THE ROLE OF HIV-1 TAT AND ANTIRETROVIRALS IN CATHEPSIN MEDIATED ARTERIAL REMODELING

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THE ROLE OF HIV-1 TAT AND ANTIRETROVIRALS IN CATHEPSIN MEDIATED ARTERIAL REMODELING

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Dedicated to the loving friends and family God so graciously placed in my life to help endure this process, especially my husband Jon Parker, and my Grandma Jennie Massey.

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LIST OF SYMBOLS AND ABBREVIATIONS

ARV	Antiretroviral
CVD	Cardiovascular Disease
ECM	Extracellular Matrix
ERK	Extracellular-Signal Regulated Kinase
EFV	Efavirenz
HAART	Highly Active Antiretroviral Therapy
HAEC	Human Aortic Endothelial Cell
Hs-CRP	High Sensitivity – C - reactive protein
HSPG	Heparin Sulfate Proteoglycan
ICAM-1	Intracellular Adhesion Molecule- 1
IMT	Intima Medial Thickness
JNK	c-JUN N-Terminal Kinase
KDR	Kinase Domain Receptor
LPV/r	Lopinavir/ritonavir
LTR	Long Terminal Repeat
M-CSF	Macrophage Colony Stimulating Factor
MCP-1	Monocyte Chemoattractant Protein- 1
NNRTI	non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
RGD	Arginine-Glycine-Aspartic
TAR	Tat Activation Region

Tat	HIV-1 Transactivating Factor
TDF	Tenofovir Disoproxil Fumarate
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGFR-2	Vascular Endothelial Growth Factor Receptor-2

SUMMARY

Major advances in highly active antiretroviral therapies (HAART) have extended the lives of people living with HIV, but there still remains an increased risk of death due to cardiovascular diseases (CVD). HIV proteins and antiretrovirals (ARVs) have been shown to contribute to cardiovascular dysfunction by affecting the different cell types that comprise the arterial wall. In particular, HIV-1 transactivating factor, Tat, is a cationic polypeptide that binds to endothelial cells, inducing a range of responses that have been shown to contribute to vascular dysfunction. It is well established that hemodynamics also play an important role in endothelial cell mediated atherosclerotic development. Upon exposure to low or oscillatory shear stress, such as that found at branches and bifurcations, endothelial cells contribute to proteolytic vascular remodeling by upregulating cathepsins, potent elastases and collagenases. However, the exact mechanisms of the effects of HIV proteins on shear-mediated vascular remodeling are not well understood.

The ***objective* of this research** is to elucidate the effects of pro-atherogenic shear stress, HIV proteins, and antiretroviral therapies on the vasculature using *in vivo* and *in vitro* models. More specifically, we want to investigate pro-atherogenic shear stress conditions coupled with these HIV factors and understand their effects on the proteolytic activity of endothelial cells and monocytes to determine downstream regulatory mechanisms using *in vitro* culture systems and the HIV-transgenic mouse model. To achieve this, a two method approach was employed: 1) the proteolytic effects of HIV

proteins on arterial cells and downstream signaling mechanisms were investigated and 2) the effects of ARVs on patient inflammatory responses and mononuclear cell cathepsin activity were determined. The major goal of this work was to identify individual and synergistic contributions of pro-atherogenic shear stress and HIV proteins on endothelial cell proteolytic activity; and to parse the individual effects of ARVs on mononuclear remodeling capacity.

The results of this work i) demonstrate that upregulation of cathepsins *in vivo* and *in vitro* is caused by a synergism between pro-atherogenic shear stress and HIV-1 proteins, ii) elucidate pathways that are activated by HIV-1 Tat and pro-atherogenic shear stress leading to cathepsin-mediated ECM degradation, and iii) identify novel biomarkers to monitor the adherence of patients on efavirenz- and tenofovir-containing antiretroviral regimens. This dissertation has broad implications that will help identify therapeutic strategies to prevent cardiovascular events in patients with HIV and pinpoint targets to aid in the alleviation of HIV-mediated arterial remodeling.

INTRODUCTION

1.1 Motivation

Major advances in highly active antiretroviral therapies (HAART) have extended the lives of people living with HIV, but there still remains an increased risk of death by cardiovascular diseases (CVD) [1, 2]. Studies have been done to elucidate whether this increase in cardiovascular incidence is due to antiretroviral treatment or the virus itself. The SMART Study group in 2007 showed that HIV-positive patients' risk for CVD was increased when patients were intermittently taken off their antiretroviral therapies, even when their CD4+ counts were kept above a certain threshold [3]. These results implicated HIV itself as causative agent, even when antiretrovirals were conserved. There is also overwhelming evidence that patients on certain antiretroviral regimens have an increased risk for CVD compared to their counterparts [4]. Of the five classes of antiretroviral drugs, protease inhibitors have been the most widely implicated. However, reverse transcriptase inhibitors are also involved; patients on these drugs show increased risk of myocardial infarction, atherosclerotic lesion formation [5-8], and arterial stiffness [9, 10].

HIV proteins have been shown to contribute to cardiovascular dysfunction by affecting the different cell types that comprise the arterial wall. In particular, HIV-1 transactivating factor (Tat) is a cationic polypeptide that binds to endothelial cells and activates monocytes, inducing a range of responses that have been shown to contribute to vascular dysfunction [11-13]. It is well established that hemodynamics also play an important role in endothelial cell-mediated atherosclerosis development. When exposed to low or oscillatory shear stress, such as that found at branches and bifurcations, endothelial cells contribute to proteolytic vascular remodeling by upregulating cathepsins, potent elastases and collagenases [14]. The cooperative effects of HIV- 1 Tat

and pro-atherogenic shear stress on cathepsin-mediated vascular remodeling have not yet been elucidated. Furthermore, overlapping mechanisms between these two factors, known to cause endothelial cell dysfunction, have not been investigated. This thesis explores the complex contributions of antiretrovirals (ARVs), HIV proteins, and shear stress on cathepsin-mediated arterial remodeling.

1.2 Research Objectives

The ***objective* of this research** is to elucidate the effects of pro-atherogenic shear stress, HIV proteins, and antiretroviral therapies on the vasculature using *in vivo* and *in vitro* models. More specifically, we want to investigate pro-atherogenic shear stress conditions coupled with these HIV factors, and elucidate their effects on the proteolytic activity of endothelial cells and monocytes to determine downstream regulatory mechanisms using *in vitro* culture systems and the HIV-transgenic mouse model.

To achieve this, a two method approach was employed: 1) the proteolytic effects of HIV proteins on arterial cells and downstream signaling mechanisms were investigated and 2) the effects of ARVs on patient inflammatory responses and mononuclear cell cathepsin activity were determined. The major goal of this work was to identify individual and synergistic contributions of pro-atherogenic shear stress and HIV proteins on endothelial cell proteolytic activity; and to parse the individual effects of ARVs on mononuclear remodeling capacity.

Our *central hypothesis* is that HIV-1 Tat contributes to arterial remodeling at regions of low and oscillatory shear stress by increasing cathepsin expression even beyond that of the endothelial cell response to disturbed flow, while specific ARV's alter proteolytic activity in monocytes, both serving to modify extracellular matrix (ECM)

content and organization, ultimately leading to altered arterial biomechanics and increased arterial stiffness to promote CVD. These hypotheses are tested in these specific aims.

Specific Aim 1: Elucidate the synergistic effects of pro-atherogenic shear stress and HIV proteins on cathepsin activity *in vitro* and *in vivo*

Hypothesis: HIV proteins exacerbate cathepsin and other protease activity in the vascular wall at sites of disturbed flow and altered hemodynamics to promote arterial remodeling and cardiovascular disease.

We tested this hypothesis using an HIV-tg mouse model [15], which allowed us to investigate the effects of HIV-1 proteins on arterial cells at regions of the vasculature naturally pre-conditioned with atheroprotective and pro-atherogenic shear stress profiles. We found that cathepsin K activity was upregulated in mouse aortas, especially at regions of pro-atherogenic shear stress, using immunohistochemistry, multiplex cathepsin zymography, and ratiometric analysis. Human aortic endothelial cells (HAECs) were stimulated with HIV-1 Tat and shear stress to identify a specific HIV target responsible for this upregulation. We found that HIV-1 Tat and pro-atherogenic shear stress act cooperatively to increase cathepsin K activity in cultured HAECs using multiplex cathepsin zymography.

Specific Aim 2: Determine synergistic effects of Tat and pro-atherogenic shear stress on ECM degradation and identify relevant kinase signaling pathways.

Hypothesis: The upregulation of cathepsin K in co-stimulated endothelial cells leads to increased gelatin cleavage, mediated by ERK and c-jun phosphorylation.

We tested this hypothesis using a shear stress apparatus that recapitulates physiologically relevant shear stresses in areas of the vasculature that are more prone to atherosclerotic plaque development. HAECs were exposed to pro-atherogenic shear stress and HIV-1 Tat; subsequent cathepsin-mediated gelatin degradation was assessed, and cathepsin inhibitable activity determined using E64, a broad spectrum cathepsin inhibitor. Time-dependent activation of ERK, JNK, c-jun, and Akt (kinases either activated by HIV-1 Tat or linked to cathepsin activity) was assessed in cells co-stimulated with shear stress and Tat, Tat alone, or vehicle controls to determine the cooperative effects of shear stress and Tat stimulation on kinase activation. Lastly, phosphorylation of ERK was assessed as a kinase mediator of cathepsin expression and increased elastase and gelatinase activity during Tat stimulation by blocking its activation in endothelial cells by incubating with U0126, a MEK inhibitor. We found that cathepsin-mediated gelatin cleavage was increased in cells stimulated with Tat. Additionally, ERK and c-jun phosphorylation were highest in HAECS stimulated with both pro-atherogenic shear stress and Tat compared to all other conditions. Finally, total c-jun signal remained relatively constant and was not attenuated as seen with vehicle controls in endothelial cells stimulated with Tat.

Specific Aim 3: Determine the effects of ARV treatment on markers of inflammation and cathepsin activity in HIV positive patients and in isolated PBMCs

Hypothesis: Antiretrovirals associated with increased inflammatory markers alter mononuclear cell cathepsin activity, which can lead to or be indicative of increased cardiovascular disease in HIV positive patients.

ELISAs were used to assess levels of inflammatory markers in HIV positive patients. Patients on efavirenz (EFV)-containing regimens showed elevated levels of hs-c reactive protein (hs-CRP) compared to HIV-negative, HAART-naïve, and nevirapine (NVP)-containing regimens. Soluble vascular cell adhesion molecule (sVCAM) was elevated in HAART-naïve, NVP- treated, and lopinavir/ritonavir (LPV/r) treated patients compared to HIV-negative subjects. In addition to serum, PBMCs from patients were collected, and as shown with multiplex cathepsin zymography, those that were isolated from patients on EFV or tenofovir disoproxil fumarate (TDF) had lower cathepsin activity compared to naïve, negative, and other antiretroviral regimens. This was validated *in vitro* using THP-1 monocytes exposed to physiological doses of TDF and EFV. Human aortic smooth muscle cells (SMCs) and HAECs were also stimulated with TDF and EFV. No change was observed in SMCs for either ARV, but HAECS expressed greater cathepsin activity after EFV stimulation when compared to controls.

1.3 Significance and Scientific Contribution

The results of this work i) demonstrate that the upregulation of cathepsins *in vivo* and *in vitro* is caused by a synergism between pro-atherogenic shear stress and HIV-1 proteins, ii) elucidate pathways that are activated by HIV-1 Tat and pro-atherogenic shear stress leading to cathepsin-mediated ECM degradation, and iii) identify novel biomarkers

to monitor the adherence of patients on EFV and TDF antiretroviral regimens. This dissertation has broad implications that will help identify therapeutic strategies to prevent cardiovascular events in patients with HIV and pinpoint targets to aid in the alleviation of HIV-mediated arterial remodeling.

Cathepsins are known to play a key role in atherosclerotic plaque progression, and drugs that inhibit their ability to degrade ECM could prove to be instrumental in alleviating HIV-mediated cardiovascular diseases. Understanding the upregulation of ERK and c-jun in relationship to Tat stimulation, helps to identify downstream targets that contribute to an overall inflammatory state and could be targeted to more effectively treat patients. Previously identified endothelial activation associated with ERK and c-jun was linked to CVD progression as a result of activation of these kinases. Finally, HIV patients are prescribed antiretrovirals to be administered over their lifetime; it is imperative to understand the long-term inflammatory effects of their use to circumvent preventable issues, and provide optimized patient regimens.

CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

2.1 HIV and Cardiovascular Disease

2.1.1 Significance

In 2010**,** the World Health Organization reported that 34 million people were living with the Human Immunodeficiency Virus (HIV) globally, with 1.8 million cases resulting in death. Due to the advent of antiretroviral therapies, HIV-infected patients are living longer. However, these patients are dying from other comorbidities such as renal, hepatic, and cardiovascular diseases [16-20].These comorbidities are seemingly just as important as the onset of Acquired Immune Deficiency Syndrome (AIDS) when comparing causes of death for HIV-positive patients [1]. Compared to the HIV-negative population, HIV-positive patients are 35% more likely to have a cardiovascular event within their lifetime and this probability increases four-fold by the time they reach 60 years of age. HIV populations have been shown to have an increased risk of myocardial infarction as was demonstrated by an increase in markers of subclinical atherosclerosis such as endothelial dysfunction [21-24], increased atherosclerotic lesions [2, 4, 8, 25] and increased carotid artery intima-medial thickness (IMT) [26-31] and stiffness [7, 23]. In 2007, a Massachusetts study showed increased risk factors for heart attack in HIV-positive patients across all ages [32].

Studies have been done to elucidate whether this increase in cardiovascular incidence is due to antiretroviral treatment or the virus itself. The SMART Study group in 2007 showed that HIV-positive patients risk for cardiovascular disease was increased when patients were taken off their antiretroviral therapies, even when their CD4+ counts were kept above a certain threshold [3].Furthermore, studies done during the pre-HAART era in naive HIV-1 positive children showed coronary arteriopathy in 3 out of 6 children [33]. Another study with HIV-positive patients aged 23-32 showed atherosclerotic plaque formation in coronary arteries, further validating HIV-elicited early onset of cardiovascular disease in HIV-positive patients who were not adhering to any antiretroviral regimen [34]. Antiretroviral naïve HIV-1-positive patients have higher levels of soluble vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule (ICAM-1), and E-selectin compared to healthy controls [35-37]. This up-regulation of cell adhesion markers suggests that HIV-1 increases endothelial cell activation and dysregulation.

2.1.2 Pathology of HIV-1

HIV-1 is a member of the lentivirus family of retroviruses that primarily infects CD4+ lymphocytes. The virus recognizes and binds to CD4+ surface proteins, through a high-affinity interaction with gp120 [38-41]. This attachment of the envelope complex to the CD4+ receptor causes a conformational change, enabling fusion and entry into the cell after engaging with a co-receptor of either CCR5 or CXCR4. Once the virus enters the cell, it releases viral contents, including viral RNA, reverse transcriptase, integrase, and protease into the host cell. The viral RNA is reverse transcribed into viral DNA, and

covalently incorporated into the host cell's genome via integrase; essentially hijacking the cell's machinery to transcribe HIV mRNA and then to translate that mRNA to produce viral proteins [42]. Once this process is done the virus buds off the host cell, and matures as the viral protease cleaves newly translated HIV proteins for proper folding. The virus then proceeds to infect other cells.

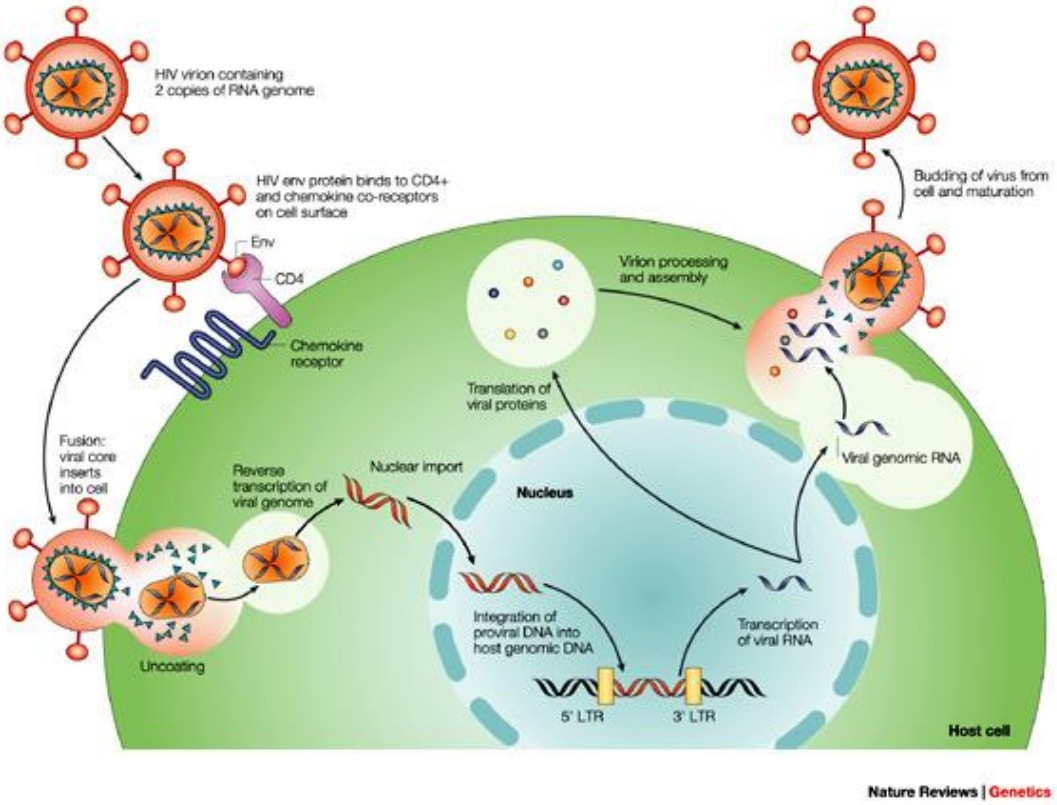

Figure 2.1: Key aspects of the HIV life cycle. Adapted from The causes and consequences of HIV evolution. Rambault. 2004. Nature Reviews Genetics.

HIV-1 proteins are encoded by nine genes located within the virion capsid. The resulting proteins are essential for HIV-1 replication and infection of target cells. *Gag*, *pol*, and *env* are three genes found in all retroviruses. In HIV, the *env* gene encodes for gp160, the precursor for gp41and gp120, envelope proteins which are necessary for virus

entry into cells. The *pol* and *gag* regions encode for the integrase and reverse transcriptase enzymes necessary for efficient HIV- replication and infection [43]. *Tat*, *rev*, *vpr*, *vpu*, *vif*, and *nef* are the remaining accessory genes that distinguish HIV-1 from other retroviruses.

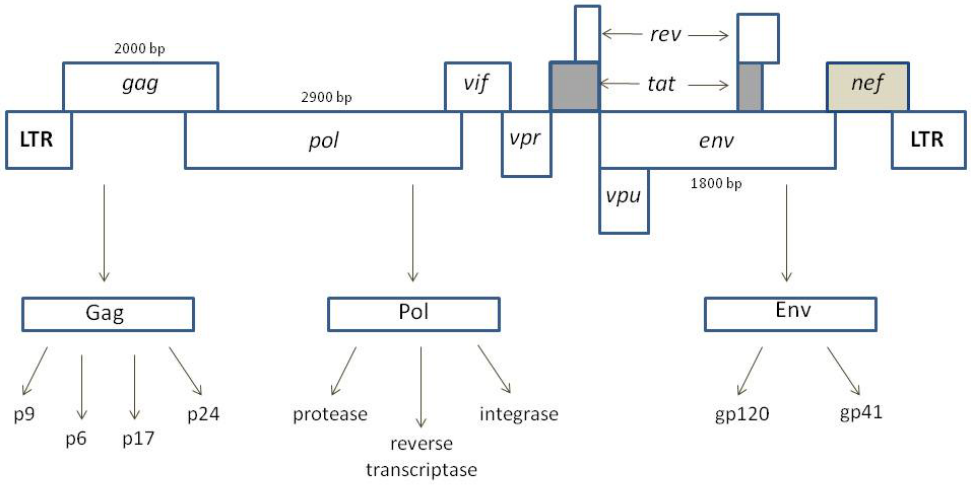

Figure 2.2: The HIV genome. Adapted from Patterns of HIV-1 mRNA expression in mice. Bruggeman et al. 1994. Virology.

