. Since vasectomized SE are devoid of contributions by the epididymis, testes, and vas deferens depending on the type of procedure, perhaps the contribution of exosomes from these organs to the makeup of SE is important for inhibition. Unpublished findings using a rat model of the male reproductive tract reveal that exosomes isolated from rat male reproductive tissues (epididymis, seminal vesicle, vas deferens, prostate, testes, and bulbourethral glands) are comparable in concentration and size. Interestingly patterns of concentration versus size profile show exosomes from rat vas deferens closely resemble that of human SE. Although limited by sample size, unpublished data show that only exosomes from rat vas deferens and epididymis inhibit HIV-1, and inhibition levels are comparable to human SE. However, exosomes from other tissues may reach significance with increased sample size. Identifying the cell origin of SE that contribute to inhibition may aid in discovery of SE antiviral factors.
Role of exosomes derived from HIV-infected donors in HIV replication

Cells infected in vitro or ex vivo with HIV-1 released exosomes that facilitated HIV infection [112, 185, 286]. Our lab showed that SE from healthy donors inhibit HIV replication [63, 64, 139]. Importantly, we show SE from HIV-infected ART-naïve donors with high viral loads (>30,000 copies/mL) restrict infection (Chapter IV). Our data indicate that despite viral loads that may indicate a proviral status of exosome-producing cells, exosomes contained in semen are antiviral. Therefore, regardless of HIV viral load, SE contain conserved inhibitory molecules that are stable even in a pro-HIV cell environment. In addition, we show BE from HIV-infected ART-naïve donors do not affect HIV infection (Chapter IV). These results suggest that unlike exosomes from in vitro infected cells, exosomes derived from an in vivo infection may not directly contribute to HIV pathogenesis. It remains to be determined whether in vivo-derived exosomes facilitate infection other than through the transport of proviral factors. For example, does exosome biogenesis mediate HIV receptor-independent infection in vivo or suppress antiviral immune responses? Proteomic analysis of BE from HIV-infected donors’ revealed increased exosome-associated immune activation and oxidative stress markers compared to HIV-negative donors [130]. Perhaps in vivo-derived exosomes function as biomarkers rather than facilitators of infection.

Interestingly, unpublished assessment of RT activity in BE and SE show RT activity in SE, but not BE. Unpublished results identify RT activity in SE from HIV-negative and HIV-infected donors (ART-naïve and ART-suppressed). Although the role of HERVs or HERV-elements during virus infections has yet to be studied, HERV-elements are found in human exosomes that can facilitate transfer of HERV components to cells [76]. Perhaps HERV components released in SE have functional implications.
Anti-retroviral drugs are incorporated in body-fluid exosomes

Treatment of HIV-infected cells in vitro with ARVs alters exosome cargo, and synthetic exosome-like particles loaded in vitro with ARVs deliver ARVs to cells [140, 237]. We show, for the first time, that semen and blood derived exosomes contain ARVs (Chapter IV). However, there currently lack technique and validation criteria to cleave surface associated ARVs from exosomes to determine whether ARVs are enwrapped in exosomes, or interacting with exosome surface molecules. Development of techniques to examine exosome association with ARVs is important for understanding how ARVs affect the biogenesis and cargo of exosomes and their function during HIV infection. We hypothesize that body-fluid exosomes may incorporate ARVs as cargo during biogenesis and facilitate transfer of ARVs to biologic sites. Our data suggest trends of body-fluid specific distribution of ARV drugs. We show an increased abundance of particular ARV drug class (NRTIs) in seminal fractions, and a separate class (NNRTIs) in blood fractions (Chapter IV). This theory is supported by a report that showed that NRTIs ARV drug class accumulate in the female reproductive tract, but NNRTIs poorly accumulate [236]. Alternatively, our observations may suggest that exosomes prolong the half-life of ARV drugs in particular bio-fluids. Synthetic exosome-like particles loaded with ARVs enhanced intracellular ARV retention and prolonged ARV drug release and inhibition of HIV infection [237].

Semen exosomes target viral factors

SE antiviral factors restrict HIV at multiple steps of viral replication. Viral lifecycle data showed that during HIV infection, SE reduce reverse transcriptase activity, levels of integrated proviral DNA, and interfere with transcription [63, 139]. Although interference in any of these viral lifecycle steps generates additional lifecycle consequences, we show that SE block
activities of purified Tat protein, indicating at least one viral target that is independent of additional targets (Chapter V, [139]). We speculate that SE antiviral mechanism(s) are largely targeted to virus lifecycle events, as SE reduced expression of viral genes but not of host housekeeping or host transcription factor genes (Chapter V, [139]). Our unpublished data support this hypothesis as addition of SE to primary cells ex vivo did not induce virus replication of HIV-infected ART-suppressed PBLs, or induce activation of quiescent or TCR-activated HIV-negative CD4+ PBLs regardless of HIV-1 infection. However, SE may modestly affect host responses; our unpublished data show that SE reduce cytokine production of IL1β, IL8, and TNFα and increase CXCL10 in TCR-activated HIV-1 negative PBLs infected with HIV-1. It is not yet known how SE-mediated cytokine levels effect infection.

