MECHANISMS OF HIV-NEF INDUCED ENDOTHELIAL CELL STRESS: IMPLICATIONS OF HIV-NEF PROTEIN PERSISTENCE IN AVIREMIC HIV PATIENTS

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Cellular and Integrative Physiology,
Indiana University

May 2019

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I dedicate this thesis to my family, who were instrumental in giving me the strength to pursue my dreams and my teachers who made the world of science magical.

ACKNOWLEDGEMENT

I would like to thank my advisor Dr. Matthias Clauss for his guidance, support and friendship. His mentoring was instrumental in shaping me to be the scientist I am today.

I would like to thank my committee members, Dr. David Basile, Dr.Richard Day, Dr.Keith March and Dr. Andy Yu. They were always forthcoming with constructive criticism, ideas and encouragement.

I thank the former and present lab members of the Clauss lab; Dr.Linden Green, Dr.Ru Yi, Noelle Dahl and Tyler Colbert who were incredibly helpful with my experiments and at the same time made this PhD journey fun and exciting.

I would like to thank the following professors for being fantastic collaborators, extremely supportive of my research and providing me with many of the tools needed to complete my project and grow as a scientist; Dr.Homer Twigg, Dr.Samir Gupta, Dr. Roberto Machado, Dr.Tim Lahm, Dr.Natalia Bogatcheva, Dr.Bernhard Meier, Dr. Dr.Alexander Obukhov and Dr.Nathan Alves.

Most importantly, this journey was only possible due to unwavering support of my parents – Dr. Chelvanambi Narayanasamy and Dr. Sulochana Chelvanambi and my brother – Manoj Chelvanambi. Finally, I would like to thank my family and friends who were always extremely supportive of my ventures and instilled in me the core values that define who I am today.

Sarvesh Chelvanambi

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HIV-associated cardio-pulmonary vascular pathologies such as coronary artery disease, pulmonary hypertension and emphysema remain a major issue in the HIV-infected population even in the era of antiretroviral therapy (ART). The continued production of HIV encoded pro-apoptotic protein, such as Nef in latently HIV-infected cells is a possible mechanism for vascular dysfunction underlying these diseases. HIV-Nef persists in two compartments in these patients: (i) extracellular vesicles (EV) of plasma and bronchoalveolar lavage (BAL) fluid and (ii) PBMC and BAL derived cells. Here I demonstrate that the presence of HIV-Nef protein in cells and EV is capable of stressing endothelial cells by inducing ROS production leading to endothelial cell apoptosis. HIV-Nef protein hijacks host cell signaling by interacting with small GTP binding protein Rac1 which activates PAK2 to promote the release of pro-apoptotic cargo containing EV and surface expression of pro-apoptotic protein Endothelial Monocyte Activating Polypeptide II (EMAPII). Using this mechanism, Nef protein robustly induces apoptosis in Human Coronary Artery Endothelial Cells and Human Lung microvascular endothelial cells. Endothelial specific expression of HIV-Nef protein in transgenic mice was sufficient to induce vascular pathologies as evidenced by impaired endothelium mediated vasodilation of the aorta and vascular remodeling and emphysema like alveolar rarefaction in the lung. Furthermore, EV isolated from HIV patients on ART

was capable of inducing endothelial apoptosis in a Nef dependent fashion. Of therapeutic interest, EMAPII neutralizing antibodies to block EMAPII mediated apoptosis and statin treatment to ameliorate Nef induced Rac1 signaling was capable of blocking Nef induced endothelial stress in both *in vivo* and *in vitro*. In conclusion, HIV-Nef protein uses a Rac1-Pak2 signaling axis to promote its dissemination in EV, which in turn induces endothelial cell stress after its uptake.

Matthias Clauss, PhD., Chair

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I Introduction

1 Vascular disease in HIV patients

1.1 HIV in the era of combined and highly effective anti-retroviral therapy

Before combined and highly effective anti-retroviral therapy (ART) was developed in the 1990s infection with HIV in most cases would lead to acquired immune deficiency syndrome (AIDS). Back in the 1980s, HIV infection comprised a near death sentence with only few exceptions such as elite controllers – individuals who could mount an immune response against the virus without the aid of ART. The discovery of combined ART transformed this illness to a completely treatable condition by the late 1990s/ early 2000s. However, it soon became obvious that although ART could protect against development of AIDS, it failed to prevent the increased risk for developing so-called comorbidities, such as cardiovascular, pulmonary and renal diseases as well as disturbing pathologies within the central nervous system which were classified under HIV-associated neurocognitive disorders (HAND). This work will focus on pulmonary ad cardiovascular complications in association with treated HIV infection

1.2 Vascular disease in HIV patients

Although anti-retroviral therapy regimens resulted into good control of viral replication, this patient population continues to remain at higher risk for developing a wide variety of cardio-pulmonary vascular diseases than the general population. Initially, ART itself was a contributor to this heightened risk in HIV positive patients. But despite the switch to regimens with low metabolic syndrome effects and vascular toxicity, this elevated risk of developing cardiovascular pathologies continues to persist in this

population. In the following, I have collected examples of studies addressing these cardiovascular complications starting out with studies using untreated (viremic) patients:

HIV infection itself serves to increase the risk of developing cardiovascular diseases (CVD) which continues to persist despite the initiation of antiretroviral therapy. A study of 255 HIV infected adults cataloged carotid intima-media thickness (CIMT) and coronary calcium (CAC) scores at baseline and 3 yr after initiation of antiretroviral therapy and observed higher CIMT and CAC progression rates in HIV-infected patients [1]. These findings are supported by the lack of normalization of other risk markers like triglycerides, LDL cholesterol, hemoglobin, or FIB-4 in another study of 297 HIV infected veterans after bringing viral loads below detectable levels [2]. Similarly, a meta-analysis comprising of five studies and 89,713 subjects provided further support of cardiovascular disease risk persistence in the era of ART. While RNA viral load [log of odds ratio=1.10 (1.04-1.17)] and CD4+ cell count less than 200/μl were related to stroke, ART therapy did not normalize the increased risk of stroke [3].

HIV infection itself acts as an independent predictor for developing cardiovascular diseases. Priscilla Hsue and colleagues established HIV infection as an independent predictor of increased Carotid Intima-Media Thickness (CIMT) when looking at blood lipids, inflammatory markers and IMT in 148 HIV-infected adults [4]. Furthermore, HIV infection was associated with a younger age at the time of first cardiac intervention when compared to uninfected patients. These patients also had a higher prevalence of symptomatic carotid artery atherosclerosis [5]. Not only do HIV patients get cardiac events at younger ages and with more frequency, but their rate of disease progression is also worse. The rate of progression was not reversed in 121 HIV patients

with a follow-up measurement 1 yr later using CAC and CIMT as predictors of CVD risk in HIV patients [6]. For these comparisons, carotid IMT was found to be better capable of detecting subclinical atherosclerosis in patients with HIV after analyzing 258 HIV infected patients. Therefore, using CIMT as a lens to visualize cardiovascular disease progression in HIV patients, multiple reports have portrayed how cardiovascular health is not restored simply by bringing viral loads back to normal using ART.

Using CIMT to study the progression of cardiovascular health in 166 HIV patients, Fitch et.al found an interesting interaction between smoking, age and HIV infection [7]. Increased CIMT was associated with smoking burden and age to a greater degree among HIV-infected vs. HIV-uninfected participants. These patients also show evidence of arterial stiffening. In another study following the progression of CIMT amongst 63 HIV patients showed that in addition to CIMT, arterial stiffness markers like carotid compliance were also heightened when compared to control ($P<0.01$) [8]. A similar study using echocardiography to study 77 HIV infected men observed elevated CIMT. But they also observed arterial stiffness as measured by looking at distensibility and compliance in brachial, femoral and carotid arteries [9]. Another study of 105 HIV infected patients also observed reduced ascending aortic distensibility when compared to controls [10].nThis increased risk of developing cardiovascular diseases was also observed in HIV infected children. Both left and right common carotid artery IMT, and left and right internal carotid artery IMT were significantly higher in the HIV infected children [11].

1.3 Cardiovascular diseases in patients on ART

Given all evidence that there is an independent role of HIV infection as a risk factor for CVD development it would be interesting to know this increased risk is altered when patients were successfully treated with ART. In the following I will provide an overview addressing CVD risk in HIV patients on ART.

There remains the need to study possible sources of vascular dysfunction in HIV patients because bringing viral replication under control using ART is insufficient to normalize the increased risk of developing vascular disease. Volpe et al could show in a 6-year long longitudinal study with 211 patients that CIMT and CAC progression continues to worsen in the HIV infected population even after bringing viral replication under control by using anti-retroviral therapy [12]. A larger study of 389 HIV infected patients showed that at baseline levels HIV patients had elevated levels of carotid intima-media thickness (CIMT) when compared to uninfected controls [13].

Even the immediate prescription of anti-retroviral therapy upon diagnosis does not help alleviate all of the increased risks for CVD development in HIV patients. Analysis of vascular health from the Strategic Timing of Anti-Retroviral Therapy (START) trial shows a possible link between systemic inflammation and vascular health. An analysis in HIV patients on ART with preserved CD4+ counts showed a strong correlation between high levels of IL-6 and C-reactive protein in patients with impaired small arterial elasticity [14].

In fact, patients from a wide variety of demographics display this increased risk of developing cardiovascular diseases. The incidence of cardiovascular disease like stroke

and transient ischemic attack (TIA) was determined in patients living with HIV in the AIDS Clinical Trials Group after ART therapy initiation [15]. In this cohort, women and non-Hispanic blacks were shown to have the highest incidence of stroke and TIA showing that these at risks groups need to be closely monitored. Another interesting observation was that at lower ages, women had a higher risk of developing stroke/TIA while at older ages, men had a higher incidence of stroke/TIA. The interplay between age and sex in determining cardiovascular health in HIV patients remains unexplained. Even young, asymptomatic women with HIV infection (n=60) were shown to have higher amounts of noncalcified coronary plaque and inflammatory monocyte activation measured through both levels of soluble CD163 and the percentage of intermediate CD14+CD16+ monocytes. [16]

Together, these studies from several groups worldwide paints a picture where HIV patients have elevated the risk of developing cardiovascular diseases even placed on anti-retroviral therapy and demonstrate good viral control. Not only do these patients get sicker at a younger age and are more likely to develop CVD, but the rate of progression of this risk is also not abrogated with ART initiation.

HIV patients also suffer from other diseases affecting the vasculature suggesting a global source of vascular stress in this patient population. A study of 40 HIV infected men on ART evaluated their forearm blood flow [17]. These patients had impaired microcirculation as evidenced by lower maximal and percentage (%) change in forearm blood flow than control patients. HIV patients also had a higher prevalence of peripheral arterial disease as measured through brachial artery index <0.9 [18] in a study of 908 HIV infected patients and 11,106 controls. Another study looked at 122 older (>50yrs) HIV

patient cohort on ART and found an elevated prevalence of hypertension (54% vs 38%), hypertriglyceridemia (51% vs 33%), low bone mineral density (BMD) (39% vs 0%), and lipodystrophy [19]. A study in an Italian cohort of 1229 deceased HIV+ patients found an abnormally high rate of circulatory disease associated mortality [20]. Amongst the leading cause of death were diseases such as hypertension and ischemic heart disease. This elevated risk of cardiovascular disease development appears to be independent of viral load or the impact of antiretroviral therapy drugs.

Elite controllers are HIV patients who are capable of mounting an immune response against the virus and therefore do not need ART to control viral replication. Hence, these patients are a good group to evaluate whether HIV infection in the absence of confounding factors like ART regimen could contribute towards HIV associated cardiovascular diseases. Pereyra et.al found an increased prevalence of atherosclerosis (78 vs. 42%, $P < 0.05$) and markers of immune activation in elite controllers compared with HIV-negative controls. Similarly, the monocyte activation marker - sCD163, was increased in elite controllers compared with chronic HIV-1 ($P < 0.05$) and compared with HIV-negative controls ($P < 0.05$) [21].

The endothelium lines the inside of blood vessels and plays an important role in regulating proper vascular function. In this regard, HIV associated increased risk of developing vascular diseases displays a strong endothelial dysfunction component. Even in patients with well-controlled viremia after an extended time (12 years) of anti-retroviral therapy administration, HIV patients had elevated markers of endothelial dysfunction [22] characterized by endothelial stress markers like β2-microglobulin, IL-8, TNFα, and sICAM-1 when compared to HIV uninfected healthy controls. Another study

of 52 patients who had a cardiac incident and enrolled in NIH clinical protocols; identified D-dimer, soluble vascular cell adhesion molecule-1, tissue inhibitor of metalloproteinase-1, and soluble tissue factor as predictors of CVD risk [23]. However, HIV associated variables were unable to predict CVD risk.

1.4 Pulmonary diseases in HIV patients on ART

Assuming that infection with HIV impairs the immune system including the one responsible for the clearance of lungs, it is not surprising that HIV patients with COPD develop various pulmonary infections including community-acquired pneumonia (CAP), pulmonary tuberculosis (TB), and Pneumocystis jirovecii pneumonia (PCP) at a higher rate [24]. However, HIV patients were found to have a higher risk of developing a multitude of pulmonary diseases not specifically related to opportunistic infections. Using the Multicenter Aging Cohort (907 HIV-infected and 989 HIV-uninfected participants) and the Women's Interagency HIV Study (1405 HIV-infected and 571 HIV-uninfected participants) found that HIV patients were at a higher risk for developing dyspnea, cough, wheezing, sleep apnea, and incident chronic obstructive pulmonary disease [25]. This risk disregards the route of HIV infection since a study looking at perinatally HIV+ patients, HIV infection decreased the ability to reverse restrictive diseases like asthma and developed emphysema at a younger age [26]. HIV infection not only increases the prevalence of disease but also worsens outcomes. A study looking at 178 patients with Acute Respiratory Distress Syndrome showed that HIV-1-infected subjects were developed ARDS at an earlier age, had higher rates of asthma, chronic obstructive pulmonary disease, and pneumonia. These patients also had greater illness severity and marginally elevated but not significant hospital mortality rates [27].

Furthermore, HIV infection increases the risk of development of pulmonary diseases with a vascular component. Pulmonary hypertension is a rare disease of the pulmonary vasculature characterized by vascular remodeling of the small vessels in the lungs. Endothelial dysfunction and hyper-proliferative smooth muscle cells result in occlusions of small vessels that lead to high pulmonary pressures and eventually diseases of the right ventricle. This disease shows an enhanced prevalence in patients already suffering from diseases like scleroderma and HIV. A meta-analysis of three studies in Africa estimated the prevalence of pulmonary hypertension to be 14% in their HIV patients which is dramatically elevated from the global prevalence of pulmonary hypertension at 15 in one million [28]. When pulmonary arterial pressures were estimated in 220 asymptomatic HIV patients [29] showed that elevated pulmonary pressures could be determined in 0.45% of the patients studied. A more thorough evaluation of pulmonary artery pressures in 2,831 HIV-infected and 5,465 HIV-uninfected veterans from the veterans aging cohort using echocardiography showed an elevated prevalence of increased pulmonary arterial pressures. Furthermore, these patients with pressures >40mm Hg showed increased mortality [30]. A similar approach in a German cohort of 374 HIV+ patients showed the prevalence of PH as determined by echocardiography to be 6.1% compared to the earlier estimate of 0.45% [31]. This elevated risk for PH was also seen in 79 patients under the age of 16 who were HIV infected by horizontal transfer [32]. In this study, 23% of the patients analyzed had elevated mean pulmonary arterial pressures. Most patients had right ventricular hypertrophy and left ventricular hypertrophy but only 11% displayed left ventricular dysfunction.

Not only do HIV patients develop pulmonary hypertension at much higher rates, but the disease is also more severe. A comparison of 27 HIV positive pulmonary hypertension patients and 115 HIV- pulmonary hypertension patients showed that HIV patients had significantly lower ejection fraction and more patients had late-stage ventricular dysfunction [33]. Furthermore, HIV infection was associated with higher levels of inflammatory cytokines and the vasoconstrictor endothelin 1 [34]. In fact, Endothelin-1 levels were highest in the HIV patients who developed pulmonary arterial hypertension suggesting HIV's capacity to induce endothelial damage and alter functionality. This association was confirmed in an independent study that measured pulmonary arterial pressures using both echocardiography and right heart catheterization [35]. Elevated plasma endothelin 1 levels were shown to correlate with elevated mean pulmonary arterial pressure.

In addition to pulmonary hypertension, HIV patients are also at a higher risk of developing COPD/emphysema, a progressive disease characterized by non-reversible loss of microvasculature. Using the large veterans aging cohort, 1428 HIV-infected and 2104 uninfected patients with at least one acute exacerbation of COPD were analyzed [36]. Not only did HIV patients have increased the risk of developing COPD, but HIV infection also increased elevated the effect of cigarette smoking towards COPD development. Furthermore, HIV infected patients had increased emphysematous changes as measured using pulmonary function tests and fibrosis [37]. While fibrosis was associated with viral loads and CD4 counts, emphysematous changes were not correlated to viral load or CD4 cell counts and the increased risk persisted even in the patients on ART with undetectable virus. Therefore, HIV infection also increases the risk of

developing pulmonary diseases with a vascular component like COPD/emphysema and pulmonary arterial hypertension.

2 HIV Nef: Virally encoded accessory protein

2.1 Nef Persistence in HIV+ patients on ART

Negative factor (Nef) is a Human Immunodeficiency Virus (HIV) virally encoded protein that plays an important role in facilitating and enhancing viral replication and immune evasion. A protein like Nef that is capable of hijacking multiple host cell signaling pathways is a leading candidate for contributing to vascular dysfunction and endothelial damage that underlies various cardio-pulmonary vascular diseases described earlier. Especially in the era of anti-retroviral therapy where the majority of HIV+ individuals are placed on anti-retroviral therapy regimen, the lack of HIV virus in the blood begs the question how HIV infection contributes to cardio-pulmonary vascular disease. Nef protein is a candidate since it persists in HIV patients on antiretroviral therapy with well-documented control of HIV viral replication.

As early as 1989 anti-nef antibodies were detected in HIV-1-seronegative, viral antigen-negative, and virus culture-negative individuals [38]. This suggested the possibility that Nef antibodies could precede seroconversion and the presence of anti-nef antibodies could serve as a marker for early activation of proviral protein activity during latency. Nef protein persistence during viral latency itself could contribute to viral replication since Nef protein was able to stimulate HIV replication in latently infected MOLT-20-2 as well as U1 cells by altering cell signaling [39]. Similarly, Nef containing extracellular vesicles were also capable of promoting HIV-1 viral replication in quiescent

CD4+ T cells which were latently infected with HIV through an ADAM17 dependent mechanism [40].

Given this benefit to persistent Nef protein for HIV viral production even during latency, Nef protein was detected in peripheral blood mononuclear cells of both treatment-naive HIV patients as well patients on antiretroviral therapy with undetectable viral load [41]. This persistence can be attributed to the presence of Nef protein inside CD45+ extracellular vesicles in the plasma of HIV infected individuals [42]. Extracellular vesicles can be readily taken up by a variety of cells and could act as the mode of dissemination of Nef in cells throughout the body.

Furthermore, these Nef containing extracellular vesicles are capable of damaging cells that end up taking in these vesicles. Multiple studies have shown the pathogenic potential of these vesicles. Nef containing extracellular vesicles (EV) were isolated from HIV patients with HIV associated dementia was capable of produce Aβ secretion from neural cells [43] suggesting a pathogenic role for these EV. These Nef EV are capable of potently creating a pro-inflammatory environment. Addition of Nef EV to peripheral blood mononuclear cells led to TNFα converting enzyme (TACE) packaging into vesicles and subsequent secretion [44]. This Nef-induced TACE release was mediated in a paxillin/Pak2 dependent fashion and is of particular importance since TACE cleaves transmembrane TNFα to produce soluble TNFα that can, in turn, promote endothelial cell death and inflammation by interacting with its receptor TNFR1. Subsequently, Nef and TACE containing extracellular vesicles were isolated from HIV patients and this in turn correlated with immune pathogenesis in chronic HIV infected patients [45]. Uptake of

these Nef containing EV induced intracellular TNF cleavage by ADAM 17 and subsequent trafficking of TNF into endosomes which were eventually released [46].

Since Nef is capable of hijacking host cell machinery, Nef is capable of exploiting multiple signaling pathways to alter vesicular trafficking and protein trafficking in the host cells where Nef is taken up. In this regard, Notch1 signaling was shown to play an important role for the endosomal trafficking of ADAM17 that precedes Nef-induced TNF release [46]. Another signaling mechanism important for Nef-induced TACE release was Hck kinase which was isolated in Nef containing EV isolated from HIV patients. Furthermore, Nef containing EV showed protein signature reminiscent of hepatocytes suggesting that the liver could act as a site for Nef trafficking and packaging during viral latency [47].

Nef protein can be secreted in extracellular vesicles not only from HIV infected leukocytes but also from a variety of cell types. HIV infected astrocytes as well as Nef-expressing HeLa cells released Nef in the form of extracellular vesicles [48]. This suggests that Nef protein can independently mediate its own transfer into extracellular vesicles in the absence of other viral protein and infection. These Nef containing extracellular vesicles were toxic to surrounding cells as bystander CD4 T cells that absorbed these Nef containing extracellular vesicles become apoptotic [49].

Surprisingly, in a study looking at 134 HIV infected patients with well documented viremic control and undetectable HIV RNA, Nef protein in extracellular vesicles were detected in more than half the patients on antiretroviral therapy as well as in elite controllers [50]. This suggests that Nef protein persists at a much higher frequency than previously believed. As a result, Nef protein, specifically in extracellular

vesicles becomes an intriguing candidate to emerge as the driver for endothelial damage even in patients with undetectable viral particles.

2.2 Nef interacts with and hijacks host cell signaling pathways

In addition to the signaling mechanisms used by Nef to alter vesicular signaling pathways highlighted earlier, Nef has been shown to activate various kinases and miscellaneous signaling proteins in the cell. Most studies were characterized in the context of HIV infectivity and how Nef protein modulating host cell signaling enhances viral infectivity. Harada et.al described the importance of Nef towards replication potential of HIV and Simian Immunodeficiency Virus. [51] They created a short-lived Nef by fusing Nef sequence to a proteasome-mediated protein degradation sequence. As a result, cells infected with short-lived Nef containing HIV had lower levels of Nef which was restored upon addition of a proteasome inhibitor. Using this model, they found that short-lived Nef expression significantly reduced infectivity of HIV-1 when compared to WT Nef. Furthermore, the addition of both short-lived Nef and WT had a comparable increase in infectivity of SIV mac239.

2.3 HIV-Nef alters packaging of host and viral proteins into virions and extracellular vesicles

As a viral protein, Nef interacts with and hijacks host cellular signaling in order to facilitate HIV replication. By altering cellular trafficking and packaging, Nef promotes the formation of viral particles. Cornall et.al [52] showed the importance of Hck kinase in Nef-mediated increase in viral infection in both T cells and macrophages. Nef was shown to interact with HCK kinase using its SH3 binding domain containing the polyproline

motif – PxxP. Nef-Hck interaction facilitated the packaging of Hck into HIV virions which promoted HIV infection into T lymphoblasts.