Without treatment, the progression from initial infection to AIDS advances over three different stages: acute infection, chronic infection, and AIDS. According to guidelines set by the NIH, acute infection develops 2-4 weeks post infection, during which time the virus is most easily transmitted. Flu-like symptoms may develop as the virus attacks and kills off CD4+ cells[44]. During the chronic phase or latent phase, which can last up to 12 years, HIV multiplies at lower levels than during acute infection [45]. Without treatment this will develop to AIDS, characterized by HIV depletion of immune cells, leaving room for opportunistic infections. The clinical diagnosis for AIDS

occurs when a person has a CD4+ count lower than 200 cells/mm^3 and an opportunistic infection [46].

2.1.3 HIV-1 Tat and Endothelial Dysfunction

It is not definitive whether endothelial cells can become infected by HIV *in vivo*, but the effects of HIV-1 Tat on endothelial cell misregulation have been greatly studied. *In vivo* models using HIV-1 transgenic animals have shown that the presence of viral proteins alone is enough to cause alterations in vascular function [47].

Of specific interest is HIV-1 Tat, a 14-kDa protein known as the transactivating factor, which performs regulatory functions and increases infectivity of the virus [48]. It binds to the secondary structure sequence TAR (Tat activation region) and enables the recruitment of cellular factors which form Tat-associated kinases, activators of transcription elongation. HIV-1 Tat is the most studied HIV protein, critical for HIV infectivity as it binds to the HIV long repeat terminal (LTR), allowing for transcriptional transactivation of viral gene expression.

In HIV-1-positive patients, Tat circulates in the bloodstream at an estimated 2 - 40 ng/ml range, and is secreted from infected T-cells and monocytes [49-51] . Because macrophages and monocytes may act as viral reservoirs within perivascular tissues around the arterial wall, it is believed that endothelial cells are exposed to even higher concentrations of Tat secreted locally by these HIV-infected cells [11]. Tat can bind to uninfected cells and be internalized from the circulation [52, 53].

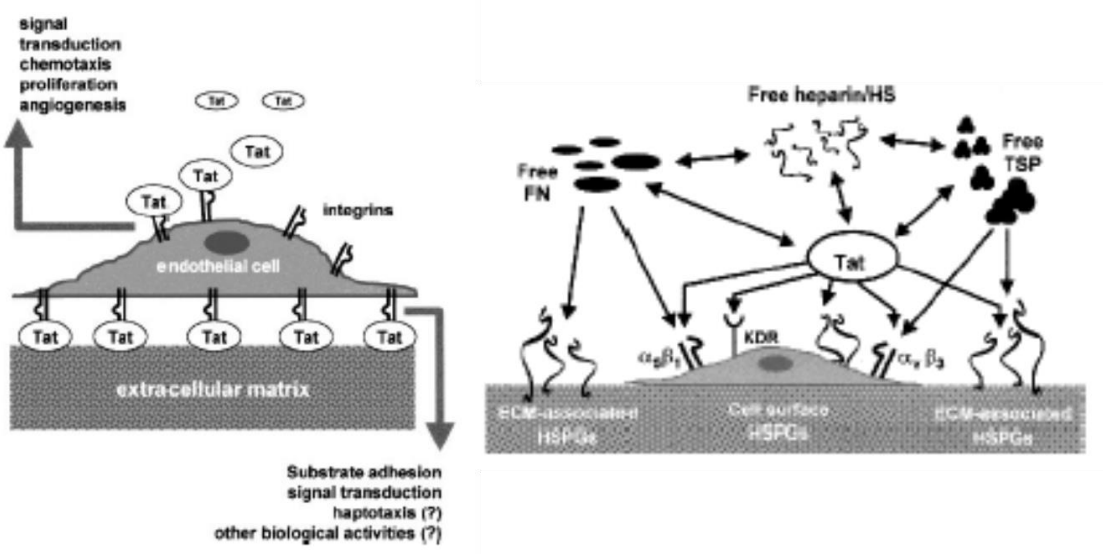

Figure 2.3: Tat affects endothelial cells by binding to surface proteins and being internalized. Adapted from HIV 1 proteins and the endothelium: From protein cell interaction to AIDS pathologies. Rusnati et al. 2002. Angiogenesis.

Five distinct functional domains have been identified in the Tat protein. These domains include the N-terminal, cysteine-rich, basic, core, and C-terminal. The Tat C-terminal domain is speculated to serve as the principal cell attachment and internalization moiety via the arginine-glycine-aspartate (RGD) sequence [54]. Integrins recognize this RGD sequence allowing Tat to bind with a high affinity to integrins α5β1 and αvβ3, transmembrane proteins expressed by endothelial cells [49, 55, 56]. Tat can also bind to cell surface heparin sulfate proteoglycans (HSPG's) and be internalized in this way, or bind kinase domain insert domain receptor (KDR) / vascular endothelial growth factor receptors (VEGFR-2) and cause signal transduction [57]. Once Tat is internalized, it can negatively alter endothelial cell biological activities by increasing cell proliferation, apoptosis, protease production, migration, substrate adhesion, angiogenesis, leukocyte adhesion, and vascular permeability [13]. Furthermore, the constant HIV inflammatory state increases IL-1beta, TNF-alpha, and IFN-gamma secretion, cytokines which upregulate receptors for Tat [37, 58,

59]. HIV-1 Tat also stimulates the adhesion molecules E-selectin, VCAM-1 and I-CAM 1 in endothelial cells [60-62].

2.1.4 HIV-1 Tat and Endothelial Cell Kinase Signaling

Although the modes of Tat binding to the endothelium have been studied, the pathways by which Tat regulates cellular function are not completely understood [63]. The most widely studied mitigators of Tat stimulated endothelial cell dysfunction have been mitogen-activated protein kinases (MAPKs) [12, 60, 63-66]. MAPKs are involved in directing cellular responses to a diverse array of stimuli and regulating cell functions - which include differentiation, gene expression, proliferation, mitosis, cell survival, and apoptosis [67].

The most studied signaling cascades in terms of Tat-mediated endothelial cell activation, are extra-cellular signal regulated kinases 1 and 2 (ERK1/2) and the c-jun N-terminal kinase (JNK) [65, 68]. Tat has been shown to increase endothelial cell proliferation, apoptosis, invasion, and angiogenesis via these pathways [56, 69, 70]. Additionally, Tat stimulation activates Ras and Rac in human umbilical vein endothelial cells (HUVECs), and subsequent Ras-mediated ERK phosphorylation results in increased cell cycle progression [63]. EC adhesion to substrate-bound Tat leads to increased pro-angiogenic activation via VEGFR2/$\alpha_v\beta_3$ complex formation and subsequent ERK(1/2) phosphorylation [71].

Similarly, Tat has been found to rapidly activate JNK in HUVECs and ECV-304 cells (an endothelial cell line). Tat-transfected HeLa cells co-cultured with ECV-304 cells resulted in persistent activation of JNK [65].

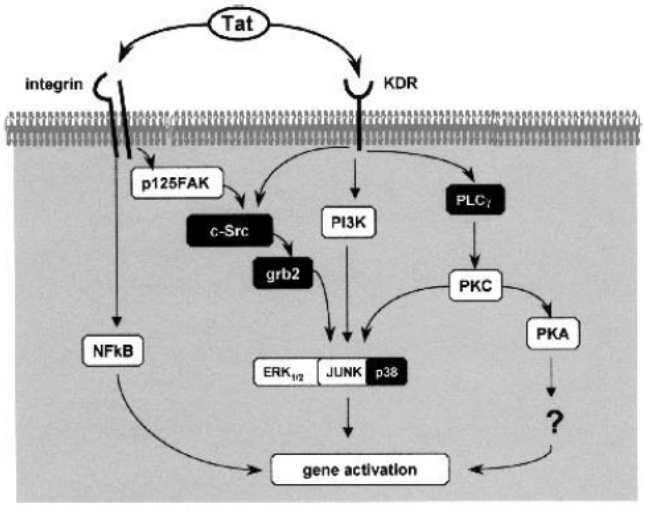

Figure 2.4: Tat activates ERK, JNK and p38 in endothelial cells. Adapted from HIV 1 proteins and the endothelium: From protein cell interaction to AIDS pathologies. Rusnati et al. 2002. Angiogenesis.

Studies have been done to explore the co-activation of ERK and JNK signaling cascades. The Feng Wes group found that Tat activated dual Nox paralogs for differential activation of ERK and JNK pathways in HUVECs, thus linking Nox 2- JNK signaling to Tat-induced cytoskeletal arrangement and Nox-4 dependent ERK signaling to Tat-dependent endothelial cell proliferation. This study showed that Tat stimulation provided specific differential control of MAPK pathways [72]. Altogether, these studies highlight the ability of HIV-1 Tat to independently modify endothelial function and induce vascular injury via the aforementioned signaling cascades.

2.2 Cathepsins and Arterial Remodeling

2.2.1 Artery Pathophysiology

Arteries are responsible for the transport of oxygenated blood to the body and have multilayered structure, categorized by three main layers: the intima, a single layer of endothelial cells; the media, comprised of smooth muscle cells and extracellular matrix proteins including collagen and elastin; and the adventitia, which is comprised of structural extracellular matrix proteins and fibroblasts [73, 74]. The unique structure of the artery allows it to be able to respond quickly to physiological factors, and each layer has a unique ability to aid in regulation of homeostatic processes of blood pressure and shear stress. Short term maintenance through dilation or constriction is regulated by smooth muscle cells, and long term maintenance is observed by arterial remodeling of the vessel structure.

The progression of atherosclerosis is multifactorial, related to the presence of excess lipoproteins within the blood and hemodynamics; in addition, cytokines within the blood and damage to the endothelium also contribute to cardiovascular disease progression [75-78]. In a healthy state, lipids pass from the endothelium to the medial layer. However, when there is an excess of lipids, this can cause vascular damage and evoke an immune response. Once endothelial cells are activated, they express adhesion molecules such as VCAM-1 and ICAM-1 to promote monocyte binding. Monocyte chemoattractant protein (MCP-1) is also secreted to further promote monocyte adhesion and migration through the endothelium [75, 79-81].

Within the arterial wall, monocytes are stimulated by macrophage colony stimulating factor (M-CSF) and other secreted factors that induce monocyte

differentiation into macrophages. The excess lipids are then cleared away by the macrophages by internalization [75, 80]. When lipids become too excessive for macrophages to digest, they become foam cells and release inflammatory cytokines such as tumor necrosis factor (TNF-α) promoting further recruitment of cells [82]. Once a plaque has formed, the major risks result from the possibility of plaque rupture and occlusion of blood flow. When plaques rupture, they release their contents into the bloodstream leading to thrombosis and eventually, occlusion of the artery.

2.2.2 Shear stress and Arterial Remodeling

Arteries are exposed to constant mechanical stresses in the form of blood pressure and shear stress at the wall as blood flows through the artery. Specifically endothelial cells are exposed to shear stress, the tangential force of the blood flowing along the vascular wall. These mechanical forces not only cause morphological changes of the endothelium and blood vessel wall, but also trigger biochemical and biological events. Multiple studies have shown that shear stress plays an important role in atherogenesis; it is well established that atherosclerotic plaque formation occurs preferentially at areas of low and oscillatory shear stress such as those seen at curves and bifurcations within the vasculature; while regions of high, unidirectional fluid shear stress, appear to be atheroprotected [83-89]. *In vitro* studies that exposed endothelial cells (ECs) to low oscillatory shear stress showed activation through upregulated cellular adhesion molecules on the cell surface including E-selectin, ICAM-1 and VCAM-1. Increases in monocyte recruitment, adhesion, and migration into the vascular wall have also been identified [84, 90, 91]. Finally, oscillatory shear stress activates shear-mediated cysteine

proteases, the cathepsins, powerful elastases and collagenases which have the ability to remodel extracellular matrix and modify the mechanical properties of the arterial wall [14, 92, 93].

2.2.3 Cathepsins in Cardiovascular Remodeling

Cathepsins are lysosomal cysteine proteases [94-96], that can be secreted extracellularly to degrade and remodel tissues. They are upregulated in many diseases such as atherosclerosis [97-103], cardiomyopathy [104-106], rheumatoid arthritis [107-110] and cancer [111, 112]. Cathepsins are synthesized in their inactive form, and the N-terminal propeptide must be enzymatically cleaved to expose the active site for substrate catalysis. Cathepsins K, V, L and S – are all potent collagenases and elastases. Cathepsins K and V are the most potent collagenases and elastases, respectively[113]. Members of the lysosomal cysteine cathepsin family have unique properties and homeostatic functions, but share 60% sequence homology [113-116].

Cathepsin K is capable of solubilizing collagen better than MMP-9, -1, and -13 [117]. It has the unique proteolytic capability of cleaving type I collagen at the telopeptides and intrahelically, which explains its characterization as the most potent mammalian collagenase. Cathepsin K also has strong elastase activity [114], as does cathepsin S. Cathepsin S is upregulated in diseases associated with elastinolytic remodeling [118], including atherosclerosis [114, 119-121], abdominal aortic aneurysms, [114, 122] and arthritis [123]. However, cathepsin V has been identified as the most potent mammalian elastase [124]. Human cathepsin V has an 80% homologous sequence

with human cathepsin L, and it has been shown that human cathepsin V is orthologous to mouse cathepsin L [115, 116].

In cardiovascular disease, when cathepsin activity is pathologically increased within the arterial wall, vascular remodeling occurs due to the altered balance of structural proteins that provide mechanical stability to blood vessels. Increased proteolytic activity by cathepsins weakens the arterial wall, allowing the migration of vascular SMCs into the sub-intimal space and increasing monocyte infiltration, thus advancing cardiovascular disease progression. The pathophysiological importance of cathepsins K, L, S and V in atherosclerosis has been demonstrated in double-knockout mice deficient in apolipoprotein E, which showed a reduction in the number and size of atherosclerotic lesions, and in some cases decreased fragmentation of the elastic lamina [98, 102, 103, 125].

Cathepsins K and S were significantly increased in atherosclerotic lesions compared to healthy vessels [14] and human atherosclerosis samples showed a positive correlation between atherosclerotic lesion development and cathepsin K levels in the endothelium [14]. Fibrous cap rupture can also be attributed to increases in proteolytic activity [101]as they can degrade or erode the ECM which contributes to plaque instability [126]. Several *in vivo* studies have shown expression of cathepsin K by SMCs and macrophages and identified their roles in vascular remodeling [14, 127, 128].

Cathepsins are dually important in the context of CVD because of their shear stress-dependent regulation. Vascular endothelial cells experienced increases in cathepsin K and L expression when exposed to oscillatory shear stress, while unidirectional shear stress inhibited gelatinase and elastase activity in a cathepsin-dependent manner [14].

Cathepsin L knockout mice showed a decrease in shear-dependent degradation of ECM proteins, implicating its human ortholog cathepsin V in shear-mediated arterial remodeling [92].

2.3 Antiretrovirals and Cardiovascular Disease in sub-Saharan Africa

2.3.1 Significance

From 1995 until 2012, new ART initiatives by UNAIDS, the Joint United Nations Programme on HIV/AIDS, averted 5.5 million deaths in low and middle-income countries[129]. By providing access to necessary HIV-treatment and educational programming to inform patients of the AIDS epidemic, there has been significant improvement in AIDS mortality rates. Although there has been significant headway in fighting the HIV/AIDS epidemic in the US, many low and middle-income countries are struggling to decrease HIV incidence. Southern Africa (including South Africa, Botswana, and Swaziland) is the most strongly affected region, with the highest prevalence of HIV infection estimated at approximately 20% [130]. Within the last 10 years, antiretroviral initiatives have been established in many of these countries and have been very effective at battling these issues [129]. With a new population using these cost-saving standard regiment antiretrovirals, including therapies no longer used in the US, the effects of ARV on toxicity and co-morbidity, especially CVD, need to be investigated to ensure the best standard of care for these patients.

2.3.2 Antiretrovirals and arterial remodeling

ARVs are divided into 5 major classes: the nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors, and entry inhibitors [24]. Although HAART fails to cure HIV, these therapies reduce HIV replication, immune activation, and chronic inflammation as well as improve CD4+ lymphocyte counts. The types and brands of antiretroviral therapies prescribed in Sub-Saharan Africa, depend on contraindications, resistance to drug therapy, and economic status.

Various studies have shown the contribution of antiretrovirals to cardiovascular disease progression in HIV positive patients, including an increase in dyslipidemia and atherosclerotic lesions [131-133]. The various HAART drugs are also known to have negative effects on vascular cells as evidenced by increased carotid intima-media thickness [134], arterial stiffening [7, 23], and endothelial dysfunction [135-137] .

Protease inhibitors have been the most widely implicated in increased lipid content, atherosclerotic plaque formation and other inflammatory factors in HIV-positive patients. *In vitro* studies have shown decreased oxygen consumption [138], and increased monocyte adhesion [139], endothelial cell permeability [140], and cholesterol uptake in macrophages [141]. Because of these adverse side effects, reverse transcriptase inhibitors have become more commonly used than protease inhibitors.

NRTIs and PIs are independent factors associated with increased arterial stiffness [7], resulting in NRTI's being the most widely studied as it relates to endothelial dysfunction. NRTI's impair mitochondrial DNA replication and repair [142]. In particular, azidothymidine (AZT) caused increased endothelial cell death, loss of adenosine triphosphate (ATP) production, and increased proliferation of endothelial cells and smooth muscle cells in

co-culture [138]. A study done in conjunction with our lab found that mice treated with AZT for 10 weeks showed increase arterial stiffness, elastin fragmentation, and intimal medial thickness [143]. Tenofovir (TDF) has also been shown to contribute to hypertension in patients [144] and increases serum adiponectin levels compared to patients on lamivudine after 48 weeks of treatment [145].

Efavirenz (EFV), an NNRTI, has pro-atherosclerotic effects on endothelial cells. HAECs stimulated with EFV had a decrease in the tight junction proteins ZO-1, claudin-1, occludin, and JAM-1and an increase in endothelial cell permeability[146]. These studies show that ARVs do impair arterial cell function, although quantifiable effects of certain drugs cannot be parsed *in vivo*. The contribution of ARV drugs to non-AIDS co-morbidities still needs to be better assessed, especially for those prescribed older ARV regiments, such as populations living in sub-Saharan Africa.

CHAPTER 3

PRO-ATHEROGENIC SHEAR STRESS AND HIV PROTEINS SYNERGISTICALLY UPREGULATE CATHEPSIN K IN ENDOTHELIAL CELLS

3.1 Introduction

Major advances in highly active antiretroviral therapies (HAART) have extended the lives of people living with HIV, but there still remains an increased risk of death by cardiovascular diseases (CVD). In 2010, the World Health Organization reported 34 million people living with Human Immunodeficiency Virus (HIV) infection globally, with 1.8 million cases resulting in death per year. HIV populations have demonstrated an increased risk of myocardial infarction, endothelial dysfunction, atherosclerotic lesions, carotid artery intima-medial thickness, and artery stiffness [2, 7, 23, 25-28, 30, 31].

Studies have elucidated whether this increase in cardiovascular incidence is due to antiretroviral treatment or the virus itself. The SMART Study showed that HIV-positive patients' risks for CVD was increased when patients were taken off their antiretroviral therapies, even when CD4+ counts were kept above a certain threshold, demonstrating that some of these factors for cardiovascular disease are due to the virus itself [3]. We have recently shown that the antiretroviral AZT stiffens arteries in wildtype mice [143], and have also shown that HIV proteins induce arterial stiffening in an HIV transgenic

[1]Portions of this section were adapted from Parker, I.K., Platt, M.O. et al., *Pro-atherogenic shear stress and HIV proteins synergistically upregulate cathepsin K in endothelial cells.* Ann Biomed Eng, 2014. **42**(6): p. 1185-94.

mouse model [47], contributing to both sides of the debate. HIV proteins enter the bloodstream after being secreted by HIV-infected cells or when shed from lysing cells without incorporation into new virions [24]. Of particular interest is HIV-1 transactivating factor (Tat), a 14-kDa protein that binds to the secondary structure sequence TAR (Tat activation region), enabling the recruitment of cellular factors to activate transcription and elongation, performing regulatory functions and increasing infectivity of the virus. Tat has been shown to bind to endothelial cells and alter proliferation, apoptosis, matrix metalloprotease-2 production, migration, substrate adhesion, angiogenesis, leukocyte adhesion, and vascular permeability [13, 55, 147, 148].

It is well established that hemodynamics also play an important role in endothelial cell-mediated atherosclerotic development. Atherosclerotic plaques preferentially form at branches and sharp turns in the arterial tree such as those found on the outer wall of the carotid artery, lesser curvature of the aortic arch, and abdominal aorta; sites where endothelial cells are exposed to low and oscillatory shear stress [149]. Among other effects, oscillatory shear stress has been shown to upregulate endothelial cell production of cathepsins, powerful elastases and collagenases that have been implicated in human cardiovascular disease, particularly cathepsins K and L [92, 150]. These proteases remodel the extracellular matrix, allowing the plaque to grow, and change the mechanical properties of the arterial wall [14, 100-103].