We speculate that the SE effects on host factors may be contingent on the involvement of host factors in infection. SE significantly reduce the transcription factor NF-kB protein levels in HIV-infected cells, but did not affect levels in uninfected cells (Chapter V, [139]). Our unpublished data show that SE do not affect NF-kB or SP1 protein levels in quiescent PBLs that are resistant to infection, but differentially regulate levels in TCR-activated PBLs infected with HIV-1. In addition, SE reduced HIV-induced LTR promoter activation but had no effect on LTR promoter activation induced by mitogen stimulating molecules (PMA and TNFα) (Chapter V, [139]). The complete host response to SE and how these effects relate to infection is yet to be determined.

**Future research of semen exosomes**

Given that SE affects multiple steps of the HIV-1 lifecycle, it remains to be determined whether these effects are independent functions or downstream consequences of an earlier mechanism. While SE appears to have at least one independent target (Tat), it is currently
unknown whether this mechanism orchestrates the other effects. Because Tat is a multi-functional protein involved in transcription [238, 239] and reverse transcription [166-168], potentially affecting integration [287, 288], it may be possible that early inhibition of Tat by SE during reverse transcription mediates effects on integration and transcription [168]. We propose future studies to define SE Tat inhibition. In order to identify if SE inhibits Tat through interaction with Tat protein domains we suggest a series of Tat protein domain mutants that target the N-terminal, C-terminal, cysteine-rich, basic, and core domain. Tat mutants have previously been developed and identified Tat domains important for transcription, RNA-binding, TAR binding, and reverse transcription [289].

SE are composed of nucleic acids, lipids, and proteins. Identifying the protective factor will aid development of novel anti-HIV therapies, and may identify additional SE-susceptible viruses. Given that SE inhibit HIV-1 and ZIKV, but not HSV-1 and HSV-2, the specificity of SE on viral infections remains to be further studied [63, 152]. SE do not appear to be restrictive to retroviruses or applicable to all sexually transmitted viruses. We suggest comparison of the proteomic, lipidomic, or nucleic acids content from inhibitory SE with SE that are less inhibitory such as SE from illicit drug users or vasectomy versus non-vasectomy donors. SE from HIV negative, HIV positive ART-naïve, and HIV positive ART-suppressed donors uniformly inhibit HIV replication (Figure 32). However, SE from HIV negative donors that use illicit drugs are impaired in inhibition and are altered in composition compared to SE from HIV negative non-illicit drug donors (Figure 32). Because reduced expression of exosome CD63 and AChE correlated with reduced inhibition, small changes in content may have substantial consequences on function. Comparison of effective and defective SE may narrow down potential antiviral factors that can be functional tested against HIV-1 and other viruses.
This thesis summarizes what is known about semen exosomes during HIV-1 infection, and future perspectives for harnessing the antiviral potential of SE.
Figure 32: Exosomes from human semen have various effects on HIV-1 replication depending on donor characteristics. Semen exosomes (SE) from HIV negative donors (blue), HIV positive ART-naïve donors (grey), and HIV positive ART-suppressed donors (black) inhibit HIV-1 replication at the steps of reverse transcription, DNA integration, and RNA transcription. SE from HIV negative illicit drug positive donors (pink) are impaired in inhibition of HIV-1 replication.
APPENDIX: GLYCEROL MONOLAURATE (GML), AN ANALOGUE TO A FACTOR SECRETED BY LACTOBACILLUS, IS VIRUCIDAL AGAINST ENVELOPED VIRUSES INCLUDING HIV

Abstract

The vaginal microbiota influences HIV sexual transmission. Colonization of the vaginal tract is normally dominated by *Lactobacillus* species. *Lactobacillus* as well as *Enterococcus faecalis* secrete reutericyclin, which inhibits a variety of pathogenic bacteria. Increasing evidence suggests a therapeutic role for an analogue to reutericyclin, glycerol monolaurate (GML), against microbial pathogens. Previous studies using a macaque-SIV vaginal transmission model demonstrated that GML reduces transmission, and GML alters immune responses to infection *in vitro*. We hypothesized that GML may also interfere with HIV replication. We sought to: i) expand understanding of how GML inhibits HIV and ii) evaluate GML antiviral effects on other viruses. We show that reutericyclin secreted by *Lactobacillus* and its analogue GML inhibit diverse enveloped RNA viruses, including HIV. These data emphasize the importance and protective nature of the normal vaginal flora during viral infections, and offer the foundation for further study of the anti-viral mechanism of GML during HIV infection and more broadly to infection of other viruses.

Introduction

Sexually transmitted infections (STIs) cover a broad range of microbial organisms. Preventing the risk of sexually transmitted infections is often centered on abstinence and barrier-form contraceptives [290]. However, a comprehensive evaluation sponsored by multiple U.S. health agencies concluded that use of barrier contraceptive by men during vaginal intercourse reduced the risk of HIV, but effectiveness was undetermined for prevention of chlamydia, syphilis, chancroid, trichomoniasis, herpes simplex virus-2, or human papilloma virus [291].
Other reports support the prevention of STIs by male barrier contraceptive, however, no study has found 100% effectiveness and contain limitations such as inclusion of sexual orientation disparities [291, 292]. In addition, many STI infections may be asymptomatic, causing significant systemic and reproductive health problems prior to initiation of treatment [293]. Therefore, preventative STI agents that do not interfere in sexual norms and protect from infections that cause no or mild symptoms are critically needed to curtail the ~340 million annual newly acquired STI infections worldwide [294].

