Characterization of the Cellular and Molecular Factors Mediating Antigen-Independent Noncytolytic CD8+ T Cell Suppression of HIV-1

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CD8+ T cells display a little understood noncytolytic activity that suppresses human immunodeficiency type 1 (HIV-1) replication in an antigen-independent and MHC-unrestricted manner. This activity specifically inhibits transcription of the HIV-1 proviral genome. Little is understood about the molecular nature of the factor(s) mediating this potent antiviral activity of CD8+ T cells. It is known that a factor secreted by CD8+ T cells can suppress the transcription of HIV-1. However, the antiviral mechanism appears most potent with cell-to-cell contact. Previous investigations by several groups into the nature of this secreted factor have been largely based on a presumption that noncytolytic suppression of HIV-1 by CD8+ T cells is exclusively mediated by a soluble protein. Based on several lines of evidence suggesting the specific involvement of cell-contact determinants in eliciting the noncytolytic CD8+ T cell effector function against HIV-1, a novel approach to the problem was utilized based on the hypothesis that a membrane-bound factor is the prime mediator suppressing HIV-1 transcription. In the ensuing investigation, evidence was uncovered demonstrating the existence of a membrane-localized HIV-1 suppressing factor that was secreted as 30-100nm spherical vesicles termed exosomes. Exosomes from a CD8+ T cell line inhibited the replication of R5 and X4 HIV-1 isolates and were shown to specifically suppress HIV-1 transcription in acute and chronic models of infection. A much greater degree of complexity to CD8+ T cell secreted antiviral activity was

found than a soluble protein alone could account for. The evidence presented in this study suggests that CD8+ T cell suppression of HIV-1 is predominantly mediated by a membrane-bound protein factor that can be cleaved into a soluble isoform with the secreted CD8+ cell antiviral activity being largely exosome-driven. The results presented in this study provide a much more concrete understanding of the mechanisms underlying CD8+ T cells suppression of HIV-1 transcription and outline new approaches to conclusively identifying the molecular factor mediating potent inhibition of the HIV-1 transcriptional promoter.

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PREFACE

"…it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice -- never at least fatally. And such persons not only received the congratulations of others, but themselves also, in the elation of the moment, half entertained the vain hope that they were for the future safe from any disease whatsoever…."

- *The Peloponnesian War* by Thucydides, describing in 430 B.C. the phenomenon of acquired immunity to disease

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1.0 INTRODUCTION

The disease progression of human immunodeficiency virus type 1 (HIV-1) infection to acquired immune deficiency syndrome (AIDS) is characterized by a progressive decline in CD4+ T cells (1,2,3), increasing viremia (4), and a downward inflection of total T-cell numbers preceding the onset of AIDS (5,6,7). The inverse correlation between patient virus loads and length of time to development of AIDS from seroconversion (4,7) demonstrates that control of viral replication is a critical factor in long-term prognosis. In the last decade, the development of highly active antiretroviral therapy (HAART) has lead to a dramatic decrease in reported AIDS cases in countries where such therapy is available as standard care (8), however, the phenomenon of HIV-1 drug resistance and side effects from therapy are becoming increasingly problematic (9,10). Of peculiar interest are a tiny percentage of HIV-1 infected subjects (<5%) who are able to maintain low or stable levels of virus replication in the absence of any intervening therapy or clinical symptoms of disease (11,12). It is clear from such patients that their immune system is able to control HIV-1 replication and prevent disease progression to AIDS. Therefore, an analysis of the immune response to HIV-1 may reveal insights into key molecular mechanisms that appear vital in controlling HIV-1 replication in these long term nonprogressing subjects.

1.1 HIV-1 REPLICATION CYCLE

HIV-1 belongs to a special subfamily of retroviruses known as lentiviruses (13). The HIV-1 virion morphology consists of a conical shaped core contained inside a spherical phospholipid bilayer envelope (14). The typical replication cycle of a virus begins with receptor-mediated attachment of the virus to the host. In HIV-1, the prime mediator of cell attachment is the viral gp120 envelope protein whose initial receptor target is the CD4 molecule expressed on CD4+ T cells and macrophages (15,16,17). Upon CD4 binding, a conformational change occurs in gp120 resulting in the exposure of a chemokine receptor binding pocket encompassing a region of gp120 between the V3 and V1/V2 loop regions (18,19). HIV-1 displays two main chemokine receptor preferences, CCR5 (20) and CXCR4 (21). Strains that bind CCR5 are referred to as R5 isolates, while CXCR4 binding viruses are classed as X4. The specificity of chemokine receptor binding defines HIV-1 cell tropism and syncytia inducing capability (20). Upon binding of CD4 and CCR5 or CXCR4, fusion of the viral phospholipid bilayer with the host cell membrane is mediated by the gp41 transmembrane protein (23,24), allowing for cytoplasmic entry of the HIV-1 ribonucleoprotein core.

HIV-1 utilizes a peculiar genomic replication mechanism shared by all retroviruses known as reverse transcription (25). After disassembly of the core, the 9.8 kB mRNA genome is reverse transcribed into double stranded cDNA by the viral reverse transcriptase, RT (26). Upon completion of reverse transcription, the ds cDNA is translocated to the nucleus and integrated into the host genome by the HIV-1 integrase protein, IN (27). After proviral integration, transcription of the HIV-1 genome is mediated with the aid of the virally encoded transcriptional transactivator, *tat* (28). Produced from an alternatively spliced HIV-1 transcript (29), Tat

mediates the recruitment of several critical nuclear proteins, such as CBP/p300 and CDK9 (30,31), by binding to a double stranded RNA stem loop known as TAR that forms on transcribed HIV-1 mRNA between the +19 and +42 nucelotide region upstream from the HIV-1 transcription initiation site (32). While the HIV-1 long terminal repeat (LTR) promoter contains binding sites for a wide array of cellular transcription factors, productive infection requires the binding of Tat to TAR in order to increase the processivity of the cellular RNApol II enzyme (33) and recruit the histone acetyl transferase activity of CBP/p300 to the LTR promoter in order to induce histone relaxation of the HIV-1 genome (34,35).

Upon active transcription of the HIV-1 genome, viral structural and accessory proteins are translated to complete the replication cycle. The nuclear export of full length genomic and singly spliced HIV-1 RNA is mediated by the virally encoded Rev protein binding to the Rev responsive element RRE (36,37), located at the 3' end of viral transcripts (38). Translation of singly spliced HIV-1 RNA results in the protein fusion products Gag, Gag-Pol and Env (39). Upon translation of the Gag-Pol fusion protein, Gag is myristolated by N-myristyltransferase (40), for targeting to the plasma membrane while the viral protease, active in Gag-Pol, functions to cleave the fusion protein (41). The viral protease proceeds in cleaving Pol into separate protease, reverse transcriptase, integrase products (41). The viral Env is cleaved by a cellular protease to yield an extracellular glycosylated gp120 and transmembrane gp41 proteins (42) which are subsequently transported to the cell surface for virion assembly (43). Virion maturation occurs during and after budding of an HIV-1 particle, where the viral protease further cleaves the Gag into p24 capsid, p17 matrix, p6 proline-rich protein, and p7 nucleic acid binding protein (44).

1.2 PATHOGENESIS OF HIV-1 AND PROGRESSION TO AIDS

1.2.1 Clinical Stages of HIV-1 Infection

HIV-1 disease pathogensis occurs in well defined stages (45). The first stage is establishment of acute infection after a transmission event whereby infection is seeded in primary lymphoid tissue (46). The acute stage, typically lasting on the order of 4 to 12 weeks (47), is characterized by a rapid proliferation of HIV-1 reaching peak viremia on the order of 105 to 106 copies/ml (49). This initial burst of viral replication leads to a large depletion of the patient CD4+ T cell pool, particularly in mucosal lymphoid tissue (50). The first detectable immune effector against HIV-1 is the CD8+ T cell response, the key immune effector mediating viral reduction during the acute stage of infection (51,52,53). An antibody response against HIV-1 is usually detected by peak viremia (54) and together with the cellular immune effectors, serves to lower HIV-1 levels eventually establishing a baseline or setpoint viral load marking a transition to chronic infection and period of clinical latency (55). It is at the onset of chronic infection that neutralizing antibody against the virus begins to develop (56). In the absence of drug therapy, the median time course of chronic HIV-1 infection before development of AIDS is 8 to 10 years with patients displaying a vast continuum of disease progression rates from patients progressing to AIDS within 1-2 years to long term non-progressing subjects who remain disease free for 20 or more years (57). In attempting to define the two clinical extremes, a striking correlation has been observed between HIV-1 viral load and the rate of disease progression to AIDS in therapy naive patients (3,4). This demonstrates clearly the importance host defenses against the virus play in the maintenance of healthy status in HIV-1 infected patients.

1.2.2 Long Term Non-Progressor (LTNP) HIV-1 Patients

In attempting to define the parameters of host immune defenses critical to disease-free status in HIV-1 infected patients, significant attention has been focused on LTNPs. Such individuals can be generally identified as therapy naïve and clinically asymptomatic for eight or more years with stable and healthy CD4+ T cell levels >500 counts per ml of blood (58). The distinguishing immunological feature of these subjects is a strong adaptive immune response (59), particularly a strong cellular immune response (60,61,62). Therapy naive LTNPs have also been observed to maintain reduced viral loads compared to rapid disease progressing HIV-1 patients (4,63) indicating stronger host defenses in LTNP cohorts. Stable CD4+ T cell counts and elevated CD8+ T cell levels distinguish LTNP subjects from rapid disease progressors (58,64). Broad neutralizing antibodies are typically found in LTNP subjects (65) in addition to high amounts of HIV-1 specific CTL activity (60,61,62) and a less understood noncytolytic CD8+ T cell antiviral activity (66,67,68). Such control of HIV-1 replication occurs in LTNPs independent of genetic factors such as previously described ΔCCR5 32bp deletion (69) and CCR2-64I (70) alleles. Therefore, immunological control of HIV-1 is the most likely determinant of delayed disease progression in LTNP subjects.

1.3 HOST IMMUNE RESPONSE TO HIV-1

Asymptomatic HIV-1 infection can be roughly characterized as an ongoing conflict between the immune system and the virus infecting it. Consequently, the breadth and quality of

the immune response during the acute and chronic stages of HIV-1 infection delineates the extent of viral replication control and consequently disease progression. The effector arms of the immune system can be broadly classified as innate or adaptive responses. The innate immune response to pathogens include effector mechanisms such as complement activation (71), phagocyte induction through toll-like receptors (72) and other non-classical pattern recognition mechanisms (73), mircrobicidal peptide secretion (74), activation of pathogen-eliminating cells such as NK cells and granulocytes (75,76) and physical-chemical barriers to pathogen transmission (77). The common feature of the innate immune system is a lack of memory of pathogenic protein sequences it encounters. The adaptive immune response on the other hand is defined by a direct association of antigen specificity and immunological memory. The adaptive immune response is comprised of two critical effector arms in pathogen control: (i) a humoral arm mediated by B cell antibody secretion to neutralize pathogen replication and induce their opsonization by phagocytic cells and/or direct lysis by complement activation (79); and (ii) a cellular arm whose prime mediators are CD4+ and CD8+ T cells that function to control intracellular pathogen replication (80).

As intracellular pathogens, most viral infections evoke a strong cellular immune response and this is especially true of HIV-1. However, HIV-1 introduces a caveat in that it infects a subset of immune cells critical for immunity. The infection of CD4+ T cells is a peculiar adaptation of HIV-1 replication, as these cells serve as a critical bridge between the humoral and cellular immune responses (80). Both of these adaptive immune responses are active in their efforts to control virus replication. However, HIV-1 has proven itself notorious in its capacity to evade elimination by the host immune system. Such persistent viremia is found even in LTNP

subjects, although these individuals maintain significantly lower viral loads than more rapid disease progressing individuals (4,63). Thus, the nature of the adaptive immune response to HIV-1 appears to be the key determinant for delayed disease progression to AIDS (81,82).

1.3.1 Humoral Immune Response

B lymphocytes are the primary effectors of the humoral immune response. Through the clonally derived specificity of the B cell receptor, these cells contribute to pathogen control via antigen specific antibody secretion. Antibodies limit pathogenic infection by several mechanisms. One function of antibody secretion is the clearance of a pathogen by direct binding to its surface, allowing for their opsonization and removal by phagocytic cells such as macrophages. Opsonization is also an important means of pathogen uptake by dendritic cells for presentation to T lymphocytes, a critical event for activating cellular immunity. Antibody coating of a pathogen is also a critical activator of the complement cascade which functions to control pathogens through direct lysis (83). In the case of HIV-1, however, the humoral response against the virus during the acute phase of infection has been found to actually play a role in the dissemination of virus as the process of antibody-dependent opsonization allows HIV-1 to specifically target lymphoid tissue sites containing the virus' primary cellular hosts (84). The most effective mechanism by which the humoral immune response can control infection is through production of high titer antibodies that specifically inhibit or neutralize protein epitopes critical for infectivity (56). In HIV-1 infection, this involves blocking or inhibiting specific antigenic sites of the gp120 envelope protein critical to CD4 receptor and CCR5/CXCR4

correceptor binding, as well as external portions of gp41 critical to facilitating virus host membrane fusion (85,86,87,88).

HIV-1, however, displays an array of measures to counter the antibody response. For instance, the HIV-1 gp120 Env displays extensive glycosolation on its exterior protein surface to hide antigenic sites (89). Epitopes critical to virus neutralization are often found buried within native interior structures of the gp120/gp41 complex (90). A high degree of amino acid sequence variability is typically found on the exterior surface of gp120, largely representing the variable or V-loops of Env (89). The extent of genetic hypervariability localized to HIV-1 Env sequences highlights the extreme capacity of the virus for mutational escape from the host antibody response (91). Indeed, temporal studies of HIV-1 quasispecies, reveal a great divergence in HIV-1 sequences from seroconversion to the onset of disease (81,82). There is evidence as well that antibody selection pressure is actually exploited by HIV-1 to enhance its replication as the variable loops in HIV-1 env appear to function as decoy antigens, promoting antibody-mediated targeting to Fc receptor expressing dendritic cells and macrophages in lymphoid tissue(92).

A broad neutralizing antibody response has been noted in studies of several LTNP cohorts (54,93,94). Neutralizing antibodies, however, have been found to be slow in their development during HIV-1 infection and do not appear at significantly potent serum titers until after seroconversion (54). This suggests that development of a potent neutralizing antibody response during asymptomatic infection is best promoted by a potent cellular response against the virus during the acute phase of infection. This notion is supported by studies comparing the

cytokine milieu of LTNPs versus rapid progressing HIV-1 patients in which a bias towards a Th1 type helper response, which favors cellular immunity, has been found to correlate with healthy status (95). A switch from Th1 to Th2 cytokine profile has been noted in several studies as a marker appearing to defining the onset of AIDS (96,97,98). Additionally, evidence has emerged that Th2 type cytokines, which help activate humoral immune responses, produce inflammatory conditions that promote Fas-mediated apoptosis of uninfected CD4+ T cells (99). Thus, shifts towards humoral immune responses that are coincident with impaired cellular immune responses appear to compromise overall immunological control of HIV-1 infection.

1.3.2 CD4+ T cell Response

CD4+ T cells are distinguished from other immune cells by the specific expression of the CD4 molecule and T cell receptor specificity for peptides expressed in the context of MHC class II molecules. CD4+ T cells, also referred to as "T helper" or Th cells, function to recognize peptide antigens derived from extracellular components of invading pathogens based on the clonally-derived specificity of their T cell receptors. These cells serve as a bridge between humoral and cellular immunity (100,101). As such, two functional subsets of CD4+ T cell can be defined: (a) Th1-type cells which mediate pathogen replication control by secretion of inflammatory molecules and (b) Th2-type cells which are critical activators of antigen-specific B cells (102). A distinction between the two cell subsets can be made based on patterns of cytokine secretion. In Th1 cells, increased levels of IL-2, IL-12, and IFN-γ are typically detected and found to promote strong cellular immune responses, while Th2 responses result in increased

IL-4, IL-5, IL-6, and IL-10 cytokine secretion which functions to promote B cell differentiation and proliferation (103).

CD4+ T cell concentration in blood been shown to inversely correlate with HIV-1 viral load (1,2,3), demonstrating the cytotoxic relationship HIV-1 infection has on CD4+ T cells. The nature of HIV-1 cellular tropism and virus dissemination puts the virus in a position to preferentially target CD4+ T cells specific for HIV-1 antigen and thereby cause their accelerated depletion by virus-induced toxicity and/or immunological elimination (105,106). While direct infection might account for much of the CD4+ T cell impairment during acute and asymptomatic infection, studies have demonstrated that HIV-1 also has more intricate adaptive mechanisms to counter a virus-specific T helper response. One indication that HIV-1 actually modulates the CD4+ T helper response is the broad Th1 to Th2 switching that has been noted at the onset of AIDS (96,97,98). While the mechanisms behind this are unclear, recent studies suggest that HIV-1 may be inducing biases towards Th2 responses by direct impairment of the Th1 response at the earliest stages of asymptomatic HIV-1 infection. In studies by Graziosi *et al.* (107) and Maggi *et al.* (108), peripheral and lymph node CD4+ T cells in unfractionated and pure populations were found to display no increases in gene expression of Th2 cytokines IL-4 and IL-10 that correlated with advanced disease. A third group corroborated this observation, however found instead a severe impairment in CD4+ T cell secretion of IL-12, a Th1 cytokine, at the earliest stages of asymptomatic disease (109). Further investigation into the matter subsequently determined that HIV-1 infection itself actively downmodulates expression of IL-12 in CD4+ T cells, thereby directly impairing a Th1 response (110). This explained data which demonstrated that infection of Th1 type CD4+ T cells promoted a switch from Th1 to Th0 (108), and it is Th0

and Th2 CD4+ T cells in which HIV-1 replication is most productive (108,111). In such a manner, HIV-1 infection appears to directly impair development of Th1 responses at the earliest stages of disease, thereby steadily biasing CD4+ T cells towards Th2 type that serve to promote humoral immune effector functions.

Recent investigations have demonstrated further intricacies in HIV-1-induced impairment of Th1 type CD4+ T cell responses that appear to be important factors in disease progression. A comparison of CD4+ T cell responses between HIV-1 and other viral infections, such as EBV and CMV, highlight dysfunctions of Th1 subsets that appear to be specific for HIV-1 infection. EBV and CMV are largely controlled by cellular immune responses. Within EBV- and CMV-specific Th1 CD4+ T cell response, three specific populations can be defined: (a) cells secreting IL-2 but not IFN-γ that are associated with conditions of antigen clearance, (b) cells secreting IFN-γ but not IL-2 that are typically associated with antigenic persistence and high viral loads, and (c) cells that doubly secrete IL-2 and IFN-γ which typifying maintenance of low viral loads in the face of persistent antigenic exposure (112). In the case of HIV-1 infection, however, investigations have found that only in nonprogressing HIV-1 infected subjects are the three distinct Th1 subsets maintained among HIV-1-specific CD4+ T cells, while a decrease in the frequency of single IL-2 secreting and doubly secreting IL-2/ IFN-γ phenotypes is correlated with disease progression (113). It remains to be determined whether the differential display of Th1 subsets between disease progressing and nonprogressing HIV-1 subjects is directly induced by virus or is simply a reflection of viral immunopathogenicity. Studies have demonstrated that HAART intervention results in enhancement of CD4+ T cell responses (114) and the restoration of polyfunctional Th1 CD4+ T cell subsets (113). This would indicate that suppression of HIV-1

replication below a certain threshold is crucial for the development of effective CD4+ T cell responses against HIV-1. Hence, other effector arms of the adaptive immune system are likely critical for the emergence of protective T helper responses to HIV-1.

1.3.3 Antigen-dependent CD8+ T cell response to HIV-1

CD8+ T cells constitute the earliest detectable adaptive immune response to acute HIV-1 infection (51,52) and are a critical factor in slowing the disease progression to AIDS (61,62). A convincing demonstration of this can be found in studies of the Rhesus macaque AIDS model where *in vivo* depletion of CD8+ T cells during asymptomatic infection using monoclonal antibodies to CD8 leads to increased viremia and a more rapid progression to AIDS (115,116). Classically, T cell receptor recognition of peptides in the context of MHC class I molecules is the distinguishing feature of CD8+ T cells from their CD4+ cousins. Presentation of antigenic peptide-MHC class I complexes and costimulation by dendritic cells is the defining event for the activation and clonal expansion of an antigen-specific CD8+ T cell immune response (117). CD8+ T cells have been well characterized for their ability to control viral pathogenesis by one of two effector functions: (i) antigen-dependent killing of virus-infected host cells by perforin/granzyme lysis (118) and Fas-mediated apoptosis (119), and (ii) release of antiviral cytokines (120). However, a special case applies to HIV-1 immunopathogenesis as CD8+ T cells are also known to possess an antigen-independent effector mechanism against lentiviral replication (121,122). Hence, a distinction is made here between the classical antigen-dependent effector mechanisms of CD8+ T cells (MHC Class I-restricted CTL activity and secretion of antiviral cytokines) and the noncytolytic antigen-independent CD8+ T cell response that is the subject of this study.

A. CD8+ cytotoxic T lymphocyte (CTL) activity

A defining feature of cellular immunity is the CTL activity employed by CD8+ T cells to kill pathogen infected cells. In CD8+ T cells, TCR recognition of antigenic peptide in the context of MHC class I molecules serves as the main trigger for a cascade of coordinated molecular interactions resulting in the polarization of the microtubule organizing center to facilitate fusion of lytic granules with the plasma membrane (123). Granular compartmental release leads to extracellular secretion of perforin, a cell membrane lysis protein with homology to complement protein C9 (124), and granzyme A and B proteins which both enter the cytoplasm of targets cells after perforin lysis (125). Granzyme B promotes the cleavage of caspase activating a caspase-dependent apoptosis pathway (126), while Granzyme A activates a caspase-independent apoptotic pathway (127). The CTLs themselves are protected from cytoxicity by cathepsin B serine protease that is expressed on the membrane of lytic granules and translocated to the cell surface upon degranulation (128). Upon TCR triggering, CTL also mediate target cell death through the cell surface expression of FasL that interacts with Fas/CD95 on target cells to induce yet another apoptotic pathway (119). The localization of FasL on the periphery of TCR clusters is noteworthy as it is coincident with the same cell surface location of lytic granule fusion, suggesting that FasL and perforin/granzyme CTL functions are interdependent (129).

B. CD8+ T cell secretion of antiviral cytokines

CD8+ T cells also elicit a second effector mechanism upon specific antigen-recognition: the secretion of inflammatory cytokines that serve in regulation of immune responses as well as

induction of pathogen-infected cells into limiting infection (130). In the case of viral infection, antigen-specific TCR triggering often leads to secretion of antiviral cytokines that induce target and bystander cells to activate intracellular pathways that recognize, and interfere with, common molecular signatures of viral replication (131). A characteristic CD8+ T cell secreted cytokine marker is IFN-γ, which has been shown to be important in the control of hepatitis B virus (130) and herpes simplex virus type 1 (120). One of the proteins induced by IFN-γ signal transduction is the ubiquitously expressed kinase, PKR (131). PKR functions in the recognition of double stranded RNA, a molecular motif present in many viral replication mechanisms, including HIV-1. PKR recognition of dsRNA leads to the protein's autophosphorylation and dimerization. In this activated state, PKR induces several signaling pathways that can directly interfere with viral replication, including phosphorylation of eIF2-α to inhibit protein translation and inactivation of IKK leading to activation of NF-κB (132), a transcriptional activator of a variety of genes involved in inflammation, apoptosis, and cell cycle control (133).

The PKR pathway is one of many antiviral pathways that can potentially inhibit HIV-1. However, the nature of this pathway illustrates a point about the nonlinearity of cytokine-induced responses that apply to all inflammatory molecules. For while PKR promotes a shut down of host translation, it also induces NF-κB, a potent transcriptional activator HIV-1 employs to enhance its proviral gene expression (134). The PKR pathway is one of several examples illustrating that the overall effect on viral replication by an inflammatory cytokine depends on the balance between intracellular signaling pathways that induce or inhibit the virus. This may underlie inconclusive results investigators have found as to the effect of IFN-γ on HIV-1 replication, as different studies have reported divergent results from stimulatory to inhibitory to

neutral for this cytokine. Another example of this differential effect of cytokines on HIV-1 infection is the TNF-α induced signaling pathway (135). While TNF-α signaling through TNFR1 activates a caspase-dependent apoptosis pathway, TNF-α signaling via TNFR2 results in NF-κB activation (136). In fact, exogenous addition of TNF-α to HIV-1 infected cultures has been found to have a stimulating effect on viral replication in CD4+ T cells and macrophages (137).

Nonetheless, several CD8+ T cell-secreted cytokines mediating antiviral effects *in vitro* have been identified. IFN-α, TGF-β, and IL-16 have all demonstrated inhibitory effects on HIV-1 replication in CD4+ T cells and monocyte-derived macrophages (137). In addition, IL-10 and IL-13 have been shown to induce antiviral effects on HIV-1 replication in macrophages (138). CD8+ T cells have also been demonstrated to secrete β-chemokines MIP-1α, MIP-1β, and RANTES (139), with their antiviral effects mediated by blocking CCR5 access to HIV-1 gp120 (140). However, these antiviral effects of CD8+ T cell secreted molecules should be taken in the context of complex network of cellular- and cytokine-mediated interactions that occur in response to HIV-1 infection in order to fully understand their overall effects on viral replication. That Th2 type environments confer a more stimulating environment over Th1 for HIV-1 infection are an example of the complexities inherent in the analysis of CD8+ T cell secreted inflammatory mediators inhibiting virus replication (141). This is in addition to the many strategies employed by HIV-1 to hijack cytokine- and virus-induced signal transduction pathways for its own replication advantage (142,143,144).

C. Evasion of Antigen-specific CD8+ T cell responses to HIV-1

With an overwhelming body of evidence clearly demonstrating the critical importance of CD8+ T cell responses in the control of HIV-1 replication and delayed disease progression, perhaps the relative importance of CD8+ T cells might further be inferred from the breadth of evasion mechanisms HIV-1 employs to thwart CTL activity. One of the classic mechanisms that HIV-1 has demonstrated in this capacity is mutational escape from antigen-specific responses. Several studies have documented instances of antigen-specific CTL-imposed selection pressures leading to subsequent HIV-1 genetic and amino acid changes that altering the presentation of the antigenic epitopes towards non-recognition by or non-presentation to CTLs (145-149). A recent analysis by John *et al.* (150) determined that at a population level, HIV-1 sequences changes tended to cluster in protein domains of minimal structural and functional constraint and were more frequently associated with patient HLA-specific MHC peptide binding domains. The same study also noted a temporal correlation between HLA-defined HIV-1 sequence changes and changes in viral load (150). Such findings are corroborated by other investigations suggesting a correspondence between heterozygosity at HLA-A, HLA-B and HLA-C loci and lower viral loads, presumably through presentation of a more diverse array of peptide antigens to CD8+ T cells than homozygosity at an HLA loci would allow (151).