Here we tested the synergistic effects of oscillatory shear stress and HIV proteins on cathepsin production and activity by measuring cathepsin levels at distinct hemodynamic regions characterized as pro-atherogenic or atheroprotective in an HIV transgenic mouse model. Synergistic effects were further examined in HAECs using a

shear stress bioreactor to actuate physiologically relevant pro-atherogenic or atheroprotective pulsatile shear stress profiles from the carotid artery, co-stimulated with Tat, an HIV protein that activates inflammatory responses in endothelial cells. Altogether, this work tests the hypothesis that disturbed blood flow with HIV proteins, specifically Tat, synergistically increase cathepsin activity on the endothelium that may participate in accelerated arterial wall remodeling.

3.2 Methods and Materials

3.2.1 HIV-Transgenic Mouse Model

Male hemizygous NL4-3Δ *gag/pol* transgenic and wild-type littermate (FVB/N) mice, 10-12 weeks old were euthanized with CO_2 and the aorta removed and cleaned free of loose perivascular tissue. Unlike the homozygous HIV-Tg mice, which are smaller at birth, have decreased food intake compared to wild-type mice, and usually die within 40 days postnatal, the hemizygous mice appear normal at birth, but develop signs of disease, specifically HIV-associated nephropathy[151]. The aortic arch, thoracic aorta, and abdominal aorta were separated for immunohistochemistry and cathepsin zymography. All work was conducted under the regulation of Georgia Institute of Technology's and Atlanta VA Medical Center's Institutional Animal Care and Use Committee (IACUC).

3.2.2 Cathepsin Immunohistochemistry

Localization of cathepsin protein expression in the aortic wall was determined by immunohistochemistry of aortic arches. Aortic arches were embedded in OCT medium

and the orientation of the greater and lesser curvature was carefully marked prior to snap freezing and storage at -80°C. Tissue sections (8 μm thick) were collected with a Leica CM3050 Cryostat and mounted on glass slides. Sections were fixed in acetone, rinsed with PBS, and then blocked in 3% BSA for an hour. Sections were immunolabeled with primary cathepsin K (Santa Cruz), cathepsin S , or mouse cathepsin L antibodies (R&D Systems) at 4°C overnight at a 1:50 or 1:100 dilution in 1% BSA and then probed with TRITC conjugated anti-rabbit or FITC conjugated-anti-goat fluorescent secondary antibodies (Life Technologies) at room temperature for an hour at a 1:100 dilution in the dark. Negative controls were incubated without primary antibody. Sections were then mounted using DAPI Pro-long Gold Antifade reagent (Life Technologies) and imaged using a Nikon Ti-E fluorescent microscope.

3.2.3 Cathepsin and MMP Zymography

Excised thoracic and abdominal aortas were separated below the diaphragm and stored in PBS on ice until they were placed in 50 μl of zymography lysis buffer (20 nM Tris–HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20) with 0.1 mM leupeptin freshly added to stabilize enzymes during electrophoresis. Aortas were homogenized using disposable sample grinders (GE Healthcare), lysates were collected and cleared by centrifugation, and total protein concentration was determined by micro-bisinchoninic acid (BCA) assay (Pierce). Multiplex cathepsin zymography was performed as described previously [127, 152] and MMP zymography according to Galis et al [153]. Gel images were captured with an Imagequant 4010 (GE Healthcare), then

the images were inverted in Adobe Photoshop and densitometry was performed using NIH ImageJ.

3.2.4 Endothelial Cell Culture

HAECs (Lonza) were cultured in MCDB medium 131 (Mediatech) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 1% endothelial cell growth serum (ECGS), and supplemental growth factors hydrocortisone (.001 mg/ml), fibroblast growth factor (.002 ug/ml), epidermal growth factor (.010 ug/ml), insulin-like growth factor (.002 ug/ml), VEGF (.001 ug/ml), and ascorbic acid (50ug/ml). Cells were maintained with 5% CO_2 at 37°C in 10 cm dishes until confluent and used between passage 6 and 8. Upon confluence, medium was changed to Endothelial Growth Medium (EGM) (Lonza) for overnight, low serum culture prior to treatment with Tat (69.4 nM) (ImmunoDiagnostix) and exposure to shear stress profiles.

3.2.5 Shear Stress Bioreactor

The atheroprotective unidirectional waveforms and pro-atherogenic oscillatory waveforms that were used in this study were first characterized in humans by Dai *et al* [154]. The atheroprotective and pro-atherogenic waveforms are generated using a cone-and-plate shear system consisting of polytetrafluoroethylene (PTFE) cones with a 0.5˚ angle. The cones are connected to the drive shaft of a SM232AQ servomotor, which actuates the user-defined motion profile. A vacuum plate is used to secure the culture plates to a base platform while the cones are in motion. The shear stress bioreactor is contained in an incubator maintained at 37ºC and 5% CO^2.

3.2.6 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates, and each data point is presented as the mean value +/- standard error of the mean. Representative images are shown. Unpaired student t-tests were used to determine statistical significance between most experimental groups.

3.3 Results

3.3.1 Cathepsin K protein expression is higher in the lesser curvature of the aortic arch in HIV-Tg mice.

Our previous findings showed increased cathepsin K activity in aortas of HIV-Tg mice as a mechanism for increased aortic stiffness [47], however we wanted to examine cathepsin expression in flow defined regions. We first investigated the aortic arch in HIV-Tg murine models since sites of atherosclerotic lesion development have been described hemodynamically, with the greater curvature being a site of atheroprotective high shear stress, and the lesser curvature being exposed to pro-atherogenic, low and oscillatory shear stress [155]. NL4-3Δ gag/pol heterozygote transgenic mice were sacrificed between 10-12 weeks and compared to littermate controls. Aortic arches were carefully embedded to denote the greater curvature and lesser curvature, cryopreserved, and immunohistochemical staining for cathepsins K, L, and S was performed. From the staining, no observed difference in cathepsin L and S protein expression was observed; however it can be seen that there was greater overall red fluorescent intensity in HIV-Tg mice compared to littermate controls, indicating greater cathepsin K protein expression (n=3, representative images are shown for each stain) (Fig 3.1). These increases in expression were strongest in the intimal layer in wildtype littermate controls, but were

throughout the medial and intimal layers within the HIV-Tg mice aortic arches. Asymmetry in cathepsin K staining was observed with the lesser curvature being higher compared to the greater curvature for both the HIV-Tg mice and the littermate controls. Taken together, these results suggest that both shear stress and HIV proteins could be contributing to upregulation of cathepsin K protein expression in artery wall.

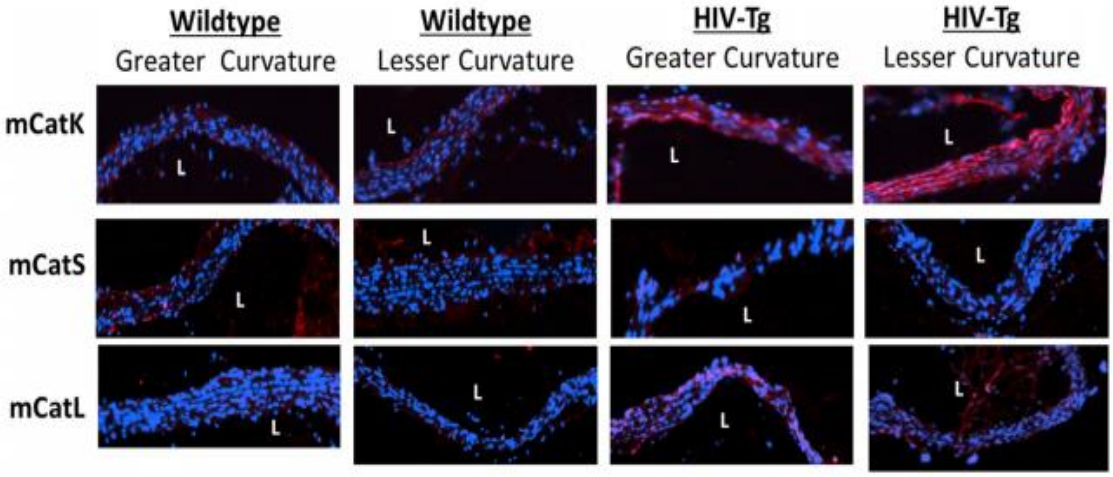

Figure 3.1: HIV-Tg mice have greater cathepsin K expression in the lesser curvature of the aortic arch than the greater curvature. HIV-Tg mouse aortic arches were excised and oriented by their regions of lesser curvature (pro-atherogenic) and greater curvature (atheroprotected), where L denotes the lumen of the vessel. Immunohistochemical staining was carried out using antibodies for cathepsin K, mouse cathepsin L, and cathepsin S. Zoomed images show increased cathepsin K staining, indicated in red, in HIV-Tg mice compared to in littermate controls with increased staining in the lesser curvature compared to the greater curvature. There were not differences in staining for cathepsin L and cathepsin S between regions of greater and lesser curvature (n=3 for wildtype, n=4 for HIV Tg, representative images shown).

3.3.2 Mature, active cathepsins K and S are increased at hemodynamically defined regions in aortas of HIV-Tg mice.

Immunohistochemistry indicated that at regions of pro-atherogenic disturbed blood flow, such as the lesser curvature, there was increased cathepsin K protein

expression. Next it was important to confirm that the cathepsin K identified immunohistochemically was the mature, active form. Antibodies used for immunohistochemistry bind to and detect both the pro (inactive) and the mature (active) cathepsins, giving an overall, total cathepsin protein level, but do not accurately quantify active cathepsins that may be involved in proteolysis within the arterial wall. To investigate this, we used multiplex cathepsin zymography, an assay that can quantify amounts of active cathepsin K, cathepsin L, and cathepsin S in cells or tissue extracts. *In situ* labeling provided by immunohistochemistry showed asymmetry at the greater and lesser curvature of the aorta whereas zymography involved homogenization of the aortic tissue for gel electrophoresis.

The aortic arch region is too small to obtain the protein amounts necessary for detection of active cathepsins K, S, or L by zymography. Therefore we used thoracic and abdominal aorta segments, which have also been defined hemodynamically [156-158]. The thoracic aorta has been characterized dominantly by atheroprotective, high unidirectional shear stress, and the abdominal aorta with regions of pro-atherogenic, low and oscillatory shear stress [149]. Aortas were excised, cut into these two hemodynamically defined regions, cleaned, and homogenized. Total protein concentration was determined, and equal amounts of protein were loaded for cathepsin zymography to quantify differences in the amount of active, mouse cathepsins K, L, and S in the aorta wall in response to hemodynamics and HIV proteins (Fig 3.2).

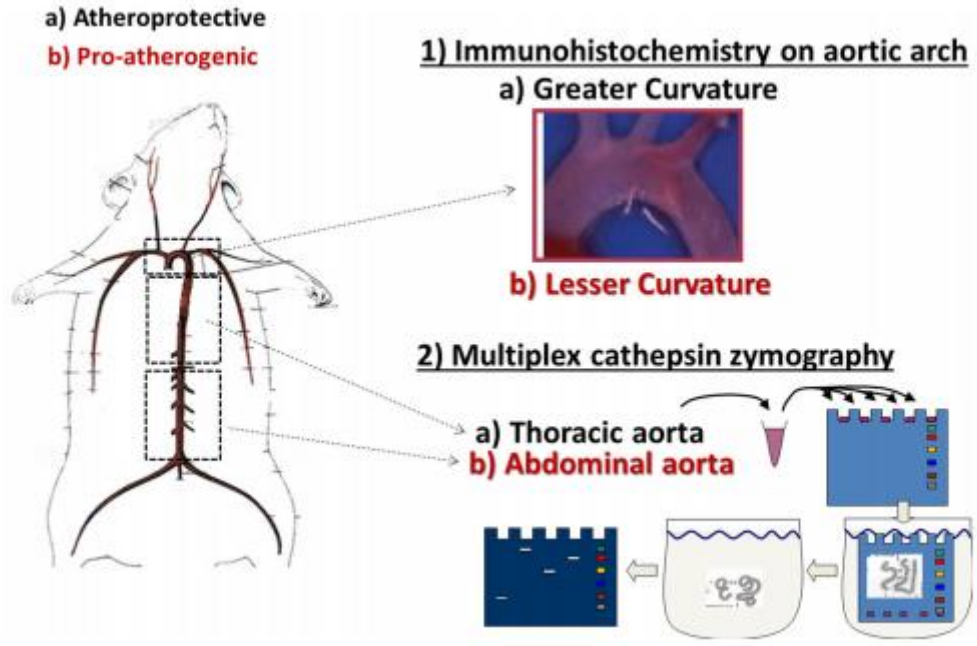

Figure 3.2: Schematic of aorta segmenting and experimental analysis. The aortic arch has distinct regions that allow for investigation of pro-atherogenic shear stress region (lesser curvature) and atheroprotected shear stress region (greater curvature) within the same animal. To obtain more protein, the thoracic (atheroprotected) and abdominal (pro-atherogenic) aorta were excised, cleaned, segmented, and homogenized. The tissue lysate was prepared for multiplex zymography to quantify the amounts of active cathepsins K, L, and S by shear stress region.

A representative zymogram of four mouse aortas tested is shown in Figure 3A with quantified densitometry in (Fig 3.3B-E). Abdominal aortas of HIV-Tg mice had higher levels of active cathepsin K compared to thoracic aortas of HIV-Tg mice and compared to the abdominal aortas of wildtype mice (7-fold increase) ($n=4$, $p<.05$). For wildtype mice, there was no significant difference in amounts of mature cathepsin K between the thoracic and abdominal aorta when averaged over the four mice (Fig 3.3B). Similarly, the highest amount of active cathepsin S was found in the abdominal aorta of HIV-Tg mice compared to thoracic HIV-Tg and wildtype mice aorta segments ($n=4$, $p<.05$) (Fig 3.3C).

Mouse cathepsin L was increased in HIV-Tg abdominal aortas by 2.4-fold compared to controls, and we observed a 3-fold decrease in activity in wildtype abdominal aortas compared to thoracic aortas, in contrast to what was observed for cathepsins K and S, indicating different baseline levels for cathepsins K, L, and S in wildtype mice. However, similarly to cathepsins K and S, there was increased cathepsin L activity in HIV-tg abdominal aortas compared to wildtype (n=4, p<.05) (Fig 3.3D). No significant differences were observed for MMP-2 due to aorta region or HIV-Tg status (Fig 3.3E).

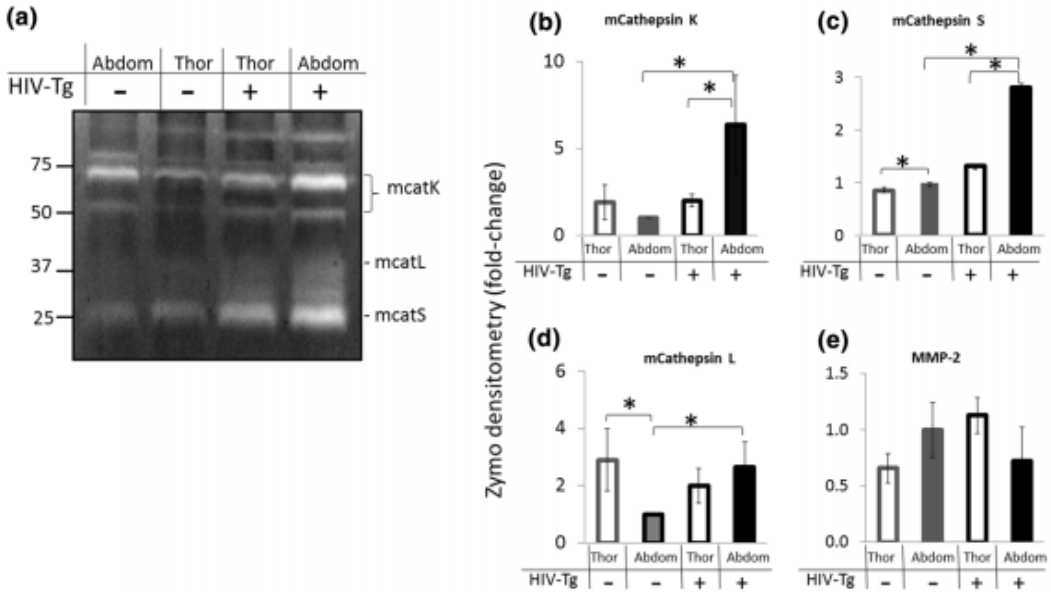

Figure 3.3. Mouse cathepsins K, S and L activity are increased in abdominal aortas of HIV-Tg mouse aortas. (a) Multiplex cathepsin zymography was performed on HIV-Tg and littermate control mouse aortas excised and separated into the abdominal and thoracic aortas. Densitometry values are shown for (b) mouse cathepsin K, (c) mouse cathepsin S, (d) mouse cathepsin L, and (e) MMP-2 (*p <0.05, n 5 4, and data is mean +/- SEM).

To parse effects on cathepsin activity regulation due to shear stress from that of HIV proteins for each mouse, ratiometric comparisons were used. The influence of shear stress region was examined by dividing cathepsin activity in pro-atherogenic regions by that of atheroprotected regions within the same animal, as quantified from densitometric analysis of the zymography. If this ratio was higher than one, then cathepsin activity was higher in the abdominal aorta where pro-atherogenic flow dominates, if lower than one, then the activity was higher in the thoracic aorta which experiences more atheroprotective shear stresses, and when equal to one, there were no shear stress dependent differences. In HIV-Tg mice, the ratio for cathepsin K activity was 3.2 indicating a 3.2 fold increase in cathepsin K activity in abdominal aortas compared to thoracic, but the ratio was close to one for the control mice (Fig 4A), indicating that the effect seen in the HIV-Tg mice was not seen in control mice ($n=4$, $p<.05$). Cathepsin S activity was 2-fold higher in pro-atherogenic regions (abdominal aorta) compared to atheroprotected regions (thoracic aorta) and also significantly higher than the wildtype ratio, which was close to one (Fig 4B, $n=4$, $p<.05$). For mouse cathepsin L we did not observe a significant increase in the ratio for HIV-Tg mice, however the HIV-Tg ratio was significantly higher than the ratio of the wildtype mice (Fig 4C, $n=4$, $p<.05$).

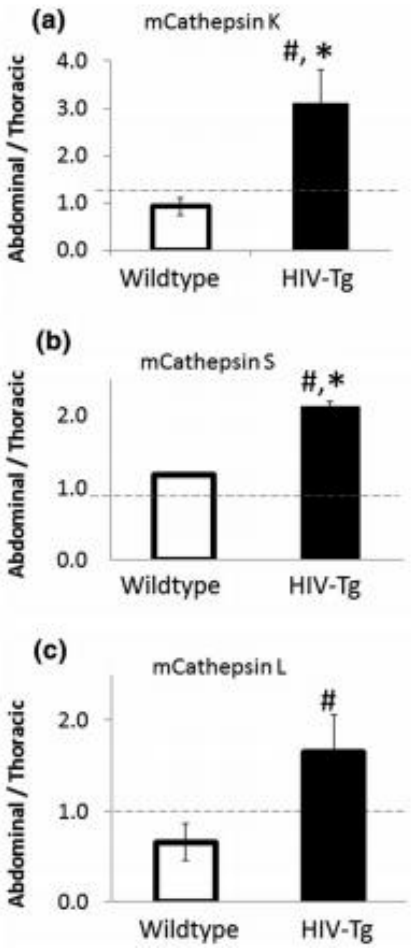

Figure 3.4: Ratiometric comparisons to parse regulation due to shear stress from that of HIV proteins. The influence of shear stress region was examined by dividing cathepsin activity in pro-atherogenic regions by that of atheroprotected regions within the same animal, as quantified from densitometric analysis of the zymography. If this ratio was higher than one, then cathepsin activity was higher in the abdominal aorta where pro-atherogenic flow dominates, if lower than one, then the activity was higher in the thoracic aorta, and when equal to one, there were no shear stress dependent differences. Analysis for A) mouse cathepsin K, B) mouse cathepsin S, and C) mouse cathepsin L are shown. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM) (# indicates ratio is statistically increased $p<.05$, n=4).

3.3.3 Co-stimulation with pro-atherogenic shear stress and Tat increase cathepsins K and V in human aortic endothelial cells.