Topical agents that maintain safety and effectively reduce transmission of multiple infectious agents are an ideal candidate to reduce the incidence of STIs. Microbicides are advantageous because of their potential activity against a range of organisms, long-term safety and effectiveness, adaptability to cultural systems, ease of use, affordability, maintenance of the normal vaginal microbiota, and tolerance at mucosal surfaces [295]. Previously developed microbicides targeted maintaining the acidity of the vaginal tract and pathogen-binding or membrane disruption [296]. However, assessment of effectiveness was incomplete as trials had to be stopped early due to safety concerns and risk of reporting bias [295].

Glycerol monolaurate (GML) is a fatty acid formed of glycerol and lauric acid with antimicrobial and immuno-regulatory properties. Currently, GML is used as a food and cosmetic additive with generally recognized as safe (GRAS) status by the FDA. Rhesus macaque safety studies showed that GML maintained localized safety and epithelial integrity during long-term vaginal use, was not disruptive of the normal lactobacillus vaginal microbiota important to maintaining vaginal pH, and did not induce inflammation [297]. *In vitro* studies showed that GML reduced T cell proliferation in the presence of activation molecules and inhibited TCR activation, consequently reducing production of TCR-induced cytokines [298]. Furthermore, *in
vitro and in vivo studies found production of IL-8 induced by S. aureus was prevented by treatment with GML [297, 299]. These findings are significant as in many STIs, inflammation can exacerbate disease.

GML antibacterial studies showed that GML inhibited the growth of gram-positive and gram-negative bacteria, reduced exotoxin production, and inhibited the formation of biofilms [299-302]. GML may target bacteria through interaction with plasma membranes and signal transduction systems [302]. Significant to STIs, GML inhibits growth of bacteria that increase susceptibility to infections, especially of HIV-1, such as Gardnerella vaginalis, Streptococcus agalactiae Group B, and Chlamydia trachomatis [302, 303]. The protective properties of GML on the vaginal mucosa and inhibitory effects on cytokine production suggest GML may confer protection during HIV-1 transmission as immune activation and inflammation increase susceptibility to HIV-1 [297]. In vivo macaque studies of SIV vaginal transmission confirm that GML protects from acute and systemic high dose intravaginal SIV infection [304, 305]. GML also effectively reduced HIV-induced secretion of proinflammatory cytokines, MIP-3α and IL-8, validating its immuno-regulatory properties during infection [304].

Purified and human milk-derived monoglycerides contain antiviral activity against enveloped viruses including herpes simplex virus-1 and -2 (HSV-1 and HSV-2), vesicular stomatitis virus (VSV), and visna virus, but are ineffective against non-enveloped viruses including poliovirus and rhinovirus [306-311]. Previous studies show that monoglycerides similar to GML inactivate enveloped RNA and DNA viruses; however, further investigation into the mechanism of action is needed. Here, we expand the understanding of GML during HIV-1 infection and show that GML effectively inhibits enveloped mumps, yellow fever, and zika virus, increasing the antiviral spectrum of GML. In addition, we develop the mechanistic profile
of GML against enveloped viruses by pinpointing specific viral lifecycle steps perturbed by GML, and show that maturation of the viral envelope alters sensitivity to GML inactivation. We show that Lactobacillus-secreted reutericyclin, an analogue to GML, protects against HIV infection. Reutericyclin, produced by Lactobacillus, inhibits a range of bacterial pathogens [312]. Lactobacillus-dominated vaginal microenvironments inhibit HIV-1 replication and reduce heterosexual transmission; here, we suggest reutericyclin produced by Lactobacillus may contribute to protection against HIV heterosexual transmission [313, 314]. We show that reutericyclin and GML effectively inhibit HIV-1 in vitro.

Materials and methods

Cells: TZM-bl and HEK293 cells (NIH AIDS Reagent Program), MRC-5 (Sigma), BSC-1 and Vero (ATCC) were maintained in DMEM (Gibco-BRL/Life Technologies) with 5% exosome-depleted FBS (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, sodium pyruvate and 0.3mg/ml L-glutamine (Invitrogen, Molecular Probes).

Viruses: HIV-1 NL4.3 was produced by Lipofectamine 2000 transfection of pNL4.3 (NIH AIDS Reagent Program) into HEK293 cells according to manufacturer’s instructions (Invitrogen). Viral titer was determined by EnzChek Reverse Transcriptase Assay (Life Technologies) and luciferase reporter relative light units (RLU) of infected TZM-bl cells (Steady-Glo, Promega) [63, 64]. HIV-1 NL4.3 assays were completed with 100,000RLU. Hepatitis virus A (HM175; ATCC), Enterovirus 68 (US/IL/14-18952; ATCC), Yellow fever virus (17D; Sanofi), Mumps virus (Jeryl Lynn; Merck), Adenovirus (Type 5; University of Iowa Viral Vector Core), and Zika virus (PR, kindly provided by Dr. Wendy Maury of the University of Iowa) titers were determined in appropriate cell lines by TCID$_{50}$ or qRT-PCR of viral RNA as previously described [315]. All virus stocks were stored at -80°C.
**Glycerol monolaurate (GML):** GML kindly provided by Dr. Patrick Schlievert was solubilized by pure ethanol to a stock concentration of 100 mg/mL. Dilutions into the appropriate working concentrations were completed in cell culture media. Dilution of pure ethanol in cell culture media was used as vehicle control. GML stock and working concentrations were stored at room temperature.