Mutational escape under selection pressure, however, is not the only strategy employed by HIV-1 to evade the CD8+ T cell response. The viral accessory protein Nef plays a very important role in this regard. Nef has been shown to selectively downmodulate MHC class I molecules expressed from HLA-A and HLA-B loci but not HLA-C or HLA-E (152). This selective downregulation of MHC class I molecules reduces the extent of HIV-1 antigen

presentation to CTLs while avoiding killing by NK cells that are triggered by the absence of HLA-C and HLA-E (153). In such a manner, Nef plays a crucial role in minimizing a combined CTL and NK cytolytic response in order to maximize viral replication. Nef appears to also be important for another mechanism of thwarting CD8+ T cell action by specifically interfering with CTL induction of apoptotic pathways. Studies have demonstrated that Nef prevents spontaneous apoptosis mediated through the TNF-α apoptosis pathway (154). Nef also interferes with Fas-induced cell death through inhibition of ASK1, a kinase participating in FAS and TNF-alpha apoptosis (155). Another point at which Nef interferes with apoptotic pathways is through the binding and inhibition of p21-activated complex and PI-3 kinase, preventing its interaction with the proapoptotic factor Bad (156). Such a diverse function for Nef in minimizing the CD8+ T cell-induced cytolysis and apoptotic responses may be why infection with *nef*-deleted virus is coincident with a dramatically delayed disease progression to AIDS (157).

While utilizing mutational escape and inhibition of host cell death for its replication advantage, HIV-1 also appears to employ more aggressive tactics against CD8+ T cells. In simian studies, Nef has been shown to increase FasL expression on the surface of infected CD4+ cells (158,159). The HIV-1 transcriptional activator Tat has also been shown to promote FasL expression (160). The viral gp120, particularly from X4 isolates, has also been demonstrated to induce expression of FasL and membrane-bound TNF-α (161). The effects of HIV-1-induced FasL expression have been demonstrated to directly induce CD8+ T cell apoptosis *in vitro* and *in vivo* (162). Through a wide array of mechanisms HIV-1 is able to not only evade the antigen-specific CD8+ T cell response, but also employ tactics that promote the elimination of these cells over the course of chronic infection. With such a chameleon-like capacity to evade antigen-

dependent immune responses, an antigen-independent immune effector mechanism may be the key to effective control of HIV-1 infection and delayed progression to AIDS.

1.3.4 Noncytolytic Antigen-Independent CD8+ T cell suppression of HIV-1

Noncytolytic antigen-independent CD8+ T cell suppression of HIV-1 replication is the least understood CD8+ T cell effector mechanisms and represents a major gap in understanding the full correlates of immune protection against disease progression to AIDS. Early in the AIDS epidemic, researchers studying clinical HIV-1 infection observed that the rate of HIV-1 disease progression appeared to correlate inversely with the ability to isolate virus from patient peripheral blood mononuclear cells (163). A decisive study into the cause of this phenomenon was made by Walker *et al.* (121) in which the investigators demonstrated that CD8+ T cells were the critical component suppressing virus replication in PBMC *in vitro*. The authors demonstrated that CD8+ T cells could induce apparently complete suppression of virus when added to CD8+ cell-depleted PBMC from an HIV-1-infected patient compared to CD8-depleted cultures. However, upon removal of CD8+ T cells, viral replication rebounded to levels higher than infected CD8-depleted PBMC cultures alone. This suggested that CTL activity was not responsible for the near-complete suppression of virus, indicating a noncytolytic mechanism was being employed by CD8+ T cells to suppress HIV-1 (121). In subsequent investigations by several groups, this noncytolytic mechanism was found to able to mediate potent suppression in HIV-1 replication in heterologous CD4+ targets in a non-toxic manner (164,165,166). A lack of MHC restriction was later confirmed for the activity (167,168,169), distinguishing it from the classical mechanism of cellular immunity. The noncytolytic antiviral activity was further found

to be able to suppress *in vitro* replication of a broad spectrum of HIV-1 isolates (170,171), including X4 and R5 tropic viruses (172), further suggesting the effector mechanism was not antigen specific like CTL activity.

A. Activation of MHC-unrestricted CD8+ T cell noncytolytic HIV-1 suppression

Several investigations have demonstrated a lack of noncytolytic HIV-1 suppression activity in CD8+ T cells from seronegative subjects that are readily detectable in most HIV-1 patients at the earliest timepoints of infection (173,174,175). More detailed temporal analysis during acute infection has demonstrated that noncytolytic suppression activity temporally coincides with the earliest detection HIV-1-specific CTL activity (51). This suggests a priming mechanism might be involved in the development of noncytolytic CD8+ T cell suppression of HIV-1. The nature of such a priming event for a noncytolytic anti-HIV-1 response is unclear, however its existence is partly supported by findings that noncytolytic HIV-1 suppressing CD8+ T cell populations exclusively display an activated HLA-DR+/CD28+ phenotype (176). It is unclear if such a development is functionally dependent on induction of CTL activity. A study by Hsueh *et al.* (175) appears to suggest that general CTL activation may remove a regulatory barrier for propagation of noncytolytic CD8+ T cell clones. These investigators reported that when primary CD8+ T cell clones were propagated by limiting dilution of bulk populations from seronegative subjects, a small percentage of clones exhibited readily detectable noncytolytic MHC unrestricted suppression activity against HIV-1 while bulk CD8+ T cell population displayed no such activity (175). The authors further discovered that mixing of CD8+ HIV-1 suppressive clones with an equal proportion of bulk CD8+ cells abrogated the suppressive activity of the clones beyond what would be expected from a dose-dependent response. These

results support the existence of a regulatory mechanism inhibiting the propogation of noncytolytic HIV-1 suppressive CD8+ T cell clones which may be removed by general CTL activation in response to primary infection (175). Corroboration for this idea is provided by one study that detected potent noncytolytic HIV-1 suppression by CD8+ T cells from an EBV-positive but HIV-1 seronegative subject (177). A second investigation additionally determined that clonal propagation of MHC-unrestricted noncytolytic HIV-1 suppressing CD8+ T cells is dissociated from CTL activity (178). Thus, if antigen-dependent CTL priming is a trigger for activation of the antigen-independent anti-HIV-1 activity, it may likely be mediated through a bystander effect as dissociation between CTL activity and noncytolytic HIV-1 suppression activity can be readily detected in CD8+ T cell clones specific for non-HIV-1 antigens (179,180).

B. Mechanism of CD8+ antigen-independent suppression of HIV-1 replication

Investigations into the molecular nature of the noncytolytic CD8+ cell HIV-1 suppression activity have revealed the dual involvement of a secreted factor and an apparent cell-contact dependent component in mediating the antiviral activity (172,181,182,183). Initial studies had demonstrated that filtered CD8+ cell-conditioned culture fluids were able to elicit suppression of virus in secondary infectious CD4+ cell cultures (171). The involvement of a secreted component was further supported by studies that demonstrated HIV-1 *in vitro* suppression of HIV-1 in CD4+/CD8+ cell co-cultures where infected CD4+ cells were separated from CD8+ T cells in a transwell system (181,183). The secreted component mediating this activity maintained all the attributes of non-allogeneic restriction and lack of toxicity displayed by the cell mediated activity (181,182,183). However, transwell experiments readily identified an apparent cell contact-dependent component of the CD8+ T cell noncytolytic anti-HIV-1 activity.

Mackewicz and Levy (184) reported that transwell-mediated HIV-1 suppression required ten times the amount of CD8+ cells as CD4+/CD8+ cell-contacted to elicit an equivalent amount of suppression. Similar findings by others (181) confirmed that cell-to-cell contact is the most efficient means for inducing maximum HIV-1 suppression. Thus, a secreted factor appears to only contribute partially to the overall noncytolytic activity. This is further indicated by findings that cell-contact mediated suppression is not necessarily coincident with secretion of an HIV-1 suppressing factor (183) with the converse also having been demonstrated as well (185). Taken together, these investigations suggest a mechanistic dissociation between cell-contact mediated suppression of HIV-1 and secretion of an antiviral factor (185).

While distinct mechanisms may underlie cell contact- and secreted factor-mediated HIV-1 suppression, a large body of evidence has revealed a single antiviral effector function linking the two. It has been widely reported now that both CD8+ T cells and a factor secreted by them are able to suppress HIV-1 by specifically inhibiting proviral transcription (172,186,187,188). Among the evidence demonstrating CD8+ cell suppression of HIV-1 transcription are: (i) CD8+ T cell Inhibition of HIV-1 gene expression but not proviral integration or reverse transcription (177,189); (ii) a temporal correlation of CD8+ T cell mediated HIV-1 suppression activity and potent inhibition of HIV-1 proviral transcription in a kinetic analysis of single cycle infectious assays (190); (iii) CD8+ T cell inhibition of spliced and unspliced HIV-1 transcripts in endogenously-infected cells (186); (iv) CD8+ T cell suppression of HIV-1 transcription in chronically infected but RT-defective 8E5 cell line (Chen and Gupta, unpublished data); and (v) inhibition of both tat-induced and PMA-induced HIV-1 LTR promoter by CD8+ T cells (190) and a CD8+ cell secreted factor (172). In particular, studies of HIV-1 suppression using LTR-

gene reporter assays define the most imperative functional link between the CD8+ cell-mediated and secreted factor antiviral activities. The suppression of PMA mitogen-induced LTR promoted transcription demonstrates that the antiviral mechanism specifically inhibits the viral LTR promoter and exclusively utilizes cellular and not viral proteins to mediate the suppressive effect since the LTR is efficiently repressed in the absence of HIV-1 Tat. This clearly explains the non-antigenic and MHC unrestricted nature of the activity as presentation of HIV-1 antigen or viral protein expression is not a prerequisite for CD8+ T cells or the CD8+ cell-secreted factor to elicit inhibition of LTR promoter activity.

The exact intracellular mechanism mediating LTR promoter suppression under induction of CD8+ T cells is not fully understood. In a study by Chang *et al.*, the secreted CD8+ cell factor suppression of the LTR was found to dependent on STAT-1 induction of secondary genes, but not through a direct effect of STAT-1 on the LTR itself (172). In a study of possible LTR promoter sequences involved in mediating the suppressive effect, 5' deletions of the LTR up to the transcription start site did not appear to abrogate the action of noncytolytic HIV-1 suppressive CD8+ T cells (Chen and Gupta, unpublished data). The non-involvement of NF-KB and sp1 binding sites in the HIV-1 LTR promoter for CD8+ T cell inhibition of HIV-1 transcription was additionally confirmed by Locher *et al* (170). These findings suggest that the LTR sequences involved in CD8+ cell-mediated HIV-1 promoter repression are located immediately downstream of the HIV-1 transcription initiation site. This may represent an important clue for defining the intracellular signaling pathways CD8+ cells use to induce suppression of the HIV-1 promoter. HIV-1 sequences overlapping the TAR region just downstream of the +1 transcription initiation site have been found to be involved in HIV-1

transcriptional silencing leading to a latent infection state in CD4+ T cells (191). Binding of nuclear factors LSF and YY1 to these DNA sequences leads to the recruitment of histone deacetylation enzymes to the HIV-1 transcription initiation site resulting in gene silencing by nucleosome compaction of LTR sequences (191). If CD8+ T cells are able to induce such a pathway in CD4+ cells, it would represent a very potent mechanism of viral suppression as the HIV-1 TAR region has been shown to be very highly conserved (192) and TAR RNA stem loop function is very sensitive to sequence changes (193) making mutational escape of for the virus conceivably difficult.

C. Molecular Characterization of the CD8+ cell secreted HIV-1 suppressing factor

In attempting to identify the molecular factors mediating the CD8+ T cell noncytolytic antiviral mechanism, many groups have focused on the soluble component of the activity, as it presents a much more practical means of biochemical elucidation than the challenges of identifying a cell-contact dependent component. However, the field of literature surrounding the secreted CD8+ cell antiviral factor (CAF) is somewhat confounded by divergent definitions of what noncytolytic HIV-1 suppression actually entails. Many investigators have defined their search for the elusive CAF based on biological assays that measure only inhibition of productive viral replication. Such approaches had identified anti-HIV molecules such as β-chemokines (139), IL-16 (194), α-defensins (195), and antithrombin III (196). However, subsequent investigations have revealed dramatic inconsistencies of these molecules to the hallmarks defining CAF activity. β-Chemokines could only explain inhibition of R5-tropic HIV-1 binding to CCR5 (20,195), and subsequent investigations demonstrated that antibody neutralization of β-

chemokines did not abrogate the suppression of both R5 and X4 viruses (197). While IL-16 can induce suppression of HIV-1 LTR promoter activity (198), neutralizing antibody to this cytokine does not abrogate CD8+ T cell suppression of HIV-1 and CD8+ T cells have not been found to secrete IL-16 at high enough concentrations to explain the observed suppression (199). α-Defensins were originally proposed to account for inhibition of X4-tropic HIV-1 as part of a multifactorial hypothesis for CAF, however, it was later demonstrated that CD8+ T cells were not the primary cellular source of the secreted α-defensins (200). The proposal of antithrombin III as a possible component of CAF activity was made upon purification of a serum factor that was specifically modified by CD8+ T cells to elicit anti-HIV-1 activity, however, this particular antiviral factor could only account for inhibition of X4 virus isolates (196).

At the present, no single known molecule has been show to fully account for CAF activity. However, in the process of attempting to define such candidate molecules, a set of anti-HIV molecules with variable inhibitory mechanisms had been identified. Thus, a hypothesis has been proposed by some that CD8+ T cell noncytolytic suppression of HIV-1 is due to multifactorial secreted components (201). Such a hypothesis envisions that β-chemokines mediate R5-tropic viruses while X4-tropic viruses are inhibited by other factors such as antithrombin III (201). However, a multifactorial hypothesis does not readily reconcile itself with evidence supporting the alternative view that the potency of noncytolytic CD8+ T cell suppression of HIV-1 is entirely due to the specific inhibition of the HIV-1 LTR promoter. The existence of such a specific and potent antiviral mechanism would argue against a divergent set of molecular factors, particularly molecular candidates which directly interfere with HIV-1 proteins. HIV-1 promoter inhibition in the absence of any other antiviral mechanism elegantly

explains all the hallmarks of noncytolytic HIV-1 suppression by CD8+ T cells without the caveats imposed by a proposal of multifactoral factors.

However, defining a single molecular determinant that accounts for transcriptional suppression as the sole mechanism underlying potent CD8+ T cell suppression would require reconciliation of cell-mediated and secreted forms of the activity. The dissociation observed between cell-contact dependent and secreted forms of the antiviral activity would argue against the factor being a purely soluble protein mediator since potent CD8+ T cell contact-dependent suppression of HIV-1 can occur in the absence of any detectable secreted activity of the same form (185). The alternative explanation would consequently be that the HIV-1 transcription suppressing factor exerts its activity through membrane-localized and secreted forms. This theory begs for a mechanistic explanation as to how the proposed factor is both soluble and membrane-bound.

To date, no cell surface molecule has been described for mediating noncytolytic HIV-1 suppression. Some attempts have been previously made to evaluate possible cell-contact determinants that mediate the antiretroviral transcription suppression activity. Barker *et al.* (202) reported that antibody inhibition of CD80 and CD86 costimulatory molecules on CD8+ T cells did not abrogate their antiviral activity, but did produce a small enhancement to the overall CD8+ cell suppressive effect. Mackewicz *et al.* (203), using a similar analysis, found that neither Fas nor FasL were involved in CD8+ cell noncytolytic anti-HIV-1 activity. The same group also reported that noncytolytic CD8+ T cell suppression of HIV-1 was not due to granzyme A/B release (204,205). The role of HLA-compatibility has also been evaluated by

several groups and found to only confer a slight enhancement of noncytolytic HIV-1 suppression activity when co-cultured CD8+ effector and infected CD4+ cells are autologous (168,169). One study also demonstrated that CD8+ T cell clones could display antigen-specific CTL activity and MHC unrestricted noncytolytic antiretroviral activity concurrently, though the two mechanisms were shown to be independent of each other (175). While these combined results are not surprising given the MHC-unrestricted nature of noncytolytic HIV-1 suppression, they remain the only reported analysis of any cell-contact factors in the context of noncytolytic HIV-1 suppression by CD8+ T cells.

The role membrane determinants might play in mediating noncytolytic CD8+ T cell suppression of HIV-1 transcription remains mostly unexplored. Defining a membrane-localized antiviral factor has its inherent complexities. Physical tethering of a molecule to cell membrane subjects it to a restricted environment dictated by the intricacies of cellular compartmentalization and lipid domain trafficking between cell surfaces and various intracellular compartments. The immune synapse, the so-called C-SMAC/P-SMAC complex (206), is a classic example of membrane protein regulation in CD8+ T cells. Another well known example is degranualtion during CTL activity, where TCR antigen recognition ultimately results in the translocation of normally sequestered intracellular membrane compartments to the cell surface (123). Therefore, if noncytolytic HIV-1 suppression was mediated by a cell surface protein, then the antiviral factor would be subject to similar membrane regulations in CD8+ T cells. In other words, protein expression of an antiviral membrane factor alone may not necessarily elicit the antiviral activity at the cellular level.

There are some paradoxical aspects for a membrane-bound suppressor of HIV-1. A mechanism must exist that allows the factor to appear in a secreted form. The trivial case would be a peripheral membrane protein, existing in equilibrium between membrane-localization and soluble molecule. A more intricate possibility is the factor being an integral membrane protein with a catalytically active domain that can be released by proteolysis. An example of this mechanism is the cleavage of membrane-bound TNF-α (207) and Fractalkine (208) into soluble cytokines. However, a more complex cellular machination that remains unexplored in CD8+ T cells may also account for secretion of a membrane-bound molecule – the extracellular release of tiny spherical lipid bilayer vesicles termed exosomes (209). This last possibility may be a more flamboyant theory for secreted CAF activity. However, it represents a phenomenon of eukaryotic cells that is gaining wider appreciation in the field of antigen presentation and tumor immunity (210). Several studies have demonstrated the ability of cell-free dendritic cell-secreted exosomes to modulate T cell responses *in vivo* (211). Such peculiar mechanisms are just beginning to be understood, as DC-secreted exosomes have been shown to harbor enriched amounts of functional peptide-loaded MHC class I and II molecules as well as other membrane-bound factors that typically help mediate antigen-specific DC activation of T cells through cell surface contacts (212). Thus, exosomes represent an intimate and functional connection between cell surface proteins and the specific endosomal membrane domains they are derived from. Accordingly, exosomes could provide a plausible mechanism for the appearance of cell-bound and cell-free membrane proteins that remains to be explored in CD8+ T cells. In the absence of any identification of a soluble factor identifying with the hallmarks of CD8+ T cell noncytolytic HIV-1 suppression, an exploration into the uncharted realm of CD8+ cell membrane proteins

might provide a novel starting point to unraveling the mystery surrounding CAF and cell-mediated suppression of HIV-1 transcription.

1.4 STATEMENT OF THE PROBLEM AND GOALS OF THE STUDY

The precise molecular mechanisms underlying noncytolytic CD8+ T cell suppression of HIV-1 transcription remain enigmatic. The importance of this CD8+ T cell effector mechanism can be inferred by its correlation to delayed disease progression and healthy clinical status in LTNP HIV-1 infected subjects. CD8+ T cell suppression of HIV-1 transcription represents a potent mechanism for inhibiting the virus due to the effector function's antigen-independence and MHC-unrestricted nature. However, a lack of insight as to the precise molecular mechanisms involved in this effector function has left a gaping hole in attempting to understand what the true correlates of protection against HIV-1 entail. No study to date has revealed the molecular identity of the factor mediating noncytolytic CD8+ T cell suppression of HIV-1. Despite clear evidence of cell-contact dependent suppression of virus, the role of membrane determinants in mediating CD8+ T cell suppression of HIV-1 has been largely unexplored. An investigation was therefore undertaken to elucidate the molecular nature of this potent antiviral activity based on the hypothesis that CD8+ T cell suppression of HIV-1 is mediated primarily by a membrane-bound factor. Preliminary work based on this postulate uncovered a membrane-localized activity that demonstrated suppression of HIV-1 replication. The goal of the ensuing study was to characterize this membrane antiviral activity and specifically determine if it identified with the hallmarks of CD8+ T cell noncytolytic HIV-1 suppression. In doing so, an investigation into how the membrane-localized activity might be secreted was undertaken to determine its possible identity to secreted CAF activity. The results of this study are presented in

the following two chapters. A broader discussion of the investigation follows with a new interpretation of the molecular mechanisms underlying CD8+ T cell noncytolytic suppression of HIV-1 based on the discovery of a novel membrane-localized and exosome-secreted antiviral factor.

2.0 CHAPTER TWO

Noncytolytic Suppression of HIV-1 Replication by CD8+ T Cell-secreted Exosomes

(U.S. Patent Application filed November 29, 2005. [Application Serial No.: 60/740,922])
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by

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2.1 ABSTRACT

It has been well established that CD8+ T cells can suppress human immunodeficiency virus type 1 (HIV-1) replication by inhibiting its transcription from the 5' long terminal repeat promoter (LTR). This antiviral activity is mediated in part by a secreted CD8+ cell antiviral factor termed CAF whose molecular elucidation remains undetermined. A potent membrane-bound HIV-1 suppressing activity was identified and found to be secreted as 30-100 nm sized endosome-derived vesicles termed exosomes. Biochemical analysis revealed the HIV-1 suppressive activity was found to be elicited by an integral membrane factor with some results suggesting the existence of a soluble isoform. Purified exosomes were found to suppress the *in vitro* replication of R5 and X4 isolates of HIV-1, inhibiting proviral transcription through the specific repression of LTR promoter driven gene expression in both acute and chronic models of infection. The results of this investigation demonstrate a significant contribution of exosomes to secreted CAF activity in CD8+ cell-conditioned culture fluids, providing for the first time direct evidence functionally linking membrane-mediated cell-contact dependent noncytolytic CD8+ T cell suppression of HIV-1 with a secreted mediator of the antiviral activity.

2.2 INTRODUCTION

In the context of HIV-1 replication, CD8+ T cells display a most peculiar antiviral effector mechanism that is distinct from its more characterized role of antigen-dependent cytotoxic T lymphocyte (CTL) activity. This rather unusual mechanism does not require major histocompatibility molecules (122,169), nor is expression of HIV-1 protein a prerequisite for the activity's elicitation (172,187,188). The target of this CD8+ T cell effector function is the repression of the HIV-1 LTR transcription promoter (170,172,188). This proviral transcription inhibition is the likely explanation for why CD8+ T cells from HIV-1 infected subjects are able to suppress lentiviral replication regardless of virus co-receptor usage (172,190). It may also be a major contributing factor to the striking correlation of long term asymptomatic status in certain HIV-1 infected subjects with the extent of noncytolytic antiviral activity displayed by their primary CD8+ T cells. A secreted CD8+ cell antiviral factor, termed CAF, has been shown to partly mediate the overall HIV-1 transcription suppression observed in cell cultures (172,181,183). However, no conclusive molecular identity has been made for this secreted antiviral activity and no known CD8+ cell-secreted molecule to date has been shown to conclusively identify with the hallmarks defining CAF- and CD8+ T cell-mediated noncytolytic HIV-1 suppression activity (182,199,200,239-241).

The identification of the molecule(s) mediating CAF activity has proven problematic, primarily due to its secretion in low amounts in CD8+ cell culture supernatants (184). A

technical development in the search for CAF has been the utilization of herpesvirus saimiri (HVS)-transformed CD8+ T cell lines. Use of these immortalized CD8+ cells in the study of noncytolytic HIV-1 suppression activity has been widely reported with demonstrations of HVS-transformed CD8+ T cell lines potently suppressing HIV-1 replication in a similar manner as primary CD8+ T cells (172,188,197,213,214). The immortalized nature of HVS-transformed CD8+ T cell lines allows for their use in generating large quantities of CD8+ cell conditioned media for biochemical purification of the antiretroviral activity. However, despite this advancement, attempts to purify the secreted anti-HIV factor from culture media using standard biochemical methods have yielded no conclusive results as to the identity of the molecule(s) involved in mediating the activity. Part of the difficulty in such an endeavor is the decreased sensitivity and reproducibility of traditional *in vitro* HIV-1 infection assays in evaluating the antiviral effects of biochemically-extracted fractions. This may be why inconsistent biological effects are often reported in the study of CD8+ T cell noncytolytic HIV-1 suppression (173,183,185). Recently, Chen *et al.* (173) described a semi-quantitative HIV-1 suppression assay using pre-infected cryopreserved acutely infected primary CD4+ T cells in which viral replication has been pre-titrated for use in a standardized and highly reproducibly acute infectious assay. This assay allows for analysis of CD8+ T cell HIV-1 suppression on a more sensitive micro-scale (1-5x10^4 infected target cells as opposed to other described assays typically utilizing 1x10^5 to 1x10^6 infected targets). In a more recent development, Chang *et al.* (172) described a 48 hour gene-reporter assay that measures secreted CAF activity specifically at the level of HIV-1 LTR inhibition. The assays described by Chen *et al.* (173) and Chang *et al.*(172) represent critical developments for quantitative detection of an antiviral activity found secreted in low amounts.

Technical limitations alone, however, may not be the primary reason why the molecular identity of CAF has remained elusive. Secreted CAF by itself may not play a significant physiological role in the overall CD8+ T cell antiviral effect as transwell studies have demonstrated that cell-contact is a far more efficient means for suppressing HIV-1 replication than a secreted mediator (184). This would imply that determinants localized to cell membranes may be primarily responsible for mediating the noncytotoxic antiviral activity, especially since cell-mediated activity can be detected in the absence of a secreted factor (183). Therefore, one reason why CAF purification has proven problematic may be the unexplored possibility that an integral membrane protein is primarily responsible for mediating the noncytolytic CD8+ T cell antiviral activity. This hypothesis, however, would require a mechanistic explanation for how the activity appears in a secreted form. One plausible model to explain the secreted component might be proteolytic cleavage of an active domain from an integral membrane protein akin to what is seen for TNF-α and Fractalkine secretion. The possibility of such a proteolysis model was studied by Mackewicz *et al.* (183), who found that certain protease inhibitors appeared to abrogate CD8+ cell- and CAF-mediated suppression of HIV-1. However, this diminishment of HIV-1 suppressive activity was incomplete and inconsistent between CD8+ cell samples and CAF-containing culture fluids from different patient samples. If the HIV-1 transcription suppressing factor is indeed an integral membrane protein, then the results of Mackewicz *et al.* (183) would suggest that the putative factor can suppress viral replication in the absence of its cleavage. If that is the case, there may be another more intricate yet unexplored mechanism to explain the secretion of an integral membrane protein with HIV-1 transcription suppression activity.

An alternative model that elegantly resolves the imparity of a secreted extracellular integral membrane protein is the cellular release of tiny membrane-limited vesicles termed exosomes (209). Exosome secretion has been described for a variety of cell types (209,216-219). Exosomes originate intracellularly through a budding process that creates vesicular invaginations within late endosomes (209). These budding lead to active formation of multivesicular bodies within the lumen of late endosomes that eventually form into the 30-100 nm diameter spherical exosomes. The exosome-containing late endosomes can undergo two fates: (i) fusion with lysosomes for degradation of intraluminal components or (ii) fusion with the plasma membrane leading to the extracellular release of vesicles (Figure 1).