HIV-1 Tat has been shown to activate human endothelial cells [13, 60-62, 147], but neither Tat's specific links to stimulating cathepsin production by endothelial cells nor any synergism with shear stress has been shown. There is a different cathepsin profile in human cells compared to mouse cells in that the nomenclature in mice is cathepsins K, S, and L, but in humans, they express cathepsins K, S, L, and L2, which is frequently termed cathepsin V (L2). Human cathepsin V (L2) and human cathepsin L share 80% homology[116], but the mouse genome encodes for only one cathepsin L, which is 75% homologous and has similar biochemical properties with human cathepsin V; consequently, mouse cathepsin L and human cathepsin V are orthologs to compare across species, and human cathepsin L has no ortholog in mice [115]. To summarize, murine cathepsin L is compared to human cathepsin V, and there is no murine cathepsin V. We have also shown that cathepsins K, L, S, and V can all be detected simultaneously by the multiplex cathepsin zymography assay with human cathepsin V appearing at 37 kDa and human cathepsin L at 20 kDa[127]; cathepsins K and S migrate as in mice.

Tat circulates in the bloodstream at an estimated 2 ng/ml - 40 ng/ml range, and is secreted from infected T-cells and monocytes. Because macrophages may act as viral reservoirs within the arterial wall, it is believed that endothelial cells are exposed to even higher concentrations of Tat secreted locally by these HIV infected cells [50]. Here we tested the hypothesis that Tat was sufficient to induce cathepsin activity in human endothelial cells cultured under fluid flow with atheroprotective or pro-atherogenic shear stress using a cone-and-plate bioreactor as described in the Methods section (Fig 5A), and lysed for multiplex cathepsin zymography (Fig 3.5B). Endothelial cells stimulated with

HIV-1 Tat and cultured under pro-atherogenic shear stress, had higher levels of active human cathepsin K (Fig 3.5C) compared to vehicle control and atheroprotective conditions (n=5, p<.05). The amount of active cathepsin V was also increased compared to Tat-stimulated atheroprotective conditions (Fig 3.5D). Endothelial cells showed no increases in cathepsin S or L activity in response to Tat and shear stress stimulation. (Fig 3.5E); n=5, p=<.05).

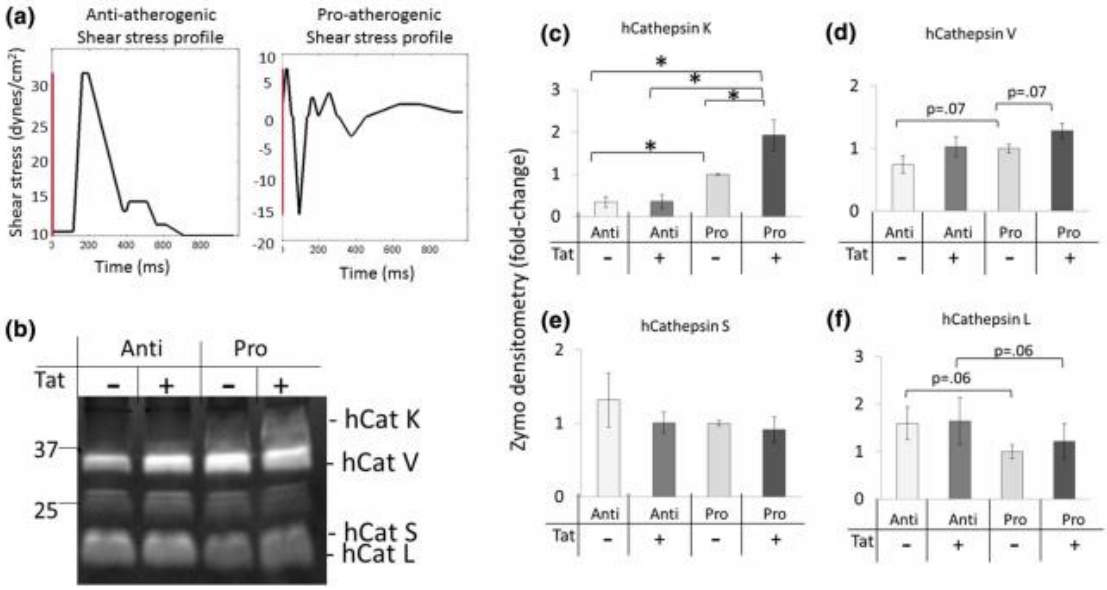

Figure 3.5: HAECs stimulated with pro-atherogenic shear stress and HIV-1 Tat have increased cathepsin K activity. A) Shear stress profiles that were programmed into Servomotors are shown. **B)** Endothelial cells were co-stimulated with HIV-1 Tat and atheroprotective (Anti) or pro-atherogenic (Pro) shear stress for 24 hours, then lysed for multiplex cathepsin zymography. Densitometry was quantified and shown for C) human cathepsin K, D) human cathepsin V, E) human cathepsin S, and F) human cathepsin L. Cathepsin K was significantly increased by pro-atherogenic shear stress and Tat co-stimulation compared to no Tat stimulation and atheroprotective shear stress. Though there was a trend, there were no significant differences for the other cathepsins investigated (* indicates $p < 0.05$ n=5 and data is mean +/- SEM).

Again, we used a ratiometric analysis to parse the effects of Tat and shear stress on cathepsin activity in HAECs. In the presence of Tat, pro-atherogenic shear stress increases cathepsin K by 11-fold compared to atheroprotective shear stress, which is

significantly greater than its upregulation in the absence of Tat (3-fold) (Fig 3.6 A, n=5, $p<.05$). No significant differences were seen for cathepsins V, S, or L (Fig 3.6 B-D). Under pro-atherogenic shear stress, Tat increased cathepsin K by 2-fold over cells under pro-atherogenic shear stress without Tat (vehicle); there was no significant difference for Tat stimulated endothelial cells exposed to atheroprotective shear stress (Fig 3.6E). Again, there were no differences for the other cathepsins V, S, or L (Fig 3.6 F-H). This suggests that Tat and pro-atherogenic shear stress synergistically increase cathepsin K beyond pro-atherogenic shear stress or Tat alone.

3.4 *Discussion*

Our results show the upregulation of cathepsin K by pro-atherogenic shear stress and HIV proteins. This is significant because both of these stimuli are present in an individual living with HIV, but their synergism, to our knowledge, has never before been studied. The role of pro-atherogenic shear stress and HIV proteins inducing cathepsin activity could add important insight as to why people living with HIV are at increased risk of CVD, as well as identify cathepsin inhibition as novel therapeutic targets.

Plaques preferentially form at sites of low and oscillatory shear stress, and cathepsin K has been shown to be upregulated in mouse endothelial cells exposed to pro-atherogenic, oscillatory shear stress compared to atheroprotective shear stress [159]. Here, we showed that HIV proteins upregulate the amount of active cathepsin K in the aortas of HIV transgenic mice, specifically at sites of pro-atherogenic shear stress such as the lesser curvature of the aortic arch and the abdominal aorta. It is important to note that

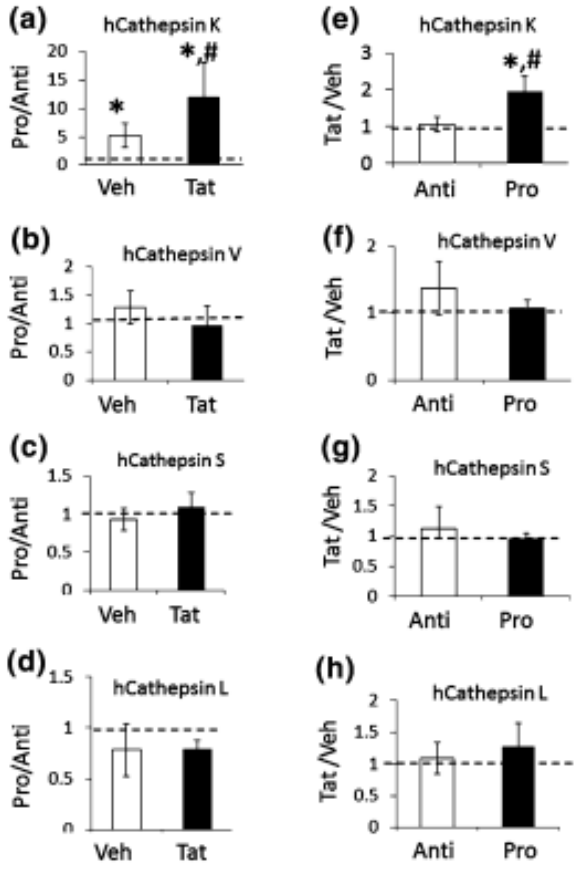

FIGURE 3.6. Ratiometric comparisons to parse human endothelial cell regulation of cathepsins K, V, S and L due to shear stress from that of HIV proteins. Influence of shear region was examined using a ratio of cathepsin activity. This parameter was determined by dividing cathepsin activity from endothelial cells cultured under pro-atherogenic shear stress (Pro) by atheroprotective shear stress (Anti) and separating the values by Tat co-stimulation for cathepsins K, V, S, and L (a–d). Additionally, the influence of HIV protein ratio was established by dividing the Tat treatment for a shear stress profile by its vehicle control (e–h). Analysis shows 11-fold increases in cathepsin K activity when comparing Tat stimulated cells exposed to pro-atherogenic vs. atheroprotective shear stress (*$p<0.05$, n =5) (# indicates ratio is statistically increased $p<0.05$, n =5). No statistically significant differences were observed for cathepsins S, L, or V.

these effects were observed in a non-atherosclerotic mouse model, as these were FVB/N background, ApoE+/+, and fed regular chow, not Western or other high fat diets. This selectively isolated the influence of HIV proteins and hemodynamics from that of lipid accumulation and inflammation associated with plaque formation. These *in vivo* studies were corroborated by *in vitro* studies with HAECs cultured under either pro-atherogenic or atheroprotective fluid shear stress in the presence of the HIV protein Tat. In both the transgenic mouse model and cultured human endothelial cells, HIV proteins and pro-atherogenic shear stress worked synergistically to upregulate cathepsin K.

Increases in cathepsin K were visualized throughout the aorta of the mice with immunohistochemistry (Fig. 3.2). It could be that the endothelial cells preferentially secrete cathepsin K basally, which was previously shown by us[14], allowing its accumulation in the medial layer of the artery wall or that smooth muscle cells may also respond to HIV proteins. Our studies with human aortic smooth muscle cells in culture showed no increase in cathepsin activity when stimulated with Tat alone (data not shown). Perhaps, other HIV proteins expressed by the HIV-Tg mouse may stimulate smooth muscle cell cathepsin expression, but this work shows that for endothelial cells exposed to shear stress, Tat is sufficient.

In this study, cathepsin K was the most responsive of the cathepsins investigated, but cathepsins K, L, S, and V have each been shown to play important roles in pathological vascular remodeling in both animal and human studies. Double knockout mice deficient in both cathepsin K and ApoE had a (41.8%) reduction in plaque area and increased collagen in plaque formation [102]. This suggests an important role for cathepsin K in atherosclerosis progression. Apo E mice deficient

in cathepsin L and S show similar trends; cathepsin L double knockout mice had significantly smaller atherosclerotic lesions compared with littermate controls, while cathepsin S double knockout mice had fewer acute plaque ruptures and smaller plaques than control [103]. Cathepsins K, L, S, and V have also been shown to be increased in human atherosclerotic plaques [14] [124]. It is interesting to note that cathepsin L was downregulated in wildtype abdominal aortas compared to wildtype thoracic aortas, which was different from cathepsins K and S (Fig.3.3); however, cathepsin L was significantly increased in the abdominal aorta of the HIV-Tg model compared to the wildtype abdominal aorta which was the same as cathepsins K and S.

This indicates that there may be differential regulation and responses for different cysteine cathepsins in the absence of HIV proteins, but with HIV proteins present, cathepsins K, L, and S were all upregulated. Differential proteolytic regulation was also shown with no upregulation of MMP-2 in the aortas of our HIV-Tg mice (Fig. 3.3). Cathepsins S and L activities were upregulated in the HIV-Tg mouse (Figs. 3.3 and 3.4), but there were not statistically significant differences in the endothelial cells stimulated with Tat (Figs. 3.5 and 3.6). This may be due to the other HIV proteins constitutively expressed in the HIV-Tg mouse model, whereas the endothelial cells were only stimulated with Tat in these experiments.

In addition, aorta homogenates from the mice contain protein from endothelial cells, smooth muscle cells, and fibroblasts, which may be contributing to cathepsins S and L activities as mouse vascular smooth muscle cells have been shown to express cathepsins L and S during CVD [101, 126] as have endothelial cells [92]. While we were able to parse the effect of Tat on endothelial cell cathepsin K activation, other HIV

proteins may play a role in cathepsin S and L activity in the murine model, and this warrants further investigation.

This study is the first to investigate co-stimulation by shear stress and HIV-proteins in the context of HIV-mediated CVD, and specifically using HAECs. These large artery human endothelial cells present a much more physiologically relevant cell type applicable to atherosclerosis, instead of the commonly used human umbilical vein endothelial cells (HUVECs) [13, 61, 62]. We observed increased cathepsin K when HAECs were exposed to pro-atherogenic shear stress and Tat stimulation, but other endothelial cells cultured from different parts of the vascular tree and smaller arteries may respond uniquely, as it has been established that endothelial cells derived from different regions have phenotypic diversity [160]. This may also provide another rationale behind the differences in cathepsin activity observed between the thoracic and abdominal aorta regions in the HIV-Tg mouse model.

Many factors accelerating CVD progression in HIV positive patients remain to be investigated. Here, we have parsed the effects of shear stress and HIV proteins, specifically Tat, on cathepsins K, S, V, and L activity of endothelial cells. These cells line the vascular wall, are directly exposed to blood fluid shear stress, and have first access to circulating HIV proteins shed from lysing, infected cells. Together, these factors may stimulate the endothelium to induce proteolytic remodeling by cathepsins and initiate earlier arterial wall remodeling indicative of HIV-mediated CVD.

CHAPTER 4

HIV-1 TAT AND PRO-ATHEROGENIC SHEAR STRESS UPREGULATE CATHEPSIN-MEDIATED EXTRACELLULAR MATRIX DEGRADATION AND C-JUN PHOSPHORYLATION

4.1 Introduction

Within HIV-infected patients, there are many factors that contribute to cardiovascular disease progression. Both ARV and HIV infection have been shown to be major contributors to increased cardiovascular risk [3, 6, 10, 20, 23, 24, 47, 143]. Specific contribution from HIV infection is not limited only to CD4+ T-cells, but also to HIV proteins shed by infected cells as they lyse. Our group has previously shown that arterial stiffness and cathepsin activity is greater in HIV-tg mice expressing HIV proteins compared to wildtype controls [47], suggesting that HIV proteins alone contribute to vascular remodeling, with upregulated protease activity being a possible mediator.

Investigating the compounding effects of hemodynamic influences on cathepsin upregulation is also an important area of study. Areas of low and oscillatory shear stress have elevated incidence of atherosclerotic plaque formation and cathepsin activity [77, 83, 84, 90], which can exacerbate HIV-induced arterial remodeling. In HIV-tg mice, areas of pro-atherogenic shear stress have elevated cathepsin activity compared to areas of atheroprotective shear stress; HIV-1 Tat is a possible culprit as these results were recapitulated *in vitro* with Tat alone (Chapter 3) [159].

HIV-1 Tat has been widely studied as an activator of endothelial cells and has been shown to increase secretion of inflammatory factors, endothelial cell proliferation,

protease production, cytoskeletal organization, adhesion, and migration [11, 12, 49, 69]. Mechanisms for Tat activation of endothelial cells are understudied, but there are reports that Tat can bind to uninfected endothelial cells and be internalized from the circulation [54, 63, 68, 161] via the C-terminal domain which binds to arginine-glycine-aspartic (RGD) sequences[56, 68].

It has been shown that Tat activates individual kinases with various physiological consequences. In HUVECs, Tat induced ERK activation leads to impaired actin stress fiber assembly [12, 63, 66], and JNK and ERK are independently activated via Nox pathways affecting cell migration and motility [72]. ERK-mediated pro-angiogenic activation encompasses increases in cell migration, invasion, and increased protease production; all of which are necessary pre-cursors for endothelial cells to traverse connective tissue [162]. In HUVECs and BAECs, oscillatory shear stress activates JNK and ERK phosphorylation [163-166], pathways also activated by Tat. Previous work in our lab has shown c-Jun and JNK activation mediates cathepsin activity, but pro-atherogenic shear stress also can increase cathepsin K activity [117, 118, 124, 167], showing possible links between Tat stimulation and cathepsin activity.

Within this study, the synergistic effects of oscillatory shear stress and HIV-1 Tat on kinase activation were tested as a mechanism for cathepsin-mediated gelatin degradation, using a cone-and-plate bioreactor to actuate shear stress on HAECs. First, gelatin cleavage due to endothelial activation by pro-atherogenic shear stress and Tat was investigated to test the hypothesis that upregulated cathepsin K activity in Tat stimulated HAECs leads to physiological consequences on ECM degradation. Tat-induced ERK, c-jun, JNK, and Akt phosphorylation were measured after preconditioning with pro-

atherogenic shear stress, and pathways stimulated by both Tat and shear stress were inhibited to understand the role of these kinases in cathepsin K upregulation. Altogether this work tests the hypothesis that ERK and c-jun regulate cathepsin activity and subsequent substrate degradation in endothelial cells co-stimulated by HIV-1 Tat and pro-atherogenic shear stress, which will help identify important mechanisms for the higher incidence of cardiovascular remodeling seen in HIV-positive patients.

4.2 Methods

4.2.1 Human aortic endothelial cell culture and shear stress studies.

HAECs (Lonza) were cultured as described previously [159]. Cells were maintained with 5% CO_2 at 37°C in 10 cm dishes until confluent and used between passage 6 and 8. Upon confluence, medium was changed to .5% serum Endothelial Growth Medium (EBM) (Lonza) for 4 hours, for low serum culture prior to treatment with Tat (69.4 nM) (ImmunoDiagnostix) or pro-atherogenic shear stress using a cone and plate apparatus to generate oscillatory waveforms characterized in humans [159].

4.2.2 Gelatinase Assay

HAECs were incubated with Tat or vehicle control, in the presence or absence of E64 (50 μM) for 24 hours under static and pro-atherogenic conditions. After stimulation, BODIPY fluorescein-conjugated DQ gelatin (25 μg/ml); (Life Technologies) in 4 ml of fresh .5% serumBM media was incubated with cells for a subsequent 24 h and 500 μl of conditioned media from each experiment was assayed for gelatinase activity using a fluorescence plate reader with background fluorescence subtracted from the no-cell negative control at 485 nm excitation and 525 nm emission.

4.2.3 Kinase Phosphorylation Studies and Western Blotting

HAECs were cultured in 10 cm dishes under static conditions or exposed to pro-atherogenic shear stress for 24 hours in 0.5 % serum EBM media. After shear stimulation, HIV-1 Tat (69.4 nM) or vehicle control was added for 0, 5, 15, and 90 minutes. Cells were subsequently rinsed with PBS , lysed on ice using 100 ul of lysis buffer (20 nM Tris–HCl at pH 7.5, 5 mM EGTA, 150 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.1% Tween-20), cleared by centrifugation, and total protein concentration determined by micro-bisinchoninic acid (BCA) assay (Pierce). 5X reducing loading buffer (0.05% bromophenol blue, 10% SDS, 1.5M Tris, 50% glycerol, 10 mM beta-mercaptoethanol) was added to all samples prior to loading. Equal amounts of cell or tissue protein were resolved by 12.5% SDS-polyacrylamide gels. Protein was transferred to a nitrocellulose membrane (Bio-Rad) and probed with phosphorylated Akt , extracellular signal-regulated kinase 1 and 2 (ERK), c-Jun N-terminal kinases (JNK), and c-jun primary antibodies (Cell Signaling) at a 1:400 dilution. Secondary donkey anti-rabbit antibodies tagged with an infrared fluorophore (Li-Cor) were used to image protein with a Li-Cor Odyssey scanner. Gels were subsequently stripped and re-probed with a 0.2 N NaOH solution for total Akt, JNK, c-jun, and ERK (Cell Signaling). Lastly membranes were reprobed for actin at a 1:500 dilution (Santa Cruz). Densitometry of labeled nitrocellulose membranes was performed using NIH ImageJ.

4.2.4 ERK and c-jun Inhibition Studies

For ERK inhibition studies, HAECs were pretreated with MEK inhibitor U0126(Millipore) at 20 μg/mL or vehicle control for one hour prior to shear stress

stimulation. For 48 hour experiments, another dose of the MEK inhibitor was added at the 24 hour mark, during media change with DQ Gelatin.

4.2.5 Gelatin Zymography

Cells were lysed as described above, with 0.1 mM leupeptin, which was freshly added to stabilize enzymes during electrophoresis. Lysates were collected and cleared by centrifugation, and total protein concentration was determined by micro-bisinchoninic acid (BCA) assay (Pierce). Multiplex cathepsin zymography was performed as described previously [127, 152]. Gel images were captured with an Imagequant 4010 (GE Healthcare), then images were inverted in Adobe Photoshop and densitometry performed using NIH ImageJ.