**Isopycnic centrifugation of HAV:** HAV virus suspensions were layered on top of a CsCl solution and centrifuged at 100,000 x g for 1 week at 10°C in SW41Ti rotor (Beckman Coulter) [316]. Approximately 20 fractions (0.5mL) were collected and tested for HAV by one-step qRT-PCR. Density-light (enveloped) particles equilibrated near the gradient top whereas density-heavy (non-enveloped) particles equilibrated near the bottom. CsCl was removed from virus fractions by dilution in PBS and centrifugation at 100,000 x g for 2 hours at 10°C in SW41Ti rotor (Beckman Coulter). Pelleted virus was resuspended in media before infection.

**One-step qRT-PCR viral RNA:** HAV RNA was extracted from infected supernatants using QiAamp Viral RNA kit (Qiagen). PCR reactions included 0.5μM each of HAV forward and reverse primer, 0.2μM HAV probe, and 11μl of viral RNA in the Platinum Quantitative RT-PCR ThermoScript One-step System (Invitrogen). PCR reactions were completed using 7500 qRT-PCR (ABI) with the following conditions: 50°C for 20min, 95°C for 2min, 40 cycles of 95°C for 15sec, and 58°C for 1min. HAV primers and probes were as follows: HAV F; 5’-GGTAGGCTACGGGTGAAAC-3’, HAV R; 5’-AACAACTCACCAATATCCGC-3’, HAV probe; FAM-5’-CTTAGGCTAATTCTTCTATGAAGAGATGC 3’-TAMARA (IDT).

**HIV-1 binding and entry:** To quantify (%) binding and entry, TZM-bl cells were maintained at 4°C for 1 hour before infection. For HIV-1 binding: prior to infection, varying concentrations of GML were incubated with HIV-1 NL4.3 for 30min at 37°C. Cells were infected for 3 hours at
4°C. After which, cells were washed 3x with ice-cold PBS before cell lysis and evaluation of cell-associated p24 by p24 ELISA (ZeptoMetrix) per manufacturer’s instructions. For HIV-1 entry: cells were infected with HIV-1 NL4.3 for 3 hours at 4°C. After which, virus was removed and cells were washed 3x with ice-cold PBS to remove unbound virus. Cells were then treated with varying concentrations of GML for 3 hours at 37°C. Cells were washed again 3x with PBS before treatment with media control or trypsin to remove cell surface-associated virus. Cells were then lysed and evaluated for p24 content (ZeptoMetrix). The % infectivity outcome of binding was determined by co-incubation of HIV-1 NL4.3 and GML for 30min at 37°C prior to addition to TZM-bl cells for 4 hours at 37°C. At which time, GML/infection media were removed and cells were cultured for an additional 24 hours in complete media. Binding outcome in the presence of GML was evaluated by RLU read-out of luciferase reporter (Steady-Glo, Promega).

**GML exposed HIV-1:** Varying concentrations of GML were incubated with HIV-1 NL4.3 for 30 min at 37°C before GML was removed from virus by 100K-centrifugal filtering (Centriprep). Remaining GML-free virus was then used for infection of TZM-bl cells for 24 hours, evaluation of HIV-1 binding as described above, and determination of HIV-1 entry as described above where here virus was incubated on cells at 37°C for 16 hours before washing, media control or trypsin treatment, and evaluation of p24 content.

**Reutericyclin:** *Enterococcus faecalis* and *Lactobacillus reuteri* were cultured overnight at 37°C in Todd Hewitt or MRS broth, respectively. Bacterial cells were removed from reutericyclin-containing supernatants by centrifugation. 4 volumes of pure ethanol were added to reutericyclin-containing supernatants and incubated at room temperature overnight to precipitate molecules >15K. The ethanol-reutericyclin supernatant was centrifuged at 2,000 x g for 20 min
to remove large molecules. The reutericyclin-supernatant was concentrated by centrifugal filtering and dried under laminar flow hood before resuspension to 10x in distilled water. Infections were completed by diluting the concentrated reutericyclin in cell culture media. HIV-1 infection of TZM-bl cells in the presence and absence of reutericyclin was assessed after 24 hours by luciferase assay (Steady-Glo, Promega). Bacterial culture broth with no microbe or exposed to non-reutericyclin producing bacteria (*Lactobacillus plantarum*; kindly provided by Laynez Ackermann at the University of Iowa) treated the same were used as controls.