Bregnard et. al used a proteomic approach to study how Nef expression alters protein cargo in virions to modulate infectivity.[53] Specifically, they identified Ezrin and EHD4 as proteins that are differentially expressed in WT virions versus Nef deleted virions. This suggests that Nef expression enhance virus infectivity by modulating virion cargo with active packaging of host proteins that are beneficial to HIV infection. Furthermore, Nef interaction with host cell proteins also increases viral replication efficiency. Another host protein modulated by Nef [54] is Protein Kinase C (PKC). They found that Nef was phosphorylated by PKC in its N terminus at residue 6. This phosphorylation was important for Nef myristoylation and PAK2 activation. PKC modulated phosphorylation of N-terminus of Nef was important for the Nef-stimulated HIV transcription and replication in resting PBMC. Qi et.al identified that Nef protein interacts with host protein cyclophilin A [55]. This cyclophilin A – Nef complex enhanced viral infectivity by its incorporation into virions via association with Gag during particle assembly. Similarly, [56] Schiavoni et.al found that Nef directly interacted with viral envelope protein env using residue 180-210 in the C terminus. This interaction occurred at the membrane and facilitated packaging of viral surface protein env into HIV viral particles to further enhance infectivity.

Not only does Nef enhance packaging of viral proteins into viral particles, but it is also capable of enhancing infectivity of particles released by altering its surface characteristics optimal for infection. Zheng et.al suggested the role of lipid rafts as a mechanism through which Nef enhanced viral infectivity. [57] They show that HIV

virions from nef-deleted virus showed decreased infectivity and reverse transcription when compared to WT HIV. Nef was important for increasing viral particle budding from lipid rafts which are microdomains on the plasma membrane rich in sphingolipids and cholesterol. Nef also increased the amount of ganglioside which is a major component of lipid rafts. This Nef-induced increase in virion infectivity was reversed when lipid rafts were disrupted in cells.

Another strategy used by Nef is to enhance the release of viral particles is through modulation of multivesicular body formation that is crucial for the release of viral particles. Costa et.al found that Nef binds to AIP1 which is an important player in the formation of multivesicular bodies [58]. Subsequently, Nef was able to increase the number of viral particles released but only when it was able to interact with AIP1. Therefore, Nef modulates multivesicular body formation to enhance egress of HIV virions from infected cells.

This ability of Nef protein to modulate pathways governing vesicular transport and release is independent of whether a cell infected by the HIV virus. In the context of aviremic HIV+ patients on ART, Nef persistence has implications on the content and amount of extracellular vesicles released.

2.4 HIV-Nef helps the virus with immune evasion

Another mechanism through which Nef is important for HIV infectivity is by aiding HIV to evade the host immune system. The presence of Nef is capable of impacting multiple host immune cell types to either deplete the immune system or impede recognition of infected cells by immune cells.

Nef's ability to enhance HIV infectivity through immune evasion was shown by Zou et.al using the BLT mouse model [59]. While inoculation with LAI strain of HIV produced 8,200,000 ± 1,800,000 copies of viral RNA/ml, inoculation with Nef deleted LAI stain of HIV only produced 1,220,000 ± 330,000 copies of viral RNA/ml. Furthermore, the WT strain of HIV efficiently depleted CD4+ T cells and CD4+ CD8- thymocytes which did not happen in the Nef deleted LAI strain. In the same BLT humanized mouse model, infection with JRCSF strain of HIV exhibited progressive loss of CD4 T cells in blood, bone marrow, spleen, lymph node lung and liver [60]. While the Nef deleted JRCSF virus had comparable peak viral loads, no loss in peripheral blood CD4 T cells was observed. Furthermore, the WT JRCSF virus induced a dramatic rise in activated CD8 T lymphocytes which was absent in the nef-deleted virus suggesting that Nef plays an important role in eliciting activated CTL response that could lead to CD4 T cell loss that precedes progression to AIDS.

In order to characterize cellular signaling pathways that play an important role in Nef's ability to impede the immune system, Pak2 kinases appear to a prominent cellular signaling axis. Nef-induced Pak2 activation was important for CD4 T cell lymphopenia as well as thymic depletion of CD4 T cells by HIV and Nef [61]. Nef-induced Pak2 activation was also important for CD3 downregulation which dramatically reduced thymic output contributing to a weakened immune system that facilitates HIV pathogenesis.

In addition to impacting CD4 T cells, Nef is capable of impacting other cell type's surface markers as well. In order to promote immune evasion, Nef was able to modulate cytotoxic T lymphocyte response [62] as Chen et.al showed that Nef-mediated HLA-I

downregulation of CD8+ T cells reduced the kinetics of infection clearance and facilitated viral immune evasion. In order to do this, Nef downregulates CD155 which is a ligand recognized by the receptor DNAM-1 expressed by natural killer cells and CD8+ T cells[63]. Nef also downregulates NKGD2 to further enhance the immune evasion of HIV infected cells. These effects were conserved between multiple lab strains of HIV as well as a patient-derived sequence of Nef. Cd1a expression in dendritic cells is important for antigen presentation. Nef downregulates Cd1a in both natural killer cells as well as dendritic cells via activation of Hck and Pak2 [64]. This Nef-induced Cd1a downregulation facilitates HIV infection and subsequent cell death of these mediators of innate and adaptive immune systems. Nef also impacts B cells [65] as Swingler et al show that hyper-gammaglobulinemia and B lymphocyte hyperactivation is seen in HIV-1 infection are attributable to Nef. Nef-expressing macrophages as well Nef + T cells were able to activate NF Kappa B mediated B cell activation which in turn lead to the secretion of B cell activation factor ferritin.

In addition to the impacting the cellular compartments of the host immune system, Nef is also capable of affecting the acellular component of our immune system. Since Nef does transfer to B cells [41] and is capable of preventing IgG class switching [66], this arises as a strong possibility. Lai et.al questioned whether Nef effects the ability of neutralizing antibodies to counter HIV infectivity through the detection of HIV envelope protein, env [67]. They tested two potent neutralizing monoclonal antibodies, 2F5 and 4E10 that target the membrane proximal external region of Env. Nef increased resistance to both neutralizing antibodies by up to 50 fold suggesting another ability of Nef to enhance HIV viral infectivity and immune evasion.

By affecting multiple arms of the host immune system, Nef is able to allow HIV infected cells to evade immune responses. Furthermore, this has implications for latent HIV infection since depletion of host immune system eventually aids HIV to break out of latency and promote viral replication.

2.5 HIV Nef downregulates surface expression of receptors

HIV Nef potently modulates the surface expression of various proteins to enhance immune evasion of HIV infected cells. As an early response gene, Nef can begin downregulating receptors even before HIV integration takes place. Sloan et.al infected cells with HIV but blocked integration using integrase inhibitor raltegravir [68]. HIV cDNA, even without integration was capable of downregulating MHC I, HLA-A, HLA-B, HLA-C, HLA A31, and HLA E. Therefore, it is possible that the early response gene Nef provide one mechanism to facilitate immune evasion even in today's era of ART where integrase inhibitors are used as the first line of defense prescribed to patients.

In this regard, Nef classically downregulates CD4 in by directly facilitating the formation of AP-2 mediated clathrin-coated pits [69]. Nef interacts with AP2 (σ2) using the hydrophobic region in the Nef C-loop with dileucine motif and the M168L170 motif and AP2 (α) using the acidic motif E174 and D175 [70]. Similarly, Nef downregulates CD36 in macrophages which corresponds to a decrease in oxidative burst capacity and ability to scavenge both GFP beads and GFP-expressing Salmonella tiphymurium [71]. This effect might not only help HIV to evade the innate immune system but also help explain the risk of opportunistic infections in HIV patients due to the weakening of the innate immune system.

In order to downregulate CD4, Nef dimerization appears to be an important first step [72]. Nef dimers were potently trafficked to the plasma membrane and the Golgi network, subcellular locations critical for Nef functionality. Dimerization defective Nef mutants had severely diminished CD4 downregulation capacity. Subsequently, HIV containing these dimerization defective Nef mutants also had lower replicative potential.

Comparable replication potential between Nef deleted HIV and dimerization defective Nef mutant containing HIV suggest that Nef dimerization is an important first step for Nef enhanced HIV infectivity. Two other motifs implicated in the Nef-induced CD4 and MHC-I downregulation are the ubiquitination K144 residue and the N-terminal tyrosine motif Y28Y39 [73]. Point mutations in either residue dramatically reduced Nef's ability to reduce surface expression of CD4 and MHC-I via upregulation receptor endocytosis. While the polyproline rich SH3 binding domain of Nef is important for a wide variety of its functionality, [74] Kuo et.al describe that overlapping domains to flanking the PxxP motif also play an important role in the regulation of Nef-mediated host cell hijacking. Logically, mutations to the PxxP motif blocked Nef-induced CD4 and MHCI downregulation in addition to enhanced viral infectivity and Pak2 activation. However, they identified G67, F68, P69 to the C terminus of PxxP and the F90 to the N terminus of PxxP were also important for Nef functionality.

There also appears to be variation in Nef's ability to downregulate receptors like CD4 between different clades of HIV-1. A study by Mann et.al [75] postulates a functional hierarchy for Nef-mediated CD4 and HLA class I downregulation. They suggest that Nef from subtype B has the greatest ability to downregulate CD4 followed by clade A/D and finally C.

The importance of Nef in HIV-1 infectivity can be gleaned by taking a look at the Nef sequence alleles in the HIV infected elite controller population. These patients are uniquely capable of mounting a strong response against HIV and limit viral replication without the aid of anti-retroviral therapies. A study by Mwimanzi et. al[76] characterized the ability of 91 Nef clones, isolated from plasma of 45 elite controllers and 46 chronic progressors to down-regulate HLA class I and CD4, up-regulate HLA class II invariant chain (CD74), enhance viral infectivity, and stimulate viral replication in PBMC. They found that while Nef in elite controllers had lower functionality in all five parameters tested when compared to Nef isolated from the chronic progressor population. In a similar study, Corro et.al compared 10 long-term non-progressors and 6 rapid progressors [77]. They found that 40% of long-term non-progressors had defective Nef alleles in their ability to downregulate MHC I and CD4. Most substitutions were seen in two domains in this small patient cohort – AWLEAQ (56-61)) and the Rxx(22-24)) domains. Specifically, they identified the Nef L58V substitution as being associated with a reduction in HIV viral loads, persistent preservation of CD4 (+) T cell counts, and lack of AIDS-related symptoms. Cruz et.al also corroborated these findings in their cohort when comparing Nef sequences from normal progressors to slow progressors [78]. Slow progressors had a variety of deletion in the c terminus of Nef, which decreased Nef activity in T cells. Hence they suggest that Nef is a progression factor towards AIDS.

Furthermore, specific point mutations characterized as a nef “allele” seems to be important for progression towards AIDS. A study by Meribe et.al found over-representation of an Ile20 allele in their HIV patient cohort when compared to the Los Alamos database where met20 is the common amino acid [79]. Interestingly, when the

met20ile mutation was introduced in the SF2 Nef strain, they observed an enhanced HLA-I surface downregulation capacity in vitro. In support of this finding, patients with Ile 20 mutation had statistically significant higher plasma viral load suggesting naturally occurring Nef polymorphisms that support enhanced HIV pathogenicity. In support of this, Iijima et.al describe that tripartite hydrophobic motif (Trp13/Val16/Met20) in the N terminus of Nef functioned as a noncanonical mu-1A-binding motif for the interaction with the tyrosine motif-binding site of the mu-1A subunit to Nef [80]. Another domain was identified by Meuwissen et.al when they discovered a highly conserved valine-glycine-phenylalanine (VGF) Nef allele from clinical isolates of HIV [81]. This VGF motif linked an acidic cluster to polyproline motif. Mutation of this motif in lab strains of HIV like NL4.3, NA-7 ad SF2 to AAA disrupted Nef's ability to downregulate MHC-I and CXCR4, activate PAK2 to deregulate cofilin-mediated actin remodeling, target Lck to the trans-Golgi network to disrupt TCR signaling and bind to the Hck using the polyproline motif. Subsequently, this motif was also important for Nef enhanced HIV infectivity and replication.

Therefore, in the heterogenic HIV infected population, various Nef alleles could contribute towards disease progression towards cardiovascular diseases. By studying consensus Nef sequences as well as lab strain HIV derived Nef, we can elucidate the common pathways through which Nef can impact cells that take up Nef. In contrast, Nef mutants capable of altering specific signaling pathways can serve as a tool to tease apart the hierarchy of signaling mechanism implicated in Nef-induced cell stress.

2.6 HIV Nef interacts with host cellular kinases

As a small 27kDa protein, Nef has no known enzymatic function. However, it is potently capable of hijacking host signaling proteins in order to modulate cellular function. Using its polyproline rich PxxP motif, Nef binds to the SH3 domains of SRC kinase family members to facilitate MHC-I downregulation and improve viral infectivity and replication [82]. Similarly, allelic variants of Nef isolated from a variety of HIV-1 subtypes was potently able to interact with TEC family kinases including Bmx, Btk, and Itk. This Nef-induced ltk activation was also shown to play an important role in enhancing viral infectivity and replication.

Nef is capable of potently altering T cell receptor signaling by modulating subcellular fractions of the master regulator Lck [83]. Nef transfers active Lck kinase signaling pools from the plasma membrane to the trans-Golgi network. Without Lck present at the plasma membrane, TCR signaling is downregulated. Instead, at the trans-Golgi network, Lck activates Ras-Erk signaling to promote the secretion of IL-2 that in turn further enhances viral infectivity. In addition to the polyproline PxxP motif, [84] a GLG motif at amino acid 121-137 was shown to play an important role for Nef-induced Lck signaling related CD4 downregulation. This domain is also implicated in Nef-dependent optimal infectivity of HIV-NL4.3 virus. Another effect of Nef-induced modulations of Lck signaling is in CD4 T cell differentiation and development [85]. Using a transgenic mouse model that expressing Nef in CD4 cells, Chrobak et.al showed that these mice develop CD4 T cell lymphopenia by depleting CD4+/CD8+ double positive thymocytes and blocking lineage commitment to CD4 single positive thymocytes. This effect was reversed upon constitutive activation of Lck. Hence Nef-

induced changes in Lck signaling is an important mediator of HIV pathogenesis. Nef sequesters both Lck and Rac1 in a pericentrosomal compartment by altering the Rab 11 mediated endocytic trafficking pathway [86]. In these signaling endosomes, downstream effectors of the Lck and Rac 1 were also identified TCRζ, ZAP70, SLP76, and Vav1. Nef-induced sequestering of Lck in Rab 11 endosomes altered T cell signaling and activation. On the other hand, Nef sequestering of Rac1 lead to reduced actin remodeling. This combination of Nef-induced Rac 1 and Lck sequestering potently alters T cell physiology.

Another Src family kinase, Hck also interacts with Nef by creating a hydrophobic binding pocket using residues Ala 83, His116 and Try120. In fact, Tyr120Ile substitution completely abrogated Nef-induced Hck binding and activation.[87]. Upon Nef binding, the Hck 32L regulatory domain is was shown to display resonance changes as characterized by NMR studies [88]. Using hydrogen exchange mass spectrometry, Wales et. al [89] showed that the N-lobe of the kinase domain adjacent to the docking site for Nef on the SH3 domain is activated. This small conformational change is capable of enhancing viral infectivity and replication. Interestingly, a diphenylfuranopyrimdine kinase inhibitor DFP-4AB selectively inhibits Nef-dependent Hck activity blocks HIV replication. Such approaches provide an interesting avenue for future drug design that specifically targets Nef-induced kinase activity.

Nef is also capable of activating the p21 activated kinase (Pak) 2 to modulate a wide variety of signaling pathways. Nef binds to Pak2 using small GTPases like CDC42 and Rac and that Nef-induced Pak2 activation played an important role in functions like serum response pathway as well increased HIV-1 viral production. HIV-Nef has been

shown to bind to and activate Rac 1 signaling which plays an important role in ROS production and apoptosis [90-94]. As a result, Rac 1 signaling is emerging as a target for the development of cardiovascular therapies [95]. Recent studies have suggested the use of statins, HMG CoA reductase inhibitors, as a novel way to block Rac 1 signaling by inhibiting the geranylgeranylation of Rac 1 [96-99]In this regard, Raney et. al showed that Nef binding to Rac1 using the polyproline PxxP motif was crucial for Nef-induced Pak2 activation [100]. Using a cell-free system, they showed that Nef from multiple lab strains was capable of activating Pak2 and that a point mutation in Phenylalanine 195 was capable of downregulating Nef-induced Pak2 activation. Endogenous GTPases like Rac1 and Cdc42 were needed to transiently activate Pak2. They also found that only a small fraction of Pak2 was associated with Nef and within the membrane-associated cell fraction. Interestingly, the presence of microsomal membranes was imperative for Nef-induced Pak2 activation which is supported by how Nef-induced Pak2 activation occurs at the membrane. Furthermore, this Nef/Rac1/Pak2 axis did not require other adaptor proteins like Nck or Beta-Pix for Pak2 activation [101]. Agopian et.al noted that most studies disrupting Nef-induced Pak2 activation (G2, PxxP72, and RR105) also affect other Nef functions, such as CD4 or major histocompatibility complex class I (MHC-I) downregulation making it difficult to pinpoint Nef-induced Pak2 activation and its downstream effects. In this regard, they analyzed Nef alleles with preserved CD4 and MHC I downregulation that was important for Pak2 activation - L85, H89, S187, R188, and F191 [102]. All of these amino acids cluster on the surface of Nef in a domain that is different from the SH3 and dimerization and therefore forms a unique interaction motif for Nef-induced Pak2 activation. The 62EEEE65 acidic cluster domain is also important

for Nef-induced Pak2 activation as mutation to alanine also disrupted Nef-induced Pak2 activation. [103] Similar to the endogenous furin protein, this acidic cluster stabilizes binding of Nef to Pak2's furin binding region.

Another consequence of Nef-induced PAK2 activation is the recruitment of the Exocyst complex protein EXOC1[104]. This interaction between Nef and EXOC was dependent on PAK2 activation and dependent on Rac1 binding using the PxxP motif. Nef-induced Exocyst activation modulated actin remodeling and impaired T cell trafficking capabilities. Downstream of Nef-induced Rac1 activation, PAK1 kinase is also modulated by Nef. This activation also leads to the formation of trichopodia, the activation of Jun N-terminal kinase, and the increase of viral production [105]. The ability of Nef to interact with two small GTPases, Rac1 and CDC42 and activate downstream kinases such as Pak1 and Pak 2 suggests a certain redundant capacity of Nef to hijack host cell signaling pathways. Furthermore, only a fraction of the kinases is signaling via Nef while the vast majority is involved in other pathways that are important for normal cell function. Therefore, identification of inhibitors based on studying Nef's interactions domains with kinases that Nef activates like Lck and Src similar to the strategies employed for targeting Nef-Hck activation [89] could lead to a useful tool for a refined understanding of HIV-Nef signaling.

2.7 HIV Nef induces production of reactive oxygen species

The NADPH Oxidase complex helps in the production of reactive oxygen species (ROS) that regulates both physiological and pathophysiological functions. Nef drives an increase in ROS production by facilitating the recruitment of NADPH oxidase complex subunit p22phox [106]. Subsequently, Nef expression was capable of increasing

superoxide production in neutrophils by helping increase the rate of assembly of the NADPH oxidase complex. Further studies using *in silico* modeling and functional studies using Nef mutants showed that FPDW 121-124 on Nef is important for Nef-p22phox binding that leads to increased superoxide production [107].

Another subunit of the NADPH oxidase complex, p47phox was also found to be modulated by Nef expression [108]. Nef induced Src kinase activation followed by MAPK and ERK1/2 activation lead to translocation of p47phox into the plasma membrane. This translocation of p47phox to its site of action is an important precursor to Nef induced superoxide production in U937 monocytic cell lines.

Nef induced increase in phagocytic oxidative burst capacity was further studied in macrophages transduced with Nef expressing lentivirus [94]. In this study, Nef was shown to utilize a vav/rac1/Pak2 signaling pathway since a constitutively active rac replicated Nef induced increase in superoxide production. Similarly, Nef mutants incapable of activating vav and Pak2 did not induce this effect providing further support for the role played by this signaling axis. Furthermore, the trafficking of Nef and NADPH oxidase complex proteins to the membrane, interaction of Nef with the endocytic machinery and subsequent activation of Rac1/Pak2 pathway were all shown to be important precursors for Nef induced superoxide release [109]. The small GTPase Rac1 is well characterized binding partner of Nef using its polyproline PxxP motif and is also a component of the NADPH oxidase complex. In this regard, DOCK2-ELMO1 protein complex recruited by Nef to enhance Rac1 activation [90].

2.8 Statin treatment blocks Rac1 activation

While Nef signals by hijacking host cell signaling of the small GTPase Rac1, one major challenge remains with Rac1 inhibition. To suggest therapeutic agents to counter Nef induced vascular stress, it is of critical importance that the therapeutic agent are well tolerated and have a well characterized history. In this regard, statins have been used in clinical HIV studies and more importantly to combat hypercholesterolemia all over the world for decades. Statins block the production of cholesterol through inhibition of the mevalonate pathway, they also inhibit the production of isoprenoid intermediates in the cholesterol biosynthetic pathway. As a result, prenylation of Rac1, a post translational modification important for surface trafficking of Rac1 is affected [97]. In turn, downstream signaling of Rac1 like Rho kinase and NADPH oxidase complex assembly are also inhibited as a pleiotropic effect of statin treatment [110].

Paradoxically, while membrane bound Rac1 was dramatically downregulated with statin treatment of microglia, the amount of GTP bound Rac1 was found to be upregulated [111]. This could be attributed to the reduced interaction between Rac1 and its negative regulator Rho guanine nucleotide dissociation inhibitor (RhoGDI). Therefore, statin mediated alterations in Rac1 signaling is primarily due to subcellular location of Rac1 and not its functionality.

Statin treatment of endothelial cells induced the translocation of small Rho GTPases from the cellular membrane to the cytosol, which was reversed by 100 μM mevalonate and 10 μM Geranyl Geranyl Pyrophosphates [99]. This shows that statin treatment, through inhibition of the mevalonate pathway depletes the pool of Geranyl Geranyl Pyrophosphates. In turn, this reduces the amount of membrane bound Rac1

where it needs to be in order to interact with myristoylated Nef. Furthermore, Atorvastatin's capacity to block Rac1 activation and protect neuronal cells was dependent on activity of geranyl geranyl transferase 1β and depletion of geranyl geranyl pyrophosphatase. [112]

Similarly, studies in rat vascular smooth muscle cells showed that simvastatin was capable of inhibiting RhoA and Rac1 signaling which could be reversed using geranylgeraniol [113]. In this model, simvastatin interruption of Rac1 signaling resulted alterations to actin assembly and contractile function of smooth muscle cells.

In THP-1 monocytes, lipopolysaccharide treatment resulted in Rac1 activation which could be blocked using pretreatment with 20μM mevastatin [114]. As a result, Rac1-PI3K complex formation was also inhibited suggesting that statin treatment of Rac1 could affect another kinase downstream of Rac1.

Another mechanism of statins pleiotropically inhibiting Rac1 activation is through the upregulation of small GTP-binding protein GDP dissociation stimulator (SmgGDS) [98]. SmgGDS silencing reduced the ability of statins to block rac1 activation and reverse Rac1 dependent ROS production. Furthermore, healthy subjects treated with statins showed increased SmgGDS expression levels along with a reduction in oxidative stress markers *in vivo.*

In a Rat model with transgenic Renin expression upon which there is constitutive Renin-Angiotensin activation resulting in increased systolic blood pressure, albuminuria, renal NADPH oxidase activity, and 3-nitrotryosine staining [115]. Interestingly, Rosuvastatin treatment was able to reverse these phenotypic changes via downregulation

of NADPH oxidase complex subunits including Rac1 and p22phox. As a result, rosuvastatin was capable of downregulating NADPH oxidase mediated ROS production in both *in vivo* model and *in vitro* model of immortalized podocyte cell line.