4.2.6 Statistical Analysis

Each experimental condition was repeated with a minimum of three biological replicates, and each data point is presented as the mean value +/- standard error of the mean. Representative images are shown. Unpaired student t-tests were used to determine statistical significance between experimental groups.

4.3 Results

4.3.1 Cathepsin-mediated cell-associated gelatin degradation is upregulated in Tat-stimulated endothelial cells

Our previous findings showed an upregulation of cathepsin K activity in HAECs co-stimulated by Tat and pro-atherogenic shear stress [159]. The examination of the effects of this co-stimulation on ECM degradation is also important to understand

physiological significance. Because cathepsin K is the most powerful mammalian collagenase, gelatin cleavage was chosen as a possible substrate for Tat-mediated ECM degradation. Collagen, a complex and stable supramolecule, is an integral part of ECM within arteries, providing structure and stability. Upon hydrolysis or enzymatic degradation, collagen's triple helix is converted into denatured gelatin strands [168, 169]. The helix is also strongly cleaved by cathepsin K [117, 127, 170, 171]. HAECs were cultured under static or pro-atherogenic shear conditions using a cone and plate bioreactor, and stimulated with Tat or appropriate vehicle control for 24 hours. Cells were subsequently cultured with DQ gelatin ± E64, a broad spectrum cathepsin inhibitor, for 24 hours. Conditioned media was collected, and fluorescent products indicative of gelatin cleavage were quantified using a plate reader. Figure 1 shows greater DQ gelatin cleavage in Tat-stimulated cells, cultured under static and pro-atherogenic shear conditions, with 16% and 50% increases respectively, compared to vehicle controls (n=5) (Fig 4.1). DQ gelatin cleavage was significantly lowered by E64 in all conditions. Cathepsins contributed up to 50% of this activity in Tat-stimulated cells and 35% in vehicle controls as indicated by the E-64 inhibitable signal. No differences were observed between pro-atherogenic and static controls. HAECs were lysed and intracellular cathepsin activity was measured via cathepsin zymography to investigate effects of E64 inhibition on cathepsin activity (Fig 4.2). Cathepsin L had the greatest reduction in activity by E64. Cathepsin K activity was not reduced, but actually increased in conditions where the E64 inhibitor was present.

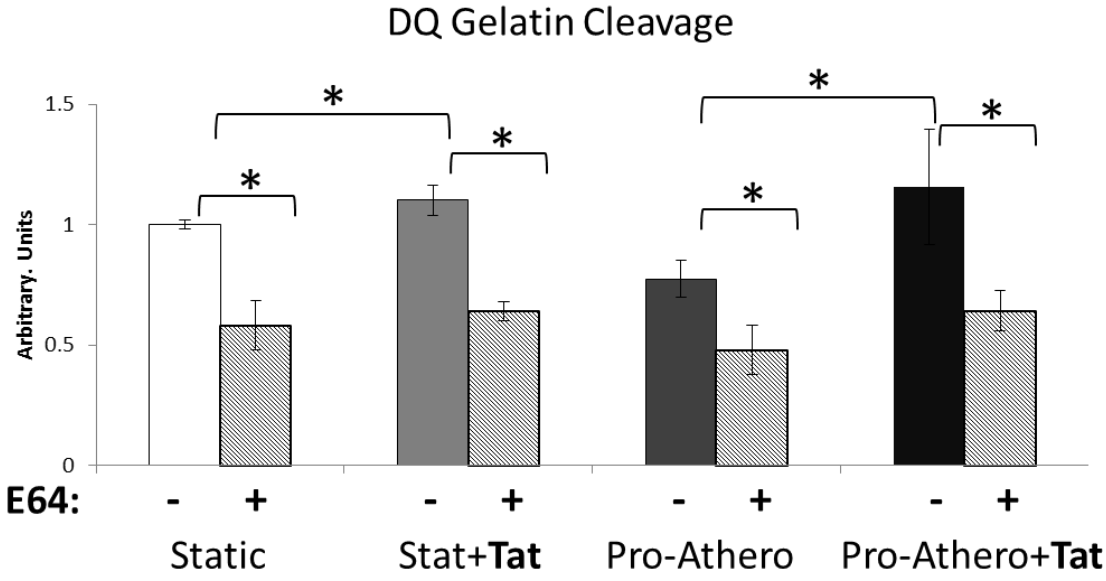

Figure 4.1: Tat stimulates cathepsin-mediated gelatin cleavage under pro-atherogenic and static culture. HAECs were cultured for 24 hours under static conditions or pro-atherogenic shear stress and co-stimulated with HIV-1 Tat or vehicle control. DQ gelatin was added and gelatin cleavage assessed using a plate reader 24 hours later. Cathepsin contribution to gelatin cleavage was assessed using E64 to inhibit cathepsin activity. Tat upregulates DQ gelatin cleavage under static and pro-atherogenic shear conditions with gelatin cleavage being lowered by E64 in each case. There is greater gelatin cleavage in cells cultured under static + Tat conditions as compared to static and pro-atherogenic shear stress + Tat as compared to pro-atherogenic shear stress alone, 16% and 30% respectively. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM)

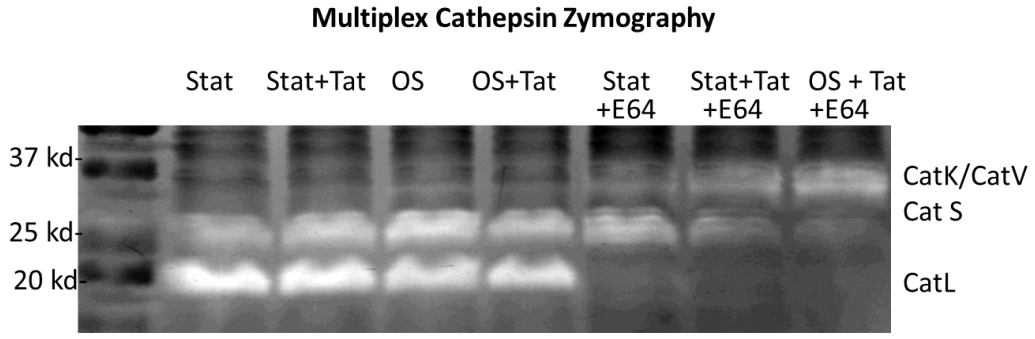

Figure 4.2: E64 differentially inhibits cathepsin L, S, V and K. After DQ Gelatin analysis, cells were rinsed, lysed, and zymographical analysis done to assess changes in cathepsin activity due to E64 inhibition. The addition of E64 inhibits cathepsin L activity, but potentiates cathepsin K and V activity. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM)

4.3.2 ERK and c-jun are activated by pro-atherogenic shear stress alone

Cathepsin K has been shown to be synergistically upregulated by HIV-1 Tat and pro-atherogenic shear stress. To understand the mechanisms behind this synergy, we probed kinases that were shown to be activated by shear stress, Tat, or linked to cathepsin K activity. ERK and JNK are widely studied kinases linked to Tat-mediated endothelial activation; c-jun is phosphorylated by JNK and is a mediator of increased cathepsin K activity. Akt, has also been linked to TNFα stimulation in HAECs [167]. To parse the effects of shear stress versus Tat stimulation, and identify the synergistic effects on kinase activation, the effects of shear stress were first investigated and later, Tat stimulation was investigated. Figure 4.3 shows a significant elevation in ERK and c-jun phosphorylation in cells cultured under pro-atherogenic shear stress. Graphs are normalized to actin as a loading control, as total c-jun was also upregulated by pro-atherogenic shear stress (Fig 4.5).

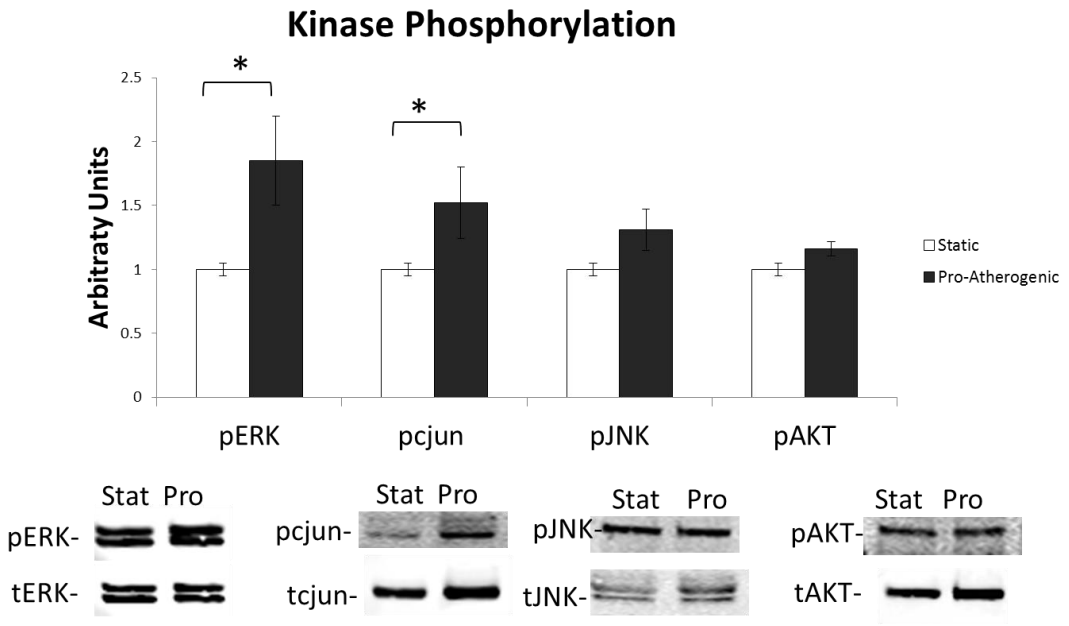

Figure 4.3. Pro-Atherogenic shear stress stimulates ERK and c-jun phosphorylation. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and lysed for Western blot analysis of ERK, c-jun, JNK, and AKT. Phosphorylated and total proteins were probed. Actin was also probed as a loading control. pERK was upregulated 1.75 fold from static controls and pc-jun 1.5 fold, when cultured under pro-atherogenic conditions as opposed to static culture. No differences were observed for JNK or AKT. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM)

4.3.3 Co-stimulation with pro-atherogenic shear stress and Tat potentiates ERK activation

To further identify potential mediators of cathepsin K upregulation established previously, the synergistic effects of pro-atherogenic shear stress and Tat on kinase phosphorylation were investigated. Shear stress alone induced cathepsin K activity, but shear stress and Tat provided the greatest differences. HAECS were pre-conditioned with shear stress or cultured under static conditions and subsequently stimulated with HIV-1 Tat or vehicle control for 0, 5, 15, or 90 minutes. Tat- induced kinase phosphorylation

was measured after static culture, or after cell pre-conditioning with pro-atherogenic shear stress. Western blotting was used to measure kinase phosphorylation in response to pro-atherogenic shear stress. Actin was used as a loading control. Phosphorylated kinases were normalized to total kinases and actin. Additionally, total kinases were normalized to actin to identify changes in total kinase levels due to pro-atherogenic shear or Tat stimulation. Figure 4.4C shows that Tat activated ERK in static controls, and in cells exposed to pro-atherogenic shear stress conditions (Fig 4.4D) when normalized to actin ($n=4$, $p<.05$). Total ERK decreased under pro-atherogenic conditions (Fig 4.4F), whereas in static culture total ERK remained relatively constant (Fig 4.4E), which explains the difference in phospho-ERK seen when normalizing to actin versus total-ERK.

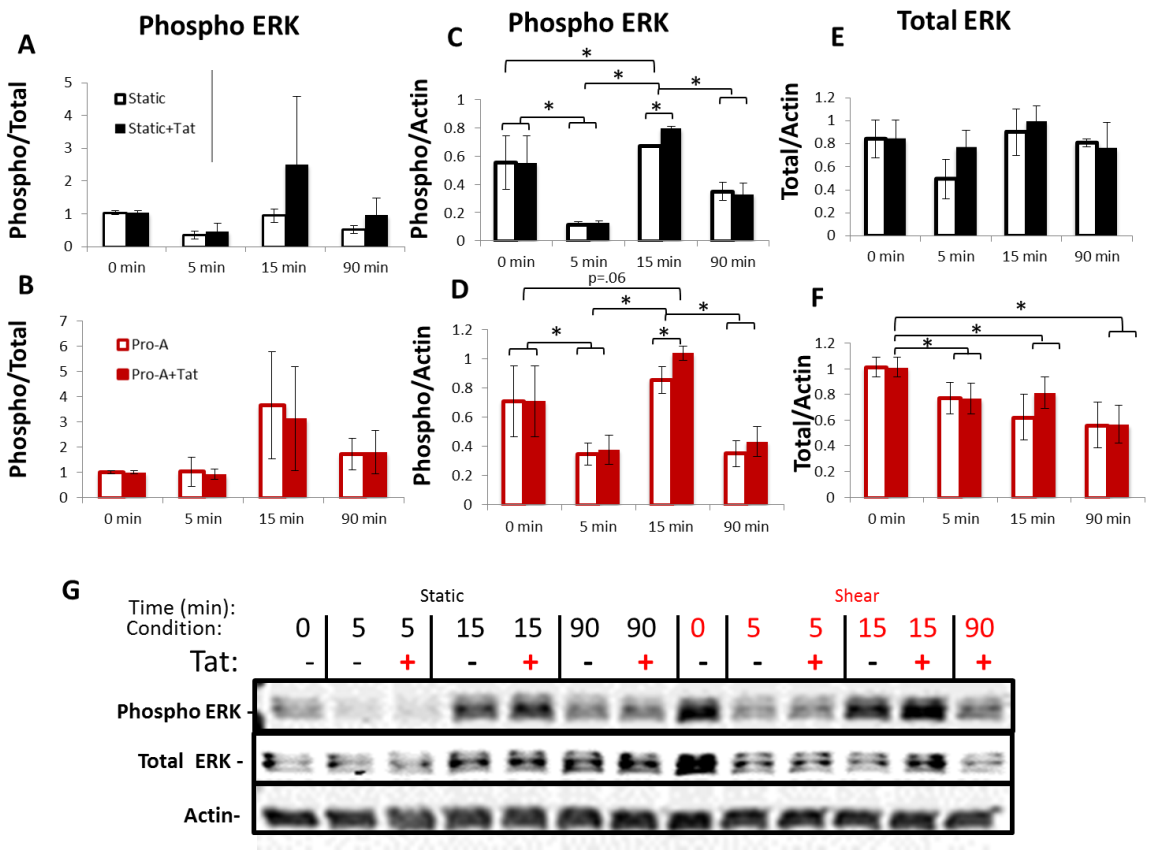

Figure 4.4: Tat stimulates ERK phosphorylation by15 minutes. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis. phosphorylated ERK was normalized to total ERK (A, B) and normalized to actin (C, D). To assess changes in total ERK cells preconditioned under static and shear conditions, protein was normalized to actin (E, F). Representative Western blots are shown (G). Phospho-ERK was potentiated by 15 minutes when compared to vehicle controls under static and pro-atherogenic shear stress conditions when normalized to actin (D). Total ERK was decreased by Tat over time, in cells stimulated with pro-atherogenic shear stress (F), with difference it total ERK between Tat and vehicle controls. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM)

When comparing all four conditions, pro-atherogenic shear stress upregulated ERK phosphorylation by 5 minutes, and Tat + pro-atherogenic shear stress increased phospho-ERK compared to pro-atherogenic shear stress alone, static +Tat, and static controls. Pro-atherogenic shear stress and static + Tat caused greater ERK phosphorylation compared to static controls (Figure 4.5).

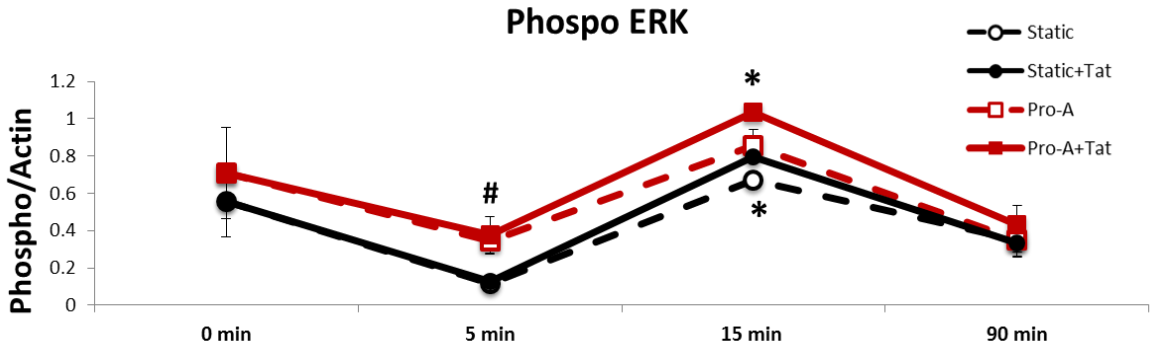

Figure 4.5: HIV-1 Tat and pro-atherogenic shear stress increased ERK phosphorylation compared to all other conditions. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis of phospho ERK normalized to actin. Pro-atherogenic shear stress activated ERK in HAECs to a greater extent than static culture at 5 minutes, and by 15 minutes cells co-stimulated with pro-atherogenic shear stress+ Tat had greater ERK activation compared to all other conditions. Additionally, ERK phosphorylation was increased in pro-atherogenic and static + tat conditions as compared to static control, although the two aforementioned were not statistically significant from each other. (n=4) ($p<.05$, * = statistically significant compared to all other conditions, #= significantly significant from both static conditions.)

4.3.4 Co-stimulation with pro-atherogenic shear stress and Tat mediate potentiated c-jun activation

The same procedures were performed to asses Tat and pro-atherogenic shear stress on c-jun and JNK phosphorylation. As observed with ERK, no significant changes were identified in phosphorylated c-jun when normalized to total c-jun, (4.6 A-B) however a 2-fold increase in c-jun phosphorylation was observed at 5 minutes in Tat

stimulated cells cultured under static conditions, 1.75 fold at 15 minutes, (Figure 4.6 C) and completely attenuated by 90 minutes. Cells exposed to pro-atherogenic shear stress and Tat showed a 1.4 fold greater c-jun phosphorylation at 5 minutes compared to pro-atherogenic vehicle controls; by 15 minutes this difference was no longer observed.

Total c-jun was also elevated by Tat under static conditions at 5 and 15 minutes, and total c-jun levels in cells stimulated with pro-atherogenic shear stress and Tat were not statistically different compared to those stimulated with pro-atherogenic shear stress alone (Fig 4.7). However, total c-jun was upregulated compared to cells cultured under static conditions in the presence or absence of Tat by 15 and 90 minutes. HAECS co-stimulated with Tat and pro-atherogenic shear stress had the greatest c-jun phosphorylation at 5 and 15 minutes compared to cells with pro-atherogenic vehicle controls and cells cultured under static conditions in the presence or absence of Tat.