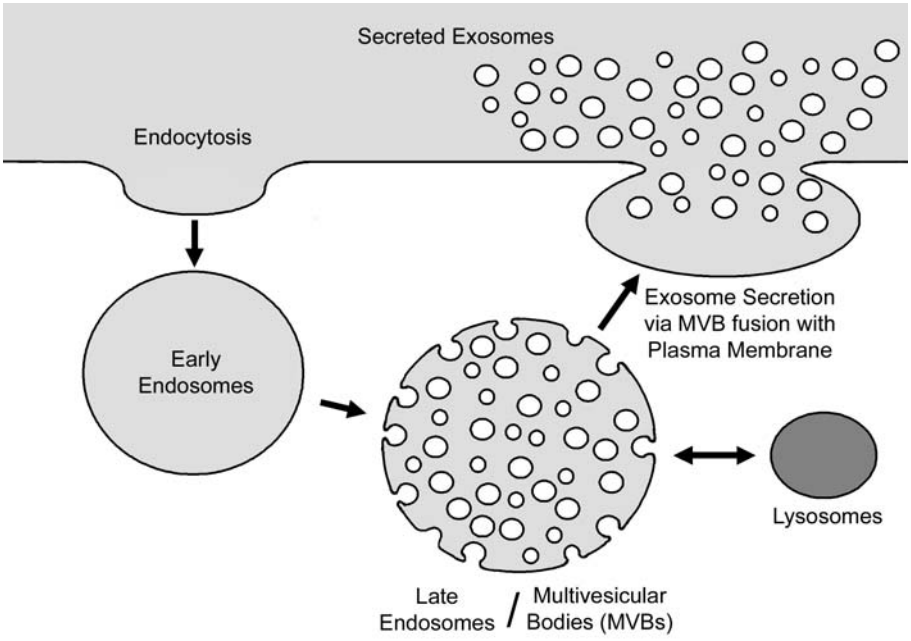


Figure 1. Endosome cycling and exosome secretion. Exosomes form by luminal invagination of late endosomes that result in the formation of multivesicular bodies (MVBs). The MVBs have two possible fates, fusion with lysosomes or fusion with the plasma membrane.

The morphogenesis of exosomes in eukaryotic cells is thought to primarily function in the degradation of cellular membranes by substantially increasing the effective lumen surface area accessible to lysosomal enzymes (220). However, an increasing body of evidence is beginning to suggest a variety of extracellular functions for these vesicles, particularly in the modulation of immune responses (218,219,221,222). Proteomic analysis of exosomes from a variety of cell types has demonstrated the specific enrichment of MHC Class I and II in the vesicles in addition to co-stimulatory molecules crucial for mediating activation of T cells (212,223). The most characteristic molecular signature of exosomes is their highly enriched tetraspanin protein content (223,224,225). These integral quadruple-spanning membrane proteins form distinct membrane protein clustering domains within cells that are analogous to but very distinct from lipid rafts (226). One tetraspanin in particular, CD63, is a specific lysosomal marker that delineates the intracellular origin of these vesicles from the same endosomal lumen through which cell surface expression of MHC Class II molecules is also effected (225). As such, exosomes provide a direct link between cell surface expression and extracellular secretion for a variety of membrane bound proteins.

Therefore, a seminal investigation was undertaken to ascertain the existence of specific CD8+ cell membrane determinants mediating noncytolytic suppression of HIV-1. Preliminary findings of potent HIV-1 activity in cellular membranes purified from CD8+ T cells prompted a more thorough evaluation of this activity's mechanisms and physiological context. In the ensuing study, evidence was uncovered demonstrating a direct mechanistic connection between cell-contact dependent and secreted factor- mediated HIV-1 antiviral activity through exosomes with the membrane-localized activity specifically inhibiting transcription from the HIV-1 LTR

promoter, a hallmark of CAF- and CD8+ cell-mediated noncytolytic antiretroviral activity. A significant contribution of exosomes to secreted CAF activity was uncovered along with evidence suggesting the concurrent existence of a membrane- and soluble- form of the HIV-1 transcription suppression activity. These results suggest that the complexity of CAF may not be the result of multifactorial cytokine secretion but due to a finite antiviral factor that is secreted in a membrane-bound vesicular form.

2.3 MATERIALS AND METHODS

Cell lines and Virus Stocks

The transformation of primary CD8+ T cells with herpesvirus saimiri (HVS) has been previously described (197). We utilized a particular HVS-transformed CD8+ T cell clone, TG, which was derived from primary CD8+ T cells purified from the peripheral mononuclear blood cells (PBMC) of an AIDS patient and transformed as previously described (197). Primary CD4+ T lymphocytes were selectively enriched as previously described (173) by immunomagnetic bead depletion of CD8+ cells from PBMC donated from an uninfected seronegative donor. Primary CD8+ T cells from two asymptomatic HIV-1 infected subjects were obtained through the Multicenter AIDS Cohort Study (MACS) at the University of Pittsburgh. The TZM-bl cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Transzyme, Inc. The 8E5 cell was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Thomas Folks. TG, 8E5, H9, Raji, U937, primary CD4+ and CD8+ T cells were cultured in growth medium consisting of 20% FCS/RPMI supplemented with 25 mM HEPES and penicillin/streptomycin. TG cells and primary CD4+ and CD8+ T cells were supplemented with 5 U/ml of recombinant IL-2 (Roche, US). TZM-bl cells were cultured in 10% FCS/DMEM supplemented with penicillin/streptomycin. The M-tropic (R5) HIV-1 isolate 33015 was derived from an HIV-1 infected long-term nonprogressor patient from the MACS. The T-tropic (X4) HIV-1 isolate 33074 was obtained from an HIV-1 infected rapid progressor

patient from the MACS. Immunomagnetic beads (Dynal, Norway) were utilized for cell separation (anti-CD8 antibody coated beads) and exosome phenotyping (anti-MHC class II antibody coated beads). For exosome phenotyping by flow cytometry, fluorescently-labeled monoclonal anti-CD9, anti-CD63, anti-CD81, anti-CD14, and anti-CD34 and control isotype mouse IgG1 antibodies (Research Diagnostics Inc., US) were utilized.

Semi-quantitative Acute Infectious Suppression Assay

Suppression of acute HIV-1 infection was assayed using a semi-quantitative acute infectious suppression assay essentially as described by Chen Y *et al.* (173). Peripheral blood mononuclear cells were isolated from an uninfected seronegative subject by ficoll-hypaque. Anti-CD8 antibody coated immunomagnetic beads (Dynal, Norway) were used for separation of CD8+ and CD8- populations. CD8-depleted cells were cultured for six days in the presence of OKT3 and rIL-2 to expand and enrich for CD4+ T cells. After stimulation, cells were pretreated for 1 h with 5 μg/ml polybrene, washed, and incubated with either HIV-1 R5-tropic 33015 strain or X4-tropic 33074 strain of HIV-1 for 2 h. Cells were washed after infection and cultured for 2 days in 20% FCS/RPMI with rIL-2. Cells were then DMSO-cryopreserved for use as target cells in an acute infectious suppression assay. A standardized protocol for measuring the HIV-1 suppression activity of a sample was performed by thawing the crypreserved HIV-1 infected CD4+ cells and coincubation of TG cells or derived biological sample. HIV-1 suppression activity of the sample was measured five days later as the percent reduction in extracellular p24 gag production, as measured by ELISA of culture fluid. This assay has demonstrated a high degree of standardization and reproducibility (173,197).

Preparation of cell membrane

TG cells were harvested from culture, and cell pellets were made containing $1x10^8$ to $5x10^8$ million cells over the course of TG cell culture and stored at -70 ºC until preparation of membrane. Frozen pellets were thawed and resuspended into STM solution (sucrose, tris-HCl, $MgCl_2$) and subjected to three additional freeze-thaw cycles using ethanol/dry ice for freezing and thawing in a 37°C waterbath. The disrupted cell suspension was homogenized using a Dounce homogenizer and the homogenate was clarified by centrifugation at 800xg at 4ºC to remove large cellular debris. Supernatant from this spin was then subjected to ultracentrifugation at 60,000xg for 30 minutes to pellet raw cell membranes. The pellet was then resuspended, overlayed on a 75% sucrose density cushion and recentrifuged at 90,000xg at 4 ºC for 1.5 h. The band above the 75% sucrose interface was extracted, washed in STM buffer, re-pelleted by centrifugation, and resuspended in HBSS or RPMI. Protein concentration was measured using the BioRad Protein assay.

Purification of exosomes

Exosomes and other membrane fractions were harvested from culture supernatants by an adaptation of methods previously described (227,228) involving serial centrifugation of culture supernatant followed by sucrose density gradient purification. Conditioned culture fluid from TG cell cultures was harvested and first subjected to centrifugation at 300xg for 10 min to remove cells. The supernatant was then subjected to serial centrifugations of increasing centrifugal force to derive depleted-supernatants and membrane pellets at 800xg for 30 min, 6,000xg for 30 min, 15,000xg for 30 min, and 60,000xg for 1 h with all spins performed at 4 ºC. In such a manner, secreted membrane vesicles were derived at each centrifugation step with

smaller vesicles pelleted at increased centrifugal force. As exosomes typically pellet at centrifugal force >10,000xg (227), the 15,000xg pellet was utilized for harvesting exosomes to avoid contamination with serum protein complexes from the culture media. A discontinuous sucrose density gradient separation was employed consisting of fractionation of the resuspended 15,000xg membrane pellet through a two layer sucrose column consisting of a 40% sucrose (1.14 g/ml) layer overlayed over a 60% sucrose (1.21 g/ml) cushion at 4ºC. After centrifugation at 28,000xg/4ºC, membrane fractions banded over the 40% and 60% sucrose interfaces and were extracted for further analysis and confirmation of exosome isolation in the 60% sucrose density fraction. Sucrose fractions were washed in HBSS, pelleted by centrifugation at 18,000xg and resuspended in HBSS. Protein concentration was measured using the BioRad assay. For other cell lines in this study, such as primary CD4+ T cells, H9, Raji, 293T, and HeLa, exosomes were prepared from culture fluids from these cells by the same method used to harvest exosomes secreted by TG cells.

Transmission Electron Microscopy

Copper grids (200 mesh) were formvar coated using 0.125% formvar in chloroform and floated on a drop of a highly concentrated exosome sample for approximately 30 seconds. The grids were removed and excess sample solution was wicked away with filter paper, then placed on a drop of 0.45 μm filtered 1% uranyl acetate in de-ionized ddH_2O (dI- ddH_2O) for 30-60 seconds. Excess stain was wicked away and samples were viewed on a JEOL JEM 1210 transmission electron microscope at 80 kV. Exosomes that were attached to immunomagnetic Dynal beads were pelleted at 500xg in a 1.5 ml microfuge tube and fixed in 2.5 % glutaraldehyde in PBS for 1 h. Pellets were washed three times in PBS then post-fixed in 1% OsO_4, 1%

$K_3FE(CN)_6$ for 1 h. Following 3 additional PBS washes, the pellets were dehydrated through a graded series of 30-100% ethanol then infiltrated in Polybed 812 epoxy resin (Polysciences Inc, US) for 1 h. After several changes of 100% resin over 24 h, pellets were embedded in a final change of resin, cured at 37ºC overnight, followed by additional hardening at 65ºC for two or more days. Ultrathin (70 nm) sections were collected on 200 mesh copper grids, and stained with 2% uranyl acetate in 50% methanol for 10 minutes followed by 1% lead citrate for 7 minutes. Sections were viewed using JEOL JEM 1210 transmission electron microscope at 80 kV.

Flow Cytometry Analysis of Exosomes

Flow cytometry analysis of exosomes was adapted from methods previously described (232). Anti-MHC class II antibody coated immunomagnetic beads (Dynal, Norway) were used to capture exosomes by incubation of high concentration vesicle sample (as determined by protein concentration) with 2.5 x 10^5 beads. Bead-captured vesicles were washed twice in cold buffer (4% FCS/PBS) and incubated with 10 μg/ml of anti-CD9, anti-CD63, anti-CD81, anti-CD14, anti-CD34, or isotype control biotinylated mouse IgG1 monoclonal antibody (R&D systems) for 30 minutes at room temperature. Beads were washed twice in cold buffer and incubated for 15 minutes room temperature with 1:50 diluted straptavidin-phycoerythrin conjugate (Invitrogen, US). After a third round of washing, beads were fixed in 1% paraformaldehyde and analyzed on a Beckman Coulter EPICS XL.MCL Flow Cytometer.

Protease Treatment

Aliquots of containing 60 μg of TG exosome were pelleted by centrifugation at 17,000xg and resuspended in 1 ml of 5 μg/ml trypsin or 1 ml of 5 μg/ml Trypsin + 5 μg/ml Chymotrypsinogen A or control exosomes resuspended in HBSS as a control. Protease treatments and controls were incubated at 37 ºC for 6 h. Protease-treated exosomes and control were then pelleted by centrifugation, washed with HBSS and resuspended in 300 μl of culture media (20% FCS/RPMI).

Delipidation of Exosomes

Exosomes were pelleted by centrifugation. In one experiment, delipidation of exosomes was performed by the Bligh and Dyer method (233). Pelleted exosomes were resuspended in a 2:1 mixture of chloroform/methanol, resulting in extraction of lipids into chloroform phase, proteins into the methanol solution and an insoluble precipitate at the chloroform/methanol interface. The three fractions were extracted and dried for further analysis. In a second delipidation method, cold acetone (-20 ºC) was used to dissolve exosomes and precipitate membrane protein. Precipitated proteins were resuspended into RPMI, centrifuged for 5 minutes at 17,000xg to separate undissolved proteins from those remaining in solution. Undissolved and dissolved proteins after acetone delipidation were analyzed for HIV-1 suppression activity.

Acute HIV-1 Transcription suppression assay

An assay for measurement of LTR promoter inhibition in a model mimicking acute infection was adapted from the methods of Chang *et al* (172). TZM-bl cells were seeded 25,000 cells/well and cultured at 37 ºC for 24 h. TZM-bl cells were then incubated with TG exosomes

or culture fluid sample for 16-24 h at 37 ºC. Cells were washed twice with media prior to LTR activation. For gene-reporter expression induced by virus infection, TZM-bl cells were inoculated with HIV-1 primary isolate 33015 and supplemented with 8 μg/ml DEAE-dextran for 1 hour, washed with media and incubated at 37 ºC for 24 h after infection. For Tat-transactivated LTR induction, TZM-bl cells were liposome-transfected with the Tat-expressing plasmid pSVtat using the Lipofectamine 2000™ reagent according to the manufacturer's instructions (Invitrogen, US). For mitogen-activation of the LTR promoter, TZM-bl cells were incubated with 100 ng/ml PMA (Invitrogen, US) for 12 hours. The extent of LTR-induced gene expression of β-galactosidase was measured using the β-GLO™ Assay (Promega, US).

Chronic HIV-1 Transcription suppression assay

8E5 cells were incubated in the presence or absence of TG exosomes over a time course of 26 days. Cell numbers were maintained between 5,000 and 50,000 cells per well in a 96 well plate and cell numbers were adjusted every 5-7 days with replenishment of media alone or media supplemented with TG exosomes. At each 5-7 day timepoint, 1000 cells were collected and intracellular HIV-1 RNA copies per 1000 cells was measured using a standardized NASBA-based HIV-1 RNA quantification assay (Organon Teknika).

2.4 RESULTS

2.4.1 Membrane from the CD8+ T cell line, TG, suppresses HIV-1 Replication

While CD8+ T cell noncytolytic suppression of HIV-1 has been previously described as mediated by soluble factors, experiments in which CD8+ T cells and HIV-1 infected CD4+ cells are separated by a semi-permeable membrane demonstrate that this antiviral mechanism is most efficient with cell to cell contact. Therefore, a seminal experiment was performed to determine if membrane protein derived from CD8+ T cells could suppress HIV-1 in a similar manner observed with cell-mediated suppression. The TG CD8+ T cell line was grown to a sufficiently large quantity for cell membrane purification. The TG cell line itself displayed potent dose-dependent HIV-1 suppression activity against acutely infected primary CD4+ T cells (Figure 2A). Cell membrane was purified from this cell line and was found to also mediate the same dose-dependent noncytolytic and nontoxic HIV-1 suppressive effect in an acute infection assay (Figure 2B). A temporal kinetic analysis of TG cell and membrane suppression of HIV-1 replication was performed by assessing the % suppression of HIV-1 replication over a time course of 0 to 96 h using HIV-1 p24 timepoints at 24 h intervals. TG cell and purified TG membrane was consequently found to suppress HIV-1 replication with equivalent kinetics (Figure 2C), suggesting a common antiviral mechanism underlying both means of noncytolytic HIV-1 suppression.

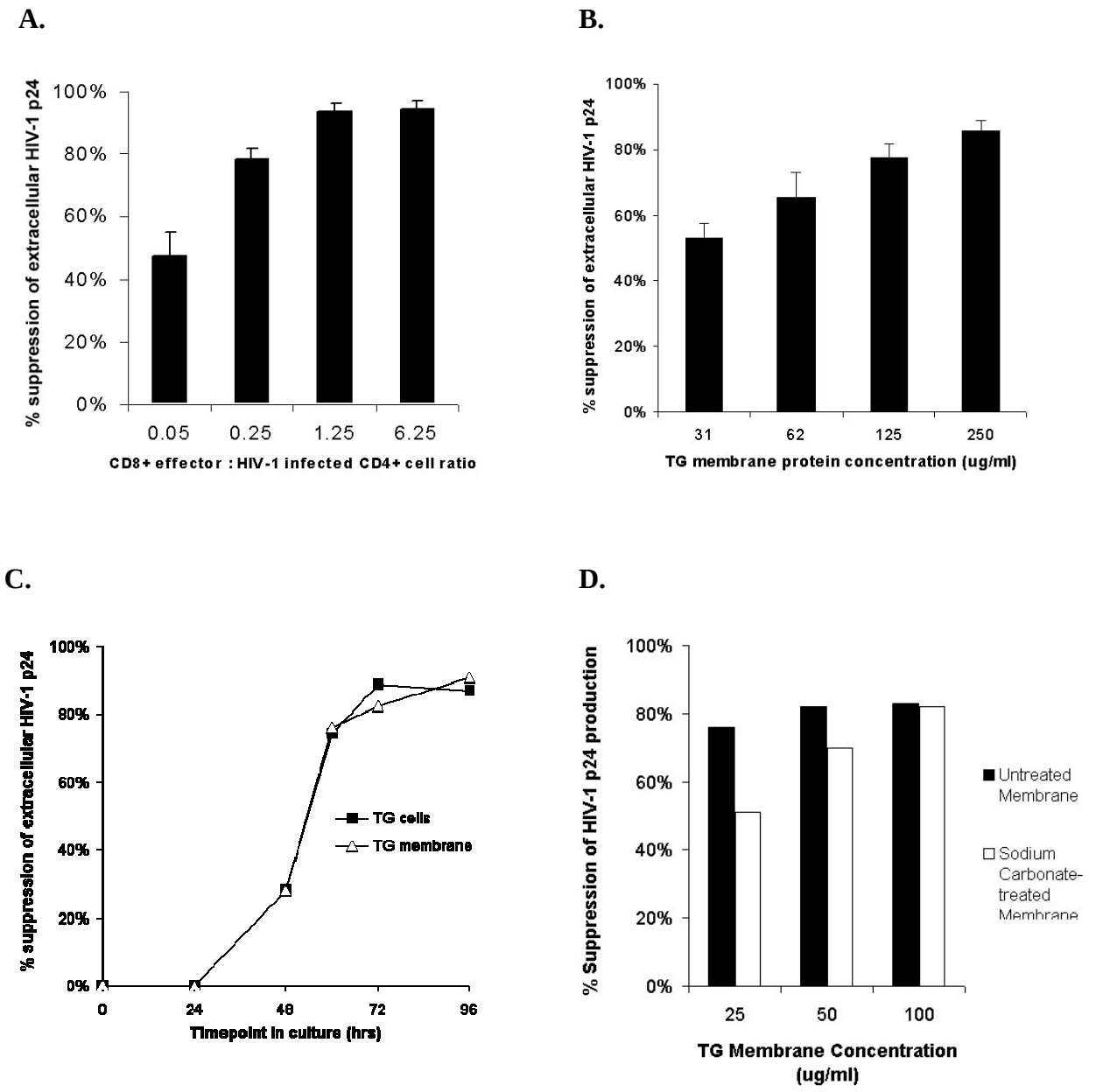


Figure 2. Dose-dependent suppression of HIV-1 replication in acutely infected CD4+ T cell by the TG CD8+ T cell line (A) and by membrane purified from TG cells (B). TG cells (Blacks Squares) and purified TG membrane (White Triangles) displayed equivalent time kinetics in suppressing replication of HIV-1 (C). The activity was tightly associated with the membrane as sodium carbonate treatment only moderately diminished membrane mediated HIV-1 suppression (D).

Since a secreted factor has been previously described as one of the defining characteristics of noncytolytic HIV-1 suppression activity by CD8+ T cells, an effort was made to discern if the TG membrane-mediated HIV-1 suppression activity was mediated by a peripheral membrane protein. Purified TG membrane was treated with 0.1 M sodium carbonate, pH 11.5, to deplete peripheral proteins from the membrane. After treatment, membrane was pelleted by centrifugation at 17,000xg, washed, resuspended in media, and assayed alongside an untreated control for dose-dependent HIV-1 suppression activity. A moderate decrease in membrane-bound HIV-1 suppression activity of 32% was detected after sodium carbonate treatment of TG membrane at the lowest dose assayed (Figure 2D), indicating that the majority of the antiviral activity still resided in the membrane after treatment, a result consistent with the presence of an integral membrane factor suppressing HIV-1.

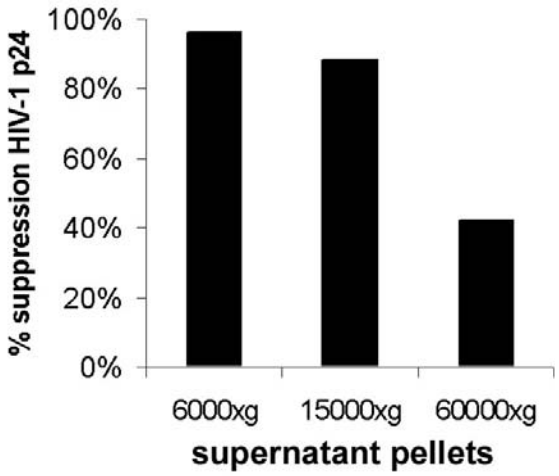
2.4.2 TG cell-secreted membrane vesicles suppress HIV-1 replication

To account for the membrane-bound nature of the antiviral activity and its reported appearance in a secreted form, we hypothesized that this activity might be released in a vesicular form by CD8+ T cells. This was based on the reasoning that if the CD8+ cell surface contained an HIV-1 suppressive activity, then vesicles secreted by TG cells would likely carry a portion of the same membrane determinants found on the cell surface and consequently also suppress HIV-1 replication. The secreted vesicles reported in the literature have been described as two general types: (i) 1 uM sized microvesicles originating from the plasma membrane and (ii) 30-100nm sized exosomes originating intracellularly from endosomal compartments (228). Therefore, an investigation was conducted as to whether the TG cell line might also be secreting similar vesicles containing HIV-1 suppressive activity. Conditioned media from our TG cell cultures

was subjected to increasing serial centrifugation to derive membrane pellets of decreasing size. In such a manner, fractions of 6000xg, 15000xg, and 60000xg pellets were collected from cell-free culture media of TG cells and standardized by volume. These fractions were assayed for suppression activity using the acute infectious suppression assay and found to contain potent HIV-1 suppression activity peaking at 96% suppression for the 6000xg fraction and 87% suppression for the 15000xg fraction (Figure 3A). To verify whether these peak TG culture supernatant membrane fractions also maintained the same integral membrane anchoring of HIV-1 suppression activity that bulk TG membrane displayed after removal of peripheral proteins, the 6000xg and 15000xg fractions were treated with 0.1 M sodium carbonate in the same manner done for bulk membrane, and after assaying for HIV-1 suppression, no significant diminishment of antiviral activity was found upon removal of peripheral proteins in either fraction (Figure 3B). This further corroborated the existence of an integral membrane factor mediating HIV-1 antiviral activity.

As these extracellular membrane fractions clearly contained a tightly bound HIV-1 suppressive activity, elucidation of their intracellular origin was sought in order to determine the functional nature of the membrane bound activity. A good candidate for these membrane vesicles appeared to be exosomes as they typically pellet at centrifugal force greater than 10000xg (227,228). Therefore, a 15000xg extracellular membrane sample was prepared and fractionated using a discontinuous sucrose gradient consisting of a layer of 40% sucrose over a 60% sucrose cushion. The sucrose gradient was based on previous methods that demonstrated exosomes being harvested within a 1.14-1.21 sucrose density gradient range (227,228).

A.



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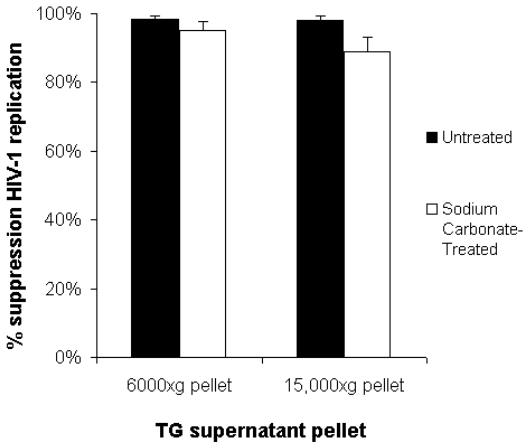

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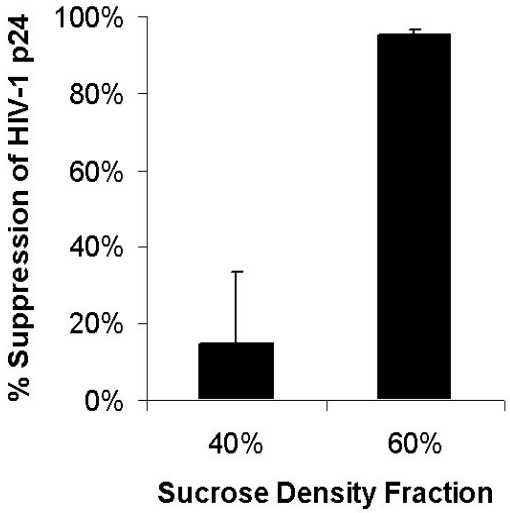


Figure 3. HIV-1 suppression activity in TG secreted vesicles: (A) Serial centrifugation of secreted vesicles. (B) sodium carbonate treatment did not diminish the HIV-1 suppressive activity of the 6000xg nor the 15000xg TG supernatant pellets. (C) Sucrose density fractionation of 15000xg pellet with peak antiviral activity in the 60% sucrose density fraction.