Similarly, angiotensin infused rats acetylcholine induced relaxation was impaired but reversed with both NADPH oxidase inhibition and anti-oxidant treatment suggesting a role of ROS production contributing to endothelial dysfunction [116]. Further studies showed that Angiotensin II increased intravascular ROS levels, induced eutrophic remodeling, collagen and fibronectin accumulation and finally resulted in elevated arterial stiffness. Treatment with rosuvastatin (10mg/kg for 14 days) blocked the elevation of phosphorylated p47phox, p67 phox and therefore lowered NADPH oxidase mediated ROS production. As a result, rosuvastatin treatment reversed angiotensin II mediated alterations in arterial resistance and function.

In addition to angiotensin II mediated ROS production, Atorvastatin treatment was also able to alter NADPH oxidase activity in ApoE deficient mice fed a high fat diet [117]. The lower levels of ROS production in aorta was attributed to the impaired membrane translocation of Rac1 upon Atorvastatin treatment.

Furthermore, Pitavastatin treatment was able to reduce angiotensin II induced ROS production, TGFβ1 secretion and smad2/3 phosphorylation [118]. This was mediated by Pitavastatin mediated downregulation of Rac1 activity resulting in protection from hypertension induced atrial remodeling.

Similar effects of statin treatment on Rac1 mediated NOX dependent ROS production were also observed in a db/db murine mouse model of type II diabetes [119].

In this model, NOX dependent ROS was elevated in plasma accompanied by elevated cytosol to membrane trafficking of NADPH oxidase subunits p47phox and Rac1 in the vessels. Interestingly, 2 week treatment of 10mg/kg Atorvastatin effectively reduced NOX dependent ROS production as well limiting the amount of membrane bound Rac1 and p47phox. Atorvastatin treatment was also capable of reversing signaling downstream of ROS including VCAM-1 surface expression in aortas, phosphorylation of redox sensitive MAPK and pro-inflammatory NFκB signaling. Furthermore, Atorvastatin was capable of reversing acetylcholine induced vasorelaxation.

Another diabetes model with impaired vascular function was Zucker fatty rats which had elevated angiotensin II mediated ROS production, endothelial dysfunction and reduced eNOS production [120]. However, Pitavastatin treatment was able to reverse these effects and restore normal vasodilation function by reducing NADPH oxidase activity as evidenced by lower gp91phox subunit localization in the membrane.

Further support of Atorvastatin treatment being beneficial to endothelial cells was provided in TNFα treated human aortic endothelial cells. TNFα treatment promoted cytosol to membrane trafficking of Rac1 which resulted in increased NOX mediated ROS production [121]. As a result, these human aortic endothelial cells showed NFκB activation followed by increased expression of VCAM-1 and ICAM-1. Atorvastatin treatment potently reversed these effects as well as upregulated ERK5 activity which helps further inhibit endothelial cell activation.

In canine models of tachycardia induced chronic heart failure, femoral blood flow responses to acetylcholine was impaired suggesting impaired vasodilations [122]. Congestive heart failure resulted in elevated vascular superoxide production and NADPH

oxidase activity due to increased membrane expression of NOX4 and p47phox. NOX4 inhibitor apocynin and Pitavastatin treatment were capable of inhibiting ROS production and restoring acetylcholine mediated vasodilation.

Pitavastatin treatment improved vascular function also improved heart function in rat model of hypertensive rats with heart failure [123]. 3mg/kg administration of Pitavastatin improved end systolic elastance and percent fractional shortening seen in rats with heart failure. Pitavastatin treatment reversed Rac signaling evidenced by ROCK kinase signaling resulting in reduced ROS production and reversing PI3K-Akt signaling.

Statin treatment was even shown to be protective in pulmonary vascular diseases like pulmonary hypertension [124]. In the aortic banding model of for pulmonary hypertension secondary to left ventricular dysfunction, simvastatin at 30mg/kg was able to reduce mean pulmonary arterial pressure and pulmonary arterial remodeling. Further, simvastatin treatment reduced pulmonary expression of reactive oxygen species, the NADPH oxidase 2 regulatory subunits, p47phox and p67phox while increasing pulmonary expression of p-eNOS and cGMP.

Vascular modeling in pulmonary hypertension is characterized by hyperproliferation of pulmonary arterial smooth muscle cells. The ability of stains to reverse vascular remodeling could also be attributed to its effect on smooth muscle cells. A study to determine the mechanism behind increased proliferation of smooth muscle cells induced by Lysophosphatidic acid (LPA), a by-product of LDL oxidation that is isolated from atherosclerotic plaques [125]. LPA dose-dependently increased NADPH oxidase mediated ROS production which was blocked using Pitavastatin treatment as well as overexpression of RacT17N dominant negative in aortic smooth muscle cells.

Furthermore, Pitavastatin treatment reversed LPA induced proliferation and MCP-1 secretion by abrogation of ROS production.

Pitavastatin treatment was also able to have a protective effect on the vasculature of chronic smokers [126]. In a study of 30 chronic smokers, Pitavastatin treatment showed significant restoration of endothelial function as measured by flow mediated dilation and oxidative stress levels quantified using malondialdehyde-low-density lipoprotein-cholesterol and free radical levels. These were only some examples taken from a wealth of literature on beneficial effects of stating in preventing vascular disease independent of its cholesterol lowering activity.

3 Endothelial-monocyte activating polypeptide II (EMAP II)

3.1 Induction of EMAPII

Endothelial monocyte activating polypeptide II (EMAPII) was identified from the supernatant of murine methylcholanthrene A-induced fibrosarcomas based on its ability to activate endothelial cells [127]. The mature form was characterized as a 25 kDa protein based on SDS-PAGE of the purified protein and Western Blotting, but cloning predicted a smaller MW of ca 18kDa and a precursor protein of a predicted 34 kDa single polypeptide chain [128]. Addition of this purified and recombinant mature EMAPII to endothelial cells lead to increase in cytosolic free calcium, secretion of von Willebrand factor and expression of the adhesion molecules E-selectin and P-selectin suggesting an activated endothelial cell phenotype. Furthermore, the addition of purified mature EMAPII to neutrophils and mononuclear phagocytes increased cytosolic calcium concentration and stimulated chemotaxis. EMAPII delivery into mice resulted in

systemic toxicity, pulmonary congestion, and the appearance of TNF, interleukin-1 and -6 in the plasma. The effects of EMAPII were due to an N-terminal binding site between residues 6-20 [129]. This peptide sequence was capable of inducing both cytosolic calcium release and peroxidase release in polymorphonuclear leukocytes. *In vivo*, injection of this peptide into the footpad of mice was capable of promoting migration of inflammatory cells. Therefore, release of mature EMAPII peptide can act as medium for cross talk between endothelial cells and inflammatory cells.

However, EMAPII is generally found to be a part of the aminoacyl-tRNA synthetase complex and needs to be processed before it can have these effects. Mature EMAPII is released from the aminoacyl-tRNA synthetase complex after digestion by Caspase 7 indicating that mature EMAPII release is tightly associated with cells undergoing cellular apoptosis[130] [131]. In a physiological setting of embryonic development, association of EMAPII with cells undergoing apoptosis was also observed since EMAPII gene was also found to be upregulated around apoptotic cells in mouse embryo throughout development [132].

Once it is cleaved and released, extracellular EMAPII acts as a ligand that binds to CXCR3 receptor based on blocking and binding studies using radiolabeled interferon-gamma-induced protein (IP-10). This ligand-receptor interaction between EMAPII and CXCR3 was necessary for endothelial cell chemotaxis towards EMAPII gradient [133]. Furthermore, this interaction of EMAPII and the CXCR3 receptor in endothelial cells is also capable of inducing endothelial cell apoptosis in a p38 MAPK dependent fashion [134].

Given these observations that the release of EMAPII is associated with a pro-apoptotic, anti-angiogenic environment; multiple reports have identified EMAPII release associated with various disease states. As pertaining to HIV, Green et. al, showed that other HIV proteins can indeed upregulate EMAPII surface expression. HIV envelope protein GP120 binds to its receptor CXCR4, activates p38 MAPK signaling and induces surface expression of EMAPII in endothelial cells [134]. This gp120 induced EMAPII surface expression induces endothelial cell apoptosis which was reversed with EMAPII neutralizing mab.

An important aspect of cancer signaling is immune evasion and since EMAPII is capable of damaging immune cells expressing the CXCR3 receptor, it would be interesting to evaluate whether EMAPII is upregulated in the setting of cancer. Elevated EMAPII levels were detected in the blood of colon cancer patients. Tumor-secreted kisspeptin was capable of inducing surface expression of EMAPII by activating G protein-Coupled Receptor 54. This Kisspeptin induced lymphocyte apoptosis could be neutralized using an EMAPII neutralizing ab [135]. Another possible regulator of EMAPII expression in tumors may be hypoxia. In this context, both EMAPII mRNA and protein expression is upregulated in TNFα fibrosarcomas and in B16 melanomas near areas of tissue necrosis [136]. These areas were shown to overlap with injected chemical indicators of hypoxia using confocal immunochemistry. In support for the hypothesis that EMAPII is regulated by hypoxia, EMAPII expression was also found to be elevated in hypoxic and apoptotic tumor cells. Furthermore, EMAPII levels in the supernatant of hypoxic tumor cells were also elevated. In support of this, EMAPII mRNA levels were elevated in hypoxic endothelial cells and colon cancer cells like DLD-1 [137]. However,

normal colon cancer cells did not upregulate EMAPII transcription when exposed to hypoxia.

Furthermore, EMAPII is also upregulated in diseases with an inflammatory component. 23 patients undergoing allogeneic stem cell transplantation therapy had elevated expression levels of EMAPII. Furthermore, EMAPII levels correlated with endothelial inflammatory markers like sIL-2R, sVCAM-1, sE-selectin, and sFasL [138]. This suggests EMAPII could contribute to Graft Vs Host disease in patients receiving stem cell transplantation. Looking at the gingival crevicular fluid from the mouth, EMAPII was shown to be upregulated in patients with generalized aggressive periodontitis when compared to healthy subjects or patients with just gingivitis [139].

Furthermore, elevated EMAPII levels could act as a biomarker for vascular complications in disease states. When EMAPII serum levels were analyzed in 80 Type I Diabetic children with/without microvascular complications, patients with microvascular complications had the highest serum EMAPII levels (1539 +/- 321.5 pg/ml) followed by Type I diabetics without microvascular complications (843.6 +/- 212.6 pg/ml). Both Type I diabetes groups had higher serum EMAPII than healthy controls (153.3 +/- 28.3 pg/ml) [140]. Similarly, EMAPII upregulation is also witnessed in diseases involving damage to the endothelium. Following the percutaneous coronary intervention, EMAPII was upregulated in distal coronary artery segments [141]. Interestingly, oral rapamycin treatment was able to successfully downregulate surface EMAPII expression.

Another mechanism of EMAPII upregulation was chronic cigarette smoke exposure. Mice exposed to cigarette smoke showed elevated levels of EMAPII protein in their bronchoalveolar lavage when compared to air control exposed mice. This cigarette

smoke exposure induced EMAPII release was mediated by apoptosis is driven feed-forward loop since inhibition of caspase 3 blocked EMAPII upregulation. [142]

3.2 EMAPII: Cell death and anti-proliferation marker

Given it's associated with apoptotic cells and anti-angiogenic environments, extracellular EMAPII, either at the surface or when secreted can act as a cell death and anti-proliferation marker. Studies in a wide variety of pathophysiological contexts have been conducted to describe these effects.

Within the setting of tumor microenvironments, EMAPII's anti-proliferative capacities were co-opted to try to fight back against tumor cells and enhance effectiveness of cancer drugs. Glioma stem-like cells (GSCs) are believed to provide drug resistance to chemotherapeutics like temozolomide (TMZ) in patients with glioblastoma. In this study by Yu et.al, EMAPII was able to enhance the cytotoxicity of TMZ, suppressed mTOR signaling, facilitated autophagy-mediated cell death as well as induced G2/M a cell cycle arrest [143]. Furthermore, the combination of EMAPII and TMZ downregulated miR-24a-3p which results in elevated levels of its target genes including Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) [144]. This EMAPII induced miR-24-a-3p downregulation resulted in elevated mitophagy, apoptosis, and impaired migration. EMAPII inhibited cell viability and decreased the mitochondrial membrane potential in both human glioblastoma cell and GSCs and served to increase effectiveness of rapamycin [145]. Upon EMAPII addition, autophagic vacuoles were observed. EMAPII also inhibited PI3K/Akt/mTOR signaling and induced mitophagy and ER stress in these cells. In combination with rapamycin, EMAPII synergistically inhibited cell proliferation, migration, and invasion of glioblastoma cells.

In pancreatic ductal adenocarcinoma (PDAC), EMAPII's anti-proliferative capacities on endothelial cells worked in combination with other front-line chemotherapeutic agents like gemcitabine and sorafenib by inducing endothelial cell apoptosis and enhancing survival from pancreatic ductal adenocarcinoma [146]. Even though EMAPII did not contribute towards killing PDAC tumor cells, EMAPII addition further enhanced median survival when given in combination with docetaxel-gemcitabine [147] or bortezomib-gemcitabine [148].In this regard, EMAPII reduced fibronectin production by PDAC. The interaction between fibronectin and integrin alpha3, alpha5, alpha6, and alphaV was crucial for tumor-associated vessel formation and endothelial proliferation [149]. Immune evasion of tumor cells is facilitated by EMAPII induced apoptosis of tumor invading lymphocytes [150]. In fact, hypoxia-exposed tumor cells not only showed increased surface EMAPII expression but also potently induced lymphocyte apoptosis. The role of EMAPII in facilitating cell death was used as a prognostic biomarker in cancer patients to track treatment response. Intralimb perfusion of TNF-α is used as a cancer therapeutic to limit tumor cell proliferation. High EMAPII levels in melanoma patients receiving intra-limb perfusion were found in patients showing complete tumor response. This EMAPII expression was seen in both melanoma cells as well as endothelial cells and macrophages near the tumor site [151]. EMAPII primes cells for TNF- α induced apoptosis by promoting the redistribution of TNFR1 from the Golgi to the cell membrane [152]. Furthermore, EMAPII promoted TNF- α mediated pro-apoptotic signaling by increasing TNF-R1-Associated Death Domain (TRADD) protein recruitment to the cell membrane. Within 2hrs upon EMAPII addition, HUVECs also had elevated levels of TNF-R1. Similarly, the addition of conditioned media from tumor cells

secreting EMAPII also produced elevated levels of TNF-R1 but not TNF-R2 in HUVECS [153].

3.3 EMAPII as a vascular permeability factor in tumors

Lowered vascular permeability in the blood-brain barrier provides another avenue for drug resistance in brain tumors like glioblastoma. Chen at.al found miR-429 to be expressed at lower levels [154] in glioma vascular endothelial cells and that high-grade glioma had even lower expression levels. However, the addition of EMAPII to glioma endothelial cells increased expression of miR-429 which in turn lead to decreased expression of tight junction proteins including ZO-1, occludin, and claudin-5. Subsequently, EMAPII addition resulted in increased permeability as well as decreased transepithelial electrical resistance. Another miRNA regulating EMAPII mediated increased brain tumor vascular permeability was described to be miR-330-3p [155]. miR-330-3p is also downregulated by EMAPII addition to Glioma endothelial cells which resulted in higher levels of its target PKC-α. PKC-α upregulation/activation results in lower levels of tight junction proteins like ZO-1, occludin, and claudin-5 in these cell types.

In addition to miRNAs modulating EMAPII induced effects on cell signaling, small signaling GTPases also play an important role. While EMAPII increased blood-tumor permeability in rat brain microvascular endothelial cells, the addition of RhoA inhibitor (C3 exoenzyme), as well as ROCK inhibitor, was capable of inhibiting this effect [156]. Similarly, these inhibitors also blocked EMAPII induced downregulation of tight junction proteins like occluding, ZO-1 as well as stress fiber formation mediated by phosphorylated myosin light chain and actin assembly protein cofilin. Upstream of

RhoA/ ROCK signaling, the cAMP/EPAC1/Rap1 signaling axis was also impacted with low dose EMAPII addition. Subsequently, elevation of cAMP levels with forskolin and Epac/Rap1 activation by 8-pCPT-2'-O-Me-cAMP reversed EMAPII induced rise in permeability [104].

Furthermore, EMAPII was also shown to impact transcellular permeability since EMAPII addition to rat brain microvascular endothelial cells resulted in increased pinocytic vesicles observed via transmission electron microscopy [157]. Within 1h of EMAPII treatment, protein expression of caveolin-1, caveolin-2, p-caveolin-1, p-caveolin-2, and p-Src were elevated. This suggests that using a tyrosine kinase-mediated pathway, EMAPII increases levels of phosphorylated caveolin proteins which in turn facilitates transcellular permeability.

3.4 EMAPII neutralization as a therapeutic strategy for protecting the endothelium

An anti-EMAPII rat monoclonal antibody was described by Rajashekhar et.al with the ability to neutralize both full length and mature EMAPII. This monoclonal antibody was named M7/1 and shown to detect EMAPII in western blot, to block migration of PBMCs towards EMAPII and ameliorate EMAPII induced tumor cell and endothelial cell apoptosis. [158] Lung-specific overexpression of EMAPII in transgenic mice resulted in the simplification of alveolar structures, apoptosis of endothelial cells and accumulation of pro-inflammatory macrophages [142]. Similarly, cigarette smoke-induced emphysema in mice was also accompanied by elevated EMAPII levels. Neutralizing antibodies targeting EMAPII reduced alveolar cell apoptosis, inflammation and emphysema-associated structural changes in alveoli and small airways as well as improved lung function.

In fact, cigarette smoke exposure also upregulated EMAPII's receptor, CXCR3 in both human lung microvascular endothelial cell *in vitro* and murine lung endothelium *in vivo*. This receptor upregulation primed endothelial cells for EMAPII induced apoptosis which was effectively blocked by using neutralizing antibodies against the CXCR3 receptor [159]. Furthermore, CXCR3 mediated endothelial apoptosis was dependent on p38 MAPK signaling. Furthermore, EMAPII was shown to be a key mediatory of Acute Lung Injury in the setting of murine Influenza A virus infection. Within this context, EMAPII neutralizing antibody was capable of blocking pulmonary epithelial and endothelial damage. [160]

In addition to modulating pulmonary diseases, EMAPII was found to be elevated following cardiovascular diseases like myocardial infarction. Interestingly, treatment with a different EMAPII neutralizing antibodies after permanent coronary occlusion in mice leads to an improvement in survival rate, reduced scar size, and prevention of heart failure. The antibody treated group also showed decreased fibrosis, improved myocyte viability in the infarct area and increased capillary density [161].

II Materials and Methods

1 Tissue Culture

Human lung microvascular endothelial cells (HMVEC) and Human Coronary Artery Endothelial Cells (HCAEC) were obtained from Lonza (MD, USA) (CC2527) and cultured in EGM-2MV. SupT1 and Nef-ER-expressing SupT1 cells were obtained from the AIDS Reagent repository and cultured in RPMI with 10% FBS. Jurkat T cells and human-derived PBMCs (Indiana Blood Center, IN, USA) were cultured in RPMI with 10% FBS. HEK 293T were cultured in DMEM with 10% FBS. Primary alveolar macrophages were isolated from BAL fluid of healthy volunteers and cultured in RPMI with 10% FBS.

2 Patient Enrollment and Consent

13 HIV- healthy volunteers, 4 HIV+ patients on anti-retroviral therapy and 13 HIV+ patients not on anti-retroviral therapy were enrolled after Internal Review Board approval and obtaining informed consent**.** PBMC was isolated from patient blood using Ficoll separation. Over 80 patients were enrolled as part of the Investigating HIV Associated Lung Disease (INHALD cohort) after Internal Board review. BAL derived cells and acellular BAL fluid was obtained from this study.

3 Extracellular Vesicle Isolation and Characterization

EV were isolated from acellular BAL fluid and supernatant of control/Nef expressing cells using ultracentrifugation .The number and size of the EV released was assessed by NanoSight (Malvern, UK).

4 Detection of Secreted Cytokines

Cytokine levels in acellular BAL fluid of HIV patients and supernatants of alveolar macrophages and HIV-Nef transfected HEK 293T cells were measured using BD Cytometric Bead Array.

5 Apoptosis Detection

Apoptosis was measured in HMVECs using Flexstation (Molecular Devices, USA) by detecting caspase 3 activity (APO Logix Caspase 3/7, Cell Technology, USA) and mitochondrial depolarization (JC-1 Ab113850, Abcam, USA). TUNEL staining was performed using Apo-BRDU apoptosis detection kit (88-6671-88, Thermo Fisher, USA) and analyzed using flow cytometry.

6 Animals

HIV-Nef transgenic mice containing the tetracycline (tet)-responsive element were crossed with the VE-Cadherin promoter controlled tet-off-transactivator mouse line [24] and housed at the Indiana University Laboratory Animal Resource Center (LARC) according to IACUC guidelines and protocols. The resulting offspring were kept without tetracycline to induce endothelial HIV-Nef. Of note, we could only use female mice, because there were extremely low numbers of male transgenic littermates for reasons unknown.

7 Physiological Assessment of Lungs in Nef Transgenic Mice

Blood oxygenation levels were measured in alert animals using MouseOx Plus neck sensor (Starr Life Sciences, Oakmont, PA). Lung inspiratory capacity was measured with the flexiVent system (Scireq) as previously described [142].

8 Reagents

Anti-Nef SN20 antibody, anti-Nef SN43 antibody [162], anti-Nef 3D12 (Abcam), anti-Nef rabbit polyclonal serum and anti-Nef EH1 antibodies (AIDS reagent repository) were all used to detect HIV-Nef. The anti-EMAPII neutralizing monoclonal antibody M7/1 was produced and purified, as recently described [158]. Anti-CXCR3 (Clone:MAB160) was purchased from R&D Systems, Inc. Anti-Nef Elisa was purchased from Immunodiagnostic Systems. Recombinant EMAPII was purchased from Sino Biological Inc. Alexa Fluor 647-C_2-maleimide (A20347) and Alexa Fluor 488-C_5-maleimide (A10254) were purchased from Thermo Fisher Scientific Inc. NHS-EG12-thiol-acetyl (10852) was purchased from Quanta BioDesign, Ltd. Apologix Caspase 3/7(Cell Technology, FAM200-2) and the Apo-BrdU apoptosis detection kit (Thermofisher, USA, 88-6671-88) were used to stain for apoptotic cells.

The following reagents were purchased from the vendors listed: Atorvastatin (Sigma Aldrich, USA), 4-Hydrotamoxifen (Sigma Aldrich, USA), NSC23766 (Cayman Chemical, USA). Calcein AM, Cell Tracker Deep Red, Dihydroethidium and C12FDG were purchased from Thermo Fisher, USA.

The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Nef-ER #31 Clone from Drs. Scott Walk, Kodi Ravichandran, and David Rekosh; Anti-HIV-1 SF2 Nef Monoclonal (EH1) from Dr. James Hoxie; Cat #2949; Anti-HIV-1 Nef Polyclonal from Dr. Ronald Swanstrom; pcDNA3.1SF2Nef (Cat #11431) from Dr. J. Victor Garcia; 1SF2NefF195R (Cat#11430) from Dr. J. Victor Garcia and pNL4-3 from Dr. Malcolm Martin.