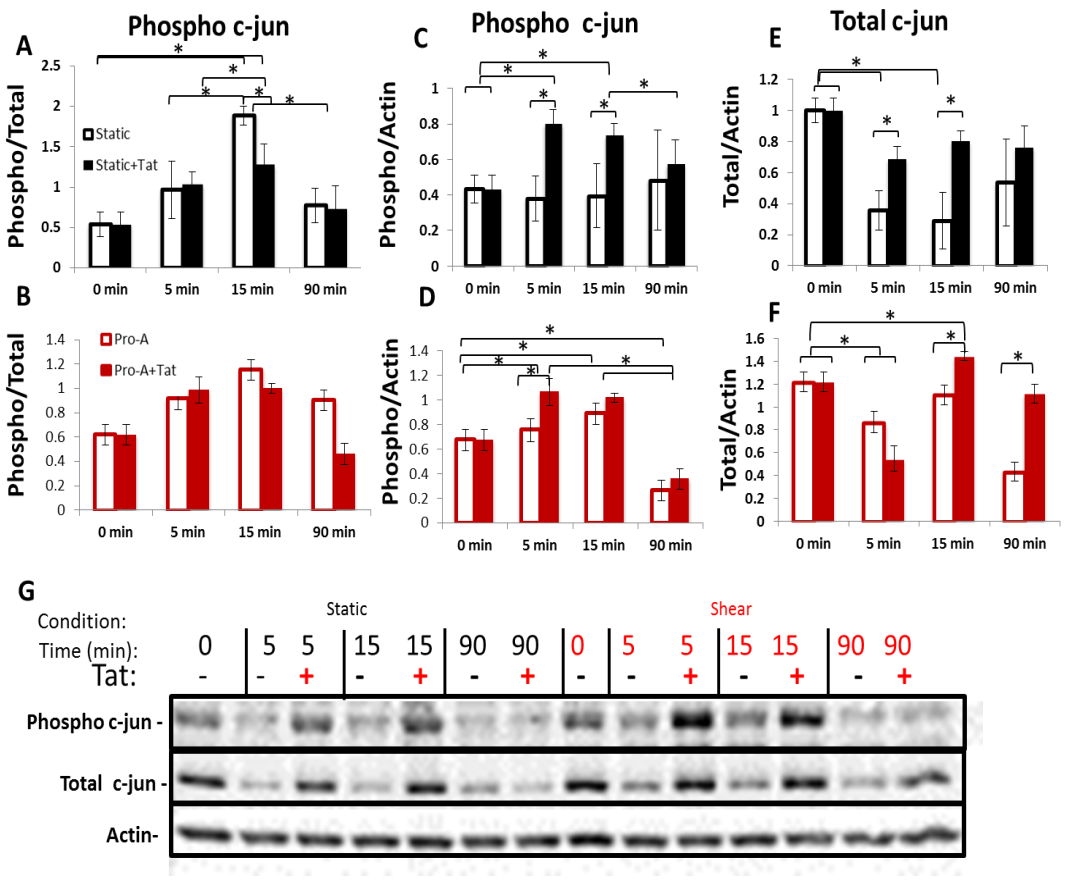

Figure 4.6: Tat potentiates total c-jun compared to vehicle controls and c-jun phosphorylation under static and pro-atherogenic conditions. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis of phospho c-jun normalized to total (A, B) and normalized to actin(C, D). To assess changes in total c-jun cells preconditioned under static and shear conditions was normalized to actin (E, F). Representative Western blots are shown (G). C-jun activation was increased at 5 minutes compared to vehicle controls under static and pro-atherogenic shear stress conditions when normalized to actin. Total c-jun was upregulated by Tat at 5 minutes in static conditions, and at 15 minutes in pro-atherogenic conditions compared to vehicle controls. (* indicates $p < 0.05$, n=4 and data is mean +/- SEM)

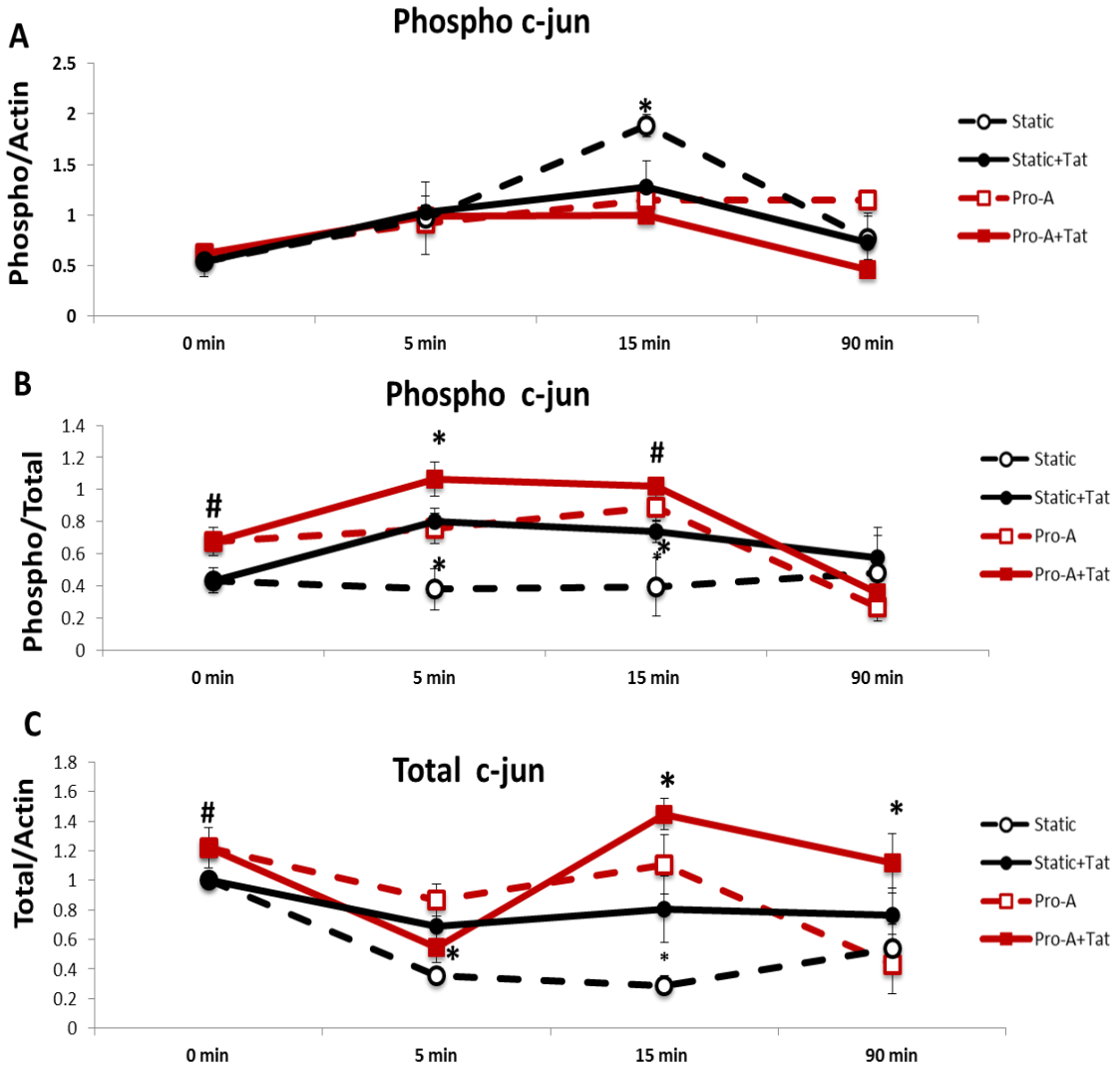

Figure 4.7: HIV-1 Tat and pro-atherogenic shear stress induce c-jun activation greater than all other conditions. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis of phospho c-jun normalized to total c-jun (A) and normalized to actin (B). To assess changes in total c-jun cells preconditioned under static and shear conditions was normalized to actin (C). There were no changes in phosphorylated c-jun between static and pro-atherogenic shear stress groups when normalized to actin or total c-jun (A, B). Pro- atherogenic stress activates p-c-jun, and tat further potentiates this phosphorylation compared to pro-atherogenic and static controls, and compared to static + Tat conditions (B). Total c-jun is also potentiated by co-stimulation of Tat and pro-atherogenic shear stress at 15 minutes compared to all other conditions (C). Pro-atherogenic and static + Tat conditions had elevated total c-jun compared static controls, but were not different from each other. (n=4, * means statistically significant ($p<.05$) compared to all other conditions, #= significantly significant from static conditions.)

JNK and Akt activation by Tat were also investigated. We found no differences in JNK activation in HAECS due to shear stress or Tat stimulation (Figure 4.7). There was elevated Akt phosphorylation by Tat under static conditions at 15 minutes, but no other differences were observed (Figure 4.8).

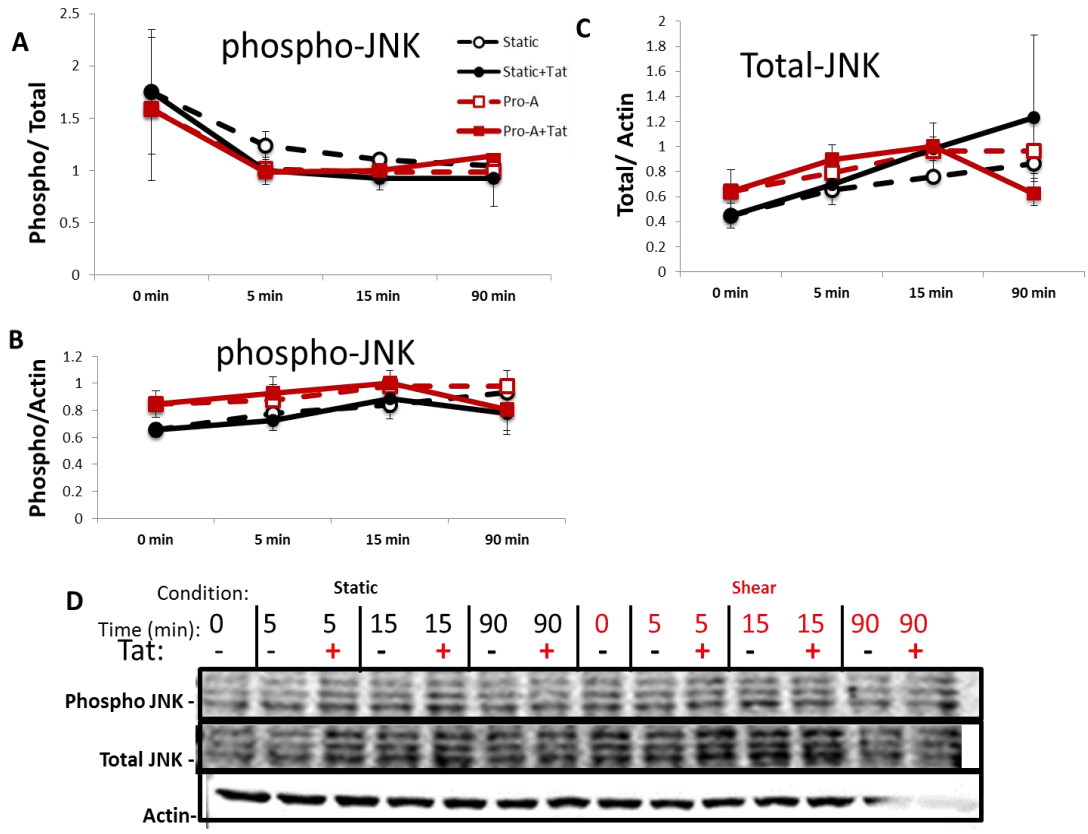

Figure 4.8: HIV-1 Tat and pro-atherogenic shear stress do not cause changes in JNK phosphorylation. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis of phospho c-jun normalized to total c-jun (A) and normalized to actin (B). To assess changes in total c-jun cells preconditioned under static and shear conditions was normalized to actin (C). No differences were observed for any conditions. (n=4, * means statistically significant ($p<.05$) compared to all other conditions, #= significantly significant from static conditions).

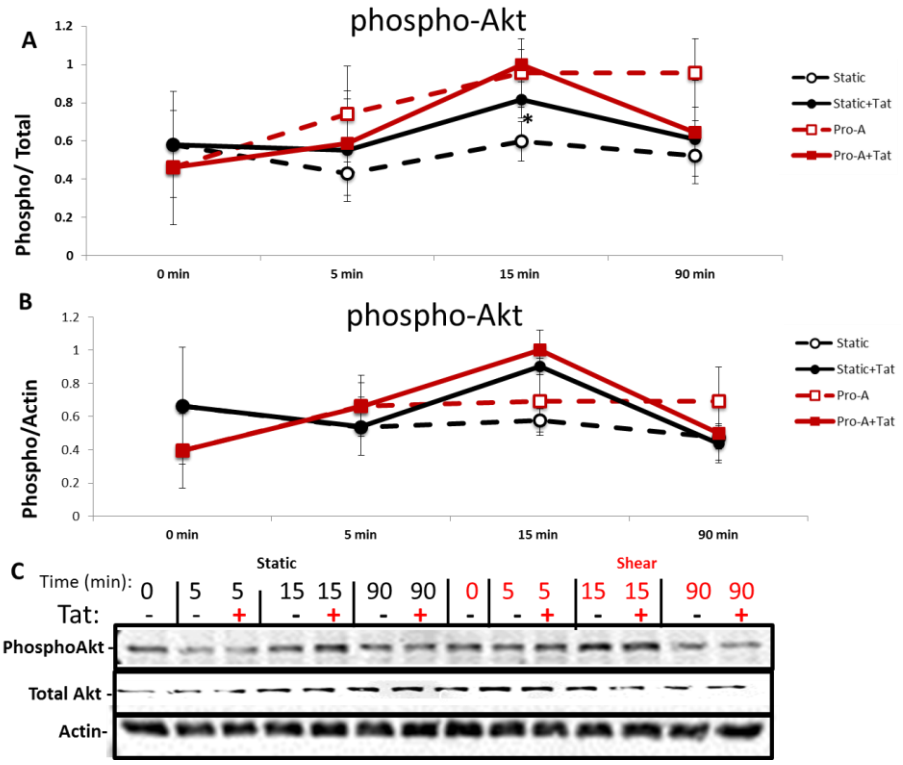

Figure 4.9: HIV-1 Tat activates Akt by 15 minutes under static conditions. HAECs were cultured under static or pro-atherogenic conditions for 24 hours and stimulated with Tat for 0, 5, 15, and 90 minutes. Cells were then lysed for Western blot analysis of phospho Akt normalized to total (A) and normalized to actin (B). Under static culture, AKT activation was observed by 15minutes. (n=4, * means statistically significant ($p<.05$) compared to all other conditions, #= significantly significant from static conditions).

4.3.5 ERK inhibition abolishes cathepsin activity in HAECs

Finally to elucidate the regulatory effects of ERK on cathepsin activity, a small molecule inhibitor was used to block ERK activation. HAECs were incubated with MEK inhibitor, U0126, and then stimulated with Tat or vehicle control while cultured under static conditions or pro-atherogenic shear stress. After 24-hours, DQ gelatin was incubated with the cells for an additional 24 hours. After this total of 48 hours, differences in DQ gelatin cleavage were not statistically significant between groups,

however there was decreased amounts of active cathepsin K, V, L and S in cells treated with the MEKi versus vehicle controls.

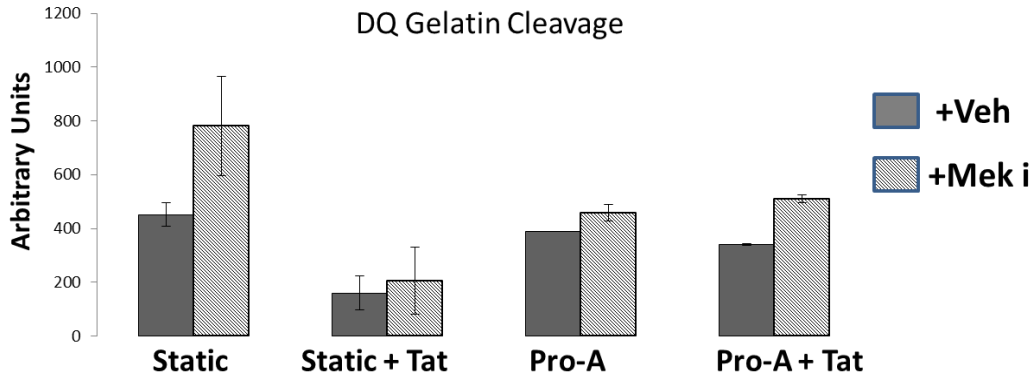

Figure 4.10: Inhibition of ERK1/2 pathways eliminates Tat-mediated increases in gelatin degradation. HAECs were pre-incubated with MEKi U0126 for 1 hour and subsequently cultured for 24 hours under static conditions or pro-atherogenic shear stress and co-stimulated with HIV-1 Tat or vehicle control. DQ gelatin was added and gelatin cleavage was assessed using a spectrophotometer 24 hours later. There were no differences in gelatin degradation with the addition of the MEKi. (n=2)

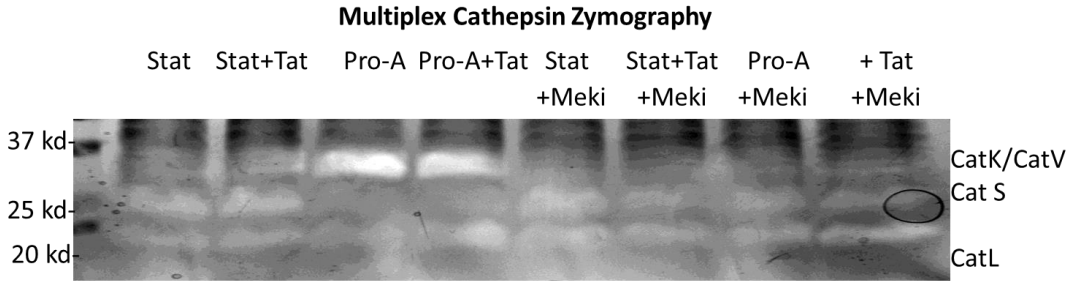

Figure 4.11: Inhibition of ERK 1/2 pathways downregulates cathepsin K and V activity in HAECs stimulated by pro-atherogenic shear stress and Tat. After DQ Gelatin analysis, cells were rinsed, lysed, and zymographical analysis done to assess changes in cathepsin activity due to MEK inhibition. Decreases in cathepsin K and V were observed when inhibiting these pathways. (n=2)

4.3.6 The role of c-jun in Tat-mediated cathepsin upregulation in HAECs

For this aim, we were not able to perform direct studies assessing c-jun's role in Tat-mediated gelatin degradation or increased cathepsin activity. However, previous studies done in our lab, linked c-jun and JNK to cathepsin K activity, stimulated by TNFα, but not by pro-atherogenic shear stress [167]. The role of this kinase needs to be thoroughly investigated as we observed the greatest increases in c-jun expression and phosphorylation by Tat, among the kinases we studied.

4.4 Discussion

These results imply that the upregulation of cathepsin K by pro-atherogenic shear stress and HIV-1 Tat may be regulated by ERK and c-jun activation. This identifies these kinases as putative mechanisms behind Tat-mediated increases in proteolytic degradation, a potential mechanism for the higher incidence of cardiovascular diseases in individuals living with HIV, where both Tat and pro-atherogenic shear stress are present. This work has shown that c-jun and ERK are upregulated by shear stress; c-jun has been linked to increased superoxide production and apoptosis in endothelial cells [172, 173], and is a major component of the AP-1 transcriptional complex, forming homodimers or heterodimers with other AP-1 components from the Jun family (Jun B and Jun D) or the Fos family (c-Fos, Fra-1, Fra-2, and Fos B) [174]. Thus, the different c-Jun dimers can exhibit distinct transcriptional properties with diverse biological consequences. ERK is also of importance in various signaling pathways, and has been shown to be important for many endothelial cell functions. In the context of Tat, ERK has been most associated with pro-angiogenic activation [12, 63, 66]. Understanding how these two kinases work

in parallel when activated by pro-atherogenic shear stress will provide insight into Tat- and shear-mediated vascular remodeling.

Increased proteolytic activity by cathepsins can degrade the internal elastic lamina, which allows migration of SMCs into the sub-intimal space, and increases monocyte infiltration, advancing CVD progression [14, 127, 128]. Human atherosclerosis samples showed a positive correlation between atherosclerotic lesion development and fragmentation of elastic lamina and cathepsin K levels in endothelium, implicating cathepsin K in elastin degradation [14]. The results from this study implicated Tat-stimulated cathepsin activity in HAECs to gelatin degradation, with greater gelatin degradation under static and pro-atherogenic shear conditions attributed to cathepsins by comparing the effects in the absence and presence of E-64, the cathepsin inhibitor (Figure 4.1). Also of importance is the differential intracellular down regulation of cathepsin L and the upregulation of cathepsin K by E64 (Figure 4.2). This could be because cathepsin K secretion was inhibited and subsequently sequestered within the cell, or due to some other complex interactions between cathepsins and their inhibitors that need to be explored. These results indicate that the synergy between pro-atherogenic shear stress and Tat stimulation on increased cathepsin K, are not transferred to gelatin cleavage, but other proteolytic enzymes could be contributing (Figure 4.10) . Cathepsin L, S, and V are also gelatinases that contribute to arterial remodeling, providing complex interplay between these cathepsins. To truly examine the effects of cathepsin K, a cathepsin K specific inhibitor would need to be used. Odanacatib is such an inhibitor that was recently approved by the FDA as a therapy for patients with osteoporosis, or other diseases with pathological collagen remodeling [175-180].

Cathepsin K activation has been linked to JNK and c-jun phosphorylation [167, 181], although ERK has not been studied as a mediator of cathepsin activity. However, ERK has shown to be activated by shear [166, 182-184] and by Tat. Akt has also been shown to be activated by shear, although not activated by Tat [184]. This study selectively isolated the effects of HIV proteins and hemodynamics from that of lipid accumulation and inflammation associated with plaque formation on the phosphorylation of ERK and c-jun. C-jun showed the greatest response to Tat and pro-atherogenic shear stress (Figure 4.6), and although ERK was also activated (Figure 4.4), the increases were less than observed for c-jun, showing that pro-atherogenic shear stress activates certain kinases that can be sustained or increased by Tat (Figure 4.7).Tat sustained total c-jun levels under static and pro-atherogenic conditions (Figure 4.7). JNK was not phosphorylated in the same manner, although many studies have shown JNK activation by Tat in HUVECs or BAECS [65, 163, 165, 185]. Many of these studies pre-conditioned cells with inflammatory cytokines IFN-γ or TNF-α to induce JNK activation, which could explain the different findings between our studies and theirs [65, 68].