After fractionating the 15000xg sample, two distinct bands were harvested, one pelleting at the 40% sucrose interface representing densities of 1.0-1.14 g/ml and a second band pelleting at the 60% sucrose interface representing densities in the 1.14-1.21 g/ml range. The two fractions were washed, pelleted, resuspended in HBSS, standardized to equivalent protein concentration, and assayed for HIV-1 suppression activity. Potent antiviral activity suppressing HIV-1 replication by 95% was specifically observed in the 1.14-1.21 g/ml extracellular membrane fraction purified from the membrane band floating above the 60% sucrose density cushion, with only 15% HIV-1 suppression observed in the 40% fraction (Figure 3C).

2.4.3 Identification of HIV-1 suppressing TG vesicles as exosomes

The specific localization of HIV-1 suppression activity to the 60% sucrose density fraction is significant as it corresponded to the sucrose densities previously reported for exosomes secreted by other cell types (223,225,227,242). We therefore sought to elucidate the identity of these TG secreted particles. A fresh 15000xg/60% secreted membrane sample was prepared for analysis by transmission electron microscopy (TEM). TEM revealed the highly enriched presence in the 15000xg/60% sample of vesicles resembling the 30-100nm size and spherical morphology of exosomes, as previously described for a variety of other cell types (Figure 4).

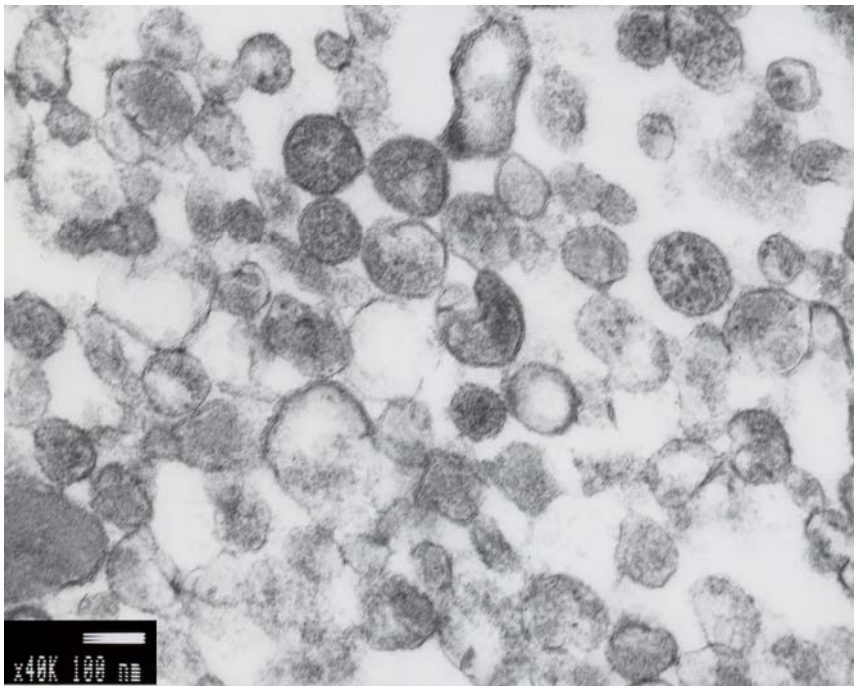


Figure 4. Transmission Electron Micrograph of the 15000xg / 60% TG supernatant vesicle fraction. The legend in the bottom left corner indicates magnification at 40,000 X with the white bar indicates a 100 nm measure.

In order to confirm the identity of these TG vesicles as exosomes, a recently described exosome bead-capture technique (232) based on the enriched presence of MHC Class II molecules on the endosomally derived vesicles was employed. The bead-capture technique utilizes immunomagnetic beads coated with antibodies specific for MHC Class II molecules. By saturating the surface of the 4.5 μm diameter beads with the nanovesicles, the antigenic content of these vesicles can then be probed to confirm the presence of specific exosome markers. The 15000xg/60% vesicle fraction was incubated with anti-MHC class II immunomagnetic beads at 4°C overnight after which the beads were magnetically isolated and washed. Two aliquots of beads after vesicle incubation were made, one for electron microscopy analysis to confirm bead capture and the second aliquot for determining the antigenic content by flow cytometry.

Analysis of the immunomagnetic beads by ultrathin section electron microscopy revealed the saturation of the bead surface with the tiny vesicles, confirming their attachment to the beads (Figure 5A). A separate aliquot of the bead-captured vesicles was subsequently analyzed by flow cytometry to dissect their antigenic content using specific monoclonal antibodies specific for exosome markers. The specific presence of CD9, CD63, and CD81 was detected on the TG vesicles attached to the beads (Figure 5B, 5C, and 5D respectively) with CD63 producing the highest fluorescence shifts (Figure 5C). CD14, a macrophage marker, was not detected for these vesicles (Figure 5E) while only moderate amounts of CD34 (Figure 5F) were observed. In addition, antibody staining of control beads did not produce any fluorescence shift in control experiments (data not shown) indicating that the fluorescence shift relative to isotype control detected for CD9, CD63, and CD81 were specifically due to the presence of markers expressed on the vesicles attached to the beads. CD9, CD63, and CD81 belong to the tetraspanin family of proteins and have been found to be highly enriched in exosomes from a variety of cell types (212,243). Additionally, CD63 is a specific lysosomal marker that also traffics to endosomal compartments (229,230), so its high expression on the vesicles relative to other markers indicates their specific endosomal origin. Accordingly, the combined tetraspanin enrichment, endosomal origin, density in sucrose, size and morphology of these HIV-1 suppressing vesicles specifically identify them as TG cell-secreted exosomes with potent HIV-1 suppressive activity.

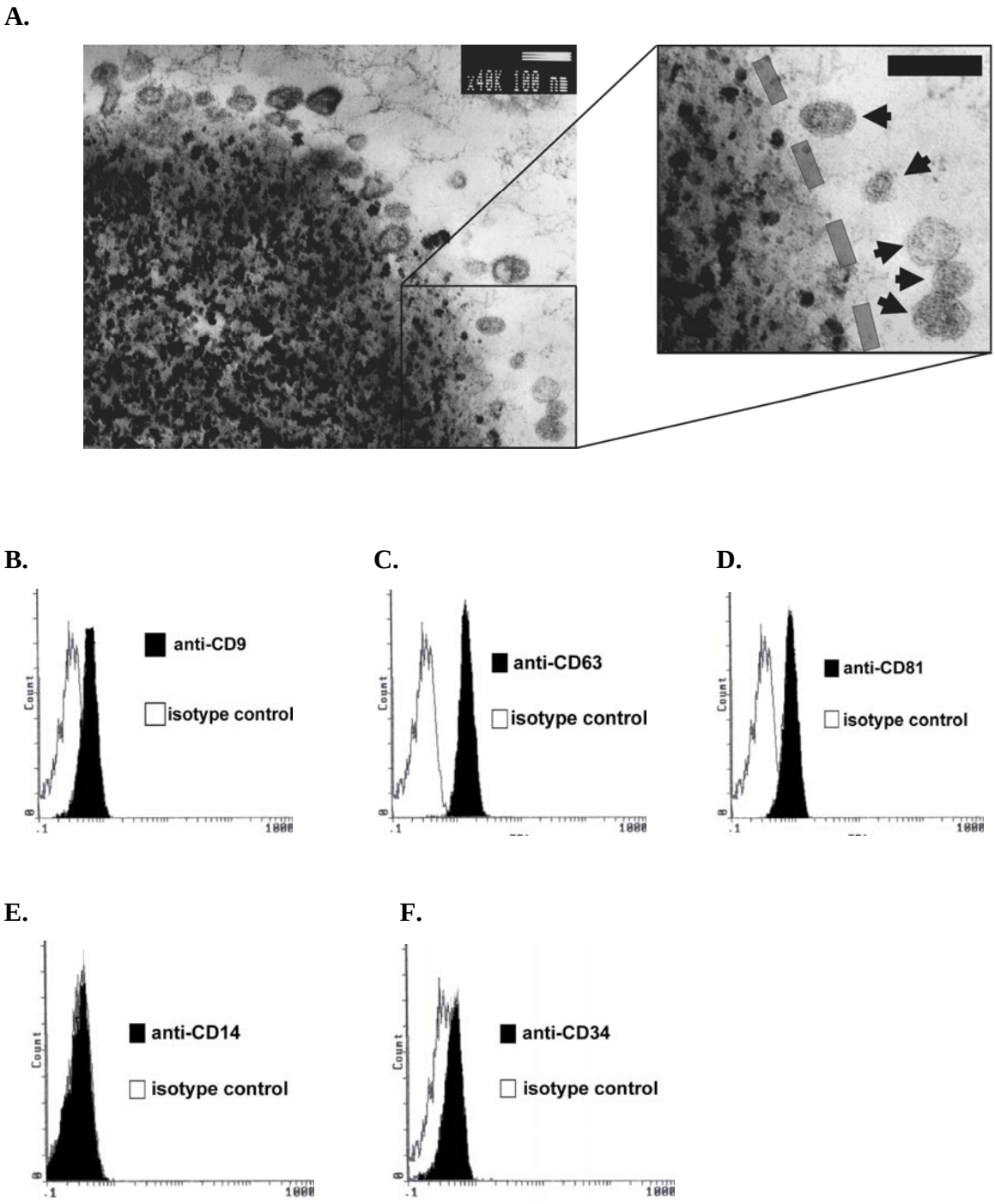
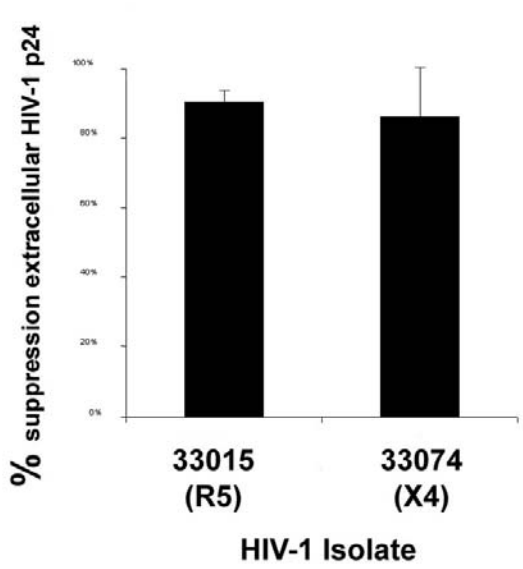


Figure 5. Analysis of 15000xg / 60% sucrose gradient TG vesicles by immunomagnetic bead capture. (A) Ultrathin section electron microscopy of anti-MHC Class II antibody coated immunomagnetic beads after incubation with the TG vesicles – an enlarged section is presented on the right with arrows pointing to exosomes, the broad dashed lines representing the bead perimeter, and the black bar as a 100 nm measure. (B to F) Flow cytometry analysis of the same vesicle-coupled beads for detection of (B) CD9, (C) CD63, (D) CD81, (E) CD14, (F) CD34.

2.4.4 TG exosome suppression of R5 and X4 isolates is protein mediated

A hallmark of noncytolytic CD8+ T cell suppression of HIV-1 is the inhibition of CCR5-tropic and CXCRX4-tropic HIV-1 replication (172). The TG exosomes were, therefore, assayed for their ability to suppress two patient derived HIV-1 isolates: (i) 33015, an R5 clinical isolate and (ii) 33074, an X4 clinical isolate. Using an acute infectious suppression assay, TG exosomes suppressed replication R5 and X4 HIV-1 isolates by 87% and 83% respectively (Figure 6A), demonstrating suppression of HIV-1 regardless of co-receptor usage. The same analysis was made for exosomes from a second and third independent TG cell culture, this time assaying both exosome- and cell-mediated suppression of R5 and X4 HIV-1 replication. In the second independent TG culture, both exosomes and cells were found to suppress HIV-1 replication regardless of viral tropism (Figure 6B), with exosomes suppressing R5 and X4 replication at 44% and 42%, respectively, and with cells suppressing R5 and X4 replication at 85% and 81%, respectively. However, in the third independent TG culture, only the exosomes were found to suppress both R5 and X4 HIV-1 replication at 35% and 40%, respectively, with no antiviral activity displayed by the cells from this culture (Figure 6C). This third independent TG culture demonstrated a clear dissociation between exosome- and cell-mediated HIV-1 suppression activity (Figure 6C) in a similar manner to what has been observed for CAF (183,184).

A.



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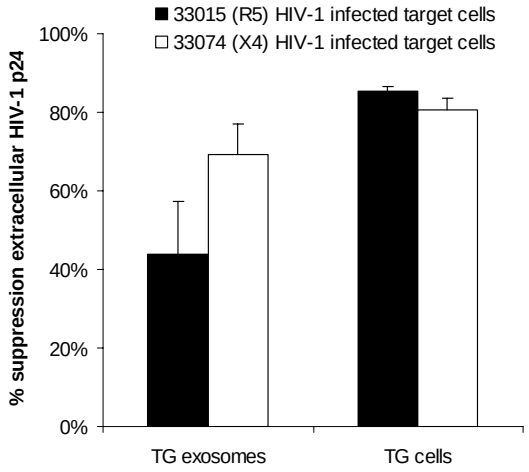

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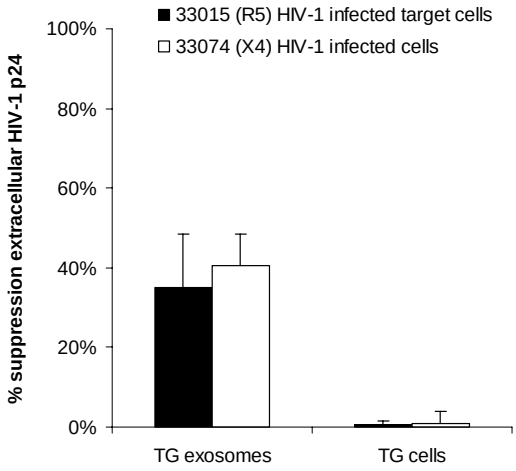

Figure 6. TG exosome suppression of R5 and X4 isolates. (A) TG Exosome suppression of HIV-1 isolates 33015 (R5) and 33074 (X4). (B) TG exosomes and cells from a second independent culture suppress replication of R5 and X4 HIV-1 isolates. (C) TG exosomes from a third independent culture suppress R5 and X4 HIV-1 isolates, however, cells from this same culture demonstrate no such suppression of HIV-1 replication.

In order to determine whether the antiviral action of TG exosomes was specifically due to a protein factor, separate exosome samples were subjected to treatment with trypsin or a combination of trypsin and chymotrypsinogen A for six hours for comparison with untreated exosomes. After protease treatment, exosomes were pelleted by centrifugation, washed and resuspended in media and assayed for HIV-1 suppression activity. Exosome treatment with trypsin alone did not diminish exosome-mediated HIV-1 suppressive activity, suppressing HIV-1 replication by 78% with untreated exosomes suppressing at 83% (Figure 7A). However, treatment of exosomes with a combination of trypsin and chymotrypsinogen A abrogated the antiviral activity of the vesicles, displaying only 7% (Figure 7A). The proteolytic inactivation of exosome-mediated HIV-1 suppression activity suggested that the active domain of the putative factor mediating the antiviral activity is expressed ectopically on the external surface of the TG exosomes. To corroborate this, a series of membrane delipidation experiments was performed to determine if a protein mediator could be extracted into solution from the exosomes. Such experiments were crucial to determining whether the exosome membrane or some lipid-mediated mechanism was involved in inducing HIV-1 suppression and to rule out a possible nonspecific lipid inhibition of HIV-1 replication.

The first exosome delipidation experiment was performed by dissolving exosomes in 2:1 chloroform/methanol, which extracts lipids into the chloroform phase, proteins into the methanol phase and as a precipitate at the chloroform-methanol interface (233). After subjecting TG exosomes to this treatment, the methanol soluble phase, the chloroform fraction and precipitated proteins were harvested, lyophilized using a speedvac, resuspended in media and assayed for inhibition of HIV-1 replication. HIV-1 suppression activity was specifically localized to the

precipitated proteins and the methanol soluble protein fraction, suppressing HIV-1 replication at 64% and 56%, respectively (Figure 7B). However, the extent of antiviral activity was greatly diminished in the chloroform fraction, suppressing HIV-1 replication by only 16%, indicating that the lipid moiety of exosomes was not involved in mediating HIV-1 suppression (Figure 7B). To further confirm this result, a second delipidation experiment was performed, this time using cold acetone to deplete exosome lipids. In this method, lipids are extracted into the organic phase producing a protein precipitate. Upon resuspension of the acetone precipitate protein, it was observed that not all the protein entered into solution, so a separation of the insoluble proteins from those that remained soluble was performed and both protein samples were assayed for HIV-1 suppression activity with the insoluble protein fraction assayed as a suspension. While a small amount of HIV-1 suppression activity was detected in the insoluble proteins (34% compared to 90% for untreated exosomes), most of the acetone-precipitated activity resided in the soluble fraction, suppressing HIV-1 replication by 66% (Figure 7C). Thus, the results of the two delipidation experiments corroborated the proteolytic sensitivity of the exosome-localized HIV-1 suppressing activity, demonstrating that the antiviral activity was specifically mediated by a protein expressed on the external surface of exosomes. Furthermore, this antiviral factor was able to mediate noncytolytic HIV-1 suppression independent of membrane anchoring.

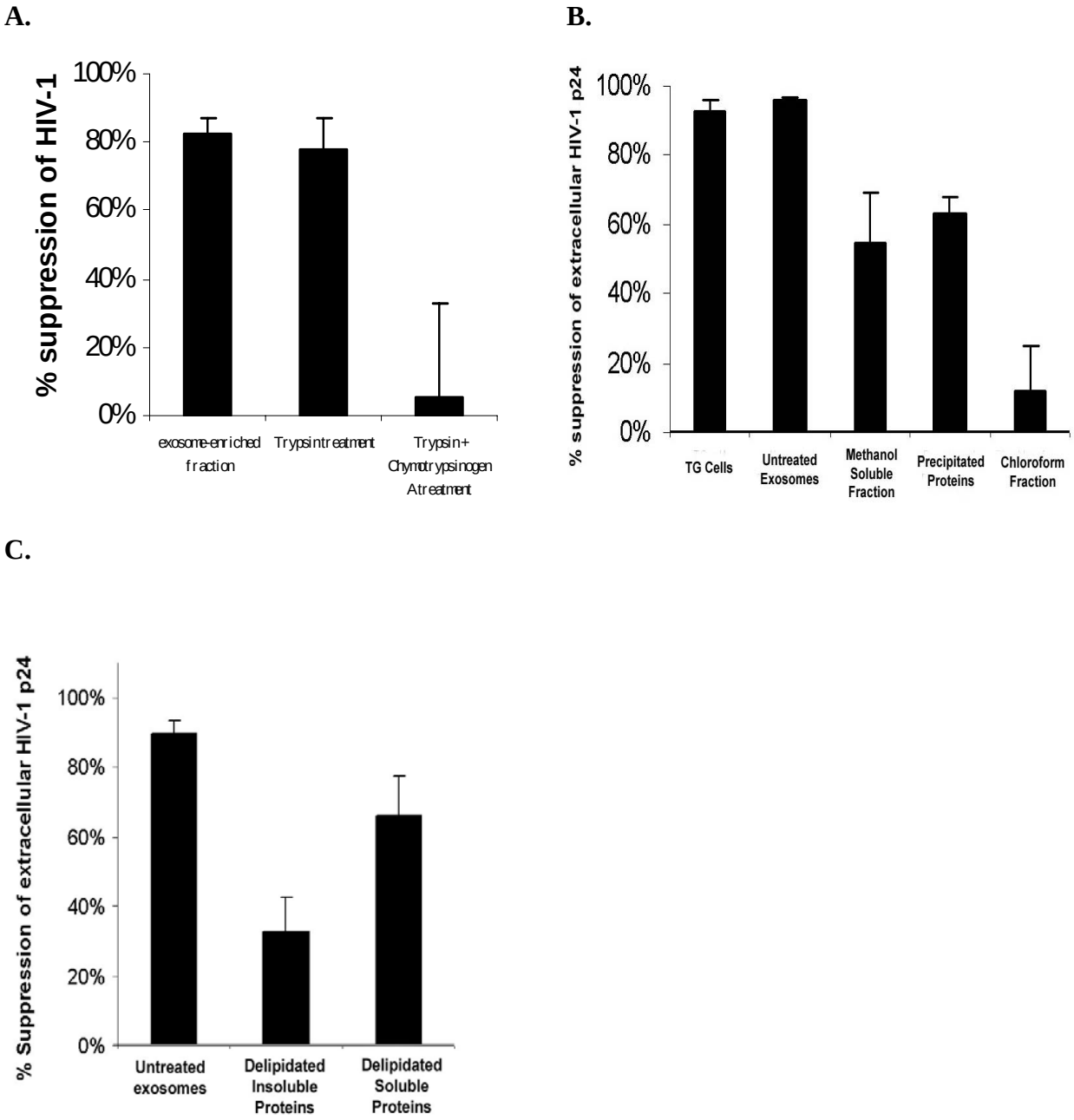

Figure 7. Exosome suppression of HIV-1 is protein mediated and does not involve exosome membrane fusion for activity. (A) Exosome suppression of HIV-1 is abrogated by proteolysis. (B) HIV-1 suppressive activity is extracted into protein phases after 2:1 chloroform/methanol delipidation. (C) Exosome delipidation with cold acetone precipitates protein containing HIV-1 suppressive activity.

2.4.5 TG exosome suppression of HIV-1 transcription

With clear evidence of an exosome-localized antiviral activity, the question of whether the TG exosomes specifically suppressed HIV-1 through inhibition of proviral transcription was subsequently explored. HIV-1 promoter suppression activity was first assessed in an LTR-activated gene-reporter assay that essentially mimics an acute infection model. Towards this aim, the HeLa-derived TZM-bl cell line that has been genetically engineered for stable expression of CD4 and CCR5 was utilized (234). This cell line contains two stably integrated LTR-reporter genes consisting of one construct with the 5'LTR fused to the β-galactosidase gene and a second construct with the 5'LTR fused to a luciferase gene. Expression of the gene-reporters can be activated in the cell line by HIV-1 infection, transfection of a tat-expressing plasmid, or by mitogen stimulation by PMA. The implementation of this cell line in developing an acute HIV-1 LTR suppression assay was based on the methods of Chang *et al* (172) who demonstrated the utility of HeLa cells for assaying HIV-1 transcription suppression activity in CD8+ cell culture fluids. These investigators subsequently explored the kinetics of CAF induction of LTR promoter inhibition and determined that maximum suppression of the LTR by CAF required a pre-incubation time on the order of 16 h before maximum suppression of transcription was observed (172). Therefore, in adapting the TZM-bl cell line for assaying acute LTR suppression, a similar kinetic titration was performed by preincubating TZM-bl cells with TG exosomes for 3, 6, 12, or 24 hours prior to LTR induction of gene reporter by HIV-1 inoculation. After LTR induction, cells were cultured for 24hrs upon which, intracellular β-galactosidase was assayed. Maximum suppression of LTR-induced β-galactosidase expression only occurred when exosomes were preincubated with TZM-bl cells for at least 6 hours (Figure 8A). This was in agreement with previous studies demonstrating a delayed induction of

transcriptional inhibition by CAF (172). To confirm that the exosome-induced block in β-galactosidase expression was specifically due to HIV-1 LTR promoter repression, TZM-bl cells were pre-incubated with TG exosomes for 12 hours, upon which β-galactosidase expression was activated by either virus inoculation, liposome-transfection with the Tat-expressing pSVtat plasmid, or mitogen activation with 100 ng/ml PMA. After 24 hour post-induction incubation of TZM-bl cells, potent suppression of the LTR promoter by TG exosomes was observed regardless of whether the LTR promoter was virus-, Tat-, or PMA-induced (Figure 8B).

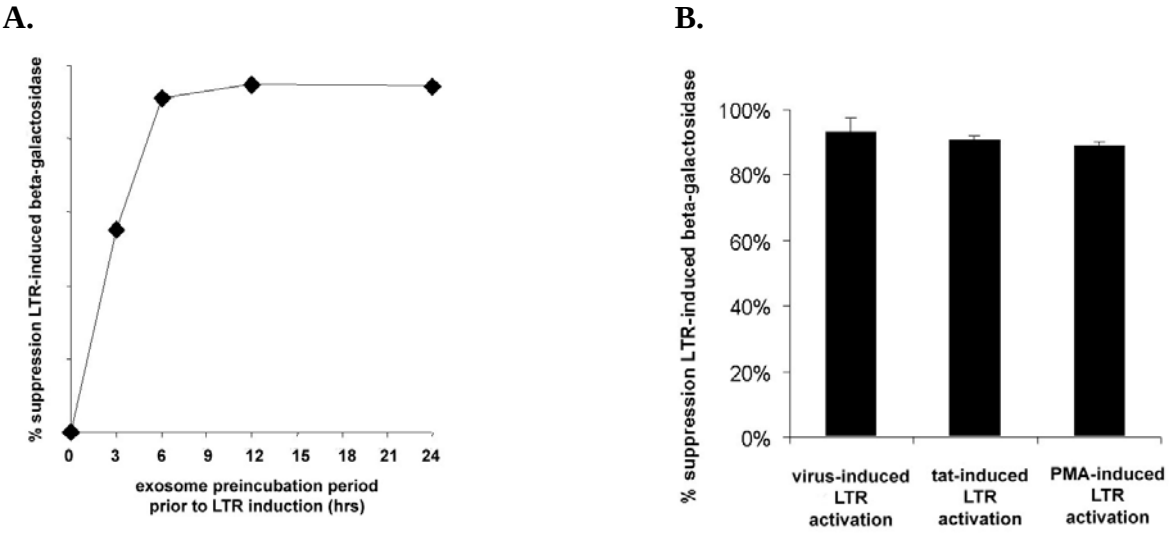

Figure 8. Exosome-mediated suppression of the HIV-1 LTR promoter. (A) TG exosome mediated suppression of TZM-bl cells requires at least a 6 hour preincubation for maximum suppression. (B) Exosomes specifically suppress the HIV-1 LTR promoter regardless the method of virus-, tat-, or PMA- induced LTR-gene reporter expression.

Since the LTR gene-reporter assay mimicked an acute infection model, the question of whether TG exosomes could also suppress HIV-1 transcription in a chronic model of infection was next investigated. The chronically-infected 8E5 CD4-negative T cell line (244) was utilized as a target to assay exosome-mediated HIV-1 transcriptional repression. The 8E5 cells contain a

single full-length copy of an integrated HIV-1 LAV genome with a null mutation in its reverse transcriptase that results in the production of non-infectious virions (244). Since no cell-to-cell transmission of virus occurs, any suppression of HIV-1 in the 8E5 cell line is specifically directed at a post-integration step of the virus life cycle. 8E5 cells were therefore cultured in the absence or presence of TG exosomes in a time course experiment. Total HIV-1 RNA copies per 1000 cells were measured every 5-7 days and cells were replenished at each time point with media alone or media supplemented with TG exosomes in addition to adjusting cell concentrations to maintain healthy cell growth. After measuring an initial transient spike in HIV-1 RNA at day 5 in 8E5 cells cultured in the presence of exosomes, a dramatic and sustained exosome-induced reduction of intracellular HIV-1 transcripts was observed with no such reduction for controls with a near 2 $\log_{10}$ reduction in viral between day 5 and the last timepoint of the experiment (Figure 9).