**Infectivity assays:** 100,000RLU HIV-1 NL4.3 virus was co-incubated in the presence and absence of varying concentrations of GML for 30min at 37°C before infection, or where indicated, GML or NL4.3 virus were added to TZM-bl cells for 24 hours before removal, washing, and GML treatment or infection for an additional 24 hours. After which, cells were lysed and measured for luciferase reporter activity at the indicated time-points by Steady-Glo (Promega). All other viruses were incubated with varying concentrations of GML for 30min at 37°C before the following cell infections: Mumps virus (Vero MOI=0.1), Yellow fever virus (Vero MOI=1), Zika virus (Vero MOI=1), Adenovirus (HEK293 MOI=1), Enterovirus 68 (MRC-5 MOI=1), and HAV (BSC-1 MOI=0.5). GML/infectious media was replaced 24 hours post-infection with complete media containing 2% FBS. Infectivity was assessed by CPE once CPE was observed in 100% of infection control wells for CPE-producing viruses. HAV-infected cultures were maintained for 14-21 days before one-step qRT-PCR of viral RNA. Viability was determined at the time of infectivity assessment, and treatments were normalized to infection control. 50% inhibitory concentration (IC₅₀) doses were calculated from three independent experiments.
**Furin treated viruses:** HIV-1 NL4.3 (100,000RLU), zika (MOI=1), yellow fever (MOI=1) were incubated in the presence and absence of 50U recombinant furin (New England Biolabs) for 2 hr at 37°C pH 5.8 [317]. Furin-negative (-Furin) and furin-positive (+Furin) viruses were then incubated with GML and infections carried out as described above.

**Viability:** Cell viability was assessed by MTT assay as previously described [111].

**Statistics:** Two-tailed t test p-value (GraphPad Prism) calculations determined statistical significance where p<.05=*, p<.01=**, p<.001=***, ns=not significant. Error bars represent standard error of the mean (SEM) of triplicate experiments.

**Results**

**GML protection against HIV-1 infection requires contact with the virus, in vitro**

*In vivo* macaque-SIV vaginal transmission studies show that GML administered 1 hour prior to SIV challenge along with daily treatments over the course of the study protects against high-dose viral challenge [304, 305]. Here, we examined the importance of the timing of GML treatment during protection of infection. Co-incubation of GML with HIV-1 at the time of infection significantly inhibited HIV-1 infection at all concentrations of GML tested (Figure A.1A). Although high concentrations of GML reduced cell viability, even non-cytotoxic concentrations of GML significantly protected against infection (Figure A.1A-B). Because of the immuno-regulatory profile of GML, we hypothesized that the addition of GML prior to infection may condition the cells to an antiviral state and further enhance its protective effect. GML treatment of cells prior to infection as well as co-incubation with virus at the time of infection significantly protected against HIV-1 infection at concentrations that did not reduce cell viability (Figure A.1C-D). However, there does not appear to be an enhancement of protection by pre-treating the cells with GML prior to infection. This observation was verified by treating cells
with GML prior to exposure to HIV-1. Non-cytotoxic concentrations of GML were not protective when cells were pre-treated before infection (Figure A.1E-F). Additionally, treatment of cells with GML post-infection did not confer protection at concentrations that were not cytotoxic (Figure A.1G-H). These data may suggest that GML-mediated cell conditioning occurs at earlier time points, or may also suggest that, in vitro, GML does not condition the cells to protect against infection and that contact of GML with HIV-1 may be required to inhibit infection.

**GML modestly restricts HIV-1 binding, but completely blocks viral entry**

These data indicate that GML-mediated protection to infection requires GML to be present at the time of infection, suggesting that the protective mechanism conferred by GML is virus-mediated rather than host cell-mediated. This hypothesis was evaluated by studying the early viral lifecycle events, binding and entry. The ability of GML to perturb HIV-1 binding to the cell surface was determined by allowing HIV-1 in the presence and absence of GML to bind to cells for 4 hours, before removal of virus and GML and evaluation of infection. Here, cell infectivity is measuring the outcome of binding before replication events downstream of binding could occur. Evaluation of infection showed no significant protection by GML at non-cytotoxic concentrations, indicating that GML does not disrupt the ability of the virus to bind to the cell-surface, and once GML is removed, bound virus can successfully infect cells (Figure A.2A-B). This was further verified by exposure of cells to cold temperature during HIV-1 infection in the presence and absence of GML, which facilitates viral binding to the cell surface, but the cold temperature inhibits viral entry and downstream events. These results show that GML modestly reduced virus bound to cells at only the highest concentrations tested (Figure A.2C). In addition, when virus was allowed to bind to cold exposed cells before the cells were warmed to 37°C,
facilitating viral entry or cell internalization, at the time of GML treatment, only the highest concentration of GML reduced levels of surface-associated and intracellular virus (Figure A.2D). However, when surface-associated virus was proteolytically cleaved from cells leaving only intracellular virus, GML completely ablated virus levels (Figure A.2D). These results indicate that GML modestly reduces viral binding, but completely blocks viral entry. Under these conditions, when GML is present, HIV-1 appears to bind to and collect on the cell-surface but is unable to facilitate entry into cells.

Because our results show GML appears to target early viral lifecycle events, we further verified this was a virus- rather than cell-mediated mechanism by exposing HIV-1 to GML before removal of GML from virus and assessment of the ability of GML-exposed virus to infect, bind to, and enter cells. GML-exposed virus show significantly reduced cell infectivity; these results do not appear to be a result of residual GML as typically cytotoxic concentrations of GML showed no reduction of cell viability (Figure A.3A-B). Reduced infectivity could not be attributed to a reduction in the ability of virus to bind to cells (Figure A.3C). However, similar to our previous results, GML-exposed virus showed a significant impairment of virus internalization but not of surface-association (Figure A.3D). Taken together, these results show GML acts on the virus, altering the ability of HIV-1 to facilitate viral entry.