UV-NBS Antibody Conjugation and Indole-EG12-Thiol Synthesis were performed as previously described [163, 164]. Briefly, Indole-EG12-Thiol was synthesized by incubating excess tryptamine hydrochloride with NHS-EG12-thiol-acetyl in PBS for 2 hours at room temperature. The thiol group was deacetylated by incubating with 0.5M hydroxylamine and 25 mM EDTA for 2h at room temperature. The resulting reaction mixture was purified via RP-HPLC on a Zorbax C18 column and mass verified using ESI-MS. The purity was confirmed using RP-HPLC on an analytical Zorbax C18 column (>95%), and the yield was 80%. For the UV-NBS conjugation, the antibody was first buffer-exchanged using 10 kDa molecular weight spin concentrators in PBS pH7.4 with no stabilizers. Purified anti-Nef antibodies (SN20, 3D12, and EH1) were then incubated with 300 μM of Indole-EG12-Thiol for 15 min at room temperature and were UV crosslinked in a Spectroline Select Series crosslinker (254 nm) for a total of 1.1 J/cm^2 energy exposure. Excess thiol linker was removed via a molecular weight cutoff filter. The NBS site-specific thiolated antibody was then incubated with either Alexa Fluor 647 C_2 Maleimide or Alexa Fluor 488 C_5 Maleimide (100 μM) for 3 hrs at room temperature, protected from light. Excess fluorophore was removed via a molecular weight cutoff filter, resulting in site-specific fluorescent labeling of the antibody at the conserved antibody NBS. Conjugation and labeling efficiency were confirmed via absorbance spectrum with a resulting conjugation yield of 1 fluorophore per antibody.

9 Adhesion Assay (Static Conditions)

HIV-Nef expressing SupT1 T cells were labeled with Calcein AM dye and allowed to adhere to HCAEC monolayer labelled with Cell Tracker Deep Red for one hour. After washing away non-adhered cells, the remaining T cells and HCAEC were

harvested with trypsinization. The cellular mix was analyzed using flow cytometry and adhesion defined as the percentage of green T cells compared to red HCAECs.

10 Microfluidic Device Fabrication and Seeding

For the flow studies we used a microfluidic device with channel dimensions of 1000 μm x 100 μm (width x height), using standard soft lithography techniques as we described previously [165]. Briefly, first the device master was fabricated using SU-8 2075 (MicroChem Corp., USA) photoresist and standard photolithography, and then the microfluidic device was fabricated using this master and casting PDMS (Corning, USA) at a 10:1 base to curing agent ratio. Prior to cell seeding, the microfluidic devices were coated with fibronectin (50 μg/mL, Sigma-Aldrich, USA) for one hour at room temperature. Endothelial cells were trypsinized and resuspended at a density of 2.0 x 10^7 cells/mL in EMG2 (Lonza, Switzerland). Approximately 20 μL of the cell suspension was flowed through the device and kept under static conditions in EGM+ for 24 hours to allow for cell attachment.

11 T-Cell Adhesion Assay (Flow conditions)

Before the perfusion assay T-cells (either Nef-ER or SupT1) were incubated with 1μM 4-hydrotamoxifen at 37°C for 6-12 hours. In drug testing experiments, cells were incubated with 10μM Atorvastatin for 12 hours. After incubation, t-lymphoblasts were resuspended at 2.0 x 10^6 cells/mL and stained with 5μmol/l Calcein AM for 30 minutes at 37°C. After incubation, T cells were spun down and the Calcein AM was removed and were again resuspended at a density of 2.0 x 10^6 cells/mL. The endothelial cell seeded microfluidic device was place in a microscopy incubation chamber (Zeiss, Germany) at

37°C with 5% CO2. Suspended T cells were then perfused using a syringe pump (Pump 11 Elite Series, Harvard Apparatus) at a rate of 18 µL/hr (giving a wall shear stress of 3 dyne/cm^2) for 25 minutes. Fluorescence and brightfield timelapse imaging (6 images/min) was performed during perfusion using a fluorescence microscope (Zeiss Z1, Hamatsu C11440 digital camera). Images were exported and analysis was performed using NIH ImageJ software. The number of attached cells in the field of view (FOV) was counted and the attachment to the entire device was calculated by multiplying the attached cells in the FOV by the length of the channel divided by the length of the FOV. Percent cell attachment was calculated by dividing the total number of attached cells by the total number of cells perfused over the 25 minutes of perfusion.

12 Cytoplasmic Transfer

Control SupT1 T cells or Nef expressing SupT1 T cells were labelled with cytoplasmic dye Cell Tracker Deep Red and co-cultured with Calcein AM labeled HCAECs. After six hours, T cells were removed with 5x washes with PBS. HCAECs were then analyzed using flow cytometry and cytoplasmic transfer quantified as the percent of HCAECs that were double positive for Cell Tracker Deep Red and Calcein AM. Confocal images were taken using Leica SP8 Resonant-scanning confocal/multiphoton microscope.

13 Co-culture Conditions

HMVEC were grown to 70-80% confluence in EGM-2MV. T cells were co-cultured, along with HMVEC, in a 2:1 ratio overnight. The T cells were washed away 3

times with PBS. HMVEC were harvested with trypsinization, followed by neutralization with serum-containing media.

14 HIV Viral Infection

Frozen viral supernatants were obtained from HEK293T cells transfected with pNL4-3 and pNL4-3ΔNef plasmids. The supernatants were used to infect Jurkat T cells. 20ng/ml of virus was used to infect 500,000 cells, and HIV+ was determined with p24 ELISA of supernatant.

15 Bronchoalveolar Lavage Samples

Acellular bronchoalveolar lavage (BAL) fluid and cells derived from BAL were obtained from 18 individuals. 14 HIV-1+ patients were on ART for a minimum of three years. One patient was HIV2+ and three were HIV uninfected volunteers. Acellular BAL fluid was centrifuged at 10,000g for 30 min, followed by 400,000g for 1 h, to isolate extracellular vesicles. Anti-Nef Elisa (Immuonodiagnostic Systems, USA) and western blot were used to quantify and detect HIV-Nef protein, respectively. Non detectable virus was extended to newer (more sensitive) assessments with <20 copies per ml blood.

Primary alveolar macrophages were isolated from BAL fluid and allowed to adhere to plastic for 2h in RPMI+10% FBS. The non-adhered cells were washed away and the adhered alveolar macrophages were cultured overnight in the same medium prior to the addition of extracellular vesicles.

Bronchoalveolar Lavage Fluid was collected from the lung of WT and Nef transgenic mice using 0.8ml PBS. The BALF was then centrifuged at 600g to remove cell pellet. The RBC were lysed using RBS lysis buffer and the remaining cells counted using a

hematocytometer. Protein content in BALF was quantified using Pierce BCA kit (Thermo Fisher, USA).

16 Isolation and Characterization of Extracellular Vesicles from Nef-expressing Cells

Extracellular vesicles were isolated from culture supernatants obtained from control and Nef-ER-expressing SupT1 cells and HEK293T cells transfected with either empty plasmid, Nef-expressing plasmid, or Nef F195R mutant. For isolation of EV, the cells were maintained in exosome-free FBS-containing media. Briefly, the culture supernatants were centrifuged for 10 min at 2000 g, followed by filtration through a 0.22 μm syringe filter (Merck Millipore, USA) to remove cell debris. The supernatants were centrifuged at 100,000g for 70 min to isolate EV. The EV obtained were washed once with phosphate buffered saline (PBS) and re-suspended in PBS for use in further experiments. To estimate the amount of protein carried by the EV, lysis was done in a 1:1 suspension of EV and RIPA buffer. Bicinchoninic assay (BCA) was performed on the lysate and the total protein was calculated. Furthermore, the number and size of the extracellular vesicles released by the cells was assessed by NanoSight (Malvern, UK), which captures and analyses videos to determine vesicular particle size distribution and particle counts. For this, 10 μg of EV were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by ultracentrifugation at 100,000g for 70 min. The EV were then suspended in 200 μl PBS and appropriately diluted for NanoSight analysis. The number of particles per μg of EV protein was also estimated. 3ug of EV (based on protein concentration) were added per ml media to test the effects of EV on endothelial cells.

17 Fluorescence-activated Cell Sorting

FACS was performed as previously described [20]. BAL-derived cells were fixed in 1% PFA for 15 min at room temperature. Cells were washed with PBS prior to the addition of 2 µg anti-EMAPII M7/1-AF488, 5 µl CD45-AF700 (Clone 2D1, BioLegend), 2.5 µl CD3-BV510 (clone OKT3, BioLegend), 5 µl CD4-PE/Cy7 (Clone RPA T4, BioLegend), 2.5 µl CD11b-BV711 (Clone ICRF44, BioLegend), 2.5 µl CD206-BV421 (Clone 15-2, BioLegend), 5 µl CD8-PercP/Cy5.5 (Clone HIT8a, BioLegend), and 2.5 µl HLA-DR-BV 650 (Clone L243, BioLegend) per $1x10^6$ cells. The cells were incubated for 45 min at room temperature and washed with PBS. Next, the cells were permeabilized using the FoxP3 intracellular staining kit (00-5523-00, eBioscience). 1 µl of UV-NBS AF-647 conjugated anti-Nef EH1 antibody, 1 µl of UV-NBS conjugated anti-Nef SN20 antibody, 1 ul of UV-NBS AF-647 conjugated anti-Nef 3D12, and 2 µl of p24-PE (clone KC57, Beckman Coulter, Inc.) was added to 1 X 10^6 cells for 1 hour at room temperature. Similar to our previous publication [20]. Cells from HIV negative individuals were used to draw the gates for HIV-Nef protein detection in HIV positive patients since HIV negative individuals have no HIV-Nef protein. Cells were acquired on BDFortessa and data analyzed on FlowJo V10.

For detecting surface expression of EMAPII in HMVECs, the cells were fixed in 1% paraformaldehyde for 15 min and stained with 2 µg anti-EMAPII M7/1 in 50 µl FACS staining buffer for 45 min at room temperature and washed with PBS. Anti-rat AF488 (Life Technologies Corporation) was used as a secondary antibody to detect surface EMAPII.

HCAEC were stained for VCAM (Clone: STA), ICAM-1(Clone: HA58), P-Selectin (Clone: AK4) and E-Selectin (Clone: HCD62E) after O/N exposure to HIV-Nef containing EV or control EV.The cells were acquired on BD FACSCalibur and data analyzed on FlowJo V10.

Surface EMAPII and apoptosis via cleaved caspase 3 protein was measured as previously described [166] . Lung tissue from Nef Transgenic mice or their WT littermates were digested with 0.25% collagenase Type I (Stem Cell Technologies, Canada) 60 min at 37ºC. Homogenates were stained with CD31-APC (Biolegend: #102409) /CD326-PerCP-Cy5.5 (118219)/CD45-PE (BD: 561087)/EMAPII antibody mixture, or permeabilized (FoxP3 staining kit, eBioscience, San Diego, CA) for FITC-cleaved caspase 3 mab staining (R&D Systems IC835G025). The cells were acquired on BD Fortessa and data analyzed on FlowJo V10.

18 HIV-Nef Transgenic Expression, Stereology, and Volume-Related Stereology for Calculation of the Total Number of Alveoli

HIV-Nef protein was induced in endothelial Nef transgenic (VE-Cadherin-Nef) mice for 3 months and the mice were euthanized. After perfusion of the pulmonary artery, the lungs were fixed by airway instillation using 4.5% paraformaldehyde. Lung volume was assessed by water-displacement method. The lungs were embedded in 3% agar and cut into commensurate parallel slices (3 mm each), then dehydrated and embedded in paraffin. After staining 3-mm sections with resorcin/fuchsin and nuclear fast red (Weigert's elastin staining), measurement of the number of alveoli was performed on 2 alternate sections 3-mm thick, with a 3-mm distance from each other, which were placed adjacent on the same slide and examined using a light microscope (Leica) equipped with

newCast software for stereology (Visiopharm). The physical dissector method was used for counting, as previously described [167]. Respective counts were related to the right-lung volume.

19 Atorvastatin Gavage Feeding

C3H mice with transgenic expression of HIV-Nef under Tet promoter were crossed with FVB mice expressing Tet Transactivator under VE-Cadherin promoter.The resulting Nef-VE Cadherin double transgenic animals were gavage fed 5mg/kg Atorvastatin [168] daily for three weeks according to IACUC guidelines.

20 Aortic Ring Dilation

Mice from each group were anesthetized by isoflurane and sacrificed by decapitation. The aortic arch was isolated from the connective tissues and cut into 2.5 mm rings in a Ca^{2+} and Mg^{2+}-free phosphate buffered saline solution. The rings were then hung between two wires of the myograph placed in a tissue bath containing the Krebs solution of the following composition: 131.5 mMol/L NaCl, 5 mMol/L KCl, 2.5 mMol/L $CaCl_2$, 1.2 mMol/L NaH_2PO_4, 1.2 mMol/L $MgCl_2$, 25 mMol/L $NaHCO_3$, and 10 mMol/L glucose, that was saturated with a gas mixture of 95% O_2 and 5% CO_2; pH 7.4, 37 ˚C. The rings were preloaded to 0.4-0.6 g and preconstricted by 10 μMol/L phenylephrine. The force of 70 mM KCl-induced contractions was regularly assessed to monitor the "health" of the aortic ring during the experiments. All of the experiments were performed in the presence of 10 μMol/L indomethacin to avoid the contribution of endogenous endothelial vasoconstrictive prostanoids and the SNAP (S-Nitroso-N-acetyl-DL-penicillamine), a NO donor, was used to be a positive control.

21 Inspiratory Capacity Measurements

Inspiratory Capacity measurements were performed with the flexiVent system (Scireq, Canada). Mice were anesthetized with inhaled isoflurane in oxygen and orotracheally intubated with a 20-gauge intravenous cannula under direct vision. A good seal was confirmed by stable airway pressure during a sustained inflation. Isoflurane anesthesia was maintained throughout the measurements, and the mice were hyperventilated to eliminate spontaneous ventilation. Starting at FRC, the flexiVent was programmed to deliver 7 inspiratory volume steps, for a total volume of 1 ml, followed by 7 expiratory steps, pausing at each step for at least 1 second. Inspiratory capacity was directly measured by deep inflation maneuver or estimated from minimum of three pressure volume loops.

22 Immunohistochemistry

Tissue Specimens/Processing: The lung samples were collected and placed in 10% NBF for fixation. The tissues were processed, embedded, microtomed, and stained for both H&E and Van Gieson's Elastin Stain.

Whole Slide Digital Imaging: The slides were scanned using the Aperio ScanScope CS system (360 Park Center Drive Vista, CA 92081). All images were scanned at 20x. The digital whole images were housed and stored in the Aperio Spectrum software platform.

Automatic Image Quantitation: Computer-assisted morphometric analysis of digital images was carried out using the Aperio software of the Aperio Imaging system.

The specific positive pixel algorithm was used for evaluating the smooth muscle in the small arterioles. The size that was analyzed was 30 microns to 75 microns in diameter.

Whole Slide Digital Imaging: The Aperio whole slide digital imaging system was used for imaging. The Aperio ScanScope CS system was used (360 Park Center Drive Vista, CA 92081). The system imaged all slides at 20x. The scan time ranged from 1.5 minutes to a maximum of 2.25 minutes. The whole images were housed and stored in the Spectrum software system and images were captured from the whole slides.

Automatic Image Quantitation: Computer-assisted morphometric analysis of digital images was carried out using the Aperio software of the Aperio Imaging system. The positive pixel algorithm was used for imaging the Van Gieson elastic slides.

The Positive Pixel Count algorithm was used to quantify the amount of a specific stain present in a scanned slide image.

To quantify the lungs, we selected three small arterioles per slide. The size measured was between 30 microns and 75 microns. Some slides only had 2 small arterioles on the cross-section of the lung. The individual sections were averaged for each mouse. In order to only capture the smooth muscle layer around the arteriole, a line was drawn around the outer edge of the smooth muscle layer of the arteriole and the lumen of the arteriole was removed.

23 Statistical Analysis

Samples were de-identified and difference between groups analyzed using Student T-test with Welch's correction, one-way ANOVA with Tukey post-hoc multiple

comparison and Mann-Whitney non-parametric tests as indicated. Spearman non-parametric analysis was used to determine correlation.

III Results

1 HIV-Nef protein persists in the lungs of aviremic HIV patients and induces pulmonary endothelial cell damage in an EMAPII dependent fashion.

1.1 HIV-Nef protein persists in the lungs of HIV patients on ART

In order to establish HIV-Nef protein persistence and distribution in lungs of HIV patients on ART, we analyzed cells and acellular fluid from bronchoalveolar lavage (BAL) of a well-characterized cohort (Table 1). We first stained BAL-derived cells for intracellular HIV-Nef using 3 different anti-Nef monoclonal antibodies directed against 3 unique HIV-Nef epitopes (mAb: EH1, 3D12, and SN20; **Fig.5**) to address the high mutation rate of HIV proteins. We also utilized a novel UV-NBS ab labeling method **(Fig.6 and 7)** to enhance sensitivity. In our patient cohort, we detected HIV-Nef protein persistence in at last 2% of BAL cells in 9 out of 15 HIV-positive patients on ART **(Fig.2, Fig.8 and Table 1).** Using markers for alveolar macrophage and lymphocytes, we found that HIV-Nef+ cells were distributed throughout alveolar macrophages, CD4 T cells, and CD8 T cells **(Fig.3)**, which is consistent with our previous findings that HIV-Nef protein is widespread in blood cells [41]. In none of the examined samples was the HIV virus detectable based on staining for the HIV protein p24 (data not shown) or deep sequencing for HIV viral sequences (RNA and DNA) of BAL (data not shown). In concert with recent findings that HIV-Nef persists in plasma EVs of ART patients, we demonstrated Nef persistence in BAL-derived EVs **(Fig.1, 4 and Table 1)**. This shows HIV-Nef protein is widely distributed in the lungs of HIV-patients even in the absence of detectable HIV protein and suggest that this persistence corresponds to a detrimental effect in patient lungs by promoting a pro-apoptotic environment.

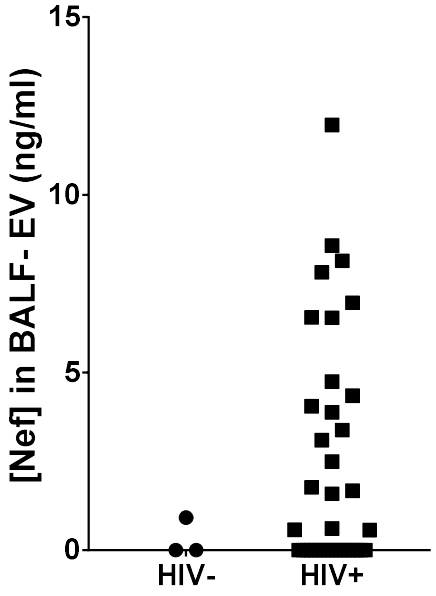

Figure 1: HIV-Nef protein persists in lungs of HIV patients on ART. HIV-Nef protein was detected using ELISA in extracellular vesicles isolated from HIV patients on anti-retroviral therapy for at least three years.

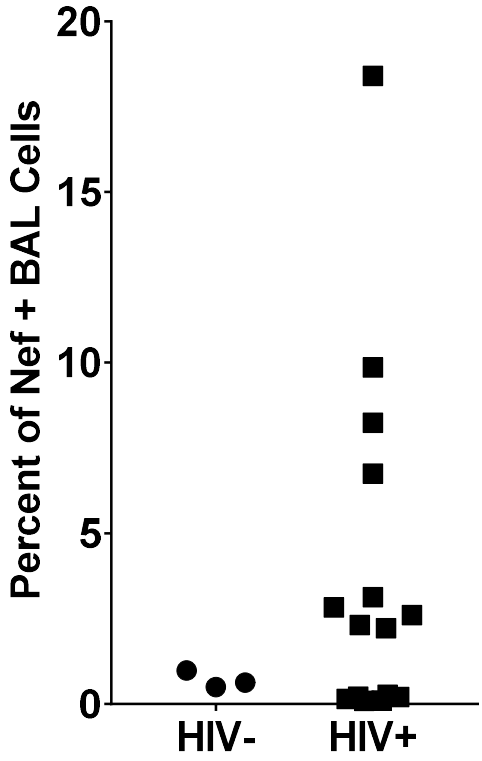

Figure 2: HIV-Nef protein persists in lung derived cells of HIV patients on ART. HIV-Nef protein was detected intracellularly in bronchoalveolar lavage (BAL)-derived cells using flow cytometry (% Nef positive cells).

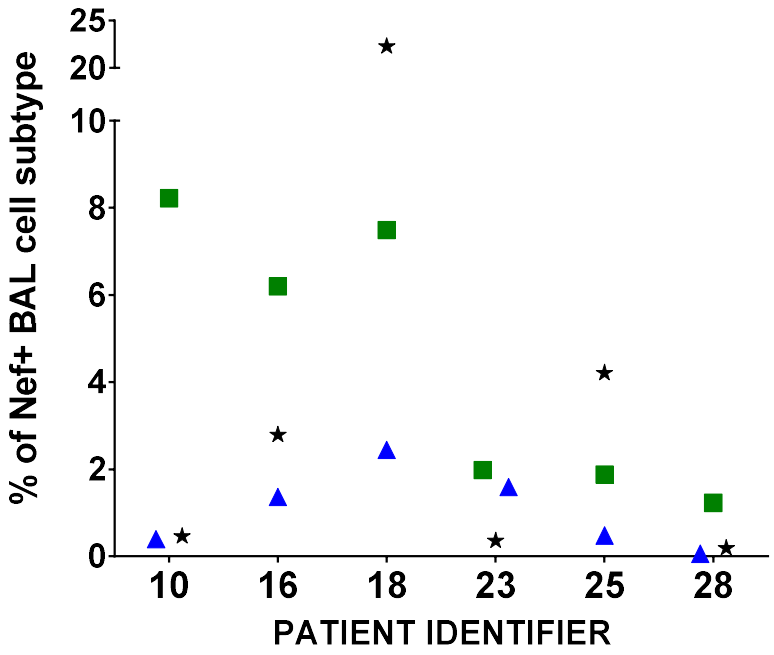

Figure 3: HIV-Nef protein persists in multiple compartments of lungs of HIV patients on ART. HIV-Nef protein was detected intracellularly in bronchoalveolar lavage (BAL)-derived cells using flow cytometry (% Nef positive cells).