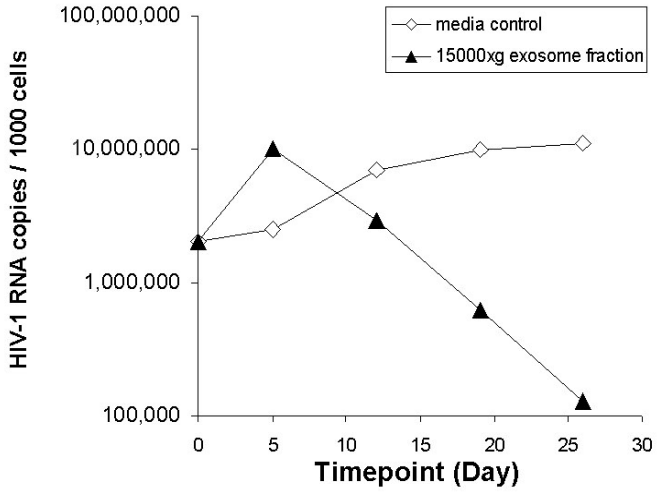

Figure 9. Inhibition of HIV-1 transcription in 8E5 cells by exosomes (black triangles) compared to controls (white diamonds).

The reduction of HIV-1 RNA only after Day 5 for 8E5 cells cultured in the presence of exosomes was consistent with a delayed kinetics of LTR promoter repression demonstrated in the TZM-bl cell line (Figure 8A) and by others for CAF (172). The potent HIV-1 transcription suppression in acute and chronic models clearly defines the mechanism TG exosomes employ to suppress HIV-1 replication, exhibiting the key hallmark defining CD8+ cell- and CAF- mediated HIV-1 transcriptional suppression.

2.4.6 Cell specificity of exosome-mediated suppression of HIV-1 transcription

Experimental results thus far appeared to identify secreted TG exosomes with the key hallmarks defining noncytolytic CD8+ T cell suppression of HIV-1, namely the suppression of R5 and X4 HIV-1 isolates and specific inhibition of the viral LTR promoter in acute and chronic models of infection. One question we wished to determine was whether these antiviral exosomes were specific only to the CD8+ T cell lineage. Previous studies have noted that cell-mediated noncytolytic HIV-1 suppression appears to be an exclusive function of CD8+ T cells (245). However, recent reports have described HIV-1 suppressive factors also being secreted by CD4+ T cells (231). The possibility that exosomes from other cell lineages might also suppress HIV-1 transcription was therefore explored. An assessment of the antiviral activity of exosomes from primary CD4+ T cells in comparison to TG exosomes was undertaken. Primary CD4+ T cells were collected from a seronegative donor and activated with OKT3 anti-CD3 antibody and recombinant IL-2 for 7 days. At Day 0 of the CD4+ T cell culture, an independent parallel TG cell culture was split into fresh media. At day 7, exosomes were harvested from both TG and CD4+ T cell culture fluids and stored at -70 ºC cells while TG and CD4+ T cells were recultured

in media supplemented with IL-2 for another seven days. At day 14, exosomes were harvested from TG and CD4+ T cell cultures. Both day 7 and day 14 exosomes from each cell type were assayed for HIV-1 transcription suppression activity using the TZM-bl cell-based acute LTR promoter suppression assay. For exosomes harvested at day 7, TG exosomes suppressed the LTR to a 2.3-fold higher level than CD4+ cell derived exosomes (Figure 10A, Black Bars). However, at day 14, CD4+ T cell secreted-exosomes were now found to suppress the LTR promoter by 90% compared to CD4+ T cell exosomes from day 7 which suppressed LTR transcription by only 34% (Figure 10A). These results indicated that exosome-mediated suppression of HIV-1 transcription was not necessarily exclusive to CD8+ T cells. A verification of this result was made by analyzing exosomes from several distinct cell lines. Large cultures of H9, a CD4+ T cell line; Raji, an EBV-transformed B cell line; U937, a monocyte cell line; and HeLa cells were made. Exosomes were purified from the culture fluids of these cell lines and assayed for HIV-1 transcription suppression. In corroboration with the previous result with primary CD4+ T cells, potent exosome-mediated LTR suppression activity was observed from H9 cell-secreted exosomes, suppressing the HIV-1 promoter activity by 87% (Figure 10B). A moderate amount of LTR suppression activity was also detected in Raji exosomes (51% LTR transcription suppression) with no LTR suppression was found in U937 exosomes, and only a small amount found for HeLa exosomes (22% LTR transcription suppression). These results demonstrate that exosome-mediated HIV-1 suppressive activity is not the exclusive domain of CD8+ T cells and suggest instead that the exosome-mediated antiviral activity is largely restricted to cells of the lymphocyte lineage, particularly T cells as only moderate antiviral activity was found in exosomes from the Raji B cell line. These results corroborate more recent findings of a CD4+ T cell secreted antiviral factor (231).

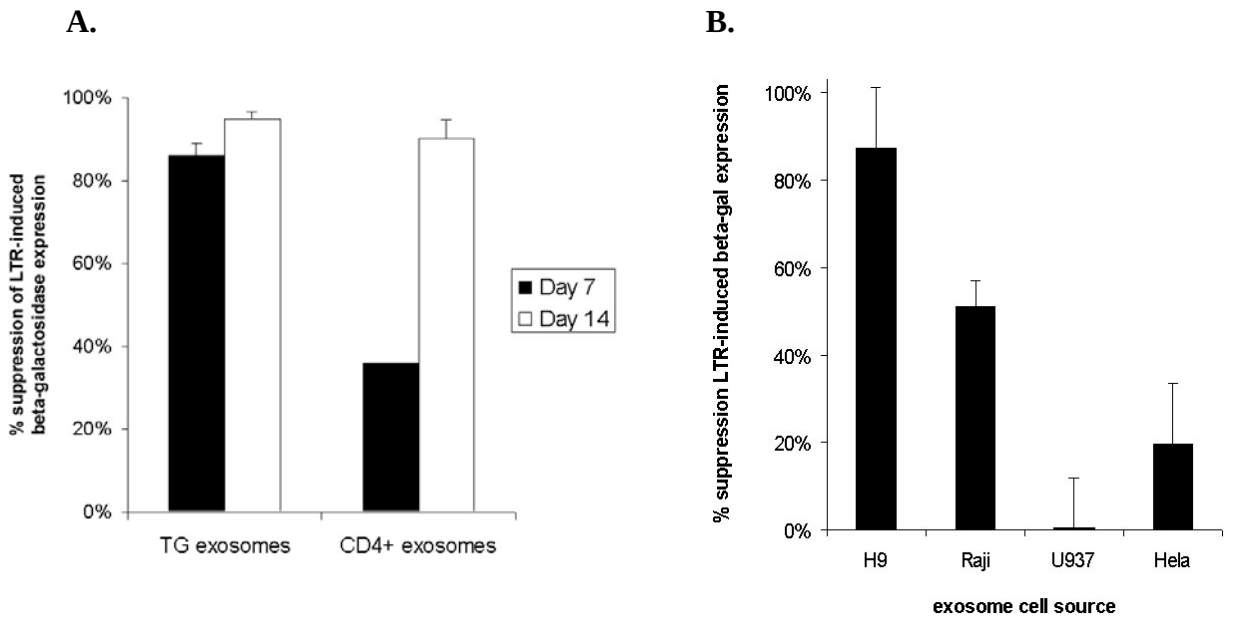

Figure 10. HIV-1 suppression activity of exosomes from other cell types. (A) Exosomes from activated primary CD4+ T cells display low HIV-1 transcription suppression activity at Day 7, but very high amounts of activity at Day 14. (B) HIV-1 transcription suppression activity by exosomes prepared from H9, Raji, U937, and HeLa cell culture fluids.

2.4.7 Contribution of exosomes to secreted CAF activity

Since TG exosomes represent a CD8+ cell-secreted antiviral factor with potent HIV-1 transcription inhibition activity, an investigation into the contribution of exosomes towards HIV-1 transcription suppression activity in CD8+ cell culture fluids was undertaken. Such an investigation was important to verify whether the secreted antiviral activity was due to CD8+ cell-secreted exosomes or to a soluble protein factor, as has been previously postulated (245). A starting point for this enquiry was taken from the original fractionation of membrane vesicles from TG culture fluids where it was observed that only moderate amounts of antiviral activity in 60,000xg membrane pellets compared to 6000xg and 15000xg pellets (Figure 3A). This would indicate that centrifugation at 15000xg depletes a majority of exosomes from cell culture

supernatant. The question consequently arises as to whether or not LTR suppression activity also diminishes in CD8+ cell culture supernatant after exosome-depletion. If a secreted factor is purely membrane bound, then reduction of the vesicles expressing the factor should be coincident with a reduction of antiviral activity. However, if vesicles are depleted but a substantial portion of the activity still remains, it would indicate the presence of a soluble membrane-free factor mediating the same activity. In an attempt to answer this question, five independent TG culture fluid samples were depleted by serial centrifugation at 300xg, followed by 800xg centrifugation, with a third centrifugation at 6000xg to deplete cells and large debris from the culture fluid samples. An aliquot of 6000xg-depleted supernatant was extracted and the remaining fluid was centrifuged at 15000xg to deplete exosomes. Both 6000xg-depleted and 15000xg-depleted supernatant were assayed for HIV-1 transcription suppression activity using the TZM-bl cell-based LTR promoter suppression assay. Only three of the five TG culture samples demonstrated large reductions in HIV-1 transcription suppression activity coincident with 15000xg centrifugation depletion of exosomes (Figures 11A, 11B, and 11C). Two of the five TG culture samples displayed no such significant reduction in LTR promoter suppression activity after exosome depletion (Figures 11D and 11E). The percentage reduction of HIV-1 transcription activity after 15000xg exosome depletion spanned a spectrum of 97%, 65%, 62%, 2%, and 0% reduction in LTR promoter activity (Figure 11F). These results indicate that HIV-1 transcription suppression activity in cell-free TG culture fluid can be mediated by either exosomes or a soluble factor, or in the case of two of the five samples analyzed where incomplete reduction of LTR transcription suppression was found after 15000xg centrifugation (Figures 11B and 11C), a combination of both.

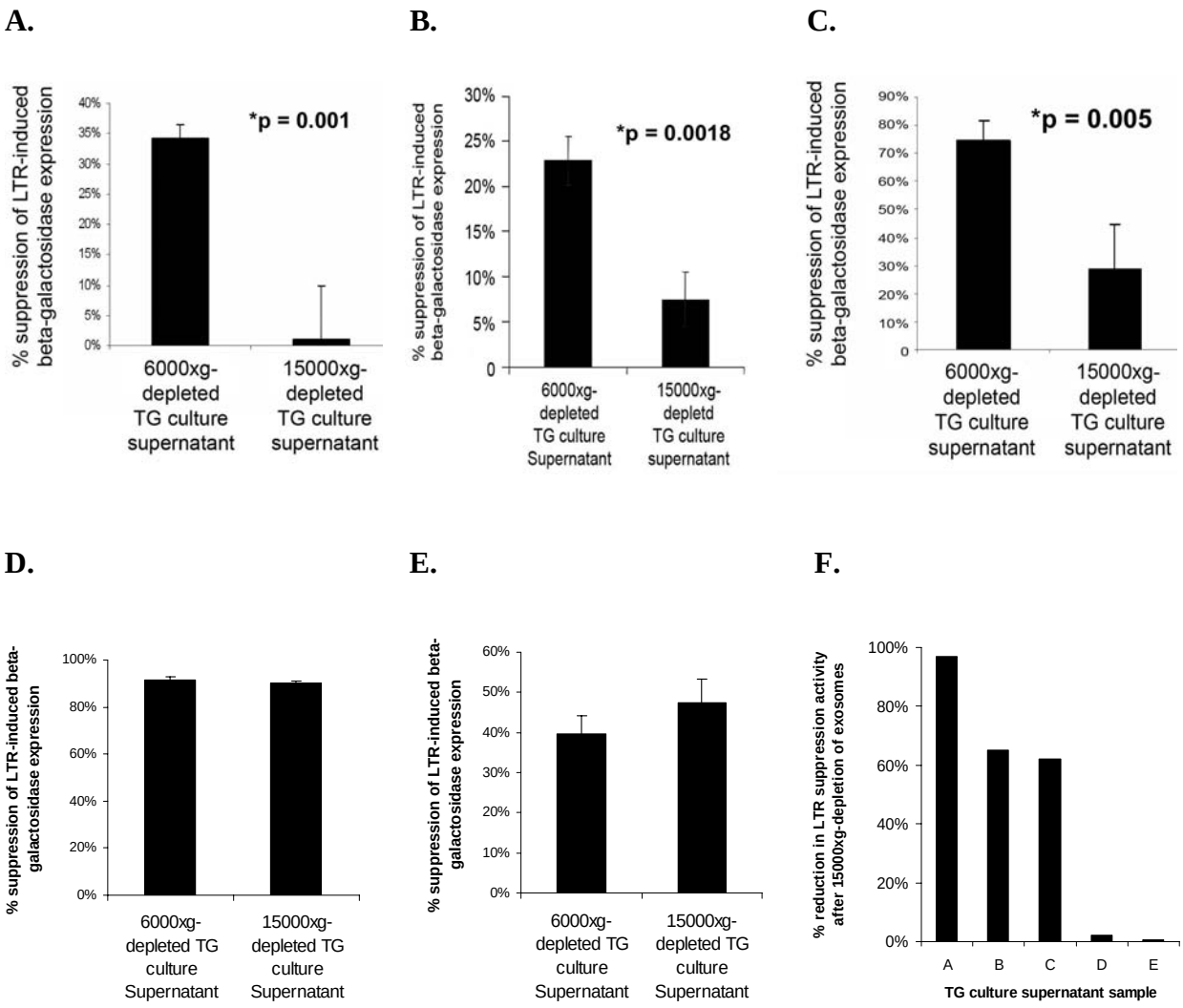

Figure 11. Analysis of physiologically secreted HIV-1 transcription suppression activity in culture supernatant taken from five independent TG cultures. Three of the five samples demonstrated reduction of HIV-1 transcription suppression activity after 15000xg exosome depletion (A-C). Two of the five samples displayed no such reduction in antiviral activity after exosome depletion (D-E). Comparison of percentage reduction in LTR suppression activity after exosome depletion for the five samples (F). In panel (F), the labels A-E for TG culture supernatant sample correspond respectively to panels (A) through (E) for this dataset.

2.5 DISCUSSION

The current study into the possible existence of membrane determinants mediating noncytolytic CD8+ T cell suppression of HIV-1 has demonstrated the existence of a membrane-bound factor secreted through exosomes which specifically inhibits transcription of HIV-1 from the proviral LTR promoter. A critical result of this investigation was the finding that TG cell-secreted exosomes can suppress HIV-1 transcription in both chronic and acute models of infection. TG exosome-mediated inhibition of HIV-1 transcription in the chronically infected 8E5 cell line is consistent with previous observations of CD8+ T cells suppression of HIV-1 in endogenously infected heterologous CD4+ cells (173). Additionally, the methodology used for demonstrating transcriptional suppression of the LTR promoter in TZM-bl cells indicates that exosomes can actually induce uninfected cells into suppressing the viral promoter before activation by viral infection, tat-expression, or mitogen induction. This may highlight a possible role CD8+ T cell secreted exosomes may play in inducing what other researchers have noted as transcriptional latency or latent infection in CD4+ T cells (191). The transcriptional suppression of PMA-induced LTR promoter observed in this study clearly demonstrates the non-antigenic requirement of this antiviral activity and easily explains TG exosome-mediated suppression of R5 and X4 HIV-1 isolates. It also confirms the involvement of only host cell proteins in mediating HIV-1 transcriptional suppression, a result previously observed for secreted CAF activity (172).

TG-secreted exosomes appear to identify strongly with key hallmarks defining CAF and the CD8+ T cell-mediated noncytolytic HIV-1 suppressive activity. However, some peculiar results were observed in this study. Exosomes secreted by primary CD4+ T cells and the H9 CD4+ T cell line were also observed to potently suppress the LTR promoter to a degree comparable to TG exosomes, with the Raji B cell line also displaying significant amounts of LTR suppression activity. This suggests that the exosome-localized factor mediating HIV-1 transcriptional suppression is not exclusively expressed by CD8+ T cells. This result should come as no surprise in light of recent reports demonstrating the secretion of noncytolytic HIV-1 suppression activity from CD4+ T cells (231). These results disagree strongly, therefore, with previously held assertions by other investigators that the antiviral factor mediating noncytolytic HIV-1 suppression is the exclusive domain of CD8+ T cells (245). Such conclusions of specificity have been largely made based on cell-mediated assays (167). The investigation performed here represented a more involved attempt to determine the possible role membrane determinants play in mediating potent suppression of HIV-1. The finding of a membrane-bound factor mediating potent HIV-1 suppression represents a new paradigm for elucidating the role cell contact-dependent determinants play in mediating noncytolytic HIV-1 suppression by CD8+ T cells.

In this respect exosome-mediated HIV-1 transcriptional repression may be the key to understanding how noncytolytic HIV-1 suppression activity is regulated within CD8+ T cells and why a dissociation exists between secreted- and cell-mediated antiviral activities. Exosomes are specifically derived from the limiting membranes of late endosomes and as such are likely subject to the same membrane-restricted compartmentalizations that other exosome proteins,

such as MHC class II and tetraspanins like CD9, CD63, and CD81 are subject to. This suggests a level of complexity in CD8+ T cell manifestation of the HIV-1 suppression activity beyond simple gene expression of an antiviral factor. This complexity is further evidenced by observations in this study of CD4+ cell-secreted exosome suppression of HIV-1 and the disconcordance between cell- and exosome-mediated mechanisms of HIV-1 suppression. Consequently, efforts to elucidate the molecular identity of the factor(s) mediating the antiviral activity based only on the premise of differential gene product expression, such as the microarray analysis of HIV-1 suppression and non-suppressing CD8+ T cells recently reported by Diaz et al (235), are unlikely to yield conclusive results as to the antiviral factor's molecular identity. As a corollary, how the factor mediating HIV-1 transcriptional suppression traffics intracellularly, particularly in the context of the tetraspanin proteins it is found to colocolize with on exosomes, may be the key to understanding why specificity of the antiviral effector function to CD8+ T cells might be found at a cellular level, but extracellular secretion of a factor mediating the same activity can be observed in other cell lineages.

Such mechanistic complexities involved in CD8+ T cell repression of HIV-1 transcription is highlighted further by an additional curious result. The analysis of LTR promoter suppression activity in TG culture supernatant samples before and after exosome depletion suggest that secreted CAF activity can be mediated by a combination of both secreted exosomes and a soluble molecule. The demonstration of membrane-bound and membrane-free mediators of an HIV-1 transcription suppression activity represents an additional intricacy to the dissociation found between cell- and secreted-factor mediated noncytolytic antiviral activity by CD8+ T cells (183,185). Since both exosomes and an apparent soluble factor mediate their

antiviral action at the level of inhibiting HIV-1 transcription, an argument could be made that the exosome-localized antiviral protein and the soluble antiviral factor are either molecularly related or share a common target to effect repression of the LTR promoter in CD4+ cells. If the soluble and membrane-bound antiviral factors are indeed molecularly related, an inverse relationship between exosome-mediated and exosome-free mediators of the antiviral activity in CD8+ cell culture fluids might be detected in a more thorough analysis of the activity. If such a result were found, it would suggest that the membrane-bound HIV-1 suppressive factor serves as a precursor for the formation of a membrane-free soluble form of the antiviral activity. Such a postulate would be consistent with the slight decreases in antiviral activity observed after sodium carbonate treatment of raw TG membranes, as this is consistent with the dislodging of a soluble mediator from a membrane-bound precursor. The precise relationship between an exosome-localized HIV-1 suppressing factor and an apparent soluble mediator is an area of active research at the present moment. Elucidation of the molecular factor(s) mediating either means of HIV-1 suppression would ultimately determine the overall mechanistic nature of noncytolytic HIV-1 suppression displayed by CD8+ T cells. For such an undertaking, CD8+ cell-secreted exosomes represent a potentially valuable source for purification of this elusive antiviral activity. The discovery of a novel membrane-bound factor inhibiting HIV-1 transcription and secreted through exosomes provides a new frame of thought for understanding, and possibly solving, the enigma of CD8+ T cell noncytolytic suppression of HIV-1.

3.0 CHAPTER THREE

Biophysical Characterization of the Exosome-Localized Factor Suppressing HIV-1 Transcription

(U.S. Patent Application filed November 29, 2005. [Application Serial No.: 60/740,922])

by

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3.1 ABSTRACT

CD8+ T cells express a factor capable of suppressing of HIV-1 transcription in CD4+ cells. This CD8+ cell antiviral factor (CAF) has been shown to be secreted, but does not identify with any known lymphokine. In the previous chapter of study, it was discovered that exosomes secreted by a CD8+ T cell line, TG, were able to mediate potent suppression of HIV-1 transcription. In the previous investigation, evidence was uncovered that secreted CAF activity could be mediated by secreted exosomes and by some soluble CD8+ cell secreted-factor. This dual nature to CAF activity led to the speculation that the exosome-localized factor mediating HIV-1 transcriptional suppression has a mechanistic relationship to the soluble protein mediating the same antiviral action. An investigation was undertaken to characterize the biophysical nature of the membrane-localized and membrane-free HIV-1 transcription suppression activities. Through a series of biochemical and proteomic analyses, evidence was uncovered indicating that a soluble antiviral mediator is derived from a membrane-bound precursor on the exosomes. Furthermore, evidence was uncovered suggesting a possible CD8+ cell exosome-specific enzymatic activity functioning in the conversion of the membrane-bound antiviral activity into a soluble form. The results of this study mechanistically reconcile exosome- and soluble protein-mediated suppression of HIV-1 transcription observed in CD8+ cell culture fluids and strongly suggests CAF secretion is an exosome-driven phenomenon.

3.2 INTRODUCTION

The molecular identification of the factor(s) mediating CD8+ T cell noncytolytic suppression of HIV-1 has been an enigma in the field of AIDS research. Elucidating the antiviral activity is of much interest due to its potent inhibition of genetically divergent HIV-1 isolates and its nontoxic action via inhibition or proviral transcription (172,186-190). While the involvement of a secreted CD8+ antiviral factor, termed CAF, has been noted in several investigations (172,181,182), more recent studies have revealed a CD8+ cell expressed integral membrane factor with potent HIV-1 transcriptional suppression activity (Chapter 2, Tumne *et al*). In this previous investigation, the secretion of this antiviral activity in the form of spherical membrane-limited 30-100nm vesicles termed exosomes was demonstrated along with evidence demonstrating that exosomes were a functional component of secreted CAF activity. Initial biophysical analysis had revealed the exosome-localized factor mediating the antiretroviral activity to be a protein expressed on the external surface of the CD8+ cell-secreted nanovesicles. In the analysis of CAF activity in CD8+ cell culture fluids, instances were observed where exosomes constituted almost all of the CAF activity, while in other CD8+ cell culture fluid samples, CAF activity appeared mediated almost exclusively by a soluble factor. Since the antiviral action of the membrane-bound and membrane-free mediators specifically inhibited LTR promoter activity, it raised speculation that this apparent dichotomy may due to the soluble mediator deriving itself from the membrane-bound precursor also mediating the same activity.

Initial biochemical data was consistent with this idea where it was observed that sodium carbonate treatment appeared to remove a small fraction of the activity from purified CD8+ cell membrane. However, the majority of the antiviral activity appeared to be tightly associated to

the membrane, indicating for the first time the existence of an integral membrane factor with noncytolytic HIV-1 suppression activity. Proteolytic analysis had demonstrated the membrane-bound factor to be expressed on the external surface of exosomes, with delipidation experiments demonstrating that the protein factor could exert its antiviral action independently of its lipid tethering. To reconcile the combined results of the previous study, a hypothesis was raised that the HIV-1 suppressive factor might exist as a cleavable integral membrane protein. A corollary to this postulate is an inferred existence of a putative protease or enzymatic activity to release a catalytically active antiviral molecule from its membrane-anchored precursor. There are, however, alternative models that may also explain the appearance of a soluble antiviral protein factor concurrent with one that is also membrane-bound. For instance, if the two factors are expressed as isoforms through alternative splicing of a shared mRNA sequence, an enzymatic action would not be required for the appearance of soluble and integral membrane proteins. Another possibility might also be that the membrane-bound factor and soluble protein mediator are two distinct gene products mediating the same action. If this is the case, then cellular expression of the two factors would be expected to be independent of each other and an inverse correlation between soluble and membrane bound activities would not be expected. Therefore, if the membrane-bound and soluble mediators of secreted LTR suppression activity do have a common precursor protein, two phenomena should be readily detected: (i) Fluctuations in exosome-mediated LTR suppression activity with an inverse correlation between exosome-localized and soluble protein-mediated antiviral activity; and (ii) Evidence supporting a catalytic activity coincident with the release of a soluble LTR suppressing factor mediator.

An investigation into the biophysical nature of the soluble / membrane-bound dichotomy of this antiviral activity was therefore undertaken. While the focus of the previous investigation in Chapter 2 was to determine conclusively the existence of a membrane bound HIV-1 transcription suppression activity and its physiological nature, attention in this study focused on defining the mechanistic relationship between the exosome-mediated LTR suppressing activity and its appearance in a soluble form. In doing so, evidence was uncovered for the soluble LTR suppressing factor being directly produced from a membrane-bound precursor also exhibiting the same activity. These results strongly suggest that secreted CAF activity is largely exosome driven and argue against a multifactorial cytokine release hypothesis that has previously been proposed for CAF activity (201). The novel experimental techniques and analytical methods performed in this study can ultimately be applied towards conclusive identification of the elusive molecular factor(s) mediating noncytolytic HIV-1 suppression.

3.3 MATERIALS AND METHODS

About methods used in this section of the study

As the current study presented in this chapter is a direct continuation of results gleaned from the last chapter, this following section of study borrows heavily the methods used in Chapter 2. As such, the reader may reference experiments described in this Chapter 3 with some of the methods used in Chapter 2. In particular, TG cell cultures, exosome preparations, and the acute LTR suppression assay using the TZM-bl gene-reporter cell line are used in this section of the study as essentially described in Chapter 2, with some minor modifications where noted in this Chapter. The TZM-bl acute HIV-1 LTR suppression assay is used throughout the study as this gene-reporter assay has proven to be a very sensitive and reproducible assay for the evaluation of biochemically extracted samples mediating HIV-1 transcription inhibition.

Exosome Preparation

Exosomes were prepared essentially as described in Chapter 2 by serial centrifugation of cell culture supernatant followed by sucrose gradient fractionation of the 15,000xg membrane pellet. In some experiments, after the final wash and pelleting of exosomes from the 60% sucrose density gradient fraction, exosomes were resuspended 0.1 M sodium carbonate instead of HBSS.