In the JNK-c-jun kinase signaling cascade, JNK activates c-jun, but it has also been shown that ERK can also phosphorylate c-jun and JNK, although this pathway is not widely studied [172, 174, 186]. This however, does not explain Tat's sustenance of total c-jun levels. An alternative hypothesis is that pro-atherogenic shear stress upregulates receptors to which Tat can bind.

It is also interesting to note that at 90 minutes phosphorylated c-jun was still elevated in cells co-stimulated by pro-atherogenic shear stress and Tat. This shows the potentiation of these stimuli over longer time periods when HAECs are co-stimulated.

Although ERK phosphorylation was upregulated by Tat to a lesser extent, pre-incubation with the MEKi completely abolished any Tat-stimulated gelatin degradation. This result was further validated with decreased cathepsin K and V activity measured by multiplex zymography. Phosphorylated ERKs possible role in c-jun activation could explain the attenuation of gelatin cleavage and c-jun-mediated gelatin cleavage, by the MEK inhibitor.

c-jun and c-fos dimerize to form transcription factor AP-1; our lab has recently shown AP-1 as a transcription factor for cathepsin K, providing direct implications of these studies on cathepsin K activity. Additionally this upregulation of cathepsin K could be due to the synergy of ERK and c-jun activating different kinase pathways. Studies have been done to explore the co-activation of ERK and JNK signaling cascades. The Feng Wes group found that Tat activated dual Nox paralogs for differential activation of ERK and JNK pathways in HUVECs, linking Nox 2- JNK signaling to Tat induced cytoskeletal arrangement and Nox-4 dependent ERK signaling to Tat dependent endothelial cell proliferation. This study showed that Tat signaling provided specific differential control of MAPK pathways [72].

While many of these mechanisms for cardiovascular disease progression need to be explored, this work has shown interesting responses in c-jun and ERK. To our knowledge, no one has studied Tat and shear stress-mediated responses of these kinases in HAECs. In this study, we used primary HAECs to investigate these changes in phosphorylation, as they are exposed to HIV-1 Tat as infected cells lyse, in addition to pro-atherogenic shear stress *in vivo.* Together c-jun and ERK may offer therapeutic targets for cardiovascular remodeling in HIV-positive patients.

CHAPTER 5

EFAVIRENZ DECREASES CATHEPSIN ACTIVITY IN MONOCYTES *IN VIVO* AND *IN VITRO* AND UPREGULATES HUMAN AORTIC CELL CATHEPSIN ACTIVITY

5.1 Introduction

Highly active antiretroviral therapies have proven to be a very effective treatment for people living with HIV - reducing mortality, viral load, and decreasing HIV transmission. However, as patient life expectancy increases, incidences of CVD are also increased. Included in these are myocardial infarction [6, 132] atherosclerotic lesions [187], arterial stiffness [7, 21, 23, 31] and carotid artery intimal media thickness [27, 29]. Protease inhibitors are the most widely implicated ARV in CVD progression [5, 8, 27, 29, 188, 189] , however nucleoside reverse transcriptase inhibitors (NRTI's) and non-NRTI's (NNRTI's) have also been implicated [10, 190, 191].

Because HIV is also an inflammatory disease, treated with a triplicate cocktail at various stages of disease progression, it is difficult to distinguish the effects of individual ARV's on cardiovascular disease. Although some correlations have been made for increased inflammatory markers [192-194], patient data is difficult to obtain and at times difficult to analyze due to compounding factors.

To truly understand the effects of these factors on proteases, such as cathepsins and MMPs, individual contributions from endothelial cells and monocytes must be explored. These proteases are upregulated in many diseased states by both cell types. It is

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[2]Portions of this section were adapted from Gleason RL Jr., C.A., Seifu D., Parker IK,Vidakovic B, Getenet H., Assefa G., Amogne W., *Current efavirenz (EFV) or ritonavir-boosted lopinavir (LPV/r) use correlates with elevate markers of atherosclerosis in HIV-infected subjects in Addis Ababa, Ethiopia*

important to parse the effects of HIV infection versus antiretroviral stimulation of cathepsin activity, and to distinguish the contribution from monocytes as they are directly exposed to antiretrovirals and bind to the endothelium. As seen in the previous aims, cathepsins are upregulated in arterial cells by individual HIV proteins; we have shown endothelial cell upregulation of cathepsin K by HIV-1 Tat (Figure 3.5). However, these findings do not take into account the effects of the constant inflammatory state on endothelial cells and monocytes *in vivo*. In this aim, we investigate the effects of ARV regimen on inflammatory markers in patients and PBMC cathepsin levels by investigating a unique subset of HIV positive patients. Patients in sub-Saharan Africa represent 69% of all people with HIV [12] and cost-effective therapies are limited in these areas. Because many patients are receiving the same antiretroviral cocktail, it is more feasible to assess specific combinations of antiretroviral therapies on CVD markers for these individuals.

This data is then validated *in vitro,* adding EFV and TDF to mononuclear cells exogenously to investigate effects of individual drugs on cathepsin activity. Essentially this study provides insight into the individual contributions of ARV's in an HIV infection state in cocktail and individually *in vitro* and *in vivo*. Ultimately, we were able to identify changes in sVCAM, sICAM, and hs-CRP based on ARV regimen, parse the subset of PBMCs that respond to ARV stimulation, and identify information on arterial cells' response to ARV stimulation.

5.2 Materials and Methods

5.2.1 Patient consent and approval by Ethics board

All protocols were reviewed and approved by the Georgia Institute of Technology Institutional Review Board and the Human Ethics Committee of the University of the Witwatersrand, and informed consent was received from all participants. This study was approved by the Institutional Review Board Committees at Addis Ababa University and Georgia Institute of Technology.

5.2.2 Study subjects

All work was performed in accordance with the Declaration of Helsinki. All participants provided written informed consent and this study was approved by the Institutional Review Board Committees at Addis Ababa University and Georgia Institute of Technology. Eighteen- to 65-year-old HIV-negative, HIV-positive HAART naïve, and HIV-positive subjects on EFV-, nevirapine-, or lopinavir/r-containing regimens for at least two months prior to the exam were recruited from Tikur Anbessa (Black Lion) Specialized Referral Hospital in Addis Ababa, Ethiopia to participate in this study. Subjects were excluded if they had active AIDS defining illnesses or diabetes mellitus. Subjects fasted and refrained from tobacco products for at least 8 hours prior to the test and refrained from exercise in the morning of the test. Participant age, sex, HIV-serostatus, date of first HIV-seropositive test, initial CD4+ cell count, last CD4+ cell count, and any viral load determinations, and date of initiation of current and all previous HAART regimens were obtained from the 114 participants hospital cards. Questionnaire-driven interviews were performed by the local recruiting nurse at the Black Lion Hospital HIV clinic under the direction of the research team. Self-reported personal and familial

(mother, father, brothers, or sisters) history of heart attack, angina, stroke, kidney disease, diabetes, or lipid disorders and self-reported alcohol and cigarette use were recorded.

5.2.3 Blood Sample Analysis

High-sensitivity C-reactive protein (hs-CRP), soluble vascular cell adhesion molecule-1 (sVCAM-1) , intercellular adhesion molecule-1 (sICAM-1), and leptin analyses were performed on blood serum using commercially available ELISA kits (Life Technologies Corporation). Analysis for HIV viral load was performed on 70%, hsCRP on 80%, sVCAM-1 on 85%, sICAM-1 on 80%, and leptin on 80% of the subject pool, equally distributed among groups.

5.2.4 Isolation of PBMCs from whole blood

Blood (5 ml) was collected in tubes containing EDTA, and then centrifuged at 800 g for 20 min to separate plasma, buffy coat, and red blood cells. 800 µl of plasma was mixed with 200 µl of dimethyl sulfoxide and this was added to the buffy coat sample to a final volume of 1 ml and stored at -80ºC [33]. After thawing, the isolated cells were then washed with a red blood cell lysis buffer (0.83% ammonium chloride, 0.1% potassium bicarbonate, and 0.0037% EDTA) for five minutes to remove any contaminating RBCs, then resuspended in PBS. This was repeated as necessary until the suspension was clear and any residual red/pink color was removed. Then cells were lysed in zymography lysis buffer, sonicated, and supernatant was assayed for total protein concentration with Micro-BCA kit (Pierce).

5.2.5 Multiplex cathepsin zymography

Multiplex cathepsin zymography was performed as described previously [127, 159]. Gels were imaged on a light box with a digital camera. Images were inverted in Adobe Photoshop and densitometry was performed using ImageJ.

5.2.6 Stimulation of THP-1, endothelial, and smooth muscle cells *in vitro* with efavirenz and tenofovir.

To investigate the effects of ARVs on monocytes, a dose-dependent study was performed on THP-1 monocytes incubated with 25 μM of TDF, EFV, or their respective vehicles for 24 hours. To further investigate any time-dependence of the antiretroviral drugs on THP-1 monocytes. Additionally, confluent HAECs, cultured as noted previously [159], were stimulated with EFV and TDF. Human Aortic Smooth muscle cells (Lonza) were also cultured to confluence using the SMC growth media and bullet kit (Lonza) and stimulated with TDF and EFV in this manner. Cathepsin protease activity of incubated monocytes was analyzed by gelatin zymography and quantified using ImageJ.

5.2.7 Statistics and data analysis

Patient demographics and characteristics at ART initiation were summarized using means with standard deviation for normally distributed continuous variables, medians with associated interquartile range for not normally distributed continuous variables and proportions for categorical variables. All statistical analyses were performed using MATLAB© (MathWorks). A one-way analysis of variance (ANOVA) was performed to determine statistical significance across groups ($p < 0.05$) on continuous variables that satisfied Bartlett's test for equal variances and Pearson Chi-

square test for or normality of residuals; a Kruskal-Wallis non-parametric one-way ANOVA was performed on continuous variables that did not show equal variances or normality.

5.3 Results

5.3.1 Inflammatory Markers in Ethiopian patients

Blood was collected from patients, serum separated using centrifugation, and ELISAs were used to assess differences in high sensitivity C-reactive protein (hs-CRP), soluble intercellular adhesion molecule-1 (sICAM), soluble vascular cell adhesion molecule-1 (sVCAM), and leptin based on ARV treatment. We founds that s-ICAM-1 was increased in all HIV-positive groups including naïve patients and those on EFV, NVP, and LPV/r (Figure 5.1 A). hs-CRP was increased in LPV/r-treated patients compared to HIV-negative subjects, and in EFV-treated compared to HIV-negative, HAART-naive and NVP treated subjects (Fig 5.1B). Compared to HIV-negative subjects, sVCAM-1 was elevated in HAART-naïve, LPV/r-treated, and NVP-treated patients, sVCAM was also increased in HAART-naïve subjects compared to patients treated with EFV and NVP (Fig 5.1 C). Leptin was lower in EFV and NVP-treated groups compared to HAART-naïve subjects and in EFV-treated compared to HIV-negative subjects (5.1 D).

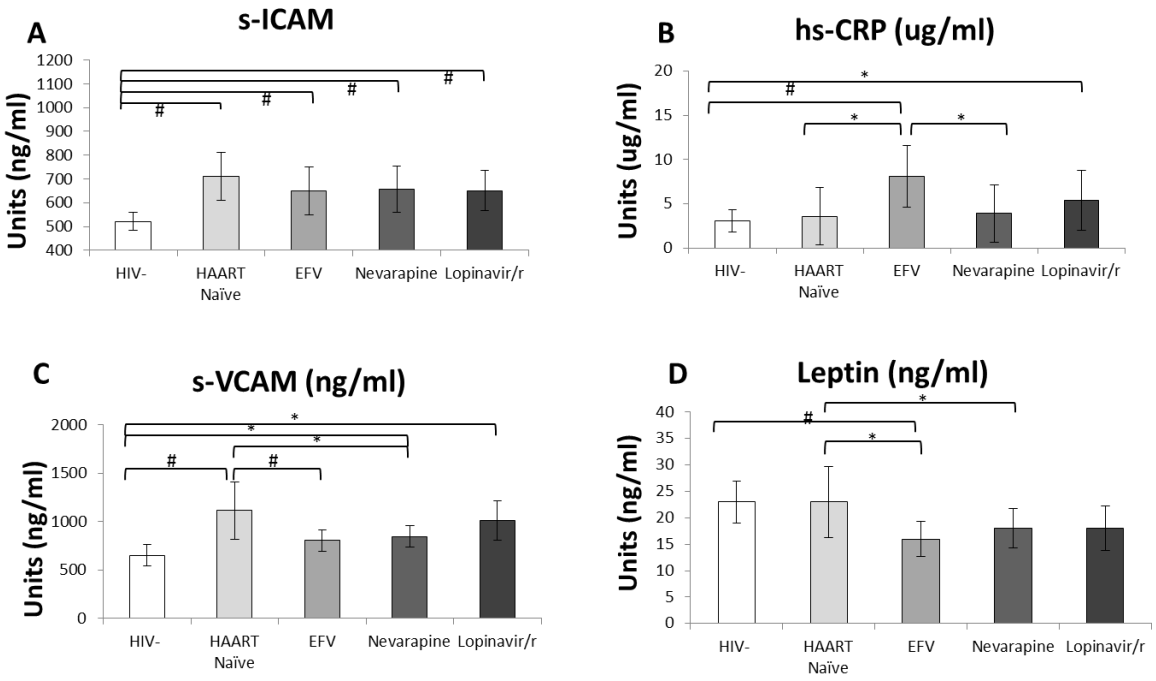

Figure 5.1: Effects of antiretroviral regimen on sICAM, sVCAM, Leptin, and hs-CRP in HIV positive patients. Analysis using serum from patients and respective ELISAs show increased sICAM in HIV positive patients compared to HIV negative (A), Increased hs-CRP in EFV treated patients compared to all others, except LPV/r and an increase in LPV/r compared to HIV negative patients (B). HAART-naïve patients have increased s-VCAM compared to HIV-, EFV, and NVP subjects. NVP and LPV/r were also increased compared to HIV- patients (C). Leptin was decreased in EFV patients compared to HAART- naïve and HIV negative patients, and in nevirapine patients compared HAART -naïve as well.(n= 36 HIV-negative patients, 51 HAART Naïve patients, 91 patients on EFV, 95 patients on Nevirapine, and 44 patients on Lopinavir/r. (#=p<.005, and *=p<.05.)

To investigate the influences of ARVs on cathepsin activity in patients, 32 samples from males and female patients were collected and tested in Addis Ababa, Ethiopia at Black Lion Hospital. Of these, 4 were HIV-positive ART naïve, 3 were HIV-negative, and 25 were HIV-positive ART experienced patients. These patients were on different combinations of ART including nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PI), and there was a significant reduction in cathepsin activity by more than 50% if the patient was prescribed either TDF or

EFV (n=7, p<.05) (Figure 5.2 B). Initially, we hypothesized that HIV-positive individuals on ART would have higher cathepsin activity, as cathepsins are linked to arterial remodeling and certain ART regimens lead to increased CVD. In contrast, these initial results indicated that a majority of the patients on ART have suppressed PBMC cathepsin activity compared to the HIV positive, ART naïve patients or the HIV negative control, as shown in the representative zymogram (Fig 5.2A)

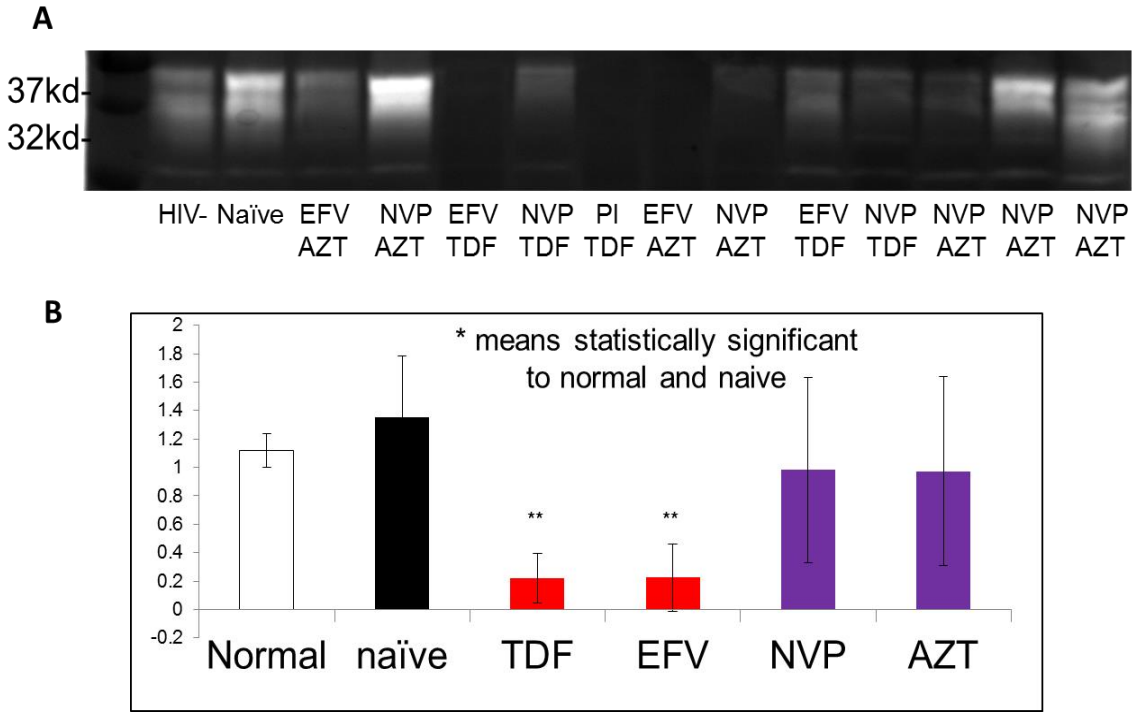

Figure 5.2: Patients taking tenofovir or efavirenz as part of their antiretroviral therapy have reduced cathepsin activity in their peripheral blood mononuclear cells from patients in Addis Ababa. A) PBMCs isolated from whole blood of HIV positive patients attending Black Lion Hospital in Addis Ababa, Ethiopia were lysed and equal amounts of protein were loaded for multiplex cathepsin zymography and **B)** quantified by densitometry. There was statistically significant reduction in cathepsin activity in patients on either TDF or EFV (*p<.05. n=7). Neg- HIV negative, NVP-nevirapine, AZT-zidovudine. Naïve- HIV positive patients that have not yet started to take ART. Data normalized to the HIV negative controls for each gel.

5.3.2 THP-1s stimulated with efavirenz or tenofovir have reduced cathepsin activity

To validate these findings without extraneous patient factors that could be contributing to decreases in cathepsin activity, THP1s were stimulated with EFV and TDF for 24 hours at physiologically relevant doses, lysed, and assayed for cathepsin activity using multiplex cathepsin zymography. Stimulation with both ARVs significantly decreased cathepsin V activity in THP-1 monocytes, similar to what was observed in patient PBMCs.

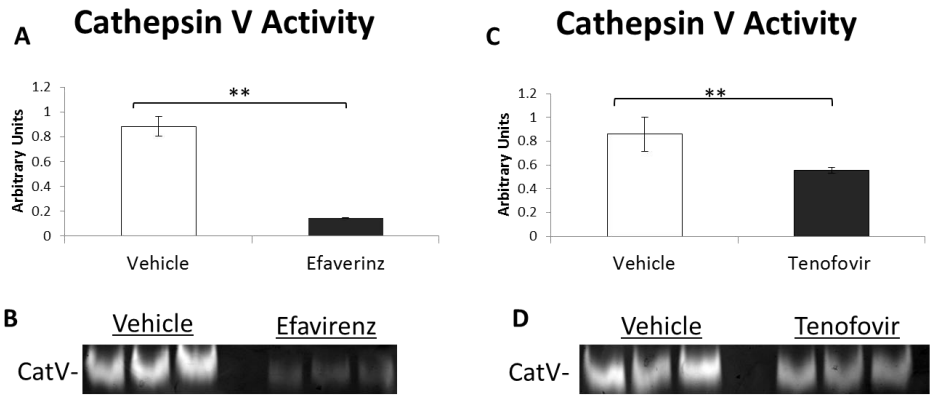

Figure 5.3: Efavirenz and tenofovir decreases cathepsin V activity in THP-1 monocytes. THP1 cells were stimulated with EFV, TDF or appropriate vehicle controls for 24 hours, rinsed, and lysed for cathepsin zymography. A) Densitometry shows an 80% decrease in cathepsin V activity for cells stimulated with EFV (A), and a 50% decrease in THP-1s stimulated with TDF (C). B) and D) Show representative zymography. (* indicates $p < 0.05$, n=3 and data is mean +/- SEM)

5.3.3 Efavirenz increases cathepsin V activity in HAECs, but does not alter smooth muscle cathepsin activity.

To further explore the effects of EFV and TDF on arterial cell cathepsin activity, HAECs, and smooth muscle cells were stimulated with EFV or TDF followed by measurements of cathepsin activity. We observed a 2-fold increase in cathepsin activity following EFV treatment of HAECs and no change for TDF or EFV in smooth muscle cells.