**GML offers broad-spectrum protection against enveloped viruses**

Our results show GML targets early viral lifecycle events, suggesting that GML-mediated protection may not be restricted to HIV-1 as many viruses use similar pathways for binding and entry [318]. Incubation of permissive cells with three diverse enveloped viruses (mumps, yellow fever, and zika) in the presence and absence of GML show GML protected against all three enveloped viruses at non-cytotoxic concentrations (Figure A.4A-F). Calculation of the half
maximal inhibitory concentration (IC$_{50}$) of GML against mumps, yellow fever, and zika virus was 31, 49, and 69 μg/mL, respectively (Figure A.4A-F). Significantly, these results verify that GML is not specific to HIV (or SIV) and may potentially function as a broad-spectrum antiviral in addition to its antibacterial and immuno-regulatory properties. However, GML was unable to protect against infection by two non-enveloped viruses, adenovirus and enterovirus 68 (Figure A.5A-D). Interestingly, isopycnic separation of hepatitis A virus into dense (non-enveloped) and light (enveloped) viral particles confirmed that GML inhibits light/enveloped particles and has no effect on dense/non-enveloped particles (Figure A.5E). These data suggest that while the antiviral mechanism of GML may be conserved among enveloped viruses, its protective mechanism is lost on nonenveloped viruses.

**Envelope maturation mediates viral sensitivity to GML**

Because we observed a range in the IC$_{50}$ against mumps, yellow fever, and zika virus we hypothesized differences in the viral structural components responsible for mediating entry may contribute to viral sensitivity to GML. Many enveloped viruses require processing of their envelope glycoproteins by furin to mediate entry [319]. Furin is a protease responsible for proteolytic activation of pre-cursor envelope (prM) proteins that are otherwise in a conformational assembly that block their ability to mediate fusion [320]. While furin cleavage enhances maturation of envelope glycoproteins and correlates with increased virulence, it is not required for the function of viral envelope proteins [319, 321]. We investigated whether viral envelope maturation by furin altered virus sensitivity to GML. Viral envelope structures are a mixed population of mature, partially mature, and immature virions where the mature form is considered infectious [322]. We hypothesized the presence of furin, and enhancement of the population of mature particles would alter the ability of GML to inhibit infection. Zika virus,
with an IC\textsubscript{50} of 63 μg/mL, showed the least sensitivity to GML. Zika virus sensitivity to GML in the presence of furin was unchanged in comparison to sensitivity in the absence of furin (Figure A.6A). However, the efficiency of prM cleavage of zika virus is currently unknown [323]. It is possible that these results are a reflection of already efficient cleavage into mature particles by zika virus. In comparison to zika, yellow fever virus showed an enhanced susceptibility to inactivation by GML with an IC\textsubscript{50} of 49 μg/mL. Yellow fever virus in the presence of furin and GML showed enhanced infectivity than in the absence of furin at the same concentration of GML (Figure A.6B). Similarly, HIV-1 in the presence of furin was more infectious than in the absence of furin, although the increase in infection was modest (Figure A.6C). The change in sensitivity to GML by yellow fever virus and HIV-1 in the presence of furin suggests that maturity of viral particles may alter the ability of GML to inhibit infection, although this requires further investigation and a more complete understanding of the efficiency of prM cleavage in these viruses.

**An analogue to GML secreted by *Lactobacillus* inhibits HIV-1 infection**

Reutericyclin, a GML analogue, is secreted from isolates of *Lactobacillus reuteri* as well as *Enterococcus faecalis* (Figure A.7A-B). Reutericyclin exhibits a broad inhibitory spectrum against bacterial organisms, but its inhibitory profile is unreported for viruses [312]. However, it has been previously shown that *Lactobacillus reuteri* enhance host resistance to viral infections, possibly through regulation of the microenvironment and through the secretion of antiviral metabolites [324]. HIV-1 challenged with varying dilutions of concentrated reutericyclin-containing media from *E. faecalis* and *Lactobacillus reuteri* showed significantly reduced infection at even highly diluted concentrations (Figure A.8A-C). Broth media in the absence of microbe or conditioned in the presence of non-reutericyclin producing strain of *Lactobacillus*
*plantarum* were used as controls (Figure A.8A-C). These data suggest reutericyclin is an effective inhibitory factor against HIV-1, and support the observation that *Lactobacillus*-dominated vaginal microenvironments are protective against HIV-1. Further, these results support the hypothesis that *Lactobacillus* may secrete protective factors that inhibit infection as well as contribute other protective functions such as maintenance of vaginal pH [325].

**Discussion**

Sexually transmitted infections are a significant global disease burden with estimates that ~1 million people are infected daily [326]. In addition, STIs often cause other health complications and increase susceptibility to secondary infections, including HIV-1 [326]. Managing the disease burden of STIs involves prioritizing prevention of new infections. Because STIs include a diverse and expansive range of pathogens, preventative strategies that incorporate microbial control agents and enable the receptive individual with protection management regardless of sexual norms are important factors for progress. The formulation of GML as a microbicide is a unique candidate to fit this need as it has an established safety profile, its antimicrobial properties include bacteria and viruses, and it regulates immune responses.