Quantitative Exosome Assay

The method of Clayton *et al* (232) was adapted to develop a quantitative assay for measurement of relative exosome concentrations between samples under nonsaturating conditions of exosome bead-capture. Immunomagnetic beads coated with antibodies specific for MHC Class II molecules (Dynal, Norway), were washed and resuspended at a concentration of $5x10^6$ beads per 1 ml in 2% FCS/PBS. A volume of 200 ul containing 10^6 beads was mixed with 50 ul of sample containing exosomes and incubated on a rotator (Dynal, Norway) at 4ºC for 12 h at 35 rotations per minute. After bead-exosome incubation, beads were washed twice with 2% FCS/PBS and stained with anti-CD63 PE-labeled or isotype control monoclonal IgG1 antibody and analyzed by flow cytometry, as described in Chapter 2. Under conditions of non-saturating exosome-bead binding, the extent of CD63-dependent fluorescence shift relative to isotype antibody controls is directly proportional to the exosome concentration of the sample (232). A proof-of-principle for this technique is described in the results of this investigation

Extraction of Peripheral Membrane proteins from the exosomes

Exosomes were pelleted by microfuge centrifugation at 20,000xg for 30 minutes. Exosomes were then resuspended in a variety of solutions for extraction of peripheral membrane proteins at 4°C. These treatments included 1M NaCl for 30 minutes, HBSS for 30 minutes and for storage of exosomes at 4°C or -70°C, 0.1 M sodium carbonate, pH 11.5 for 30 minutes, deionized double distilled H_2O (dI-ddH_2O) for 16-24 hrs. Upon treatment, exosomes were re-pelleted by microfuge centrifugation to extract supernatant containing solublized proteins. Dialysis and concentration of soluble proteins after salt extractions was performed by three successive rounds of washing and microfiltration using a 5 kDa cutoff microfilter cartridge

(Millipore, US). The final 5 kDa microfilter dialyzed concentrate was resuspended into media for assaying HIV-1 suppression activity at a volume equivalent to the original exosome preparation the extract was derived from. Dialysis using the 5 kDa cutoff microfilter was found to fully retain LTR suppression activity (data not shown).

MALDI-TOF-MS analysis of TG and H9 catalytically released proteins

TG and H9 secreted exosomes were purified, assessed for protein concentration using a BioRad assay, treated with 0.1 M sodium carbonate pH 11.5 to remove peripheral proteins from the exosomes. After a 30 minute treatment at 4ºC, the membrane fraction was separated from the supernatant by centrifugation at 20,000xg. The resulting exosome membrane pellet was rinsed three times with dI-ddH2O to remove residual salt before resuspending

the pellet in dI-ddH2O. Aliquots of the dI-ddH2O extracted proteins were assayed for HIV-1 suppression activity. A second aliquot of each dI-ddH2O extracted exosome sample was lyophilized for MALDI-TOF-MS analysis. In preparation of MALDI-TOF-MS samples, lyophilized samples were resuspended in 3 ul of a solution of 0.3% tricitric acid / 50% acetylnitrile and then mixed with 3 ul of α-cyano-4-hydroxycinnamic acid. Aliquots of 1.5 ul were spotted on a stainless steel mass spectrometer plate and dried at 40°C. The matrix-embedded samples were then analyzed by MALDI-TOF-MS on a Voyager DE-PRO Mass Spectrometer (Applied Biosystems, US).

3.4 RESULTS

3.4.1 Variability in exosome-mediated HIV-1 LTR promoter suppression activity

In the previous study of CD8+ cell culture fluids, secreted CAF suppression of the HIV-1 LTR promoter was found to be mediated by a combination of exosomes and some soluble factor (Chapter 2, Figure 11). The extent to which secreted CAF activity was due to exosomes or soluble factor suggested an inverse correlation between the two mediators of HIV-1 transcription suppression. It was not known at the time whether the LTR suppression activity fluctuated with respect to its exosome localization in the TG cell line. If it did, exosome samples displaying divergent degrees of antiviral activity would provide an important starting point for molecular dissection of the protein factor mediating suppression of the HIV-1 promoter. A time-course analysis of the exosome-mediated antiviral activity was performed for two independent TG cultures that were at late stages of culture. Exosome purifications were performed at four independent time points for each culture and exosome samples were standardized by protein content. The HIV-1 LTR suppression activity of each purified exosome sample was assayed using the TZM-bl cell line-based acute LTR suppression assay described in the previous chapter of study. In the panel of samples analyzed, two instances over a time course from day 52 to day 100 were found where exosome-mediated LTR suppression activity fluctuated in both TG cultures (Figure 12A and 12B).

A. B.

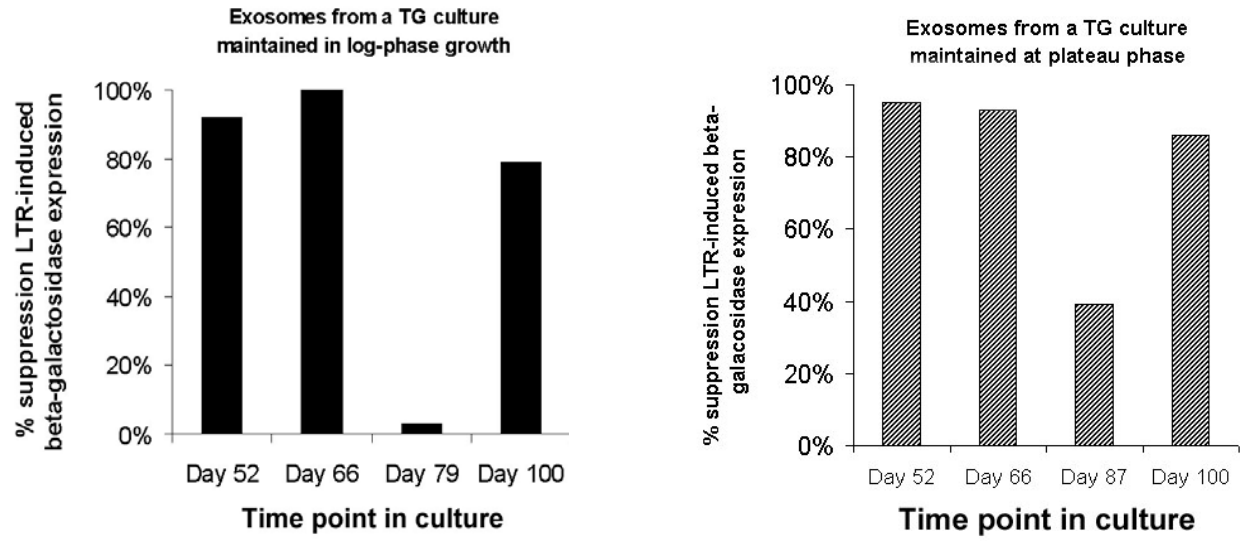


Figure 12. Measurement of exosome-mediated HIV-1 LTR suppression activity over a time course study. (A) Exosomes from a TG cell culture maintained in log-phase growth. (B) Exosomes from a TG cell culture maintained at saturating plateau conditions.

The analysis indicated that fluctuations in exosome-mediated LTR suppression do occur in the TG cell line. The possibility that this variability was due to differences in exosome concentration between the samples was addressed. An exosome titration assay based on the quantitative immunomagnetic bead-capture method of Clayton *et al* (232) was employed to determine the validity of normalizing independent exosome samples by protein concentration. The quantitative detection of exosomes using anti-MHC Class II antibody-coated beads is based on the principal that under conditions of unsaturated bead capture of exosomes, flow cytometric measurement of exosome markers produces a fluorescence shift relative to isotype control that is directly proportional to concentration of exosomes present during bead binding (232). The utility of this assay was tested in a titration experiment on three exosome samples purified from three independent TG cell cultures. A two-fold dilution series of each of the three independent

samples was made from 80 μg/ml to 10 μg/ml. Applying the quantitative exosome capture assay, a striking linear correlation was found between exosome protein concentration and CD63 fluorescence shift (Figure 13), demonstrating utility of protein concentration for standardizing exosome samples. The concordance between the three independent exosome samples indicated a high degree of reproducibility for the quantitative exosome capture assay.

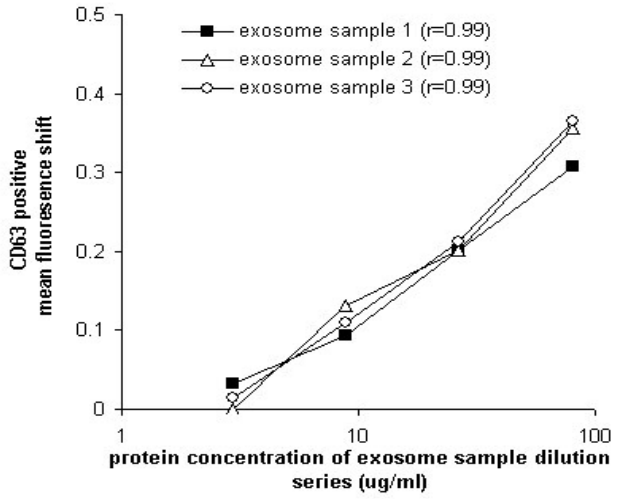


Figure 13. Demonstration of linearity in the bead-capture exosome quantitative assay on dilution series of three independent TG exosome samples.

The exosome quantitative assay was next used to analyze three exosome samples from the time-course analysis in Figure 12 that displayed high, medium and low HIV-1 LTR suppression activities (Figure 14A). Applying the quantitative exosome assay, the three samples with divergent antiviral activities were found to contain equivalent amounts of exosomes as indicated by similar CD63 dependent shifts (Figure 14B). This demonstrated that the fluctuation of exosome-mediated HIV-1 LTR suppression activity observed in TG cell cultures was

specifically due to the variable presence of the activity on the exosomes themselves and not to differences in exosome concentration or method of standardization.

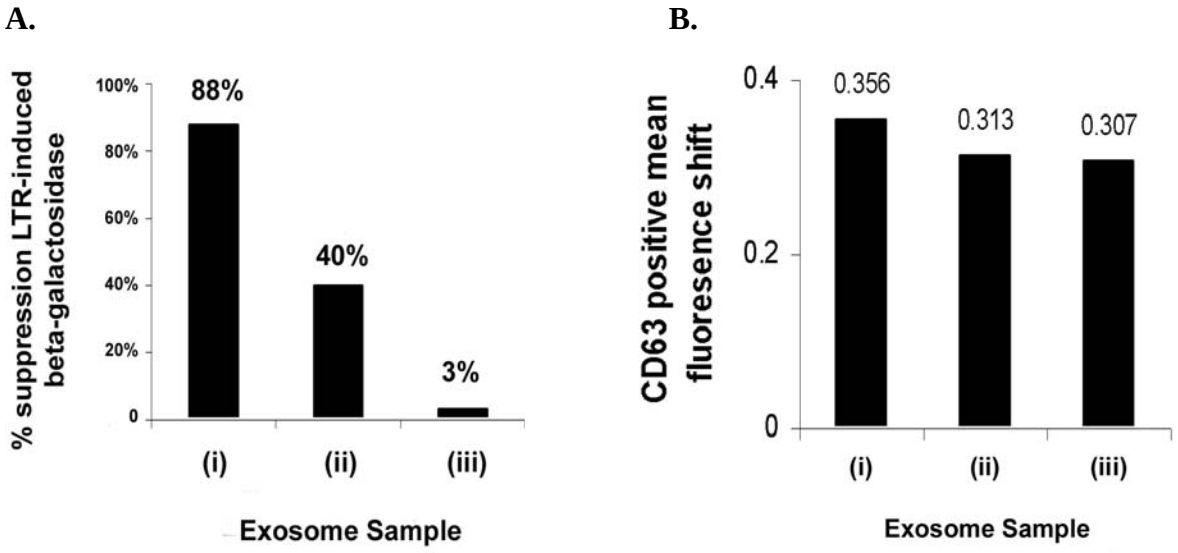
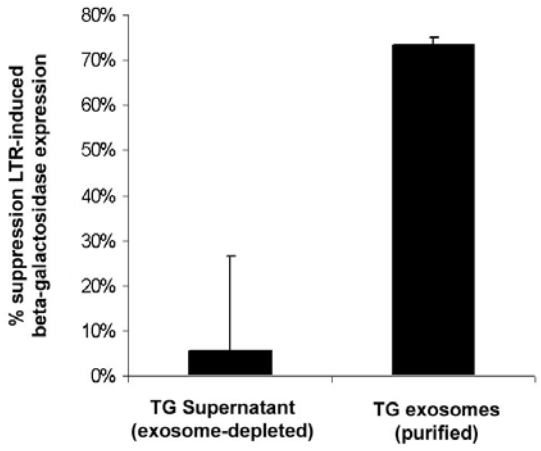


Figure 14. Comparison of LTR suppression activity and relative exosome concentrations in three exosome samples (i), (ii), and (iii). (A) LTR suppression activity of three exosome samples (i), (ii), and (iii) as standardized by protein content. (B) Measurement of relative exosome concentrations, as denoted by CD63 positive fluorescence shift, of the three same exosome samples (i), (ii), and (iii), standardized by protein content.

After determining the specific variability of a factor localizing to exosomes, the possible relationship of TG exosome-mediated antiviral activity with antiviral activity in exosome-depleted culture supernatant was next explored. Upon analysis of several independent samples, instances were observed where (i) LTR suppression activity was found exclusively in the exosomes (Figure 15A); (ii) LTR suppression activity was localized to both supernatant and exosomes (Figure 15B); and (iii) LTR suppression activity was found only in the supernatant and not exosomes (Figure 15C). These results further verified the observed fluctuations in exosome-mediated antiviral activity do occur and appeared to portray a pattern of inverse association

between exosome-localized LTR suppression activity and the appearance of a soluble mediator in exosome-depleted TG culture fluids.

A.



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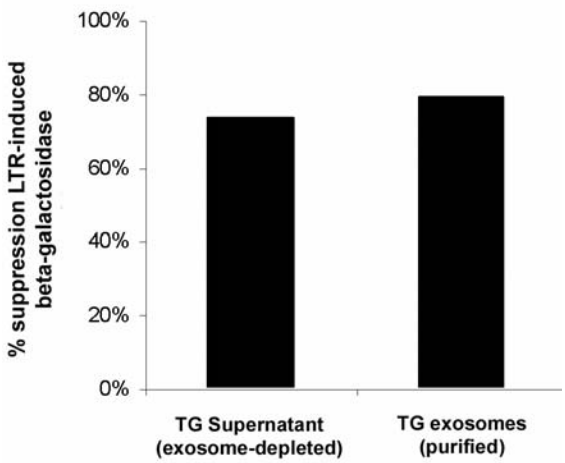


C.

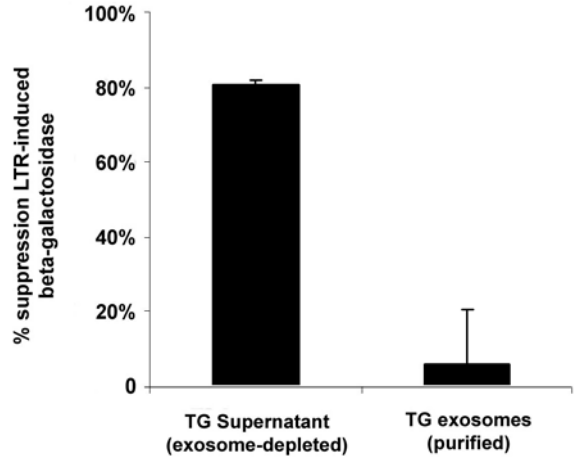


Figure 15. Comparison of LTR suppression activity in exosome-depleted TG culture supernatants to the purified exosomes depleted from them. (A) LTR suppression activity is found exclusively in TG exosomes. (B) LTR suppression activity is found in both TG exosomes and exosome-depleted supernatant. (C) LTR suppression activity is only found in exosome-depleted culture supernatants and not in the exosomes.

3.4.2 Nature of the LTR suppressing factor's localization to TG exosomes

The apparent fluctuation of the LTR suppressing activity between an exosome-localized and soluble form prompted a more precise evaluation concerning the nature of the LTR-suppressive activity's localization to the exosome membrane. If the HIV-1 transcription suppressing factor was a cleavable integral membrane precursor, then the soluble form of the activity might still be localized as a loosely bound peripheral membrane protein on the exosome surface. If this represented the means by which a soluble factor appeared in TG culture fluids, then the extent to which HIV-1 transcription suppression activity is membrane-bound should correlate inversely with the appearance of a soluble mediator on the exosomes and in exosome-depleted culture supernatant. To explore this possibility, a biochemical analysis was performed on two independent exosome samples that had been purified and stored at -70ºC from a previous study in which one culture displayed considerable LTR suppression activity in exosome-depleted culture fluid (Figure 11E) with the second culture displaying no such activity from a soluble protein mediator in exosome-depleted culture fluid (Figure 11A). The two exosomes samples were subjected to a variety of salt treatments to quantify the extent of soluble LTR suppression activity to that which remained membrane-bound after extraction.

In the first exosome sample from which significant LTR suppressing activity was found in exosome-depleted culture fluid (Figure 11E), the exosomes were subjected to a series of soluble extractions as outlined in Figure 16. After purification from cell culture fluid, exosomes were stored in HBSS overnight with an aliquot of exosome-depleted culture supernatant stored at

-70ºC prior to their analysis. Upon thawing, an untreated aliquot of the exosome suspension was saved as a control before the remaining suspension was centrifuged to pellet the exosomes and extract the storage buffer supernatant for analysis. The exosome pellet was treated with 0.1 M sodium carbonate, pH 11.5 to remove all remaining peripheral proteins. After treatment, exosomes were pelleted and supernatant of the sodium carbonate extract and the exosome storage buffer supernatant were separately dialyzed into media. The sodium carbonate-treated exosome pellet was washed and resuspended into media. LTR suppression activity was assayed for each of the fractions collected. No HIV-1 promoter inhibition activity was found in the exosomes after sodium carbonate treatment (Figure 17). The LTR suppression activity was only found in dialyzed sodium carbonate fractions and storage buffer supernatant in addition to its appearance in culture supernatant after exosome purification. In addition, The LTR suppression activity was considerably higher in exosome-depleted supernatant than that found in the purified exosomes (43% and 23% LTR suppression activity, respectively). In this particular exosome sample, the activity was found to be completely localized to exosomes as a loosely bound peripheral protein.

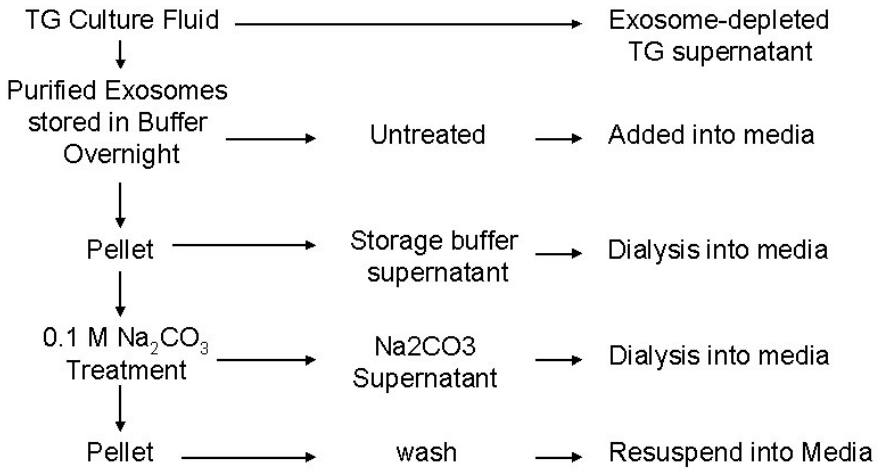


Figure 16. Schematic for the analysis of exosomes from a TG culture in which the culture supernatant displayed considerable antiviral activity after exosome depletion.

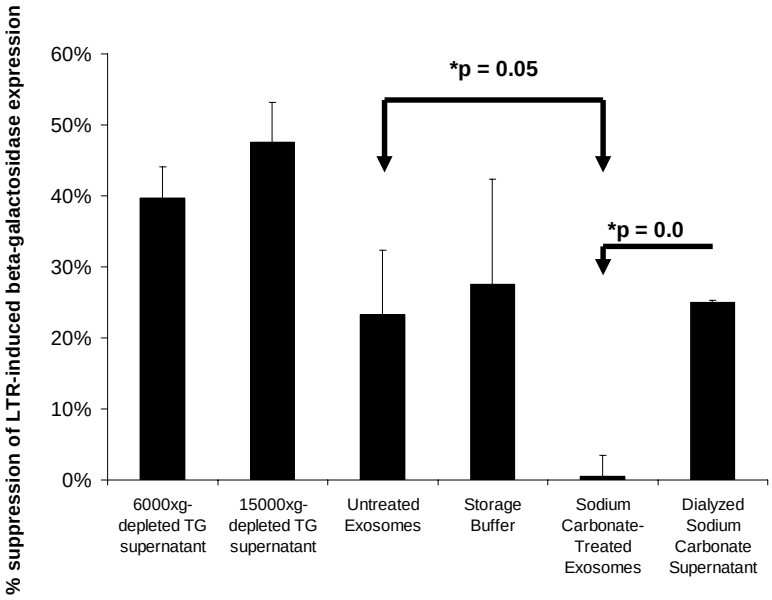


Figure 17. LTR suppression in fractions harvested from the exosome sample purified from the TG culture described in Figure 11E. TG exosome-depleted supernatant activity was considerably higher than untreated exosomes. Untreated exosomes also contained equivalent LTR suppressing activity that was eluted into storage buffer and the dialyzed sodium carbonate supernatant.

A similar analysis was next performed on a second exosome sample that was previously prepared from a TG cell culture in which no LTR suppressing activity could be found in exosome-depleted culture fluid (Figure 11A). The experimental schema for the second analysis is outlined in Figure 18. This second treatment consisted of a much more rigorous analysis to ensure that salt extraction of peripheral proteins was complete and exhaustive. This included subjecting exosome aliquots to 1M sodium chloride extraction as well as performing two serial sodium carbonate extractions on an exosome sample to ensure thorough removal of soluble proteins (Figure 18). After performing the various treatments outlined, the various soluble and the exosome fractions were assayed for LTR suppression activity.

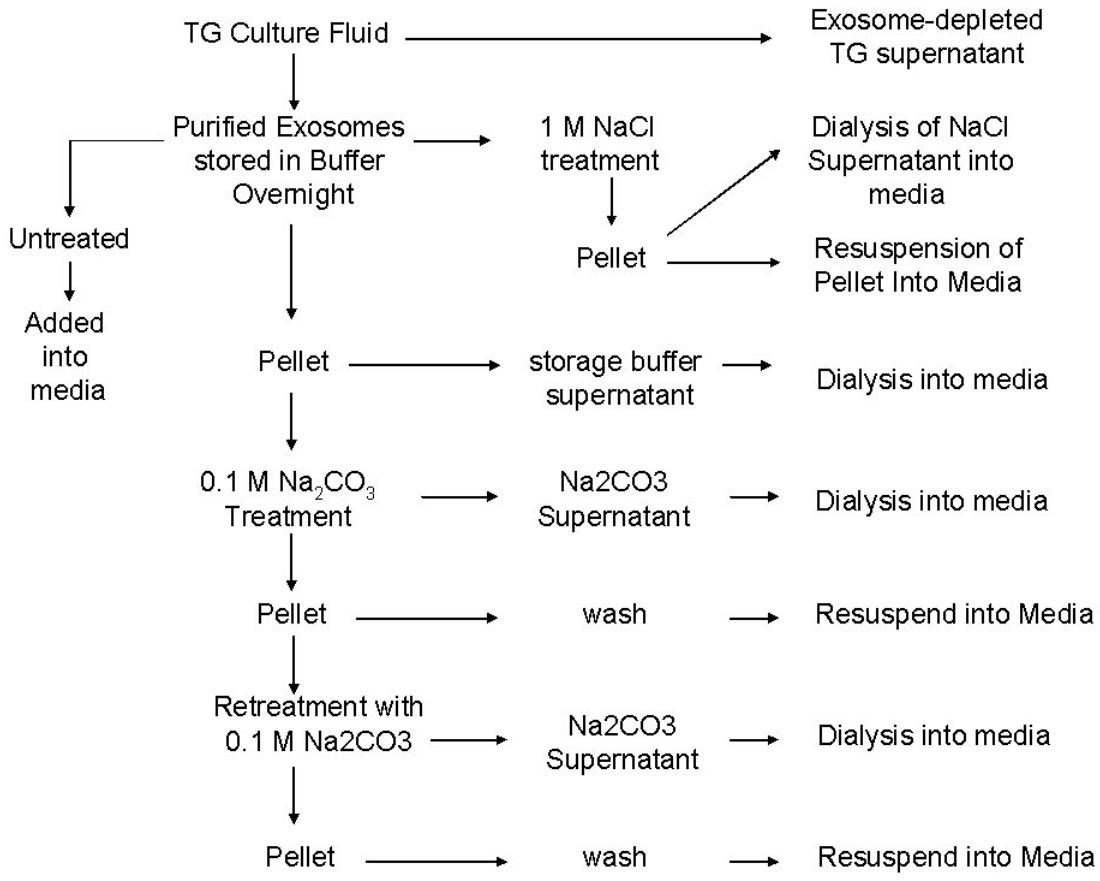


Figure 18. Schematic outline of the subsequent elaborate analysis of exosome-extracted soluble fractions. Exosome extractions performed included buffer, sodium chloride, sodium carbonate, and re-treatment with sodium carbonate.

In agreement with the previous analysis, HIV-1 LTR promoter suppression activity was eluted from this second independent exosome sample by sodium chloride treatment and sodium carbonate treatments in addition to its elution into HBSS that was used as a storage buffer for exosomes (Figure 19A). Very surprisingly, however, HIV-1 LTR suppression activity was eluted into solution after two successive rounds of sodium carbonate treatment of exosomes (Figure 19A, last bar from the left). Upon assaying the suppression activity of resuspended post-treatment exosome pellet fractions, in contrast to the previous exosome sample, this second

exosome sample was actually found to retain membrane-localized LTR suppression activity even after two successive rounds of sodium carbonate treatment (Figure 19B). In contrast to the previous exosome sample (Figure 17), a tight association of the peripheral membrane protein mediating the LTR suppression activity to exosome membranes was found in this second sample. Such results are inconsistent with the LTR suppressing factor associating to exosomes as a purely peripheral protein.