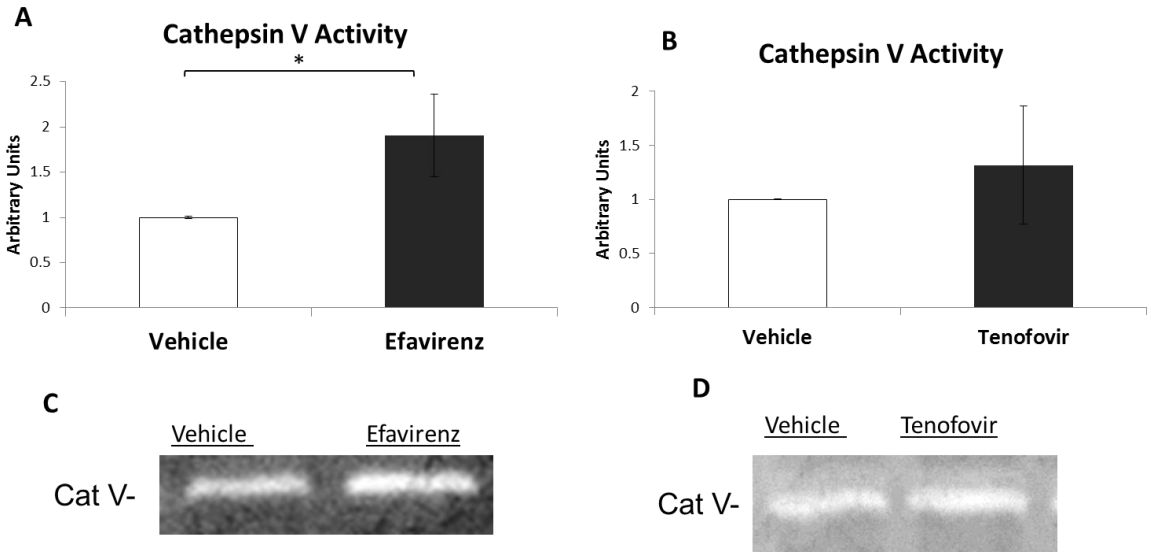

Figure 5.4: Efavirenz increases HAEC cathepsin V activity. HAECs were cultured to confluence, stimulated with 25 um EFV or TDF, rinsed and lysed for cathepsin zymography. A-B) EFV increased cathepsin V activity 2-fold. C-D) Representative zymograms. EFV significantly increases cathepsin V activity.

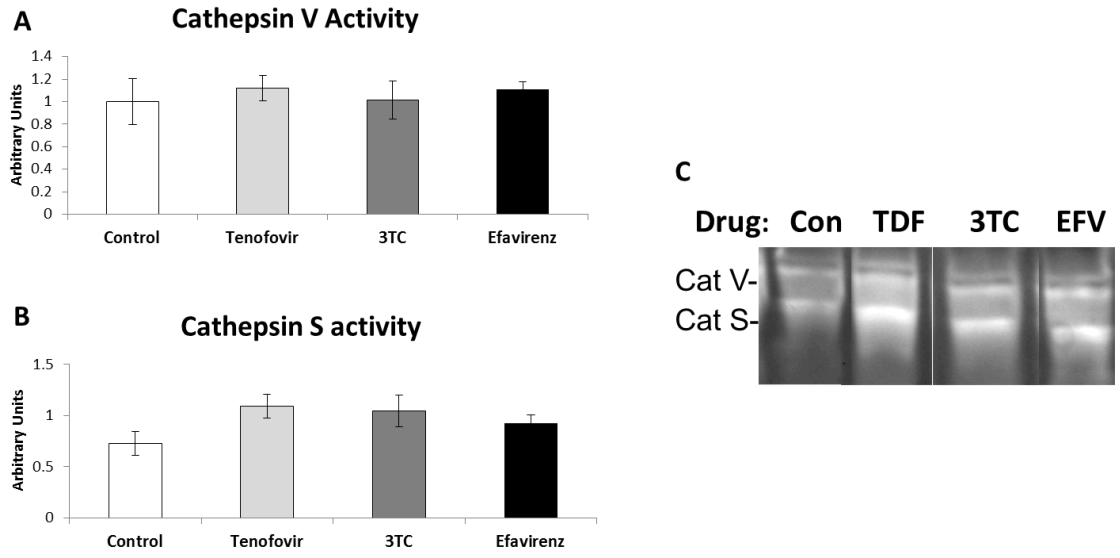

Figure 5.5: Neither tenofovir, lamivudine (3TC), nor efavirenz alter SMC cathepsin activity. Smooth muscle cells were stimulated with 25 uM TDF, 3TC, EFV, or vehicle control for 24 hours, rinsed, and lysed for cathepsin zymography. A) Densitometry shows no change in cathepsin V activity, B) no change in cathepsin S activity, and C) a representative zymogram.

5.4 Discussion

Various studies have shown the contribution of ARVs to CVD progression in HIV-positive patients including an increase is dyslipidemia [7, 21, 23], arterial stiffness [8], and atherosclerotic lesions [6, 131-133]. The various HAART drugs are also known to have negative effects on vascular cells as evidenced by increased carotid intima-media thickness, arterial stiffening, and endothelial dysfunction [135].

Protease inhibitors have been the most widely implicated in increased lipid content, atherosclerotic plaque formation and other inflammatory factors in HIV-positive patients [5, 27, 29].

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) have been associated with elevated cholesterol levels and triglycerides [191]. EFV is associated with higher

total cholesterol and triglyceride levels than NVP [4] . Less is known about the association between NNRTIs and arterial stiffness. Increased arterial stiffness is a key predictor of future cardiovascular events [187] and is elevated in HIV-positive populations [21, 195]. This could be due to a change in proteolytic degradation of arteries by different types of arterial cells. We have previously shown that AZT leads to increased arterial stiffness and increased cathepsin expression within arteries in a mouse model and hope to elucidate the effects on of certain ARVs on specific cell types[143].

Our results show that hs-CRP is elevated in EFV-treated subjects as compared to HIV-negative, HAART-naïve, and NVP-treated subjects. Hs-CRP is elevated in HIV-positive patients [192-194, 196-198] and elevated levels of this cytokine are associated with CVD in HIV-negative patients [196, 199, 200]. We show that hs-CRP was upregulated in patients on EFV, although they are not generally associated with specific HAART regimens [10, 21, 191]. sVCAM-1 and sICAM-1 are plasma biomarkers that have been associated with atherosclerotic plaque progression [201] and are generally upregulated in HIV-positive patients[45]. These values have been shown to decrease when patients initiate HAART treatment [192, 197, 202], but are still higher than HIV-negative patients. Our results are consistent with these findings. sVCAM-1 and sICAM-1 levels are elevated in HAART-naïve and in HAART-treated subjects compared to HIV-negative subjects to a lesser degree.

More interestingly, was the decrease in cathepsin activity in PBMCs by TDF and EFV. Although contrary to our original hypothesis, this is an interesting phenomenon observed not only in our Ethiopian patient cohort, but also validated *in vitro* without confounding factors, and in a South African cohort in a patient matched longitudinal

study, with HIV naïve patients tested before HAART initiation and 6 months after treatment (Platt et al, *manuscript under review*). The physiological implications of this suppressed activity is being further explored to investigate the effects on monocyte ECM cleavage, parse the difference in EFV stimulation of monocytes versus macrophages, and investigate these effects *in vivo* using a mouse model . Although these findings were unexpected, they have also led to the investigation of cathepsin activity as a novel biomarker for ARV adherence, especially for patients taking TDF or EFV. Studies done by Platt et al. have shown the specificity of a zymography negative signal for patients with undetectable viral load treatments as 86%, which makes it viable for use as an inexpensive diagnostic tool.

This study has also shown differential regulation by smooth muscle cells, endothelial cells, and monocytes in response to ARV's. It is important to understand this complex interplay to fully elucidate mechanisms for ARV-induced cathepsin-mediated arterial remodeling. For example, the inflammatory data found EFV to be linked with increased hs-CRP levels and increased arterial stiffness; however we found that EFV lowers PBMC cathepsin activity, which hypothetically would lead to a decrease in arterial remodeling. Coincidentally, HAEC cathepsin activity was increased by EFV. HAECs line the arterial wall and would constantly be exposed to these protease inducing factors, in contrast to PBMC's which have a high turnover rate.

This data, in addition to increased c-IMT, increased PWV, and other factors recently found by others in our group [203], could show that EFV creates a pro-remodeling environment when compared to others investigated in this study. EFV has been implicated in CVD progression in a few instances, but arterial cells' proteolytic

response has never been captured. We have done this in human patients, and validated *in vitro* to asses these effects. There was no difference for smooth muscle cells, indicating that the key mediators of cathepsin regulation due to ARV administration, at least for patients on regimens including TDF or EFV are monocytes and endothelial cells. In prescribing antiretroviral regiments for people at high risk for CVD, and especially those in the developing world, we have discovered factors that need to be thoroughly explored as they could add important contribution to understand increased cardiovascular risk in HIV-positive patients.

CHAPTER 6

SUMMARY AND FUTURE CONSIDERATIONS

6.1 Major Findings

The work presented in this thesis focused on developing a more complete understanding of the progression of HIV-induced CVD. Patients with HIV experience elevated incidences of cIMT, arterial stiffening, and vascular remodeling, and are exposed to a constant inflammatory state. Because of this, difficulties in parsing the effects of specific inflammatory signals in order to examine their contribution to CVD progression arise. In addition to infected cells, HIV proteins also circulate in the bloodstream and potentiate pathophysiological effects on arterial cells. It is important to identify the individual contributions of these factors to endothelial cell activation, specifically protease upregulation. Using an HIV-tg mouse model, where the contributions of HIV-1 proteins alone can be assessed, there was increased arterial stiffness, and subsequent cathepsin activity, which has been linked to initiation and progression of arterial remodeling.

There is a well-established correlation between the direction and magnitude of hemodynamic wall shear stress and the susceptibility of arteries to remodeling; specifically, high unidirectional shear stress is atheroprotective, while low, oscillatory shear stress is pro-atherogenic. Furthermore, cathepsin K is known to be up-regulated by pro-atherogenic profiles which promote arterial remodeling. Therefore, we hypothesized that naturally occurring regions pre-disposed to pro-atherogenic shear stress within the vasculature of HIV tg mice, would have increased protease activity. To achieve this, we

systematically investigated HIV protein stimulated cathepsin activity *in vivo* and *in vitro*, identifying specifically that HIV-1 Tat protein interactions activate cathepsin activity in endothelial cells. The lesser curvature of the aortic arch and the abdominal aorta, two well defined pro-atherogenic regions, showed an upregulation of cathepsin K protein expression. Cathepsin K was localized throughout the aortic arch, and the abdominal aorta showed greater cathepsin K activity when compared to the thoracic of wildtype and transgenic mice.

Physiological shear stress waveforms characteristic of arterial regions were actuated using a cone-and-plate shear stress bioreactor, and endothelial cells were either protected or predisposed to remodeling using this system. We were able to show that Tat stimulation under a pro-remodeling environment increased endothelial cell cathepsin K activity. This work demonstrates that Tat is sufficient to induce cathepsin K activity, but only when coupled with pro-atherogenic shear stress in endothelial cells. Therefore, the findings from this thesis support the idea that HIV-1 Tat promotes a pro-atherogenic environment by elevating cathepsin K, a potent collagenase and elastase, advancing pathological remodeling within the aorta. I have done this using HAECs, primary cells novel in their use to study Tat-endothelial cell interactions.

In an effort to expand these findings to a physiological context that would allow us to investigate the implications of increased cathepsin K activity in vivo, we explored the effect of Tat stimulation of cathepsin-mediated gelatin degradation using an E64 inhibitor. We saw Tat stimulated, cathepsin-mediated, gelatin cleavage in endothelial cells, without synergy of pro-atherogenic shear stress and Tat on gelatin degradation.

Although contrary to the initial hypothesis, this is still a novel and exciting finding that has not been reported elsewhere.

To develop a more detailed depiction of this upregulation in cathepsin activity, signaling pathways activated by pro-atherogenic shear stress and Tat were explored. This thesis identified c-jun and ERK activation by pro-atherogenic shear stress, with further upregulation by Tat stimulation. ERK and c-jun are phosphorylated by Tat under static conditions, but this was significantly increased under pro-atherogenic conditions. Additionally, we discovered that ERK inhibition reduces cathepsin activity in HAECs. Cathepsin activity has not been linked to ERK signaling and this provides useful information to the field on signaling linked to cathepsin regulation. Taken together, these data identify three novel and unique targets for therapeutic intervention: direct inhibition of cathepsin K activity, inhibition of ERK signaling; or inhibition of c-jun signaling.

Lastly, many of the confounding factors in patients are due to ARV adherence. ARVs have differential effects on the vasculature and many of them have been linked to arterial dysfunction. In order to ascertain a more holistic perspective of HIV and ARV-induced arterial remodeling, we also investigated the effects on multiple antiretroviral regiments on inflammatory markers and cathepsin activity. I found that EFV increases hs-CRP in human patients, but decreased cathepsin activity in PBMCs from patients on this regimen. TDF also stimulated decreased cathepsin activity in PBMCs. To study these effects without the presences of inflammatory factors, these experiments were run *in vitro* using THP-1 cells, and similar results were observed, confirming that TDF and EFV downregulate monocyte cathepsin activity. Within the vasculature CD4+ T-cells are the main targets for antiretroviral therapy, however many other cells are also exposed,

including endothelial cells. To investigate the effects of EFV and TDF as universal cathepsin reducing agents, endothelial cells were stimulated in vivo; however an increase in cathepsin V activity was observed. This demonstrates a differential regulation in cathepsin activity by endothelial cells and monocytes. Although the findings of this study were mostly observational, they are valuable as multiple stimuli are present in HIV-positive patients. Many projects have formed based on this initial data; one exploring cathepsins as novel markers to monitor adherence in resource limited countries; one establishing the effects of EFV on monocyte differentiation and macrophage cathepsin activity; and another investigating the role of EFV in arterial stiffening in ApoE knockout mice.

The cumulative findings of this doctoral thesis provide a novel foundation for understanding the dynamic interactions between the hemodynamic environment, HIV inflammatory factors, and cathepsin activity leading to increased cardiovascular risk in HIV-positive patients. Through the use of *in vivo* animal models and *in vitro* bioreactor systems, my work has identified multiple new therapeutic targets for CVD risk. However, there remains a large body of work that needs to be further validated and assessed. In addition to more complex models by which to explore HIV protein and ARV endothelial cell interactions, knowledge of specific inhibitors and a complete understanding of the biomolecular networks involved, are necessary to push the work forward.

6.2 Identifying the systemic effects of HIV-1 Tat on cathepsin activity and arterial remodeling using a Tat overexpressing mouse model

The work explored in this thesis underscored the important contribution of HIV proteins to cathepsin activation, specifically in atheroprone regions of the vasculature.

The NL4-3Δ *gag/pol* transgenic mouse (HIV-Tg) is a non-infectious model with a 7.4 kb transgene that contains the genetic sequence for the HIV-1 proteins *env, tat, nef, rev, vif, vpr,* and *vpu,* but lacks the *gag* and *pol* genes and is thus unable to replicate [15]. Although it is an important model for assessing changes in physiological function due to HIV protein stimulation, it is hard to parse the effects of specific HIV proteins. Antibodies would allow for specific protein detection, such as immunohistochemical staining, indicating co-localization of HIV-1 Tat and cathepsin K expression. However, with the exception of antibodies developed to detect gp120, other HIV proteins either have not been well characterized, or have been characterized using very high concentrations of the HIV protein of interest. Within the HIV-tg mouse it is difficult to quantify the levels of a particular HIV protein expressed in tissue and the expression is non-uniform throughout the animal model. The use of a Tat transgenic mouse model would allow us to parse the effects of Tat versus other HIV proteins on arterial stiffening to validate the *in vitro* endothelial cell studies. Additionally, kinase signaling could be established for a number of cells exposed to constant Tat stimulation in vivo, allowing for a more in depth look at signaling pathways altered by Tat. Subsequent inhibition of these pathways could provide critical information in assessing Tat-mediated co-morbidities.

6.3 Determination of cathepsin K specific contributions to elastin and gelatin cleavage in Tat-stimulated endothelial cells

Cathepsin K upregulation by pro-atherogenic shear stress and Tat is a novel finding presented by this body of work, however we were unable to identify cathepsin K specific contributions to cathepsin-mediated gelatin degradation in HAECs. Odanacatib is a potent, selective, and neutral cathepsin K inhibitor that is being further developed for

the treatment of osteoporosis [175, 176]. The use of odanacatib, would allow for greater specificity of this inhibition, and allow identification of cathepsin K contributions. Cathepsins K, V, L, and S are constitutively expressed by endothelial cells and our data shows that E64 differentially inhibits these, specifically in HAECs. To our knowledge this is the first time differential inhibition by E64 has been shown in endothelial cells. In addition, gelatin cleavage was not synergistically increased by Tat and pro-atherogenic shear stress, suggesting that the effects of cathepsin K upregulation may not be related to gelatin cleavage, but elastin cleavage. Alternatively, other proteases, such as MMPs may be counter regulated as the cell attempts to maintain homeostasis. Within the HIV transgenic mouse model, our group observed decreased elastin and no change in collagen [47], and this finding could also be true of cathepsin K in endothelial cells. These avenues need to be explored to further elucidate the effects HIV-1 protein contributions to arterial remodeling and to understand the physiological implications of this work.

6.4 Synergy of kinase activation and cathepsin upregulation

This thesis describes kinases activated by pro-atherogenic shear stress and Tat thereby identifying novel targets for cathepsin activation. C-jun and ERK were activated by pro-atherogenic shear stress, and further activated by Tat stimulation. We also identified ERKs connection to cathepsin activity, when we inhibited ERK phosphorylation via the MEK inhibitor U0126 we observed decreased cathepsin K activity. This phenomenon needs to be further explored in the context of kinase regulation of cathepsin activity and inflammatory stimuli across various conditions. These kinases could be working together synergistically, phosphorylating different targets that lead to cathepsin K activation. For example, ERK could phosphorylate c-fos,

which dimerizes with c-jun to form AP-1[174]. Chromatin immunoprecipitation assays could be useful in assessing transcription factors linked to the cathepsin K gene to further explore this phenomenon. In addition, c-jun and ERK inhibition could be studied in conjunction to understand the effects of both on endothelial cell cathepsin activity. We have explored ERK and c-jun inhibition in our studies and attempted to parse the activation of these enzymes by pro-atherogenic shear stress alone versus tat (Figure 4.5 - 4.7). However, with our current experimental setup, it can pose difficulties. Optimal experimental design would allow for addition of Tat 5, 15, or 90 minutes prior to stopping shear stress culture to achieve true co-stimulation. Unfortunately, our current setup does not allow for the addition of any factors upon initiation of the experiment. Individual changes in cathepsin activity- in response to pro-atherogenic shear stress, or Tat, after inhibition of these signaling pathways would allow us to further explore the effects of these two factors on arterial remodeling. Finally, this upregulation in ERK phosphorylation could exacerbate conditions previously noted in the literature relating to Tat-induced endothelial cell proangiogenic activation, increased cell migration, or increased apoptosis [12, 63, 66, 71, 72]; all factors which could lead to arterial remodeling.

6.5 Regulation of arterial cell cathepsin activity by EFV

This thesis investigates the effects of lopinavir/r, EFV, zidovudine, nevirapine, and TDF on inflammatory cytokines and PBMC activity in an Ethiopian cohort, linking EFV to greater hs-CRP expression in patient serum and decreased cathepsin activity in patient PBMCs. However, we also observed that EFV increased cathepsin activity in endothelial cells. This is a phenomenon that needs to be further explored in the context of

shear stress to investigate the endothelial cell response to EFV stimulation in physiologically relevant pro-atherogenic environments. Additionally, identifying relevant signaling pathways would allow for the assessment of overlap in kinase phosphorylation, although mechanisms for ARV stimulation are not well understood. Specifically from these studies, one would understand the contributions to arterial remodeling from HIV proteins and ARVs, specifically in endothelial cells. Altogether, it is imperative for the field to understand regulatory mechanisms in hemodynamically defined regions for remodeling within the vasculature where plaques form. The contribution of all of these confounding factors must be assessed to thoroughly evaluate to risk of cardiovascular disease for HIV positive patients.

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