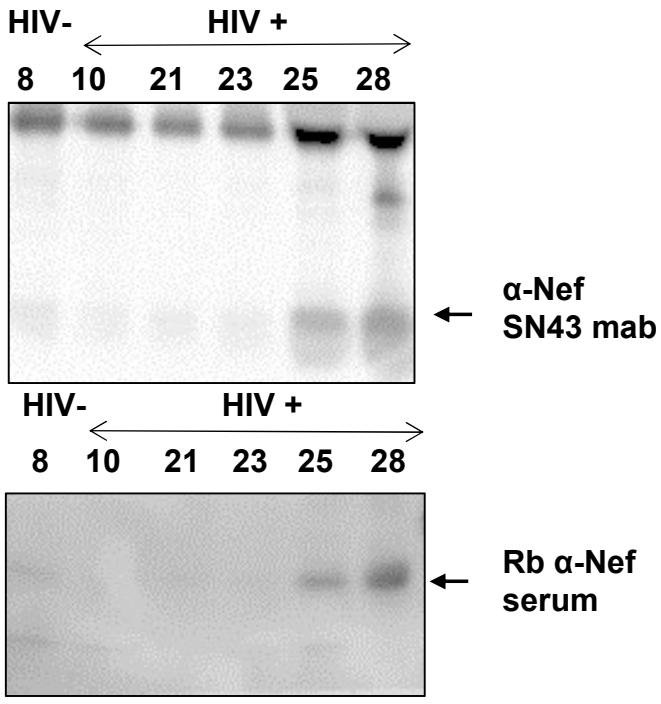

Figure 4: Western blot analysis of extracellular vesicles isolated from acellular BAL fluid (Upper: Anti-Nef SN43ab) and (Lower: Anti-Nef Polyclonal Rb Serum).

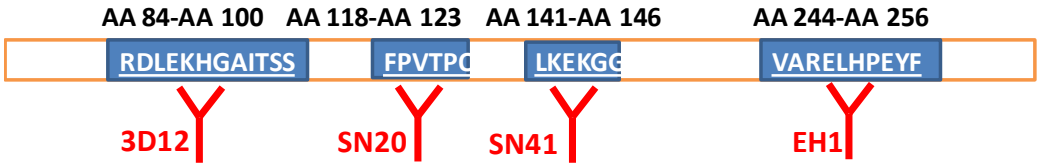

Figure 5: Schematic of the antibody cocktail used for intracellular Nef detection using conserved domains.

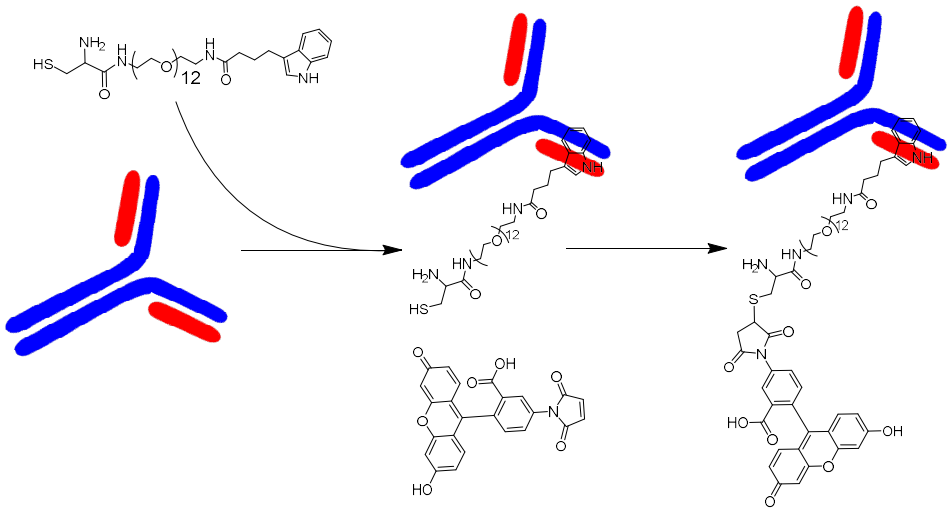

Figure 6: Scheme demonstrating the method of UV-NBS conjugation through a site-specific non-native thiol.

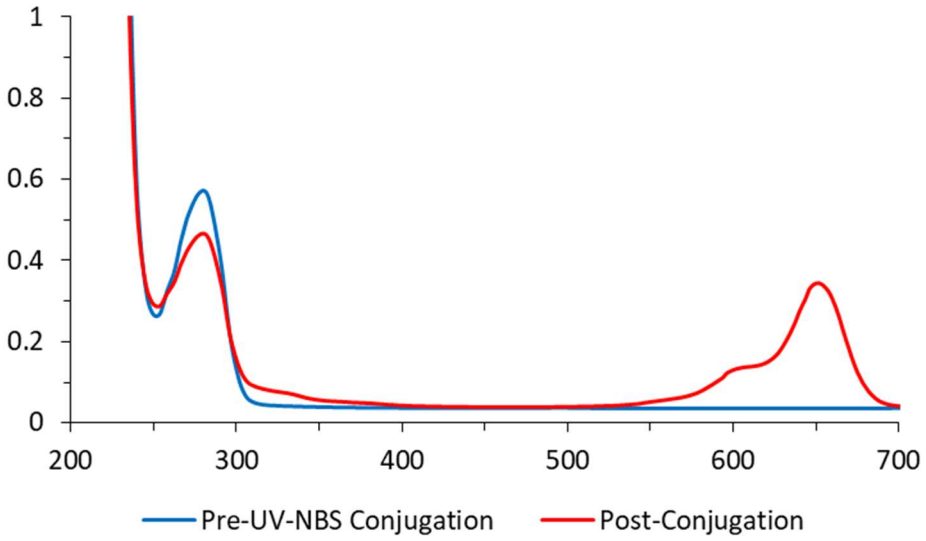

Figure 7: Spectrums of the antibodies before and after demonstrating fluorescent labelling of the antibody.

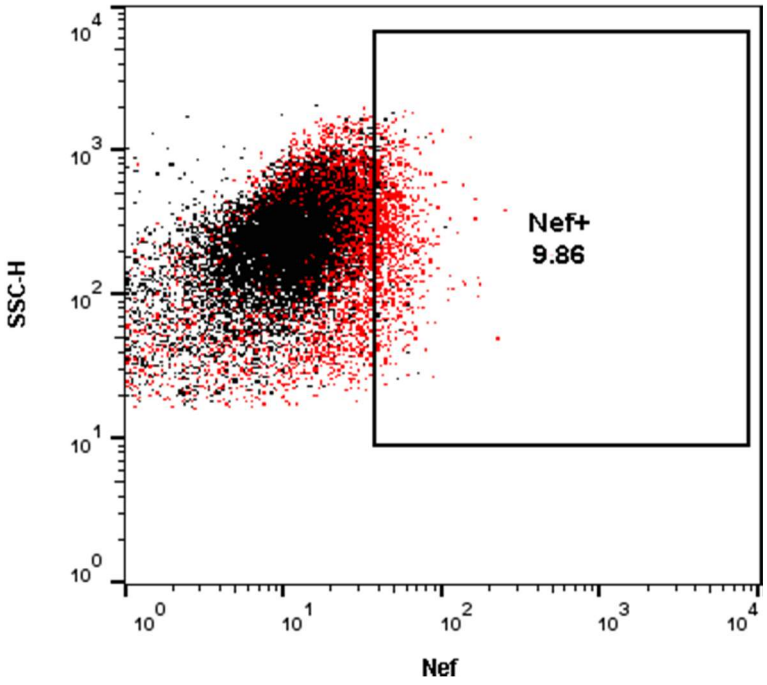

Figure 8: Representative flow cytometry scatter plot showing HIV-Nef staining in BAL-derived cells from control HIV-Patient 8 (Black dots) and HIV+ Patient 10 (Red dots, Nef+ = 9.86%).

Pt. ID	Nef+ BAL cells (%)	[Nef] in EV (ng/ml)	EMAP II + BAL cells (%)	CD4 cell count	Smoking status
8	0.63	N/A	1.41	HIV-	Yes
10	9.86	0	6.62	709	Former
16	3.13	0	6.06	707	Former
18	2.22	1.5	2.99	688	Former
19	6.75	6.5	12.3	N/A	Former
20	8.24	1.7	0.55	985	No
21	2.6	0	0.54	904	No
22	0.15	3.8	0.03	771	Yes
23	2.31	0	0.02	158	Yes
24	0.03	0	0.00	1305	Yes
25	2.83	8.5	3.23	495	No
26	0.27	6.5	0.12	737	Yes
27	0.5	N/A	0.1	HIV-	Yes
28	18.4	4.7	0.25	966	No
29	0.2	0.5	0.06	158	Yes
30	0.21	4.3	0.1	231	Former
31	0.98	N/A	0	HIV-	Yes
32	0.1	1.6	0.03	332	Former

Table 1: Bronchoalveolar Lavage fluid from 18 patients were analyzed for HIV-Nef persistence and EMAPII surface expression. CD4 cell counts and smoking status of each individual is also characterized.

1.2 BAL fluid induced endothelial cell apoptosis in HIV+ patients is Nef-dependent and mediated by EMAPII

We followed up our observation of Nef persistence in BAL by studying the role of EV in HIV patient's lung microenvironment. BAL fluid from four HIV- patients and 8 HIV+ patients were added to HMVEC in a 1:10 dilution in EGM2MV media and cultured for 16h. BAL from HIV+ patients significantly upregulated early apoptosis marker, Annexin V in HMVEC **(Fig.9)**. To determine whether HIV BAL induced HMVEC apoptosis was dependent on Nef persistence, BAL fluid from 4 HIV-, 4 HIV+ Nef+ and 4 HIV+ Nef- were added to HMVEC at 1:10 dilution. Interestingly, only the persistent BAL was capable of induced HMVEC apoptosis measured using TUNEL labeling as a late stage marker for apoptosis **(Fig.10)**. These findings were confirmed with JC-1 staining to measure mitochondrial depolarization. JC-1 **(Fig.11)**. The dye aggregates in the negatively polarized mitochondria and fluoresces red (590nm) but fluoresces green (520nm) in the cytoplasm. Therefore, a drop in red/green ratio indicated mitochondrial depolarization which is an early marker for apoptosis. Using this assay, Nef-dependent HIV BAL induced HMVEC apoptosis was confirmed. The fourth indicator for pro-apoptotic signaling was active caspase 3 signaling in HMVEC treated with BAL from 6 HIV-. 6 HIV+Nef+ and 6 HIV-Nef- patients **(Fig.12)**. Interestingly, in all four assays, HIV BAL induced HMVEC apoptosis was abrogated when 10μg/ml of α-EMAPII neutralizing monoclonal antibody - M7/1 suggesting that BAL induced HMVEC apoptosis was mediated by the pro-apoptotic protein EMAPII.

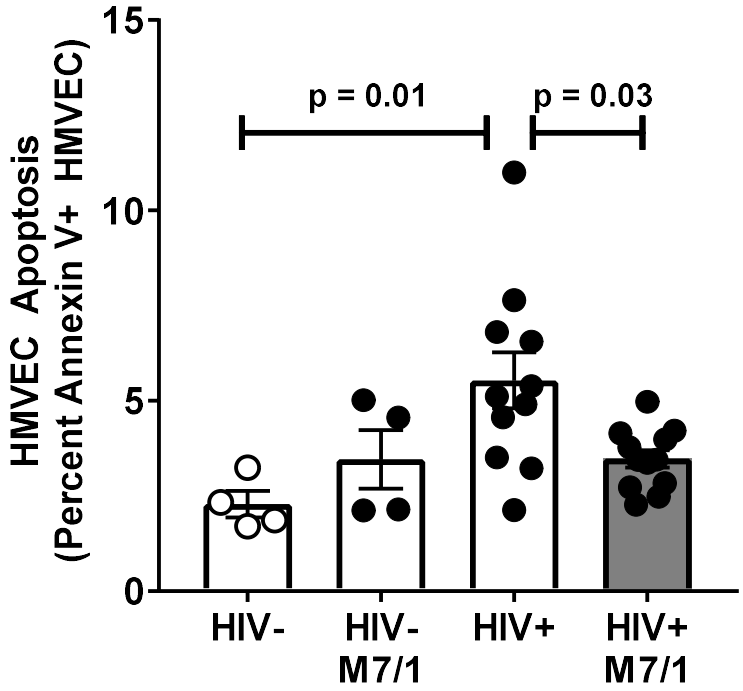

Figure 9: Addition of 50µl of acellular BAL fluid from HIV- and HIV+ patients for 16h to 450µl EGM2MV induces surface expression of early apoptosis marker –as detected using Annexin V FITC staining. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

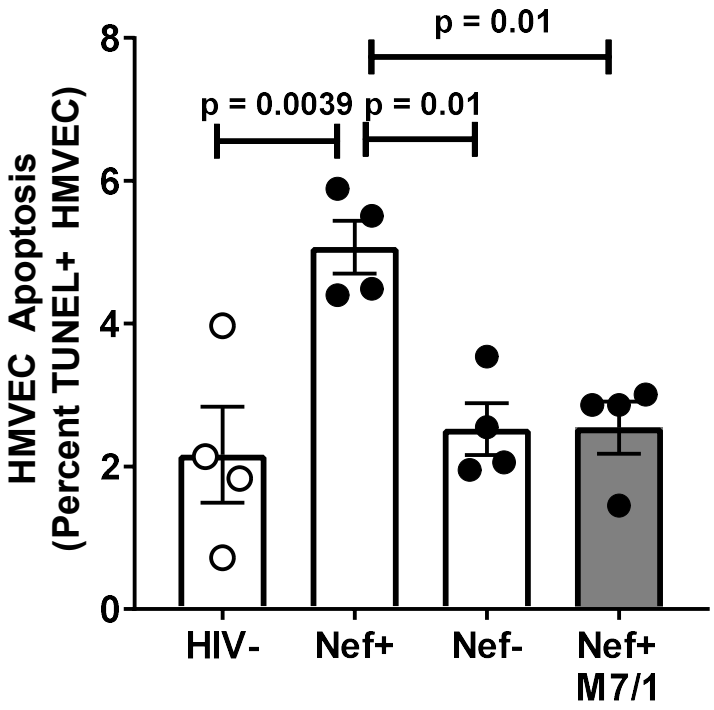

Figure 10: Addition of 50µl of acellular BAL fluid from HIV-, HIV+ Nef+ and HIV+ Nef- patients for 16h to 450µl EGM2MV induces apoptosis –as detected using TUNEL staining to identify DNA fragmentation. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

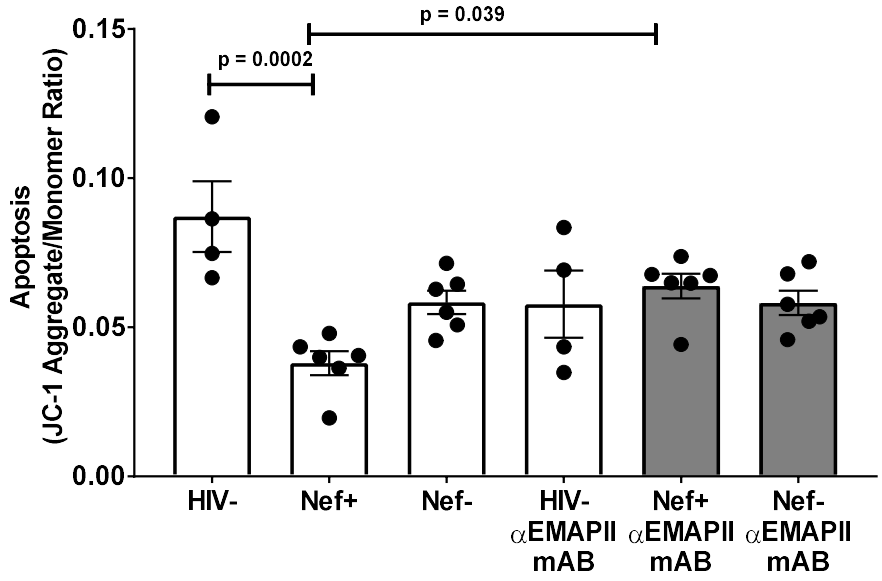

Figure 11: Addition of 10µl of acellular BAL fluid from HIV-, HIV+ Nef+ and HIV+ Nef- patients for 16h to 90µl EGM2MV induces apoptosis – as detected using JC1 staining to quantify mitochondrial depolarization, an early marker of cellular apoptosis indicated by a drop in aggregate/monomer ratio. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

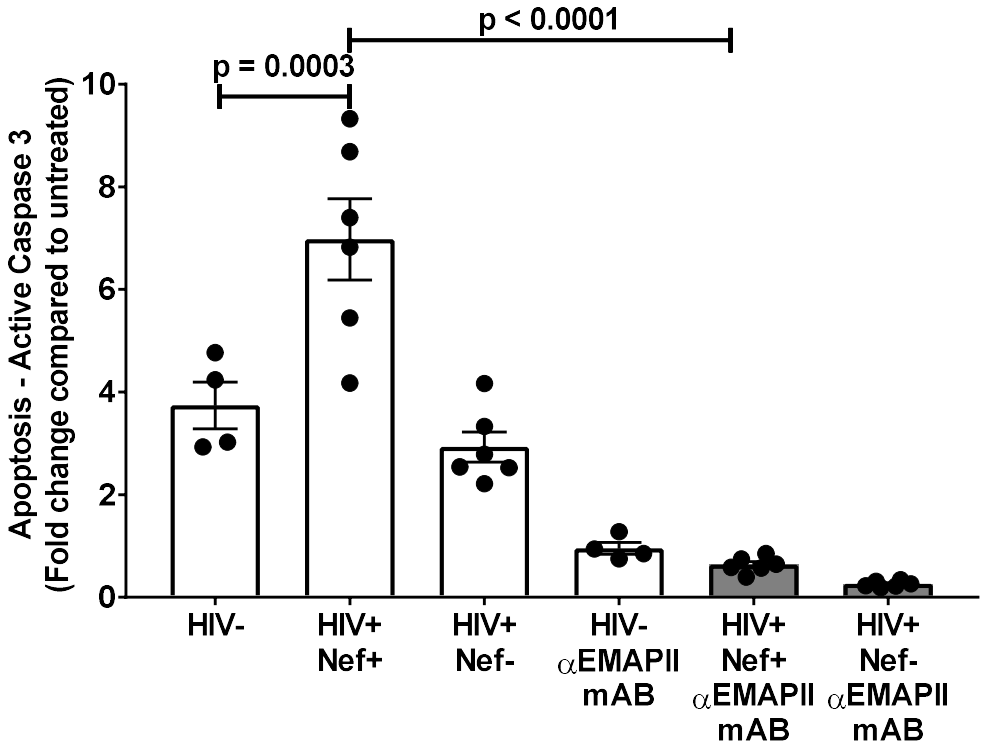

Figure 12: Addition of 10µl of acellular BAL fluid from HIV-, HIV+ Nef+ and HIV+ Nef- patients for 16h to 90µl EGM2MV induces apoptosis –as detected QVD-FVB peptide to measure active caspase 3 signaling. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

1.3 Nef containing Extracellular Vesicles mediates BAL fluid induced HMVEC apoptosis in an EMAPII dependent fashion.

Since we were able to isolate the pro-apoptotic HIV viral protein from the extracellular vesicle fraction of HIV patient BAL fluid, we tested if the extracellular vesicle fraction could induce HMVEC apoptosis in a Nef dependent fashion. We isolated EV from 50µl of BAL from 6 HIV-, 17 HIV+ Nef+ and 19 HIV+Nef- patients (classification based on Nef ELISA) and resuspended in 100µl EGM2MV. After 16h treatment, only the HIV+ Nef+ EV induced HMVEC apoptosis as measured through mitochondrial depolarization with JC-1 staining **(Fig.13)** and active caspase 3 signaling **(Fig.14)**. Interestingly, this extracellular vesicle induced HMVEC apoptosis could be completely blocked using 10µg/ml α-EMAPII neutralizing monoclonal antibody, M7/1.

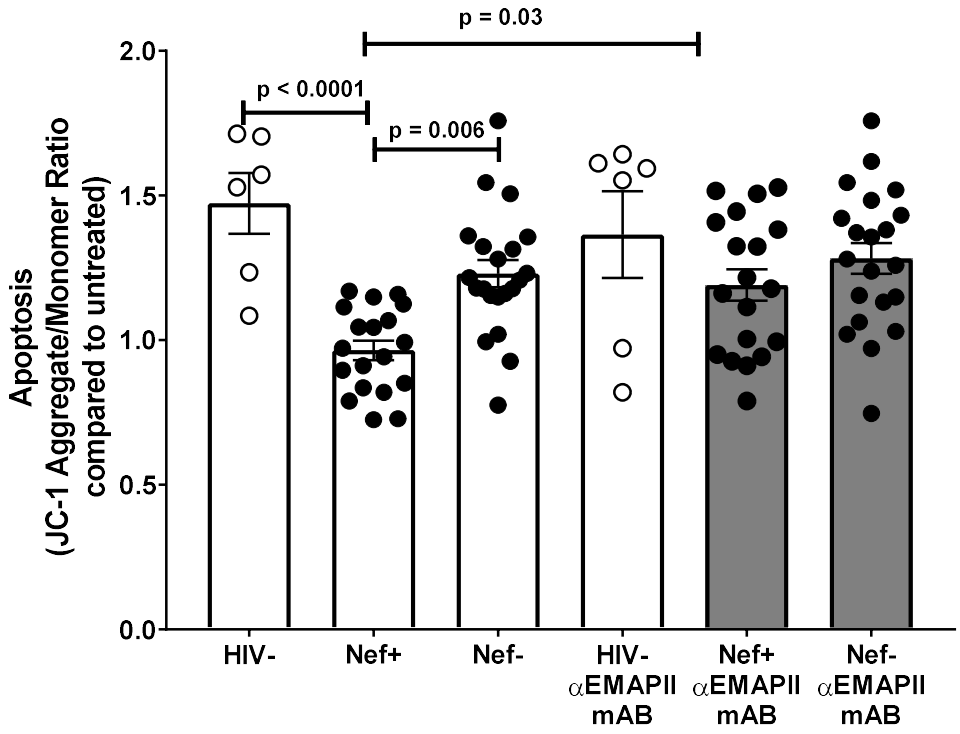

Figure 13: Addition of extracellular vesicles isolated from 50µl of acellular BAL fluid from HIV-, HIV+ Nef+ and HIV+ Nef- patients for 16h to 100µl EGM2MV induces apoptosis –as detected using JC1 staining to quantify mitochondrial depolarization, an early marker of cellular apoptosis indicated by a drop in aggregate/monomer ratio. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

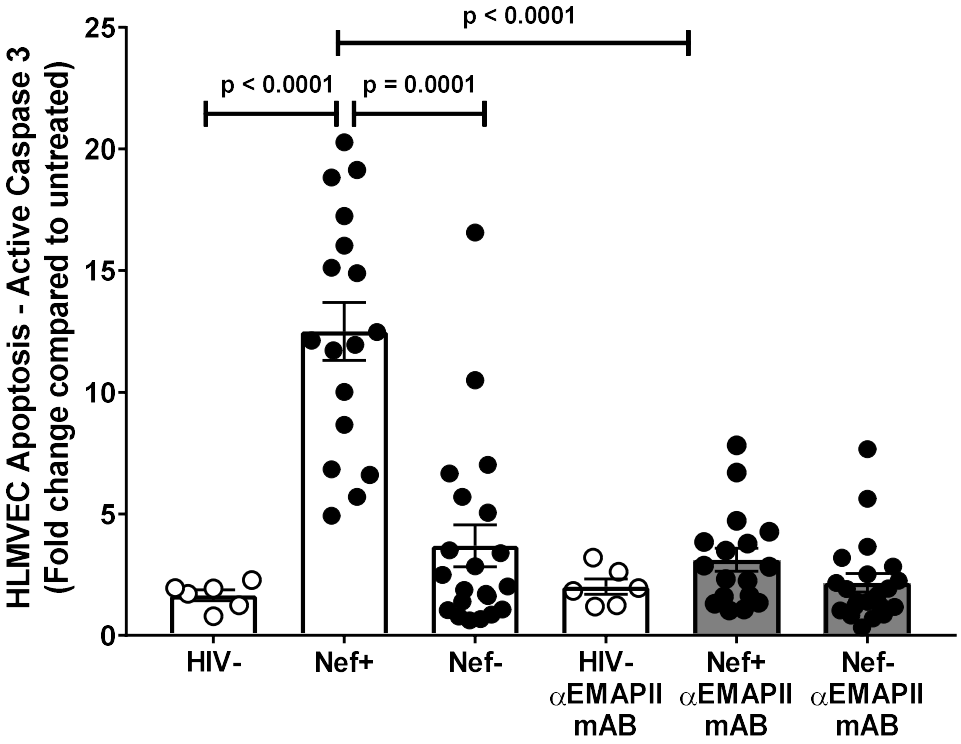

Figure 14: Addition of extracellular vesicles from 50µl of acellular BAL fluid from HIV-, HIV+ Nef+ and HIV+ Nef- patients for 16h to 100µl EGM2MV induces apoptosis –as detected QVD-FVB peptide to measure active caspase 3 signaling. Addition of 10µg/ml α-EMAPII neutralizing antibody, M7/1 abrogated HIV BAL induced HMVEC apoptosis.