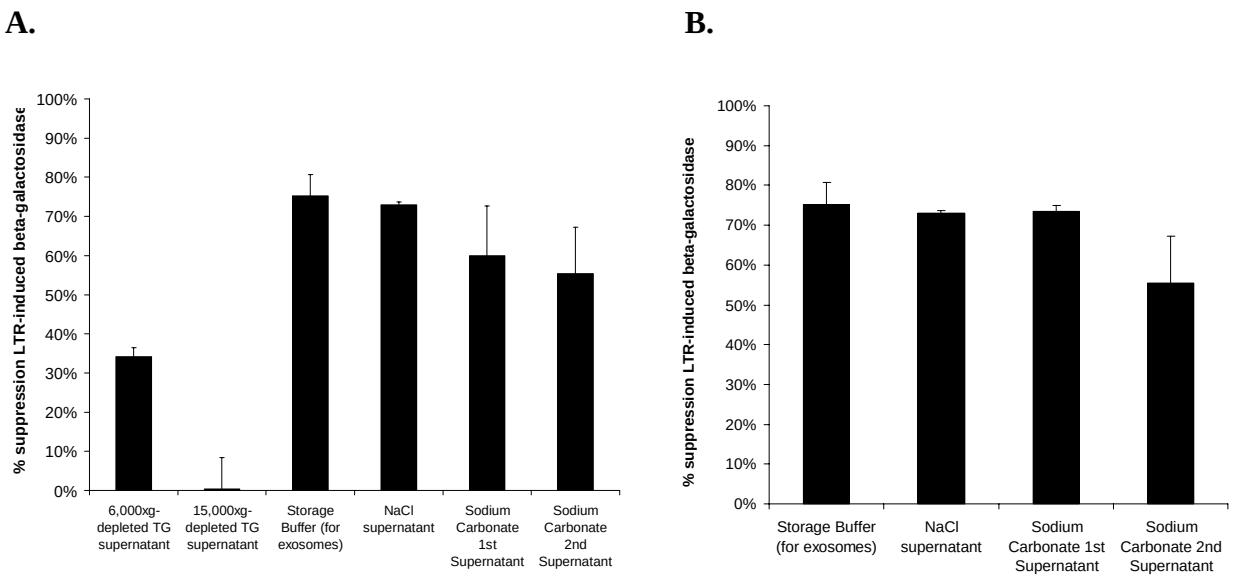


Figure 19. Sodium chloride and sodium carbonate extractions of a second TG exosomes sample. (A) Soluble fractions from 6000xg- depleted TG culture fluid, 15000xg exosome-depleted TG culture fluids, exosome storage buffer, sodium chloride, and sodium carbonate 1st and 2nd treatments. (B) Untreated exosomes or pelleted exosomes after sodium chloride treatment, or 1X or 2X sodium carbonate treatments.

The extraction of the LTR suppressive activity from exosomes into solution with the activity still also retained in an exosome-bound form appeared to hint at a possible ongoing conversion of a membrane-bound molecule into a soluble form. To determine whether such a model for the exosome-bound LTR suppressing factor was valid, exosomes from two

independent TG cell cultures were purified, resuspended in deionized double distilled water (dI-ddH_2O) and stored for 24 h at 4 ºC. Exosomes were next pelleted by centrifugation and the supernatant was extracted. Pelleted exosome were subjected to sodium carbonate treatment to deplete remaining peripheral membrane proteins followed by dialysis of the extracted sodium carbonate supernatant into buffer. The sodium carbonate-treated exosomes were then resuspended in dI-ddH_2O for a second 24 h extraction at 4 ºC. After assaying the various fractions for HIV-1 LTR suppression activity, a high amount of antiviral activity was found eluted in the dI-ddH_2O fractions even after sodium carbonate removal of peripheral protein (Figure 20). That the last extraction with dI-ddH_2O contained approximately 2-fold greater antiviral activity than after the preceding sodium carbonate removal of peripheral proteins, a result consistent with catalytic conversion of a membrane-bound molecule into a soluble form.

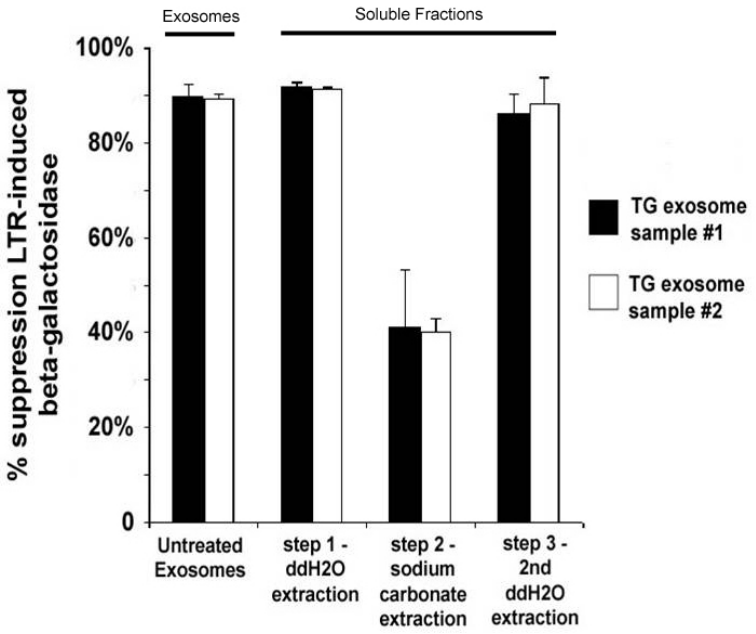


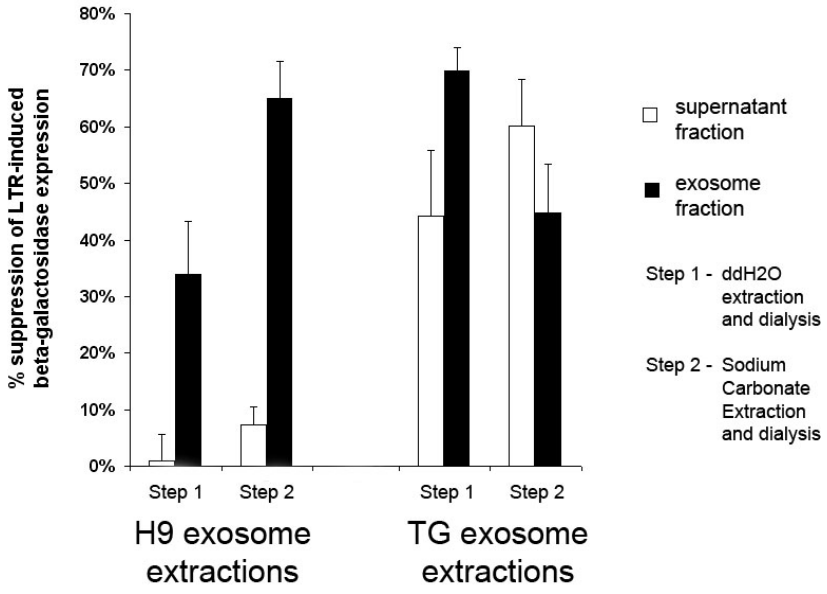
Figure 20. Experimental verification of a possible catalytically released factor. Purified exosomes were resuspended in pure water and subjected to a three step extraction of soluble protein.

3.4.3 Comparison of soluble and membrane-bound LTR suppression activity between TG and H9 exosomes

The biophysical analysis performed with dI-ddH_2O and sodium carbonate extractions was extended to determine if the same phenomenon held for exosomes from the H9 cell line, as these CD4+ cell-secreted exosomes also display potent inhibition of HIV-1 transcription (Chapter 2, Figure 10). Exosomes from H9 and TG cell cultures were purified and resuspended in dI-ddH2O for overnight extraction of soluble proteins at 4°C. Exosomes were then pelleted to harvest the supernatant and pelleted exosomes were subjected to sodium carbonate treatment upon which water-dialyzed sodium carbonate supernatant and resuspended exosome membrane pellets were prepared. The dI-ddH_2O and sodium carbonate supernatant and exosome pellet fractions were assayed for HIV-1 LTR suppression activity. HIV-1 LTR suppression activity was readily detected in TG exosomes as well as dI-ddH_2O and sodium carbonate extracts made from the TG exosomes (Figure 21A). In the H9 exosomes, however, little to no soluble extraction of the LTR suppression activity was found in either dI-ddH_2O or sodium carbonate extracts made from H9 exosomes despite considerable suppression of the HIV-1 promoter by H9 exosomes after each extraction (Figure 21A). The extent of soluble vs. membrane-bound antiviral activity for TG exosomes was comparable (44% vs. 70%, respectively, after dI-ddH_2O extraction and 60% vs. 45%, respectively, after sodium carbonate treatment), whereas H9 exosomes demonstrated a much greater discrepancy between soluble and membrane-bound LTR suppression activities (0% vs. 33%, respectively, after dI-ddH_2O extraction and 7% vs. 65%, respectively, after sodium carbonate treatment). To confirm this apparent difference, a second analysis was performed on second set of H9 and TG exosome samples, this time reversing the soluble extractions, subjecting samples first to sodium carbonate treatment followed by overnight extraction in dI-

ddH_2O at 4°C. LTR suppression activity was assessed for the soluble fractions and results demonstrated an apparent restriction in the specificity of extraction for the soluble antiviral activity to TG exosomes but not H9 exosomes (Figure 21B). Soluble extracts from TG exosomes displayed 45% and 39% LTR suppression activity, respectively, after sodium carbonate and dI-ddH_2O treatments, whereas soluble extractions from H9 exosomes only resulted in 1% and 17% LTR suppression activity, respectively, for the same extractions (Figure 21B). Aliquots of the dI-ddH_2O made in this second experiment were consequently stored at -70°C for further analysis.

A.



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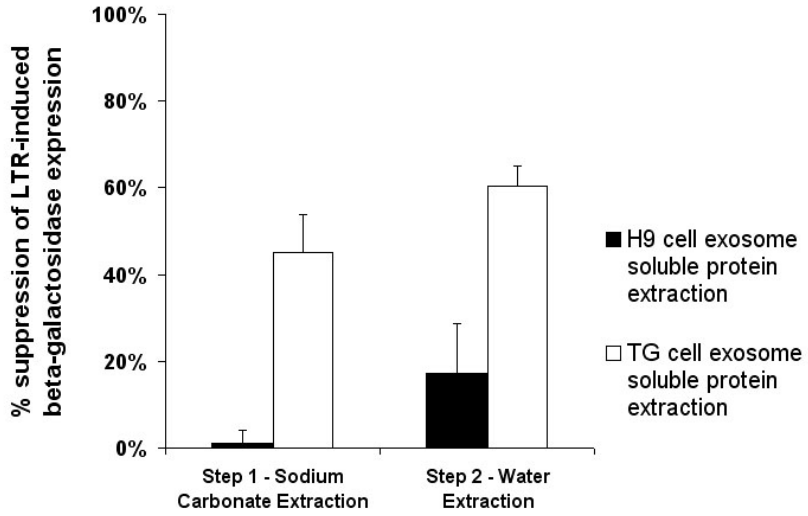


Figure 21. Soluble extractions from H9 and TG exosomes. (A) A step wise extraction procedure consisting of ddH2O extraction in the first step and sodium carbonate in the second step, assaying aliquots after each procedure for supernatant and exosome LTR suppression activity. (B) Analysis of soluble fractions after the order of extractions is reversed with sodium carbonate as a first step and ddH2O in the second. The step 2 ddH2O fraction shown in (B) was used for analysis by MALDI-TOF-MS.

3.4.4 MALDI-TOF-MS analysis of dI-ddH_2O-eluted fractions from H9 and TG exosomes

The finding that soluble extraction of the LTR suppressing activity was largely restricted to TG exosomes suggested that soluble fractions made from H9 exosomes could serve as an ideal control for differential proteomic analysis of dI-ddH_2O-solublized extracts. The proteomic analysis technique of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) was employed to determine if differences in LTR suppression activity could be attributed to differential MALDI-TOF-MS analyte signals produced from proteins in the dI-ddH2O extracted samples. The dI-ddH2O extracted TG and H9 samples described in Figure 22B were analyzed by MALDI-TOF-MS using an Applied Biosystems DE-PRO Voyager Mass Spectrometer. In the resulting spectra, a mass/charge (m/z) range between m/z 5.0 and 14.0 kDa was analyzed to identify differential and common analyte signals between the TG and H9 samples (Figure 22A and 22B, respectively). A common triplet of signals was observed at m/z 11.3, 11.7, and 12.2 kDa in both the TG and H9 solublized samples. The m/z 11.3 kDa analyte was chosen to serve as an internal control in an attempt to identify differential analyte signals between the TG and H9 samples. Since the samples analyzed were standardized for volume and were extracted from their exosome sources at equivalent exosome protein concentrations, differentially displayed analyte peaks relative to an internal control should reflect the relative levels of the protein giving rise to a particular peak. Of particular interest were MALDI-TOF-MS signals that were at higher levels in the TG sample than in the H9 sample relative to the m/z 11.3 kDa signal designated as an internal control. One such signal at m/z 8.6 kDa appeared to stronger in the TG spectra than for the H9 spectra. The ratio of the peak integration values of the m/z 8.6 kDa signal to the m/z 11.3 kDa signal corresponded strikingly with the differential LTR

suppression activity observed between the TG and H9 dI-ddH2O extracted samples (Figure 22C).

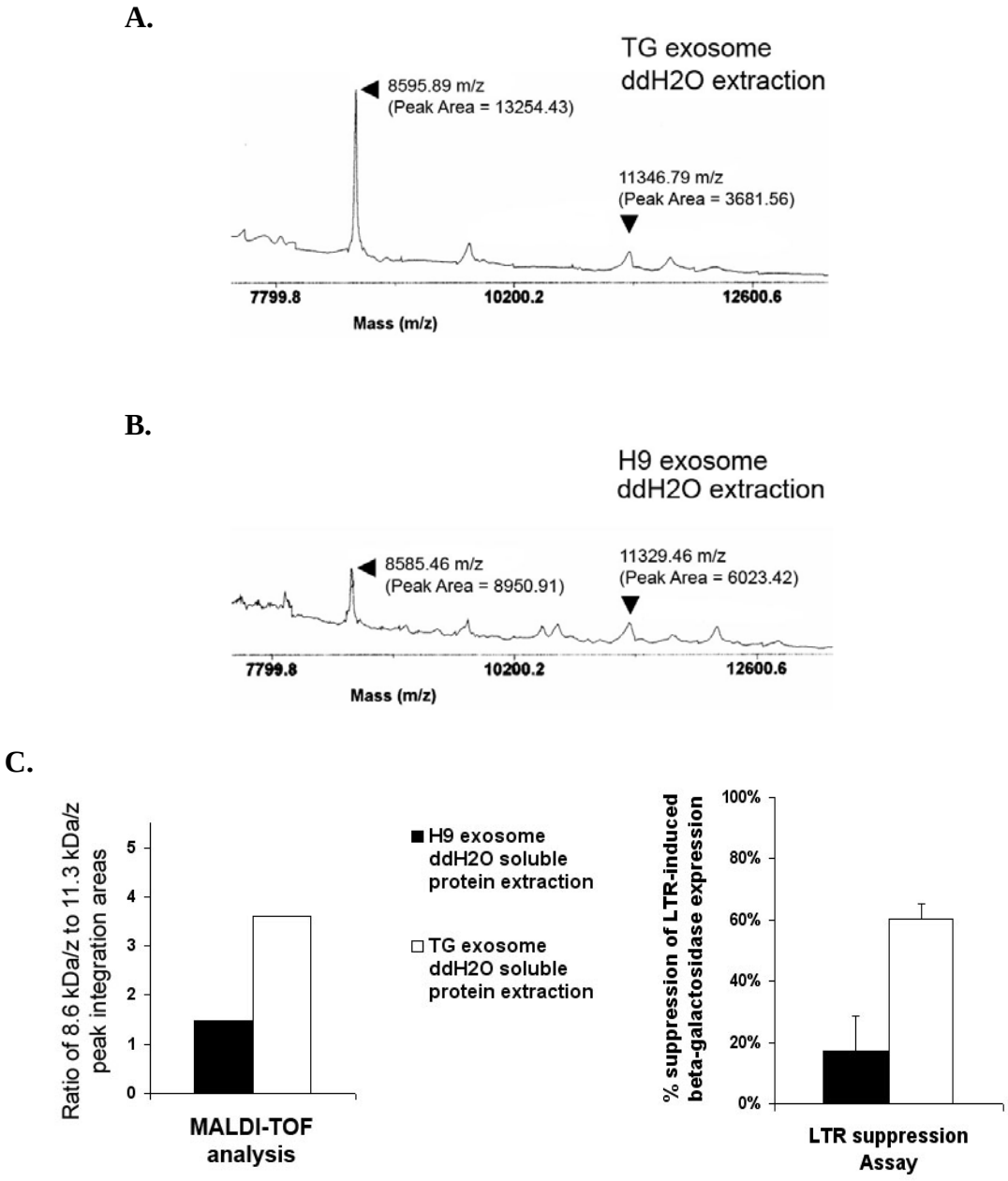


Figure 22. MALDI-TOF-MS analysis of the ddH_2O fraction from Figure 21.B. (A) MALDI-TOF-MS mass to charge spectra of TG exosome ddH2O-extracted fraction. (B) MALDI-TOF-MS mass to charge (m/z) spectra of H9 exosome ddH_2O-extracted fraction. Analyte signals corresponding to approximately m/z 8.6 kDa and m/z 11.3 kDa are denoted in the spectra along with peak integration values in brackets (A and B). (C) The ratio of m/z 8.6 kDa to m/z 11.3 kDa peak integration values shown in the graph on the left compared to LTR suppression activity in the graph on the right.

The MALDI-TOF-MS analysis was extended to a larger panel of dI-ddH_2O extractions from five TG and two H9 exosome samples. The seven dI-ddH_2O extracted samples displayed a divergent range of LTR promoter suppression activity (Figure 23). MALDI-TOF-MS analysis was performed on the seven samples (Figure 24). The triplet signals of m/z 11.3, 11.7, and 12.2 kDa were found in all seven dI-ddH_2O extractions, validating their use as internal controls. In addition to the m/z 8.6 kDa signal, differential signals of m/z 5.0, 5.4, and 6.2 kDa were also identified that appeared to correspond with the HIV-1 suppressing activity for each sample.

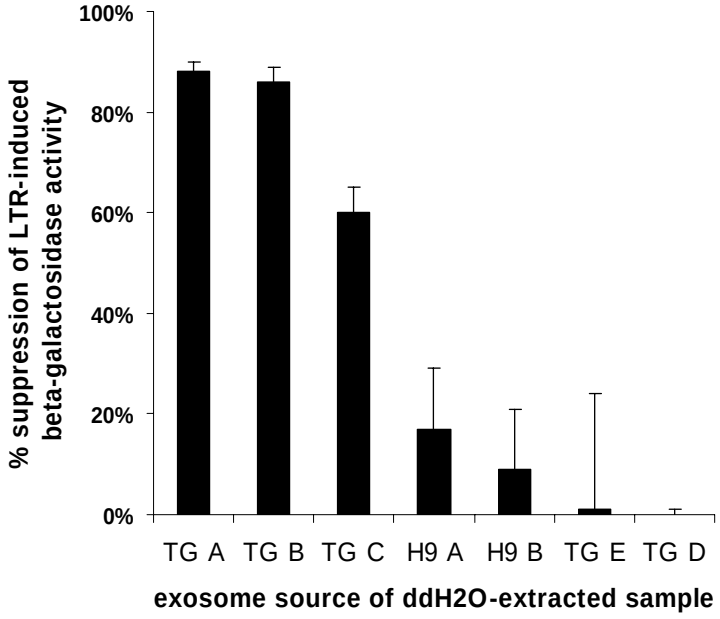


Figure 23. Panel of five TG and two H9 dI-ddH2O extracted samples with a divergent range of LTR suppression activity. The samples are arranged here in rank order from highest to lowest antiviral activity.

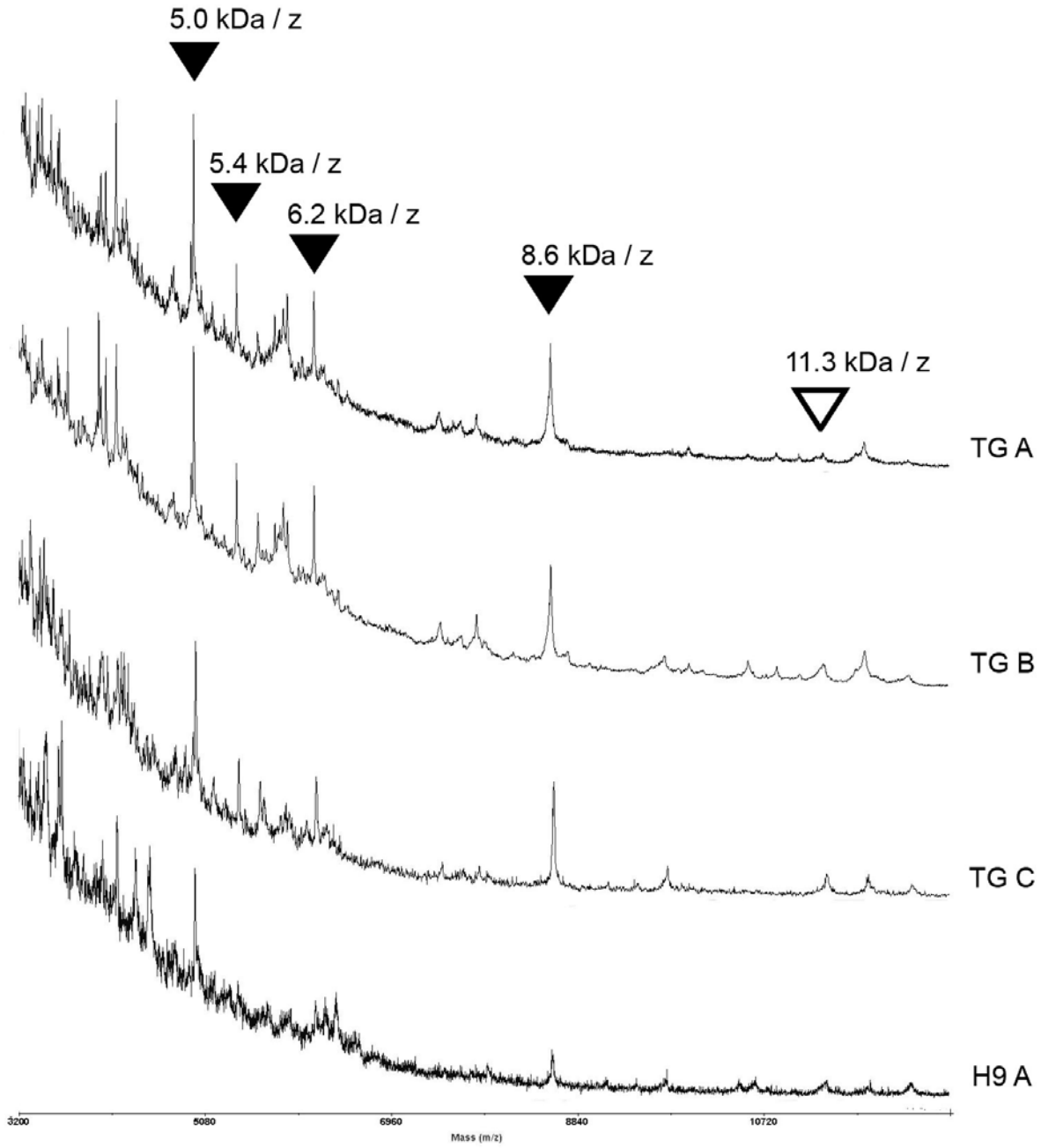
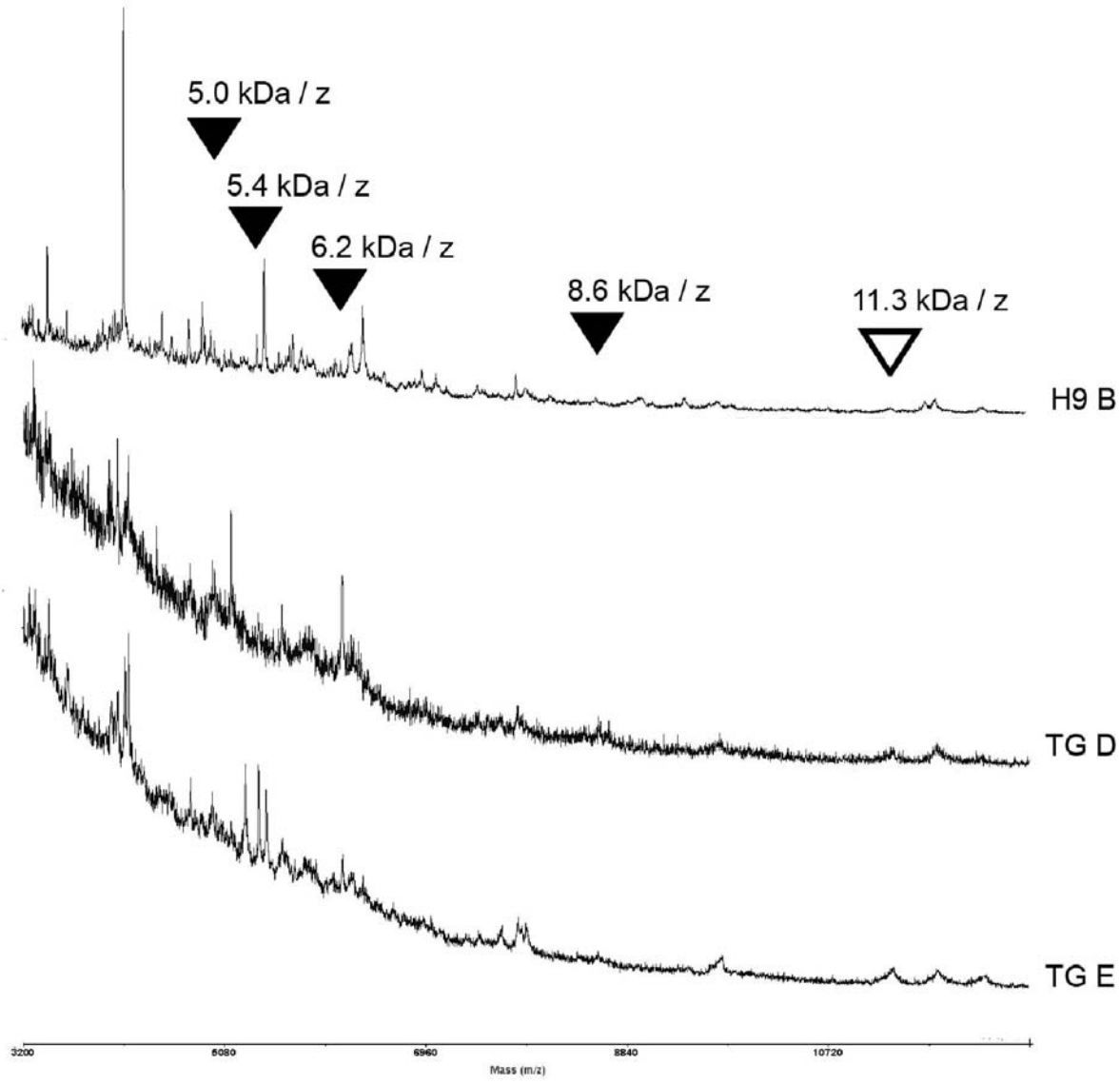


Figure 24. MALDI-TOF-MS analysis of the five TG and two dI-ddH2O extracted samples. The samples are arranged here in order of highest activity to lowest: TG A > TG B > TG C > H9A > H9 B > TG D > TG E. (continued on p.97).