GML and similar monoglycerides are effective antiviral agents against diverse viruses. However, an understanding of the antiviral mechanism is needed. Incubation of HIV-permissive cells with GML or its analogue, reutericyclin, at the time of infection inhibits HIV-1 at concentrations that did not reduce cell viability. The IC$_{50}$ of GML against HIV-1 is ~15 μg/mL, significantly lower than that observed in previous *in vivo* studies [304, 305]. GML action on HIV-1 particles was rapid, and inhibition occurred in part by reducing virus bound to cells by >30%. In addition, when GML was added after virus was allowed to bind to the cell surface, viral entry was completely blocked. Thus, GML-mediated inhibition occurs at both the binding
and entry step of the viral lifecycle. Here, we evaluated the antiviral mechanism of GML on the virus lifecycle, however, other studies have evaluated the immune mechanism of GML and identified that GML blocks immune signaling [298, 304]. It is probable that the inhibitory mechanism of GML involves multiple mechanisms.

GML was active against three additional enveloped viruses (yellow fever virus, mumps, and zika) where the IC$_{50}$ of GML is estimated at 49, 31, and 69 μg/mL, respectively, but was not active against two non-enveloped viruses (adenovirus and enterovirus 68). Interestingly, separation of hepatitis A virus into non-enveloped and enveloped virions confirmed these observations. GML inhibited the enveloped, but not the non-enveloped population. These findings are consistent with those by others that described monoglycerides inhibited enveloped HSV-1 and -2, VSV, and visna virus but were unable to restrict non-enveloped poliovirus and rhinovirus infection [306-310]. Our data, together with those by others, indicate the viral envelope as a target of inactivation by GML. We show enveloped virus sensitivity to GML may depend on envelope maturation. Furin-mediated proteolytic activation reduced virus sensitivity to GML inactivation in two enveloped viruses with known susceptibility to furin-induced maturation. However, it is unknown whether GML induces conformational changes to the viral lipid envelope or interferes with envelope interactions with host factors that mediate binding or entry, such as fusion. GML may alter viral lipid conformations as GML induced dramatic conformational changes in T cells, resulting in the formation of unusual filopodia structures [311]. Because GML alters the lipid domains of human T cells, perhaps GML also alters the lipid structures of viruses. Similarly, exposure of VSV to fatty acids caused deterioration of the envelope structural integrity that the authors speculate may occur because of lipid destabilization during fatty acid incorporation into the lipid bilayer [308].
GML is an analogue to *Lactobacillus*-secreted reutericyclin. The normal flora of the vaginal tract is dominated by *Lactobacillus* and protective against HIV-1. Our data suggest reutericyclin may contribute to this protective function as microbe-produced reutericyclin inhibited HIV-1. This is a novel finding as reutericyclin inhibits bacteria, but its activity against viruses is unreported [312]. However, our purification of reutericyclin does not exclude the presence of other molecules such as reutericin and reuterin that may contribute inhibitory effects [312]. Perhaps structural similarities of GML and reutericyclin will reveal the components required for inhibitory activities and development of even more efficacious derivatives. Compounds that resemble the protective function of the vaginal microbiota in addition to other antimicrobial functions may be the key to successful microbicide development.
Figure A.1: GML-mediated inhibition of HIV-1 requires virus contact. (A) Infectivity and (B) viability of increasing concentrations of GML co-incubated with 100,000RLU HIV-1 NL4.3 for 30 min at 37°C before addition to TZM-bl indicator cells for 24 h. (C) Infectivity and (D) viability of TZM-bl cells pre-treated with GML for 24 h before treatment with co-incubated GML and HIV-1 as described in A-B. (E) Infectivity and (F) viability of TZM-bl cells pre-treated with GML 24 h before infection with 100,000RLU HIV-1 NL4.3 for 24 h. (G) Infectivity and (H) viability of TZM-bl cells infected with 100,000RLU HIV-1 NL4.3 for 24 h before post-treatment with GML for an additional 24 h. For all GML treatments, equivalent concentration of ethanol was used as vehicle control. Vehicle treated cells are set as reference at 100% for infectivity and viability. Statistics was determined by comparing infectivity or viability values from vehicle control to treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of three biological replicates each with triplicate values. ns= not significant.
Figure A.2: GML reduces HIV-1 binding and entry. (A) Infectivity and (B) viability of GML or ethanol vehicle control co-incubated with 100,000RLU HIV-1 NL4.3 for 30 min at 37°C before addition to TZM-bl indicator cells for 4 h. After which, cells were washed and cultured in GML- and HIV-free cell culture media for 24 h. Infectivity (A) is indicative of infectivity potential of virus bound to cells in the presence of GML. Vehicle treated cells are set as reference at 100% for infectivity and viability. (C) Binding of 100,000RLU HIV-1 NL4.3 co-incubated with GML or ethanol control for 30 min at 37°C before addition to TZM-bl indicator cells for 3 h at 4°C. (D) Entry of 100,000RLU HIV-1 NL4.3 added to TZM-bl cells for 3 h at 4°C before cells were warmed to 37°C and treated with GML or ethanol control. For C-D, cells were lysed and treated with trypsin to proteolytically cleave surface-associated virus, where indicated, prior to p24 ELISA. Vehicle treated cells p24 content was set as reference at 100% for binding and entry. For all, statistics was determined by comparing vehicle control to treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of three biological replicates each with triplicate values. ns= not significant.