1.4 HIV-Nef mediates its own transfer into extracellular vesicles

HIV-Nef was suggested to exert its function through insertion into and transport through EV [43-45, 47, 49, 169, 170]. Here, we investigated how HIV-Nef impacts EV formation and mediates its own incorporation into EV. When HIV-Nef was induced in a 4-hydro-tamoxifen (4-HT)-inducible expression system using a SupT1 T-cell cell line, a strong increase in EV secretion was observed **(Fig.15)**. Likewise, HIV-Nef transfection into HEK 293T cells resulted in an increase in the number of secreted EV **(Fig.16)**. These HIV-Nef induced increase in EV secretion was abrogated when HEK 293T were transfected with HIV-Nef F195R, a mutant incapable of PAK2 activation [171, 172]. This suggests that HIV-Nef modifies EV packaging and secretion contingent on PAK2 activation, a pathway implicated in exocyst complex mediated vesicular transport and exocytosis. Similarly, PAK2 inhibition with 5μM FRAX 597 reversed HIV-Nef induced increase in EV release **(Fig.17 & 18)**. We found that PAK2 activation is also required for the incorporation of HIV-Nef into EV **(Fig.19)**. Pak2 is also known as Rac1 activated kinase and nef-Rac1 interaction is usually required for Pak2 activation. In support of this, Rac1 silencing using siRNA was able to block Nef-induced increase in EV release **(Fig.20)** Interestingly, HIV-Nef containing EV from transfected cells induces the release of pro-inflammatory cytokines like TNFα, IL-6, MCP-1, IP-10 and RANTES **(Table 2)** when added to freshly isolated primary human alveolar macrophages. Our data suggest that HIV-Nef persistence in the lung involves packaging of HIV-Nef into EV and that this EV are capable of promoting pathological activities in vitro.

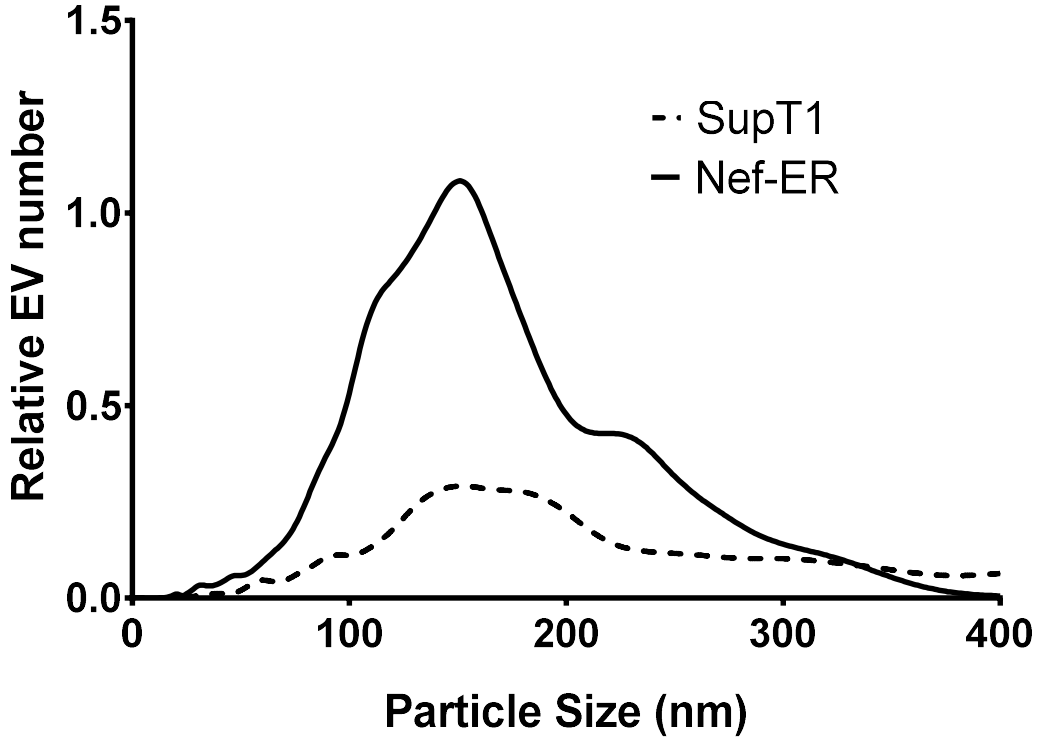

Figure 15: NanoSight based size and number analysis of extracellular vesicles secreted from Nef-ER expressing SupT1 T cells.

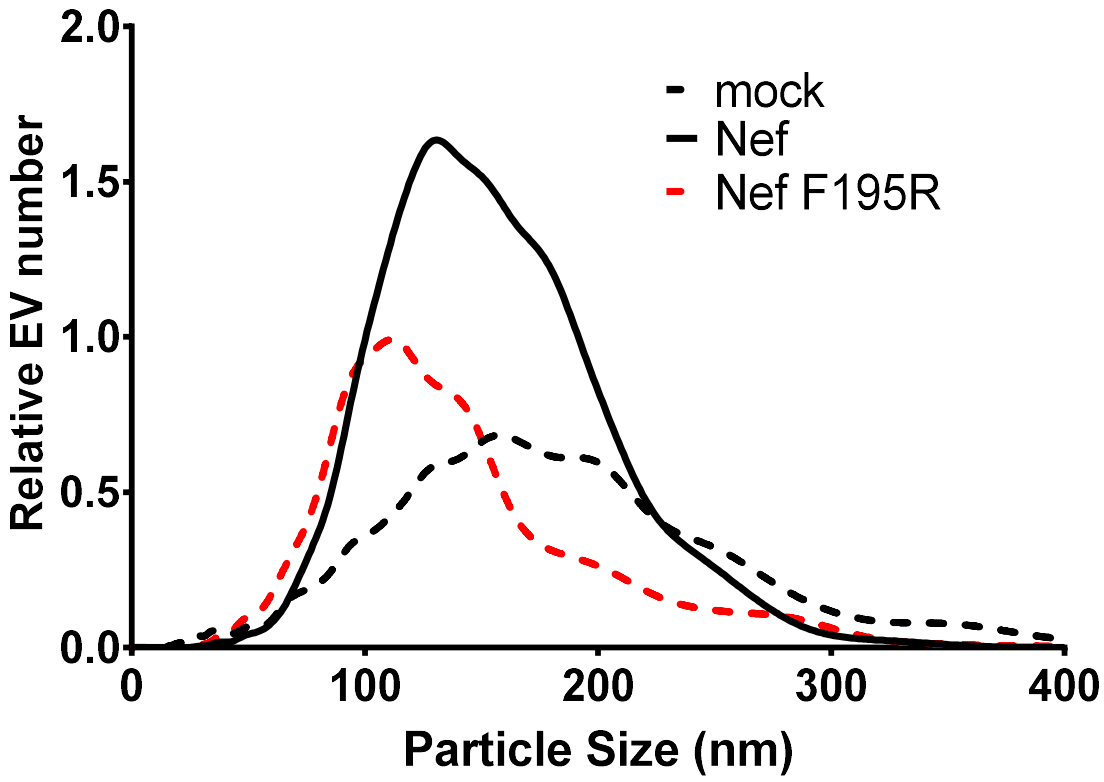

Figure 16: Analysis of HIV-Nef protein expression in extracellular vesicle. NanoSight based size and number analysis of extracellular vesicles secreted from HEK 293T transfected with either empty vector (mock), wt Nef (Nef) or Nef mutant incapable of Pak2 activation (Nef F195R).

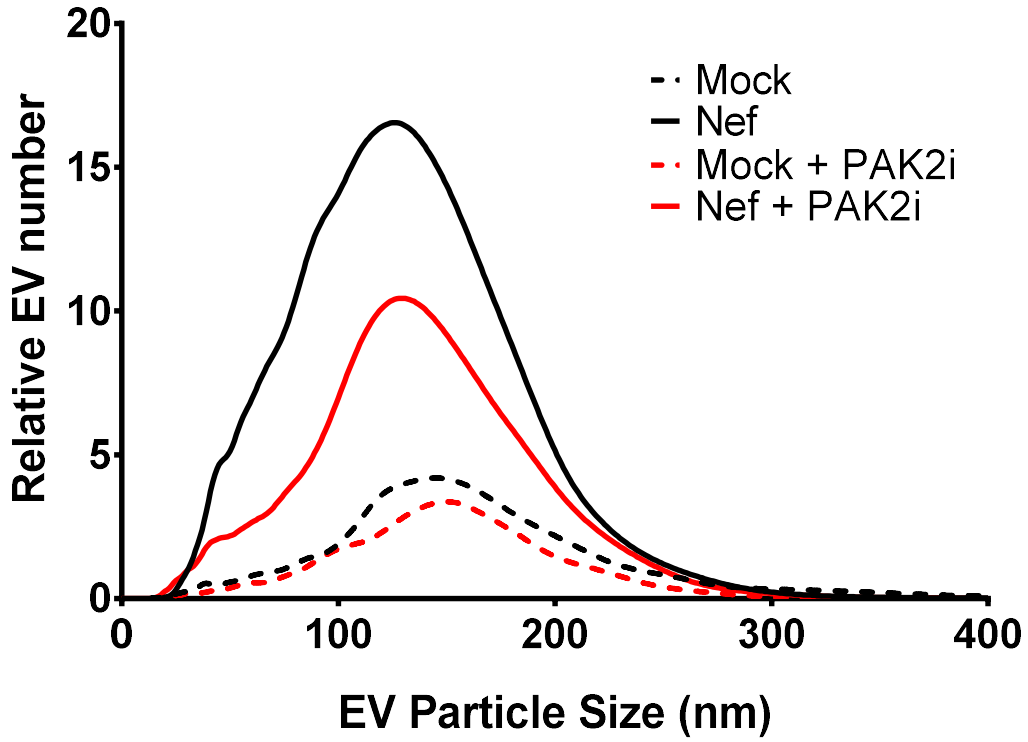

Figure 17: Analysis of HIV-Nef protein expression in extracellular vesicle. NanoSight based size and number analysis of extracellular vesicles secreted from Nef-ER (nef) expressing SupT1 T cells (mock) in the presence/ absence of Pak2 inhibitor FRAX 597.

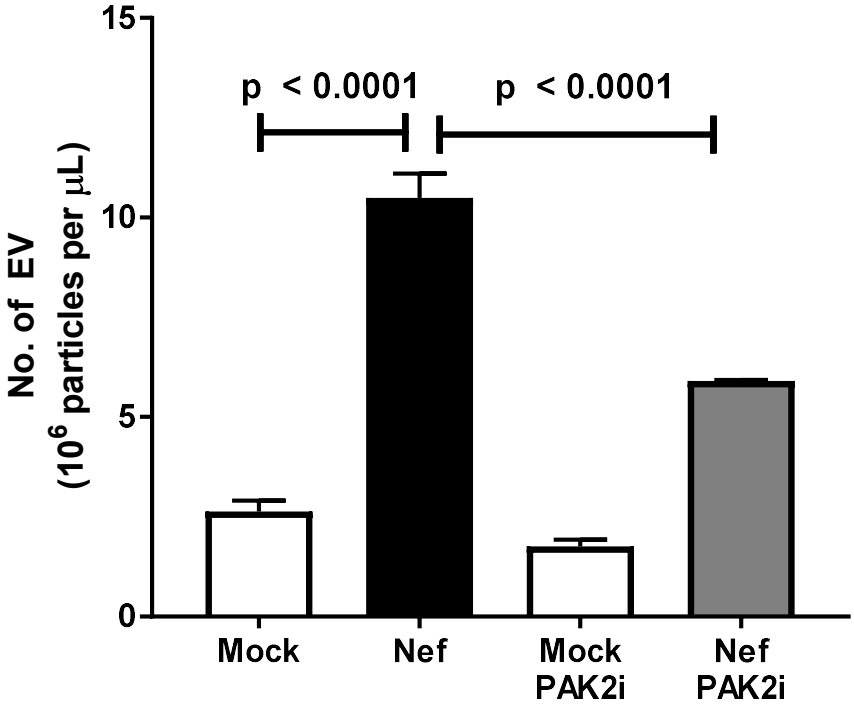

Figure 18: NanoSight based number analysis of extracellular vesicles secreted from HEK 293T transfected with Nef cDNA in the presence/absence of Pak2 inhibitor FRAX 597.

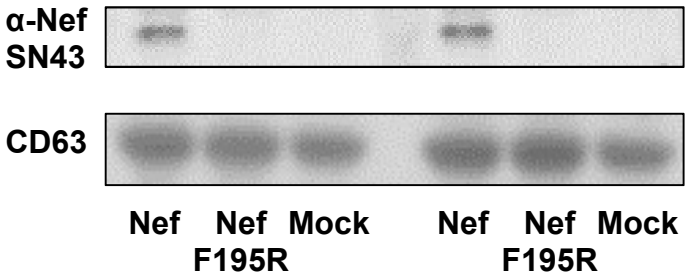

Figure 19: Representative western blot of EV isolated from HEK 293T cells transfected with empty vector (mock), wt Nef, or Nef mutant incapable of activating PAK2 (Nef F195R). CD63 antibody was used to show EV in all lanes.

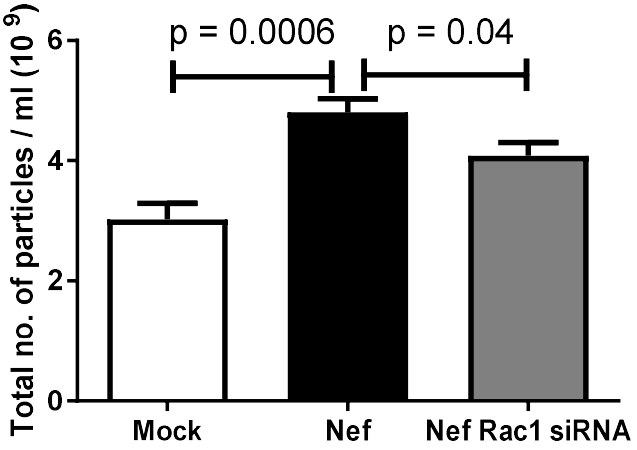

Figure 20: NanoSight based number analysis of extracellular vesicles secreted from HEK 293T cells transfected with Nef cDNA along with Rac1 silencing.

Treatment	TNFα (pg/ml)	IL-6 (pg/ml)	IP-10 (pg/ml)	MCP-1 (pg/ml)	RANTES (pg/ml)
Untreated	5.4	8.6	0	78.6	0
Mock EV	8.6	8.3	0	92.3	1.7
Nef EV	2015.1	1820.3	33.4	223.3	10.8

Table 2: The addition of Nef containing EV isolated from transfected HEK 293Ts to primary BAL derived alveolar macrophages induced the secretion of pro-inflammatory cytokines after 4h measured using BD CBA.

		Lung					Blood				
		IP-10 (pg/ml)	MCP-1 (pg/ml)	MIG (pg/ml)	RANTES (pg/ml)	IL-8 (pg/ml)	IP-10 (pg/ml)	MCP-1 (pg/ml)	MIG (pg/ml)	RANTES (pg/ml)	IL-8 (pg/ml)
HIV+ Nef+ n=12	Mean (SD)	319.8 (580.7)	33.9 (52.1)	173.8 (250.6)	2.3 (1.5)	64.9 (132.0)	243.2 (109.7)	32.3 (22.1)	154.5 (187.4)	1542.3 (818.4)	5.4 (2.8)
	Median (IQR)	83.1 (19.2, 354.1)	12.5 (6.3, 19.9)	29.75 (4.4, 223.7)	2.85 (0, 3.5)	13.05 (6.3, 44.1)	233.5 (85.7, 309.8)	32.5 (3.1, 38.3)	103.8 (34.5, 132.0)	1025.4 (639.4, 2500)	5.5 (1.7, 7.4)
HIV+ Nef- n=2	Mean (SD)	25.8 (6.3)	25. (18.1)	12.4 (4.2)	0	16.4 (2.5)	174.5 (7.8)	20.8 (6.4)	96.2 (6.3)	809.7 (299.9)	9.8 (1.7)
	Median (IQR)	25.8	25.4	12.4	0	16.4	174.5	20.75	96.2	809.7	9.8
HIV- n=15	Mean (SD)	113.6 (182.8)	20.4 (31.2)	114.5 (351.4)	3.2 (2.3)	51.3 (117.5)	190.2 (115.2)	23.2 (22.3)	76.8 (57.0)	1947.62 (718.6)	9.7 (16.5)
	Median (IQR)	64.2 (40.3, 93.9)	10 (5.1, 15.1)	20.7 (14.7, 29.3)	2.7 (1.8, 4.9)	14.6 (8.9, 36.1)	147 (117.1, 230)	15.1 (10.3, 25.8)	71.5 (30.45, 93.9)	2500 (1592, 2500)	3.4 (1.0, 10.1)

Table 3: Cytokines concentration in BAL and plasma of HIV patients on ART classified by Nef persistence in extracellular vesicles or BAL derived cells.

1.5 HIV-Nef Protein induces EMAPII surface expression

Based on our previous findings that HIV envelope protein GP 120 induces the surface expression and release of EMAPII, a central mediator of emphysema development and progression [158, 173, 174]; we raised the question whether the persistent intracellular HIV viral protein, HIV-Nef can also cause EMAPII release.

First, we investigated whether cells found in HIV-Nef containing lung microenvironments would be enriched for EMAPII surface expression. Indeed, we observed a strong correlation between the percentage of Nef positive (Nef+) cells in the BAL and the percentage of EMAPII positive (EMAPII+) cells (Spearman correlation coefficient: 0.74; p=0.0006) **(Fig.21)**.

To show that HIV-Nef expression can independently induce EMAPII trafficking to the cell surface membrane, we transfected HEK 293T cells with cDNA to express HIV-Nef protein. Compared to mock-transfected HEK 293T cells, SF2 HIV-Nef transfected cells showed increased EMAPII surface expression ($p < 0.001$) **(Fig.22)**. This EMAPII upregulation was dependent on PAK2 activation, as the Nef F195R mutant did not induce EMAPII surface expression **(Fig.22)**. Likewise, the addition of 5 μM FRAX597, a PAK2 inhibitor, abrogated EMAPII surface expression in HIV-Nef transfected HEK 293T cells **(Fig.23)**. Similarly, 4-HT activation of Nef-ER expressing SupT1 T cells also induced an increase in surface EMAPII expression **(Fig.24)**.

Next, we analyzed the impact of HIV-Nef protein persistence in the lung on EMAPII surface expression in the lung endothelium. We tested the hypothesis that Nef-containing EV or Nef-expressing T-cells can induce EMAPII expression in HMVECs.

The addition of Nef-ER-expressing SupT1 cells **(Fig.25)** and EV from HIV-Nef-transfected HEK 293T **(Fig.26)** significantly induced EMAPII surface expression in HMVECs. Again, use of a PAK2 activation incapable mutant **(Fig.26)** abrogated EMAPII surface expression in EV treated HMVECs and pulmonary arterial endothelial cells **(Fig.27)**, respectively.

We and others have reported that HIV-Nef protein can be transferred to endothelial cells and that its transfer stresses endothelial cells, which is a precursor for the development of vascular lesions [175-177]. Based on these reports, we wanted to know whether HIV-Nef in endothelial cells was sufficient to cause EMAPII upregulation in vivo. To this end, we crossed HIV-Nef transgenic mice containing the tetracycline-responsive element with the VE-Cadherin promoter controlled tet-off-transactivator mouse line [178]. The resulting offspring were kept for the duration of life without tetracycline to induce endothelial HIV-Nef, which led to a statistically significant rise in EMAPII surface expressing endothelial cells isolated from the lungs of VE-Cadherin-Nef double transgenic vs wild-type littermates **(Fig.28)**.

These data suggest that HIV-Nef persistence in the ART-treated HIV population leads to EMAPII upregulation in vitro and in vivo.

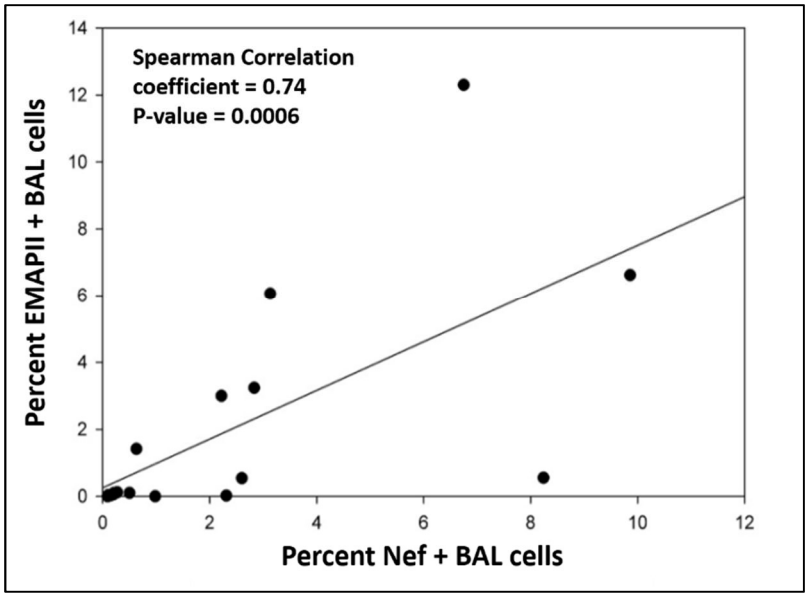

Figure 21: BAL-derived cells of 17 patients from INHALD cohort were stained for intracellular Nef and surface EMAPII. The percentage of Nef+ and EMAPII+ cells were quantified using flow cytometry. Shown is a Spearman correlation between intracellular Nef and surface EMAPII expression.