Figure 24. (continued from p.96)



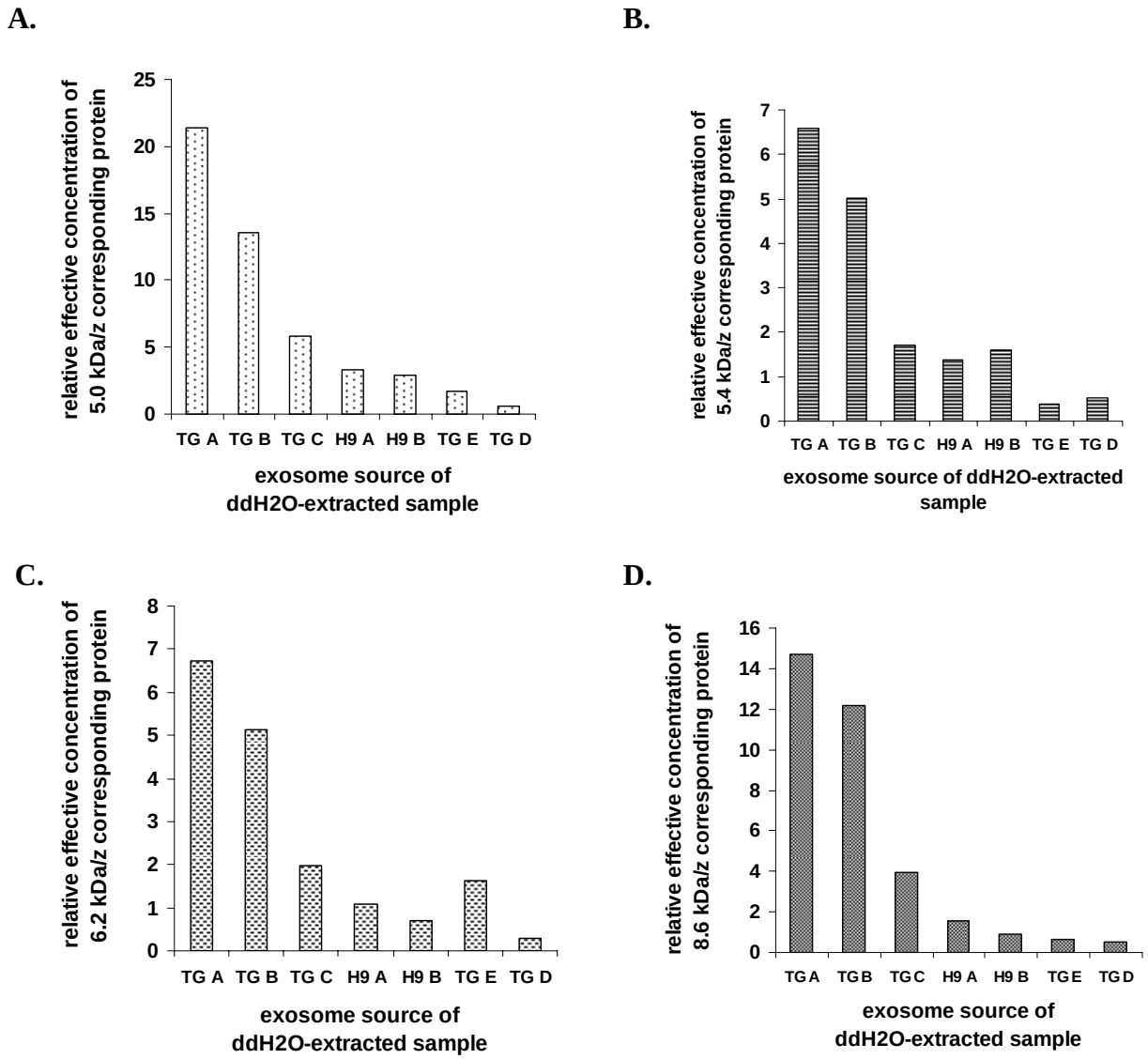


Figure 25. Relative effective concentration of proteins corresponding to various MALDI-TOF-MS analyte signals among five TG and two H9 dI-ddH2O extracted samples. Shown here are effective concentrations values for (A) m/z 5.0 kDa, (B) m/z 5.4 kDa, (C) m/z 6.2 kDa and (D) m/z 8.6 kDa analytes.

Since MALDI-TOF-MS analyte signals correspond to proteins contained in the original exosome extracts, relative peak integrations standardized by the m/z 11.3 kDa internal control signal as well as the original exosome protein concentration during dI-ddH_2O extraction of the

fractions describe the relative concentration of a protein giving rise to a specific mass/charge signal. Calculation of relative protein concentrations corresponding to the m/z 5.0, 5.4, 6.2 and 8.6 kDa signals (Figures 25A-D) were striking in their correspondence to the LTR suppression activity (Figure 24) for the panel of dI-ddH_2O extracted samples analyzed. Interestingly, the relative proportions of the m/z 5.0, 5.4, 6.2, and 8.6 kDa appeared in relatively conserved proportions averaging a ratio of 3 : 1 : 1 : 2 for samples displaying significant HIV-1 suppression activity (Table I). This would further identify these four analytes as a functional set, distinguishing them from other signals such as the m/z 11.3 kDa, which appear invariant to LTR suppression activity. The m/z 5.0, 5.4, 6.2, and 8.6 kDa signals thus serves as valuable markers indicating the presence of a soluble protein mediating HIV-1 transcriptional suppression.

Table 1. A generally conserved ratio of 3:1:1:2 for the m/z 5.0, 5.4, 6.2, and 8.6 kDa MALDI-TOF-MS signals from the dI-ddH_2O extracted samples displaying significant LTR suppression activity. (i) The m/z 5.4 kDa signal used as the normalizing factor. (ii) The m/z 6.2 kDa signal used as the normalizing factor.

(i)

Sample	**Relative Ratio**			
	5.0 kDa/z	**5.4 kDa/z**	**6.2 kDa/z**	**8.6 kDa/z**
TG A	3.240964	1	1.018072	2.230924
TG B	2.702479	1	1.024793	2.427686
TB C	3.444444	1	1.160494	2.345679
H9 A	2.428954	1	0.780161	1.123324
Average	2.954211	1	0.99588	2.031903

(ii)

Sample	**Relative Ratio**			
	5.0 kDa/z	**5.4 kDa/z**	**6.2 kDa/z**	**8.6 kDa/z**
TG A	3.183432	0.982249	1	2.191321
TG B	2.637097	0.975806	1	2.368952
TB C	2.968085	0.861702	1	2.021277
H9 A	3.113402	1.281787	1	1.439863
Average	2.975504	1.025386	1	2.005353

3.5 DISCUSSION

The investigation described here was undertaken based on some specific results uncovered in the previous chapter of study (Chapter 2). The discovery of HIV-1 suppressing exosomes secreted by TG cells was a seminal development for the inquiry presented here, as it represented a novel source of HIV-1 transcription suppression activity that could be purified at a large enough scale to probe the molecular nature of the elusive antiviral effector mechanism of CD8+ T cells. The previous demonstration in Chapter 2 of a dual soluble factor- and exosome-mediated nature for secreted CD8+ cell antiviral activity was the starting point for this investigation to determine if a mechanistic connection existed between the two components of CAF activity. The present investigation began by attempting to detect fluctuations in the potency of exosome-mediated LTR suppression activity. Using careful quantification methods, results from this first set of experiments readily detected fluctuations in LTR promoter suppression activity on the surface of exosomes. Upon side-by-side analysis of various exosome-depleted TG culture fluids and exosomes purified from them, an apparent pattern of inverse correlation was observed between exosome-mediated and membrane free antiviral activities present in TG culture fluids. This result was consistent with the idea of the soluble factor being derived from a molecule expressed on TG exosomes and consequently provided an alternative interpretation other than simple variable gene expression to explain the fluctuations observed in exosome-mediated HIV-1 transcription suppression activity.

An effort was accordingly undertaken in this study to define the precise manner in which the HIV-1 LTR suppressive activity localized to the membrane of TG exosomes in order to begin

probing the possible mechanism by which the antiviral activity expressed on the external surface of exosomes might be converted into a soluble form. Salt-extraction experiments performed in this study demonstrated for the first time that a soluble antiviral factor could be extracted from the exosomes themselves. A striking result in the ensuing investigation was the finding that the LTR suppressive activity exhibited a curious partial solubility from TG exosomes, exhibiting the property of both a membrane-bound and membrane-free molecule despite exhaustive sodium carbonate treatment of TG exosomes. This was especially demonstrated in carefully controlled soluble extraction experiments of H9 and TG exosomes, where the property of solubility was observed to be largely restricted to TG but not H9 exosomes. Such a result strongly implies a non-random mechanism by which the membrane-bound antiviral factor is converted into a soluble form, namely the potential presence of some secondary enzymatic activity in TG exosomes functioning in the cleavage of a membrane-bound HIV-1 transcription suppressing factor off its molecular anchor to produce a soluble active antiviral protein fragment. Indeed, in one experiment, after sodium carbonate depletion of peripheral proteins from two independent TG exosome samples, a significantly higher level of HIV-1 transcription suppressive activity was observed after subsequent dI-ddH_2O extraction than what was found in the previous sodium carbonate treatment (Figure 21).

These findings were further corroborated by MALDI-TOF-MS analysis of various dI-ddH_2O extracts from TG and H9 exosomes. Results of this analysis uncovered several analyte signals corresponding with the LTR suppressive activity of the set of fractions studies. This would indicate the presence of specific proteins or protein fragments released from HIV-1 suppressing exosomes, eluted into solution by simple water extraction *after* depletion of

peripheral membrane proteins from exosomes. That an apparent defined ratio between a set of MALDI-TOF-MS signals appeared preserved across independent HIV-1 suppressing soluble samples further suggests a specific non-random event giving rise to the solublized factors producing the observations described. One of these signals could very well originate from the elusive solublized factor mediating suppression of HIV-1 transcription. In addition, the number of analyte signals detected corresponding to LTR suppression activity and their non-equivalent proportions in each soluble extract sample, further hints at more than one protein source correlating with LTR suppression activity. This would be another inference to a non-random activity involved in releasing such molecules from their membrane tethering. These results strongly suggest the existence of a specific proteolytic or enzymatic activity in releasing the antiviral activity mediating transcriptional suppression of HIV-1 (Figure 26), a postulate that is consistent with indirect evidence presented by other investigators of protease inhibitor modulation of both CAF and CD8+ cell-mediated HIV-1 suppression (215).

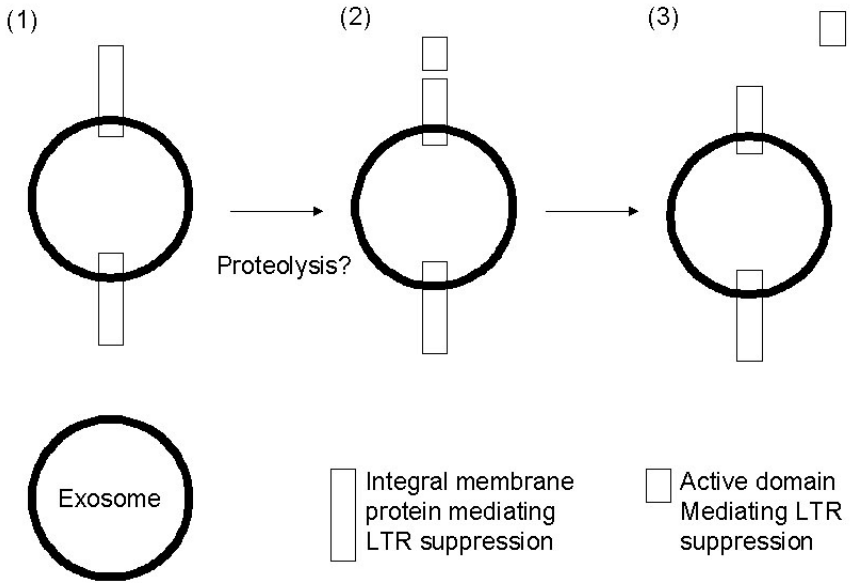


Figure 26. A proposed model for the concurrent membrane-bound / soluble nature of the LTR promoter suppression factor and its experimental verification. This model postulates an integral membrane protein mediating HIV-1 LTR promoter suppression (1) that undergoes a proteolytic event that cleaves a fragment containing the LTR suppressive activity (2) which is released as a soluble factor (3).

The possible presence of a proteolytic action releasing a membrane-free form of HIV-1 transcription suppression activity from a membrane-bound precursor would elegantly explain the results observed in this study and reconcile the dual exosome and soluble factor components found for secreted CAF activity in the previous chapter. If a proteolytic activity of this nature does indeed exist, then a number of properties for such a putative catalyst can be predicted based on the results of this study. Firstly, the secondary factor would be predicted to exist in a membrane-bound form as sodium carbonate depletion of peripheral proteins did not prevent recurring formation of an extractable soluble antiviral factor. Secondly, a putative enzymatic cleavage activity of this nature would also be expected to colocalize with the same exosome proteins as the LTR suppressive factor, such as the tetraspanin molecules found localized on exosomes (Figure 5, Chapter 2). A potential autolytic cleavage activity on a membrane-bound

HIV-1 suppressing protein can perhaps be ruled out as H9 exosomes readily demonstrate membrane-bound antiviral activity with little to no soluble fragment mediating the same action, implying the requirement of a secondary factor for releasing the antiviral activity from a membrane anchor. If a protease is involved in cleaving an HIV-1 suppressing precursor into a soluble form, then its presence can potentially be probed through the use of protease inhibitors to determine if disruption of proteolytic activity is coincident with non-extraction of a soluble fragment. If such a result was found, not only would it elegantly explain many of the results in this investigation, but it would also be consistent with the biogenesis of HIV-1 exosomes from within late endosomes, an intracellular compartment that functions in general degradation of extracellular membrane protein.

While the possible involvement of a proteolytic action in modulating the factor mediating LTR promoter suppression activity provides an interesting avenue to explore noncytolytic CD8+ T cell suppression of HIV-1, the most exciting result of this investigation is, perhaps, the development of novel techniques in this study for the serum-free purification of the HIV-1 suppressive activity into pure water. Although such a result may appear trivial at first glance, it represents an unprecedented advancement in the 20 year quest to identify the molecular factor mediating this potent but elusive antiviral activity. Previous attempts at purifying this activity have utilized methods attempting to harvest the soluble protein from bulk quantities of filtered culture media. Even when grown under serum free conditions, the presence of large quantities of recombinant growth factors in serum-free culture media make biochemical purification of the antiviral factor problematic, especially given that the antiviral activity appears typically secreted in low quantities. While exosomes are also harvested from bulk culture media, results from the

investigation performed here have demonstrated that the activity mediating HIV-1 transcriptional suppression remains intact after exosome purification and multiple biochemical treatments, allowing for elution of water soluble fractions of the activity towards protein identification. Utilization of HVS-transformed CD8+ T cell lines for exosome harvesting allows for theoretically unlimited scalability for production of water eluted fractions containing high concentrations of HIV-1 transcription suppression activity.

In the quest for ultimate protein identification of the elusive HIV-1 transcription suppressive factor produced by CD8+ T cells, several techniques described in our investigation will greatly aid in such an endeavor. MALDI-TOF-MS represents only a first tier method for more advanced proteomic methods such as protein chip profiling, ion-trap MALDI-TOF/TOF-MS/MS and electrospray injection-based multidimensional proteomic profiling. The identification of specific MALDI-TOF-MS analyte signals is particularly promising for application of the more advanced ion-trap MALDI-TOF/TOF-MS/MS, as this technique utilizes capture of specific ion masses towards direct sequence evaluation. Such a procedure would not only be valuable in identifying potential candidate molecules identifying with the HIV-1 suppressive activity, but perhaps also identify possible exosome markers co-localizing with the antiviral factor to further define its intracellular regulation and manifestation at a cellular level. The utility of traditional biochemical fractionation techniques such as HPLC and gel filtration is now made feasible with procedures outlined in this study for larger scale production of the solublized antiviral activity. Such fractionation techniques combined with biological evaluation using the acute LTR suppression assay described here can facilitate more conclusive interpretations of proteomic data. The techniques and results presented in this chapter of study

represent a major leap forward in bringing to fruition conclusive identification of the molecular factor mediating HIV-1 transcription.

4.0 FINAL DISCUSSION AND CONCLUSIONS

Before undertaking the study presented here, there were several lines of evidence that suggested a greater complexity to the antiviral mechanism behind CD8+ T cell noncytolytic HIV-1 suppression activity than a simple cytokine release model. One was the greater potency of cell-contact mediated noncytolytic HIV-1 suppression (181,183) and the second was dissociation between cell-mediated suppression of HIV-1 and secretion of a soluble antiviral factor (183,185). These results from previous investigations of the antiviral mechanism demonstrated that cell-contact played an important role in mediating the antiviral activity. These findings were never investigated more thoroughly by other investigators, save for a handful of studies evaluating the involvement of molecules known to play a role in antigen-dependent CD8+ T cell responses (202-205). Therefore the role of membrane determinants in mediating the antiviral activity remained largely unexplored, despite clear evidence of cell-contact modulation of the activity. What has ensued in the field was an effort by several groups to identify secreted mediator, CAF. While the many candidate molecules proposed did display HIV-1 antiviral activity (139,194-196), none of them were found to identify with the hallmarks of secreted CAF activity (197-200). As a result, lack of any clear identification of a single responsible element led some groups to propose a hypothesis that noncytolytic suppression of HIV-1 by CD8+ T cells is largely mediated by a multifactorial secretion of antiviral molecules (195,201). The results of the study presented here strongly refute this hypothesis.

A multifactorial cytokine secretion hypothesis for CD8+ T cell noncytolytic HIV-1 suppression not only ignores two key lines of evidence demonstrating the involvement of cell surface molecules, it dilutes the involvement of one key effector mechanism that may very well represent the "Achilles' heel" for HIV-1: the specific inhibition of lentiviral transcription by CD8+ T cells (172,177,186-190). Therefore, the present course of study was based on a central thesis that the specific factor mediating transcriptional suppression of HIV-1 is a membrane-bound protein and in the ensuing study new evidence was uncovered strongly suggesting that CD8+ T cell suppression of HIV-1 transcription, including the secretion of an antiviral effector, is a phenomenon mediated by a specific membrane-bound protein activity. The novel discovery that exosomes potently suppress transcription of HIV-1 in acute and chronic models of infection finally provides a key to defining a functional link between a cell surface antiviral protein and a secreted antiviral factor. Initial observations of CD8+ T cell culture fluids suggested that CAF was basically composed of two components: (i) CD8+ T cell secreted exosomes that suppressed HIV-1 transcription and (ii) a soluble factor that also suppressed HIV-1 transcription. That both mediated the same antiviral action prompted an investigation into whether these distinct components had a common molecular determinant.

Initial characterization of TG membrane and secreted vesicles in Chapter 2 revealed that the responsible factor mediating suppression of HIV-1 replication was an integral membrane protein that could express itself on the external surface of exosomes and could exert its antiviral action independently of its lipid anchoring. Upon discovery of a soluble protein mediating the same transcriptional suppression as exosomes, the most rational hypothesis to explore was that of a cleaved precursor since it was consistent with our initial analysis demonstrating an integral

membrane antiviral factor. Through a careful series of solvent extraction analysis described in Chapter 3, clear evidence of an HIV-1 transcription suppressing integral membrane factor being converted into a soluble form was found. By pre-treating exosomes with sodium carbonate to deplete the vesicles of all peripheral proteins, water soluble extracts of specifically cleaved proteins from membrane-bound precursors could be made. Application of MALDI-TOF revealed the presence of more than one protein that correlated with the released LTR suppression activity. Furthermore, the release of the soluble antiviral factor was due to the presence of a second membrane-bound enzyme, as determined in a comparison of H9 and TG exosomes, which functions in the cleavage of a characteristic set of proteins, including the HIV-1 LTR promoters suppressing factor. A characteristic signature of that enzymatic activity could be defined by MALDI-TOF-MS analysis. Therefore, in an effort to define the LTR suppressing protein factor, a molecular mechanism was uncovered that elegantly explained the inverse correlations observed between the appearance of a soluble protein in CD8+ T cell culture fluid suppressing HIV-1 transcription and a tight association of the antiviral activity to exosomes. The results of this study strongly refute a multifactorial cytokine secretion model for CAF and suggest instead a novel and elaborate membrane-mediated mechanism underlying secreted CAF activity and cell-mediated suppression of virus. The findings in this study support a new model of CAF secretion, one that is an exosome-driven phenomenon with the appearance of a soluble protein factor being the consequence of an exosome-localized integral membrane converting the membrane-bound HIV-1 transcription factor into a soluble form. In this new model, between an exosome-bound and soluble form of the same antiviral protein that comprises secreted CAF activity.

Such a distinction between a membrane-bound and membrane-free antiviral mediator may have important physiological consequences. Recent studies in the exosome field have demonstrated a remarkable adhesion capability of exosomes to cell surfaces (233). If such an engagement occurred with CD4+ cell surfaces by CD8+ T cell exosomes, it may facilitate specific engagement of the LTR suppressing factor with its cognate receptor to induce potent inhibition of proviral transcription. Conceivably, the converse might be true where a soluble mediator may have greater conformational freedom than a membrane bound factor for engaging a cognate receptor on CD4+ cells. The physiological role of a CD8+ cell-secreted mediator in suppressing HIV-1 transcription awaits further dissection. However, the evidence uncovered in this study of a specific molecular factor secreted via exosome biogenesis indicates a much more complex nature to noncytolytic HIV-1 suppression by CD8+ T cells than previously thought (215).

Evidence outlined in this study of an integral membrane factor with potent LTR promoter inhibition activity opens a new door to finally understanding cell-contact mediated CD8+ T cell suppression of HIV-1. The secretion of this antiviral protein via exosomes defines a very unique membrane microdomain the LTR suppressing protein factor colocalizes with. While the identification of the LTR suppressing factor is an area of active research at the present, identification of the molecules colocalizing with the membrane-bound anti-HIV factor can greatly illuminate how such a factor is regulated by CD8+ T cells, particularly in dissecting the events that lead to the apparent priming of the noncytolytic HIV-1 suppression activity and its deficiency in HIV-1 patients rapidly progressing to AIDS. The findings of HIV-1 suppressing exosomes secreted by CD4+ T cells underscore the point that simple expression of the antiviral

gene product may not necessarily translate into cell-mediated activity. In the context of cellular regulation, it can perhaps now be understood why noncytolytic HIV-1 suppressing CD8+ T cell clones are typically found displaying an HLA-DR+/CD28+ activated phenotype (176). Such a phenotype is indicative of a dynamic re-organization of cell membrane protein, particularly the translocation of endosomal membrane compartments to the cell surface, especially with increased granule release that is characteristic of CTL activity. In B cells and DCs, HLA-DR has been found to specifically associate with CD9, CD63, and CD81 tetraspanins (234,235) which are molecules we found specifically expressed on HIV-1 suppressing exosomes. Thus, the tetraspanin colocalization with the HIV-1 suppressing activity in exosomes may provide clues as to how the factor might be regulated in CD8+ T cells. Tetraspanin organization in CD8+ T cells has not been well studied. However, recent reports have described tetraspanin protein webs as critical mediators in the dynamic reorganization of T cell surfaces during antigen recognition (236,237).

Such a role for tetraspanin protein networks have been found in other cells as well, particularly in reshaping the cell surface and driving agglomeration of molecules into high concentration at points of cell-to-cell contact, with such processes occurring prior to and independently of MHC molecule-T cell receptor engagement (238). This MHC-independent membrane reorganization might represent the vehicle by which cell-contact mediated LTR suppression is effected by CD8+ T cells. Such a mechanism would presumably allow for physical aggregation of the HIV-1 suppressive factor on the surface of CD8+ T cells and therefore a more concentrated signaling for induce more potent suppression of proviral transcription (Figure 28.A). In contrast, a fluid mediator driven by exosome secretion, composed

of exosome- and soluble-mediating components, might only deliver weak to moderate signals to suppress HIV-1 transcription due to the diffusible nature of the secreted activity (Figure 28.B). Our findings of LTR suppression activity co-localizing with tetraspanin protein networks may finally resolve the dissociation observed between secreted- and cell contact-mediated forms of noncytolytic HIV-1 suppression displayed by CD8+ T cells.

A. **B.**

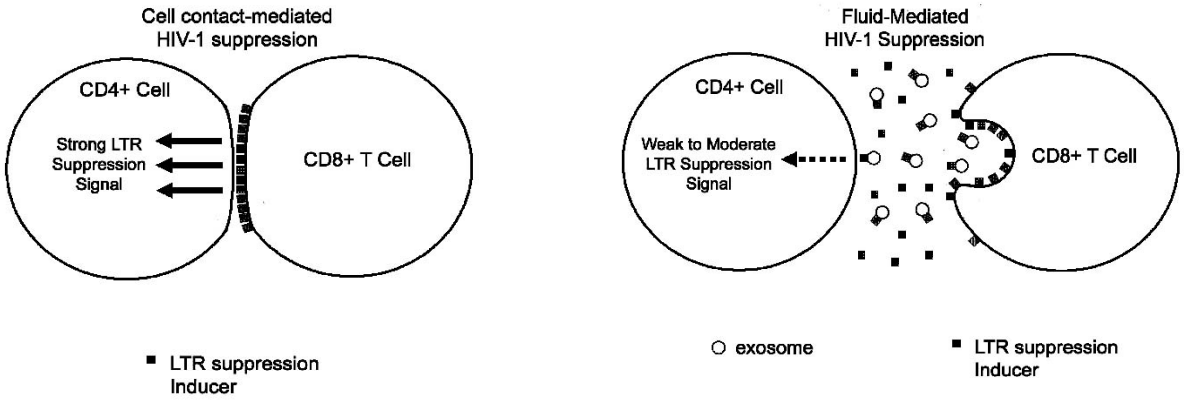


Figure 27. A model for CD8+ T cell suppression of HIV-1 transcription by a membrane-localized antiviral factor. (A) Cell-contact mediated CD8+ T cell suppression of HIV-1 might involve a purely membrane-bound factor that induces potent HIV-1 transcription inhibition due to a possible tetraspanin-dependent clustering. (B) A fluid mediator is driven by exosome secretion where the antiviral factor mediates LTR suppression either bound to the exosome or a cleaved soluble fragment.

Identification of the LTR suppressing molecule would aid immensely in unraveling the mechanism behind potent HIV-1 transcriptional suppression by CD8+ T cells. The paradigm of a membrane-localized nature for the antiviral activity has led to the development of novel methods for purifying concentrated fractions containing the activity. The elution of the activity from a serum free source will greatly facilitate conclusive identification of the activity. In

addition, immunophenotyping of exosomes by the immunomagnetic bead capture can allow for a more comprehensive understanding of the factors that might regulate the LTR suppressive factor at the cell surface and in the secreted vesicles. The evidence presented in this study of a suggested protease activity colocalizing to HIV-1 suppressing exosomes may be just one example of this antiviral factor's intracellular regulation. Further dissection of the exosomes by global proteomic profiling techniques, such as MuDPIT (246), can provide a much more comprehensive analysis of the network of proteins associating with and possibly regulating the HIV-1 suppressive factor. The results of this study have provided a much needed illumination on the true nature of this enigmatic but potent CD8+ T cell effector function against HIV-1 replication. Future study of how CD8+T cells inhibit HIV-1 transcription can now be approached with greater clairvoyance.

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