Figure A.3: Exposure of HIV-1 to GML impairs virus activity. 100,000RLU HIV-1 NL4.3 was exposed to GML or ethanol control for 30 min at 37°C before separation of GML from virus by centrifugal centrifugation. (A) Infectivity and (B) viability of GML exposed virus added to TZM-bl cells for 24 h. Vehicle-exposed virus treated cells are set as reference at 100% for infectivity and viability. (C) Binding of GML-exposed virus added to TZM-bl cells for 3 h at 4°C. (D) Entry of GML-exposed virus added to TZM-bl cells for 16 h at 37°C. For C-D, cells were lysed and treated with trypsin to proteolytically cleave surface-associated virus, where indicated, prior to p24 ELISA. Vehicle-exposed virus treated cells p24 content was set as reference at 100% for binding and entry. For all, statistics was determined by comparing vehicle control to treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of three biological replicates each with triplicate values. ns= not significant.
Figure A.4: GML is virucidal against diverse enveloped viruses. Diverse enveloped viruses were co-incubated with ethanol control or GML for 30 min at 37°C before infection on cells. (A) Infectivity and (B) viability of mumps virus on Vero cells MOI=0.1. (C) Infectivity and (D) viability of yellow fever virus on Vero cells MOI=1. (E) Infectivity and (F) viability of zika virus on Vero cells MOI=1. GML/infectious media was replaced 24 h post-infection with 2% culture media. Infectivity was assessed by CPE once CPE was observed in 100% of infection control wells. % infectivity was calculated by the number of infected wells out of eight total wells. 50% inhibitory concentration (IC_{50}) doses were calculated from three independent experiments. Statistics was determined by comparing vehicle control to treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of three biological replicates. ns= not significant.
Figure A.5: GML is inactive against non-enveloped viruses. Non-enveloped viruses were co-incubated with ethanol control or GML for 30 min at 37°C before infection on cells. (A) Infectivity and (B) viability of adenovirus on HEK293 cells MOI=0.1. (C) Infectivity and (D) viability of enterovirus 68 on MRC-5 cells MOI=1. GML/infectious media was replaced 24 h post-infection with 2% culture media. Infectivity was assessed by CPE once CPE was observed in 100% of infection control wells. % infectivity was calculated by the number of infected wells out of eight total wells. (E) Hepatitis A virus was separated into enveloped and non-enveloped particles by isopycnic centrifugation and infected on BSC-1 cells MOI=0.5 in the presence of 40 μg/mL GML. GML/infectious media was replaced 24 h post-infection with 2% culture media. Cultures were maintained for 14-21 days before one-step qRT-PCR evaluation of supernatant viral RNA. Statistics was determined by comparing vehicle control to treatment. Significance was determined by student’s t test. *=P<0.05, **=P<0.01, ***=P<0.001. Error bars are SD of three biological replicates. ns= not significant.
Figure A.6: Envelope maturation reduces sensitivity to GML-mediated inactivation. Viruses sensitive to furin-mediated envelope maturation were incubated with 50 U recombinant furin for 2 h at 37°C pH 5.8. Furin exposed and unexposed viruses were co-incubated with ethanol control or GML for 30 min at 37°C before infection. (A) Infectivity of zika virus on Vero cells MOI=1. (B) Infectivity of yellow fever virus on Vero cells MOI=1. GML/infectious media was replaced 24 h post-infection with 2% culture media. Infectivity was assessed by CPE once CPE was observed in 100% of infection control wells. % infectivity was calculated by the number of infected wells out of eight total wells. (C) Infectivity of 100,000RLU HIV-1 NL4.3 on TZM-bl cells for 24 h. Statistics was determined by comparing + furin to –furin for each treatment concentration. Significance was determined by student’s t test. *=P<0.05. Error bars are SD of two biological replicates.
Figure A.7: Molecular structures of GML and reutericyclin. (A) Glycerol monolaurate (GML). (B) Reutericyclin.
Figure A.8: An analogue to GML, reutericyclin, inhibits HIV-1. *Enterococcus faecalis* and *Lactobacillus reuteri* secreted reutericyclin was concentrated by ethanol precipitation and centrifugal filtering. Growth media incubated without microbe or with non-reutericyclin producing *Lactobacillus plantarum* were used as vehicle controls. 100,000RLU HIV-1 NL4.3 was co-incubated with increasing dilutions of reutericyclin-containing supernatants or controls on TZM-bl cells for 24 h. (A) Infectivity of *E. faecalis* reutericyclin relative to control media grown without microbe. (B) Infectivity of *L. reuteri* reutericyclin relative to control media grown without microbe. (C) Infectivity of *L. reuteri* reutericyclin relative to control media grown with *L. plantarum*. Vehicle treated cells are set as reference at 100%. Statistics was determined by comparing vehicle control to treatment. Significance was determined by student’s t test. *P<0.05, **P<0.01, ***P<0.001. Error bars are SD of three biological replicates. ns= not significant.
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