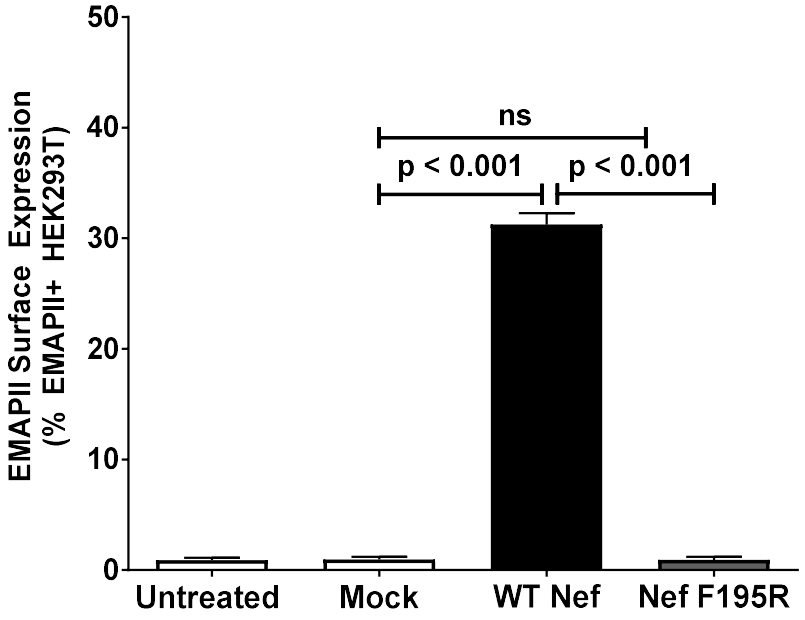

Figure 22: EMAPII surface expression was measured in HEK 293T transfected with empty vector (mock), wt Nef (wt Nef), or Nef mutant incapable of activating PAK2 (Nef F195R).

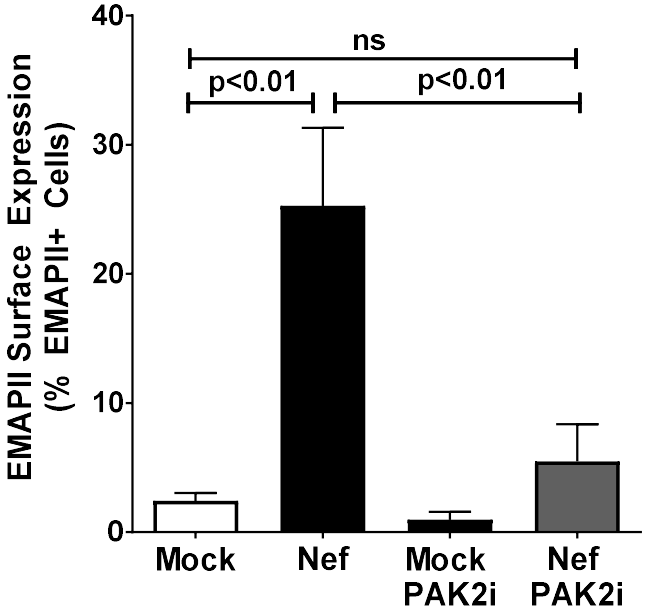

Figure 23: Transfection of HIV-Nef overexpressing plasmid in HEK293T-induced EMAPII surface expression, which was blocked using 5uM FRAX597, a PAK2 inhibitor.

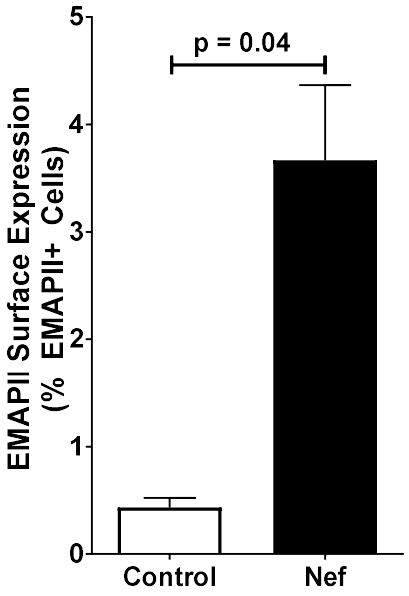

Figure 24: Nef-ER-expressing SupT1 T cells showed increased EMAPII surface expression upon activation with 4-HT.

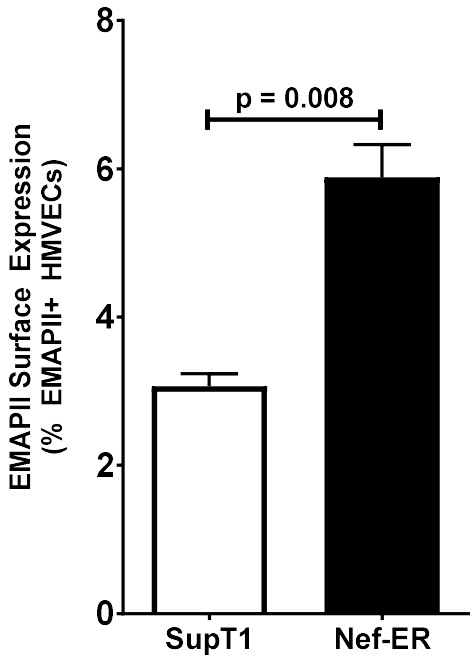

Figure 25: EMAPII surface expression in HMVEC 24 h after the addition of tamoxifen (4-HT) activated Nef-expressing SupT1 T cells (Nef-ER) or 4-HT activated control SupT1 T cells (SupT1).

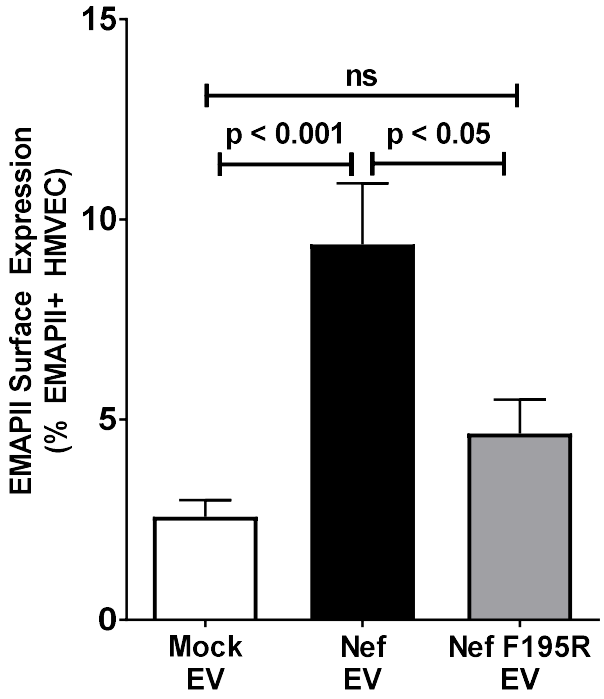

Figure 26: EMAPII surface expression in HMVEC 24 h after the addition of EV isolated from either Nef wildtype (Nef) or Nef-mutant (Nef F195R) transfected HEK 293T supernatants.

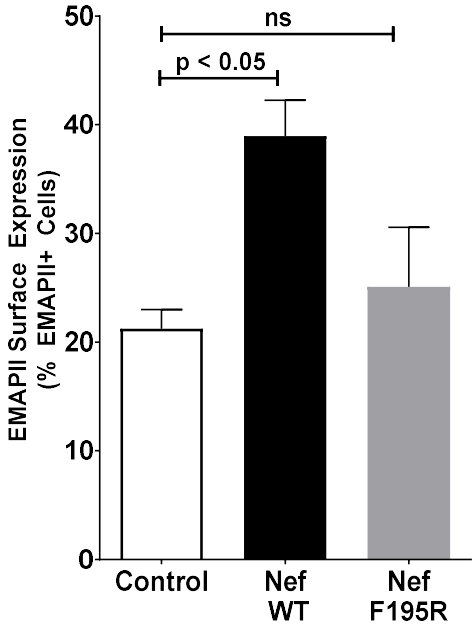

Figure 27: Addition of extracellular vesicles isolated from HEK 293T transfected with empty control vector, WT Nef, or Nef F195R, a mutant incapable of PAK2 activation induced EMAPII surface expressing in human pulmonary artery endothelial cells.

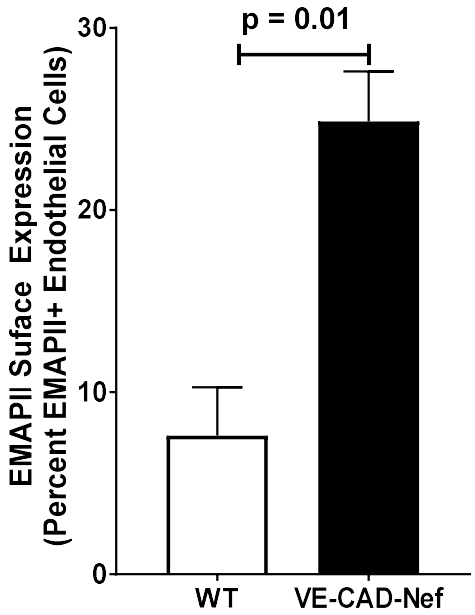

Figure 28: EMAPII surface expression was measured in CD45-/CD326-/CD31+ cells isolated from lungs of HIV-Nef transgenic mice and their littermates. The means of each group was compared using Student T-test or one-way ANOVA with Tukey post hoc comparison.

1.6 HIV-Nef Protein induces pulmonary endothelial cell apoptosis in an EMAPII-dependent fashion

We have previously shown that HIV-Nef protein can independently mediate its transfer from T cells to endothelial cells. We also found HIV-Nef transfer is necessary and sufficient to induce endothelial cell ROS production and apoptosis [177]. To address the role of EMAPII surface expression in HIV-induced endothelial cell apoptosis, we observed a significant increase in HMVEC apoptosis when co-cultured with HIV-NL43-infected Jurkat T cells **(Fig.29)** which was abolished in cells treated with anti-EMAPII antibodies. Previously, we have shown that HIV induced Human Coronary Artery Endothelial Cell apoptosis was HIV-Nef dependent [177]. Supporting this finding, HIV induced HMVEC apoptosis was also HIV-Nef dependent. Jurkat cells infected with HIV-NL4-3 mutant with defective Nef (NL4-3 ΔNef) had no significant upregulation of HMVEC apoptosis compared to uninfected Jurkat cells **(Fig.29)**.

To examine the role of EMAPII in HIV-Nef-induced endothelial apoptosis, we analyzed endothelial cell apoptosis in the co-culture model of Nef-ER-expressing SupT1 cells (Nef-ER) and HMVECs. Co-incubation with 4-hydro-tamoxifen (4-HT) activated Nef-ER increased endothelial cell apoptosis as measured by TUNEL staining ($p<0.05$) **(Fig.30)** and promoted mitochondrial depolarization, an early marker for apoptosis as measured by JC-1 staining ($p<0.01$) **(Fig.31)**. The addition of neutralizing antibodies against EMAPII and the EMAPII-receptor (CXCR3) abolished Nef-ER cell-induced HMVEC apoptosis **(Fig.30)** and anti-EMAPII antibodies prevented HMVEC mitochondrial depolarization **(Fig.31)** indicating that EMAPII surface expression plays an important role in mediating HIV-Nef induced endothelial apoptosis. Furthermore, secreted EMAPII may potentiate HIV-Nef induced endothelial apoptosis since the

addition of recombinant EMAPII to a co-culture of Nef-ER SupT1 T cells and endothelial cells further enhanced endothelial cell apoptosis **(Fig.32)**.

Finally, when HIV-Nef positive and HIV-Nef negative EV **(Fig.19)** were added to endothelial cells, only the HIV-Nef containing EV induced dose-dependent **(Fig.33)** apoptosis in HMVECs after 24h but not after 4h **(Fig.34)**. This HIV-Nef EV-induced apoptosis was prevented with EMAPII neutralizing antibody treatment **(Fig.35)**. Taken together, these data clearly demonstrate that HIV utilizes HIV-Nef-induced and EMAP-mediated pathways to induce apoptosis in endothelial cells.

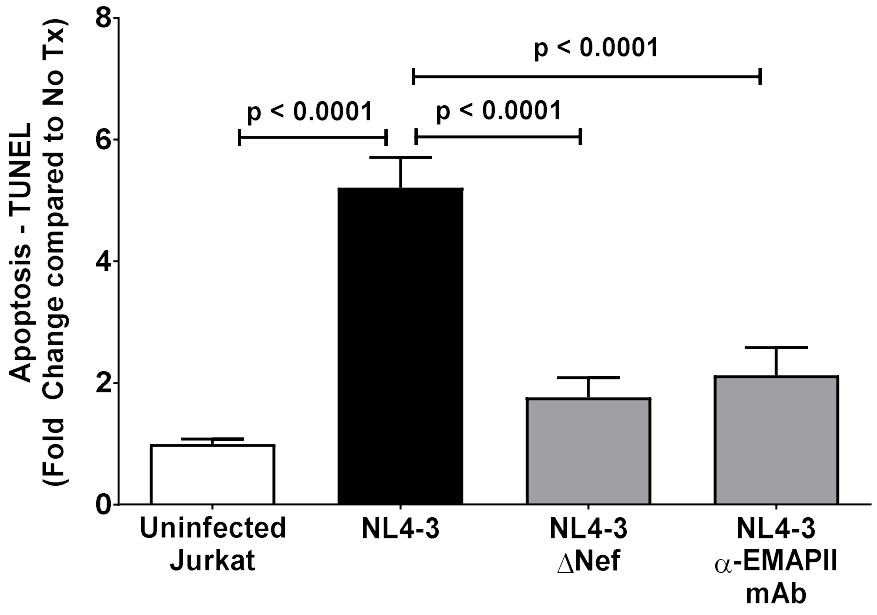

Figure 29: Apoptosis of HMVEC co-cultured with HIV NL4-3-infected Jurkat T cells with/without EMAPII neutralizing antibodies and Jurkat T cells infected with HIV-NL4-3 with defective Nef- (ΔNef) induced apoptosis, as measured by TUNEL.

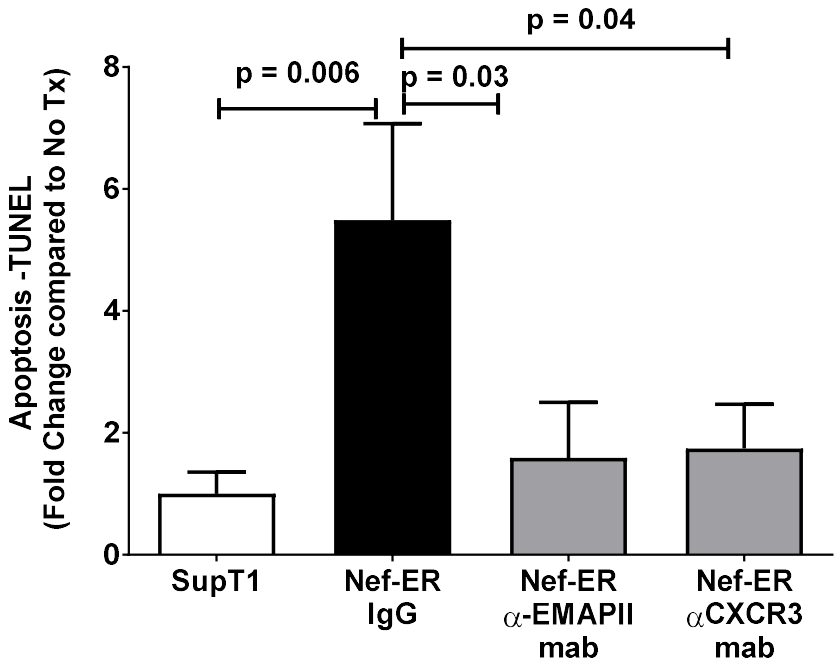

Figure 30: Co-culture of tamoxifen (4-HT) activated Nef-ER-expressing SupT1 T cells (with/without EMAPII and CXCR3 neutralizing antibodies) and 4-HT activated control SupT1 T cells induce HMVEC apoptosis as measured by TUNEL staining.

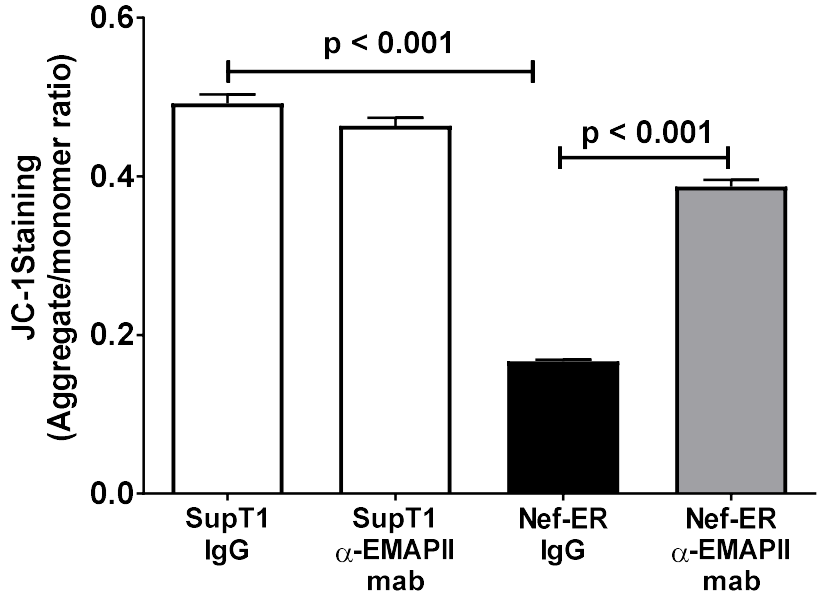

Figure 31: Confirmation of apoptosis staining through TUNEL by JC-1 staining, which is indicative for mitochondrial function. Note the decreased staining of JC-1 after exposure to 4-HT activated Nef-ER SupT1 cells.

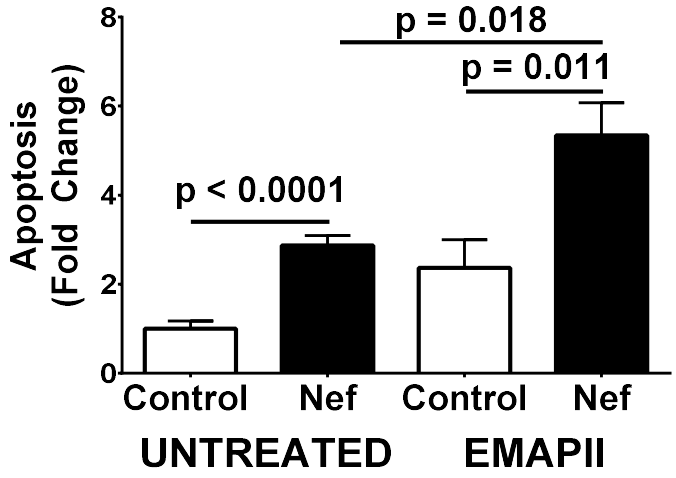

Figure 32: Co-culture of Nef expressing T cells induced increased endothelial cell apoptosis which was comparable to the amount of endothelial cell apoptosis induced by control T cells with the addition of 10μg/ml recombinant EMAPII. The addition of Nef expressing T cells and recEMAPII induced a synergistic increase in endothelial cell apoptosis suggesting that EMAPII primes endothelial cells for Nef induced apoptosis.

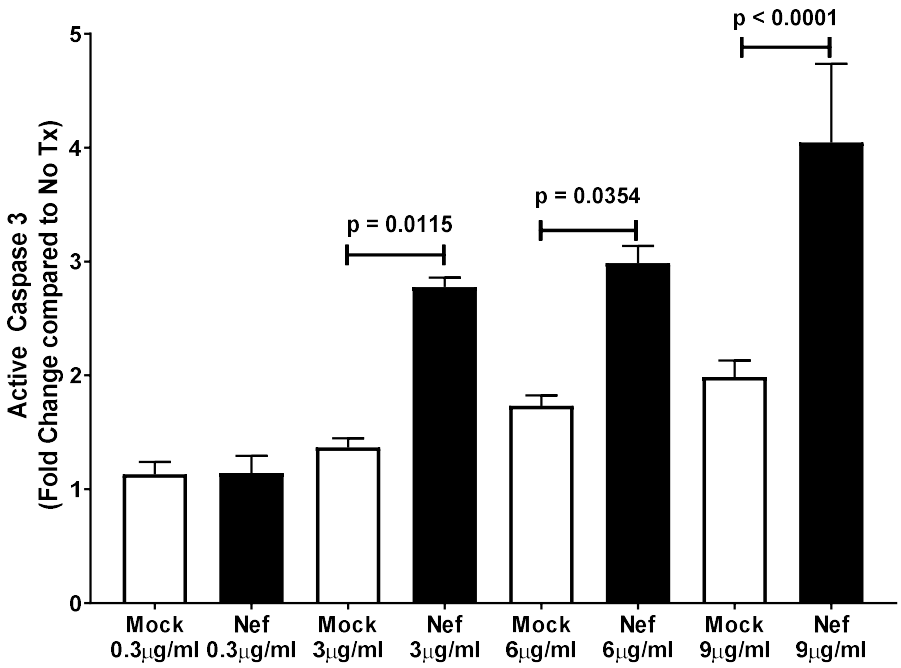

Figure 33: Apoptosis was measured in HMVEC after the addition of EV from supernatant of Mock, WT Nef or Nef F195R (PAK2 activation mutant) transfected HEK293T. Dose response of EV addition on Caspase 3 activity in HMVEC at 24hr.

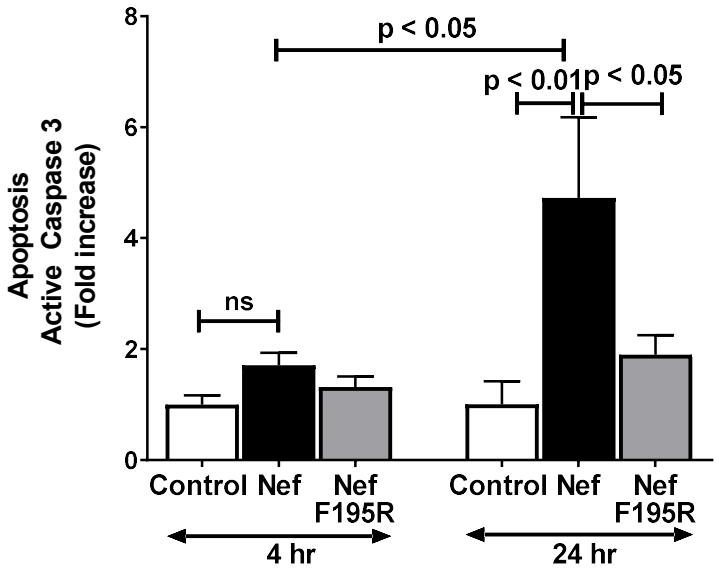

Figure 34: Apoptosis was measured in HMVEC after the addition of EV from supernatant of Mock, WT Nef or Nef F195R (PAK2 activation mutant) transfected HEK293T. Caspase 3 activity was measured at 4hr and 24hr after the addition of 3µg/ml EV.

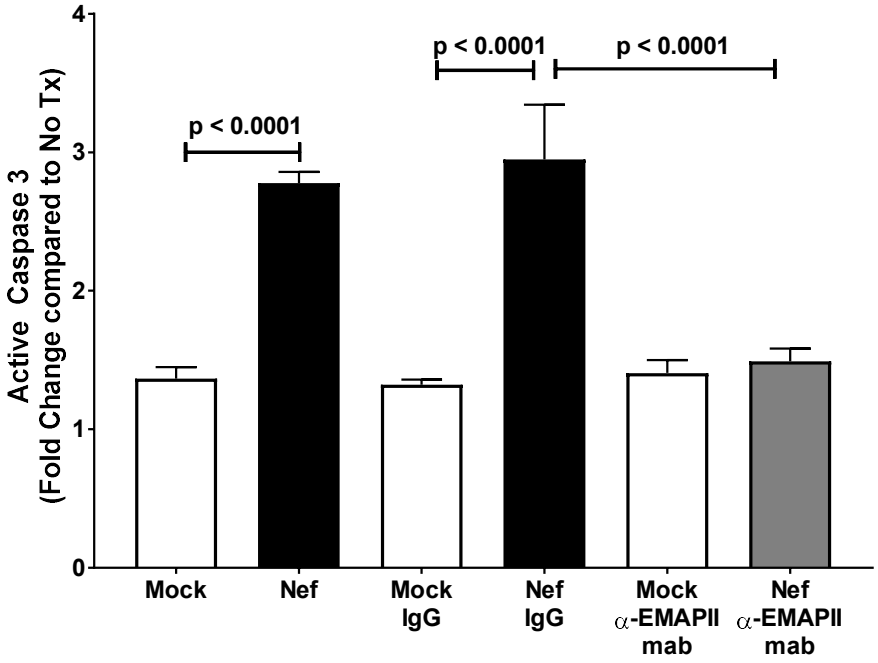

Figure 35: Effects on HMVEC apoptosis (determined by staining for active caspase 3) after addition of HIV-Nef-containing extracellular vesicles from HEK 293T cells transfected with wt HIV-Nef plasmid or HIV- Nef F195R mutant.

1.7 Endothelial HIV-Nef expression in transgenic mice induces pulmonary pathology

To test whether Nef transfer to the endothelium is sufficient to cause pulmonary pathologies, we analyzed mice in which Nef was specifically expressed in the endothelium under control of VE-cadherin promoter. At the age of 4 month, Nef-expressing mice did not manifest significant changes in weight comparing to their Nef-negative WT littermates (data not shown). However, pulse oximetry showed statistically significant reduction in blood oxygenation ($p = 0.016$) in VE-Cadherin-Nef double transgenic mice **(Fig.36)**. PFT analysis of 5 month-old animals showed significant increase in inspiratory capacity measured using both deep inflation maneuver (grey bars) and extrapolated from PV loop (red bars) **(Fig.37)** indicating lung tissue rarefaction [179]. Indeed, stereological analysis demonstrated a decrease in alveoli numbers in HIV-Nef transgenic animals compared to their Nef-negative WT littermates **(Fig.38)**. Importantly, endothelial cells from lungs of Nef-expressing mice manifested higher percentage of cleaved caspase 3- positive cells **(Fig.39)**, suggesting a possible role of Nef-induced apoptosis in alveolar loss. Interestingly, this effect was abrogated upon two-time intraperitoneal injection of 10μg/ml EMAPII neutralizing antibody, M7/1.However, we failed to observe significant changes in wet/dry lung ratio, WBC or protein infiltration in lung **(Fig.40)**. These data highlight the notion that HIV-Nef causes EMAPII mediated endothelial apoptosis, followed by lung tissue rarefaction and are in line with our previous findings in lungs of EMAPII transgenic mice [142].

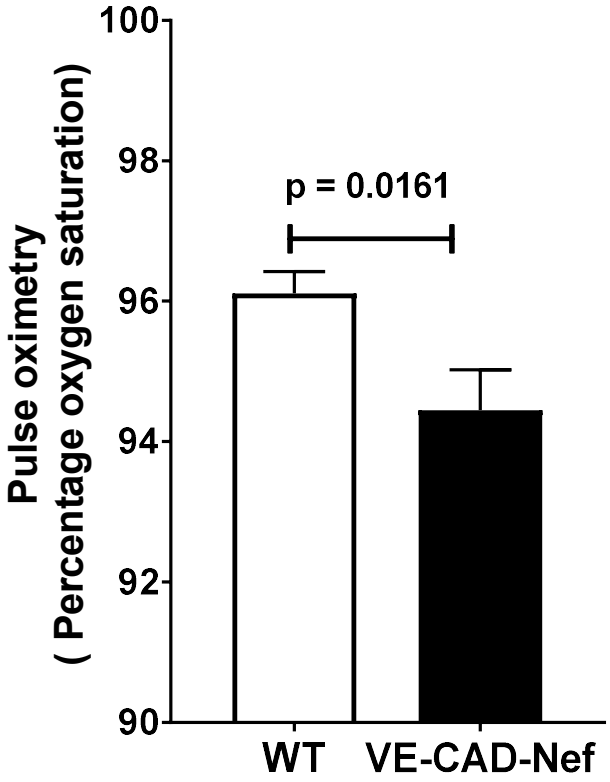

Figure 36: Oximetry showed reduced oxygenation in the blood of HIV-Nef transgenic mice.

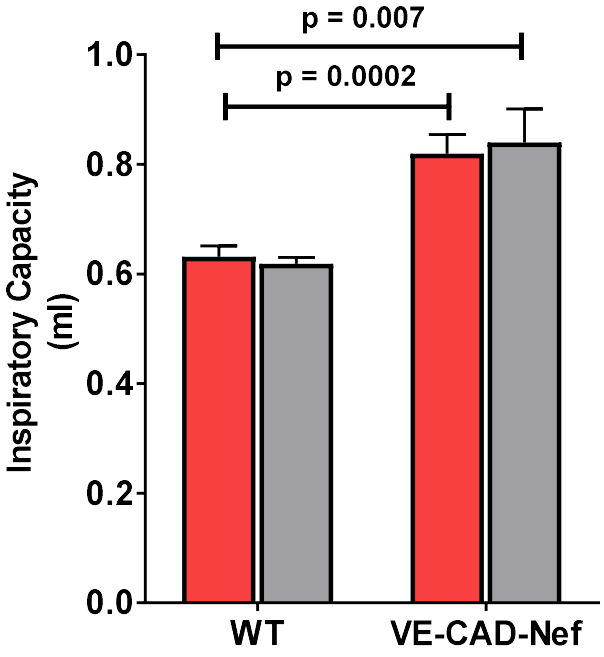

Figure 37: Inspiratory capacity was quantified using deep inflation maneuver (grey bars) and extrapolated from PV loop (black bars) using Flexivent data analysis.

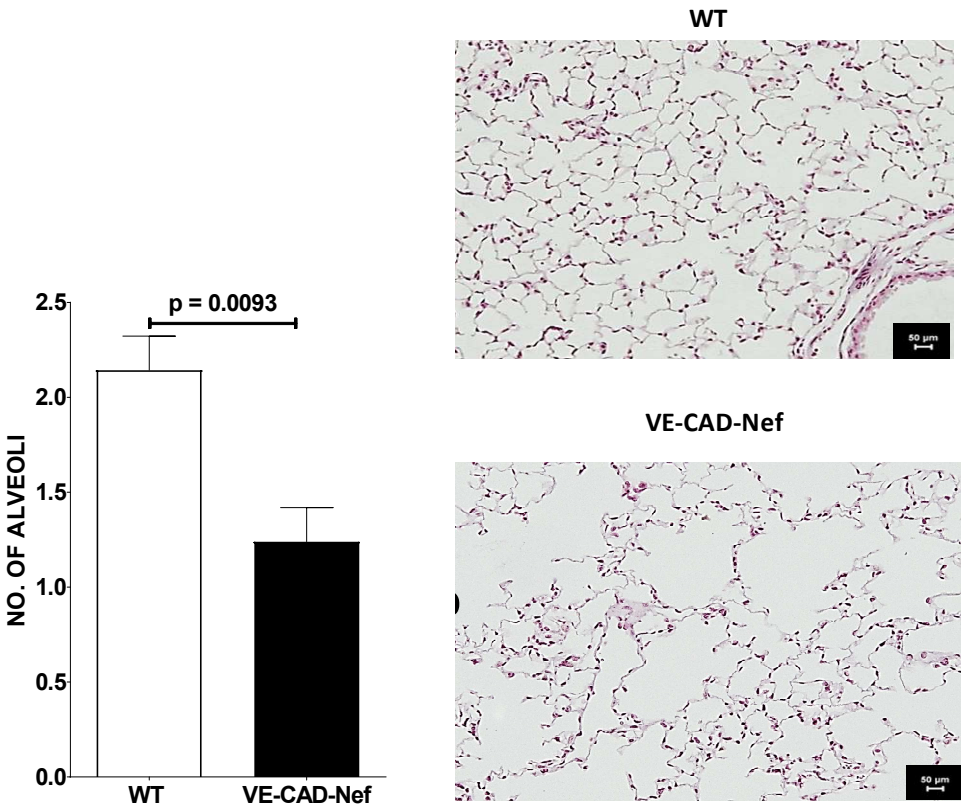

Figure 38: Stereological analysis revealed corresponding reduction in alveoli numbers VE-Cad-Nef expressing lungs compared to wildtype (WT) littermates. Representative H&E staining of VE-Cadherin Nef Transgenic mice and their WT littermates showing alveolar rarefaction characteristic of an emphysema like phenotype.

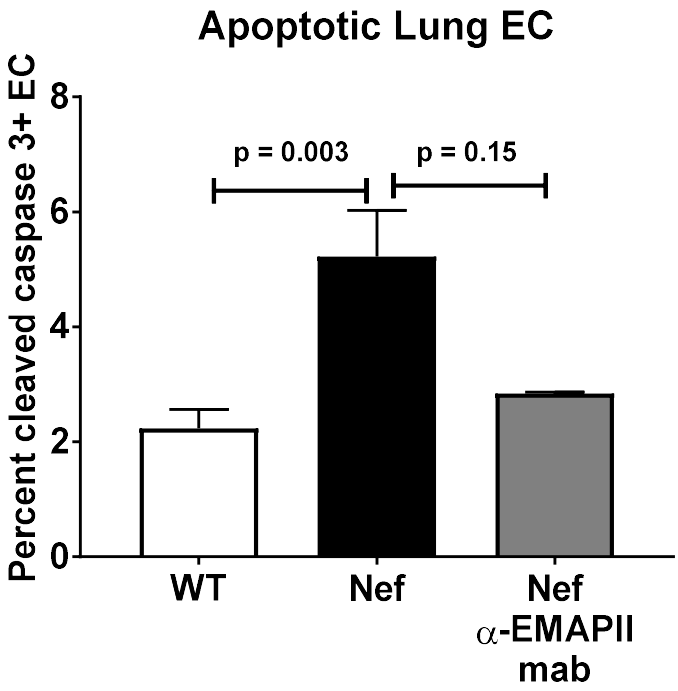

Figure 39: Cleaved caspase-3 staining in CD45-/CD326/CD31+ cells from lungs in Nef transgenic mice with sham treatment or after 2x i.p injection of anti-EMAPII neutralizing antibody 72h and 24h prior to sacrifice.

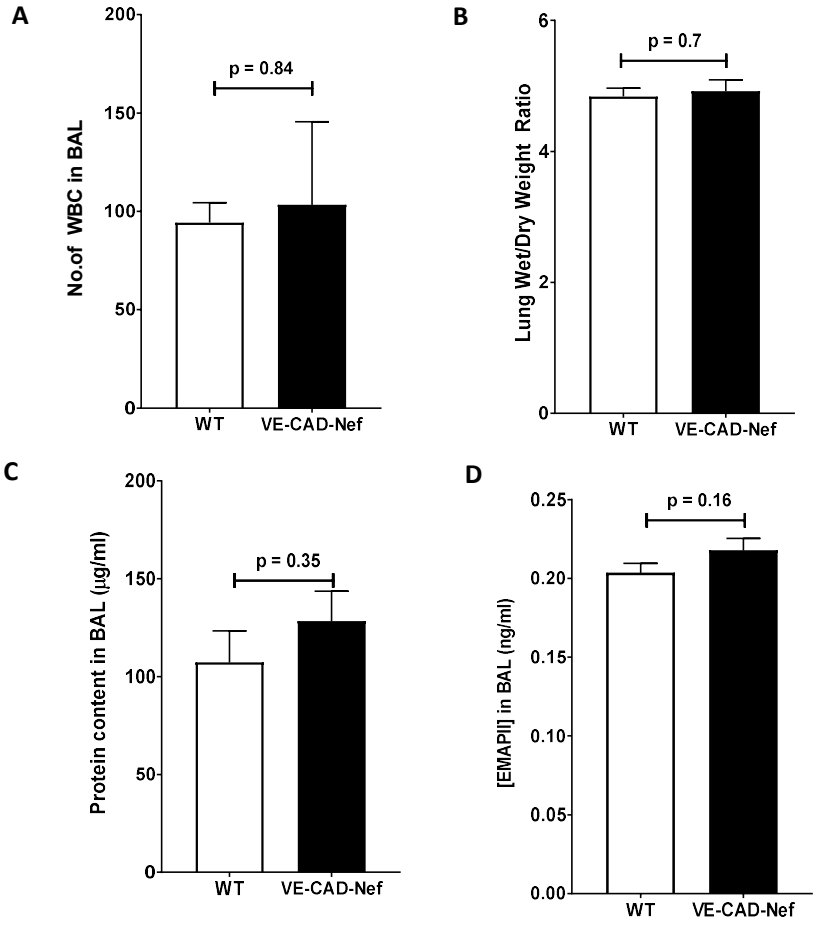

Figure 40: BAL from Nef transgenic mice and their WT littermates were analyzed for (A) number of WBC, (C) protein content using BCA and (D) EMAPII using anti-EMAPII ELISA (MyBioSource). (B) Lung edema was quantified using wet/dry weight measurements of lung.

2 HIV-Nef induces endothelial dysfunction in a Rac1 activation dependent manner: Implications for statin treatment to protein HIV-associated cardiovascular diseases

2.1 PBMC of HIV+ patients on ART are HIV-Nef positive and trigger endothelial apoptosis.

HIV-Nef protein has been shown to persist in the circulation of HIV patients on ART with undetectable viral loads both intracellularly [177] and in EV [50]. However, the high rate of mutation of the HIV virus makes it challenging to accurately quantify the amount of HIV-Nef protein. Here we used a novel approach where three antibodies (SN20, EH1 and 3D12) targeting different Nef epitopes were used in concert to detect intracellular HIV-Nef protein **(Fig.5)**. Analysis of PBMC from fifteen HIV patients on ART and four untreated HIV patients revealed that HIV-Nef protein persisted in 3 out of 4 treatment-naïve and 9 out of 10 ART-treated HIV patients **(Fig.41)**. Importantly, analysis of 13 healthy controls revealed undetectable levels of HIV-Nef supporting the specificity of our assays.

In our previously published work [177], we have shown that HIV-Nef protein is necessary and sufficient to cause HIV-induced endothelial cell apoptosis. Since HIV-Nef expression in analyzed PBMC was not affected by ART therapy, we wanted to determine if HIV-Nef positive PBMC will effectively trigger endothelial apoptosis. Added to HCAEC, PBMC from HIV patients induced increased levels of active caspase 3 **(Fig.42)** and changes in the EC mitochondrial membrane potential **(Fig.43)** when compared to control PBMC.

Since Nef protein was also identified in extracellular vesicles isolated from plasma of treatment naïve HIV patient, HIV+ patients on ART with undetectable viremia and HIV+ elite controllers with undetectable viral load in blood [50], we wanted to test if the extracellular vesicle compartment would be capable of inducing HCAEC apoptosis. Using active caspase 3 signaling **(Fig.44)** and JC-1 staining to measure mitochondrial depolarization **(Fig.45)**, we show that only the EV fraction and not the EV depleted plasma fraction is capable of inducing HCAEC apoptosis. In line with our identification of Nef protein persistence in both ART treated and untreated patients, EV from both ART treated and treatment naive HIV patients induced HCAEC apoptosis.

Taken together, our data suggests that HIV-Nef persists at a higher frequency in PBMC of HIV patients than previously estimated and can cause endothelial cell apoptosis. Furthermore, the EV fraction of HIV patient blood is also capable of inducing endothelial cell apoptosis.

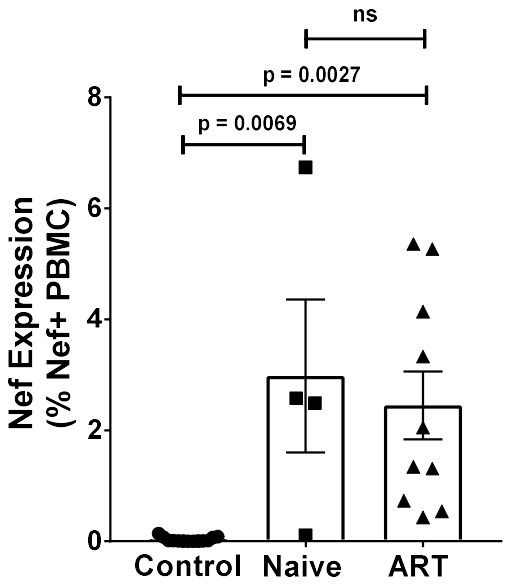

Figure 41: HIV-Nef protein persists in the circulation of HIV patients on ART and induces coronary artery endothelial cell apoptosis. Nef protein persists in PBMCs of HIV patients in both treatment naïve (n=4, square) and ART (n=10, triangle) as measured using FACS.

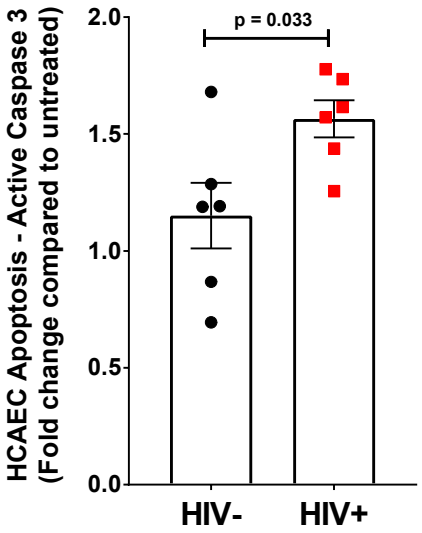

Figure 42: Addition of PBMCs from HIV patients to Human Coronary Artery Endothelial Cells (HCAEC) induced endothelial cell apoptosis as measured by Active caspase 3 levels.

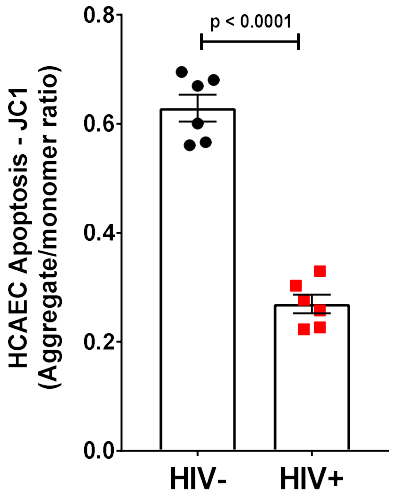

Figure 43: Addition of PBMCs from HIV patients to Human Coronary Artery Endothelial Cells (HCAEC) induced endothelial cell apoptosis as measured by mitochondrial depolarization using JC-1 staining.

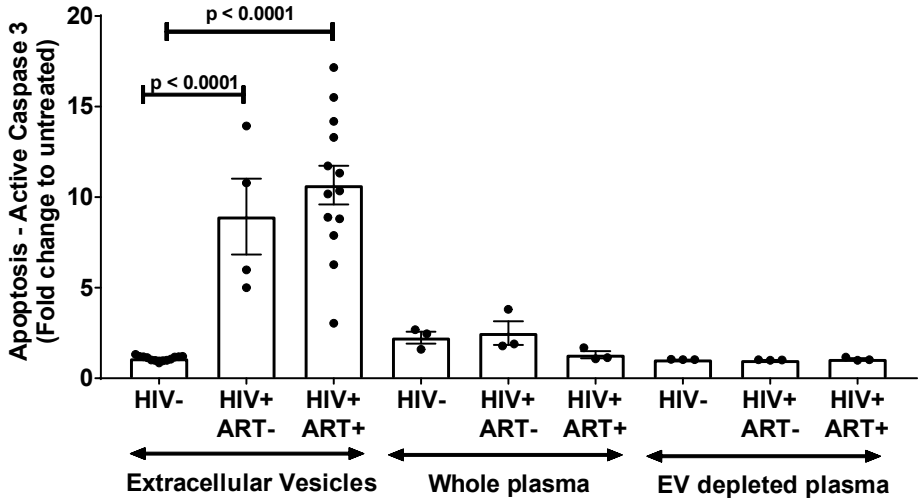

Figure 44: Addition of extracellular vesicles from 50µl plasma added to 100µl EGM2MV and 10µl of either whole plasma or EV depleted plasma to Human Coronary Artery Endothelial Cells (HCAEC) induced endothelial cell apoptosis as measured by Active caspase 3 levels.

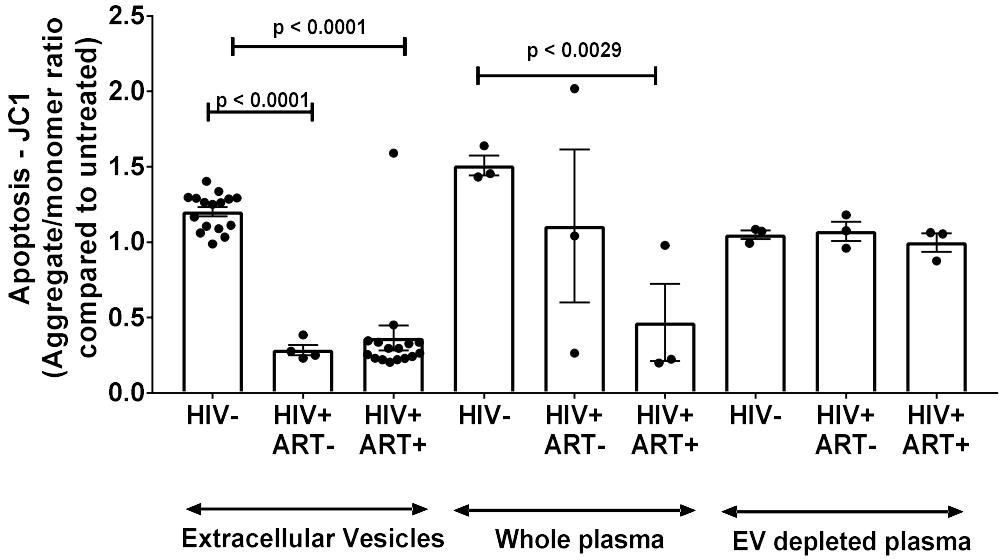

Figure 45: Addition of extracellular vesicles from 50µl plasma added to 100µl EGM2MV and 10µl of either whole plasma or EV depleted plasma to Human Coronary Artery Endothelial Cells (HCAEC) induced endothelial cell apoptosis as measured by mitochondrial depolarization using JC-1 staining.

2.2 HIV-Nef alters T cell adhesion to endothelial cells

We next characterized whether HIV-Nef could modulate T cell interaction with endothelial cells. Using microscopy, we quantified the amount of calcein AM-stained T cells adhered to an endothelial monolayer. SupT1 T cells expressing HIV-Nef showed increased adhesion to HCAEC monolayer compared to control T cells **(Fig.46)**. HIV-Nef is known to activate the small GTPase Rac 1, a regulator of actin remodeling and T cell adhesion [reference]. To elucidate the role of Rac1 in HIV-Nef-mediated adhesion, we blocked Rac1 activation using two different strategies; first using a Rac-1 GEF interaction inhibitor, NSC23766, and second by using Atorvastatin's pleiotropic capacity to inhibit Rac 1 geranylgeranylation. **(Fig.46)** shows that both inhibition strategies negated the ability of HIV-Nef to increase T cell adhesion to endothelial cells.

We further confirmed our findings in experiments when adhesion of T cell to endothelial monolayer was studied under physiological flow conditions **(Fig.47)**. Similar to static conditions, Nef-expressing T cells displayed increased attachment to endothelial monolayer compared to control T-cells; this increase was completely suppressed in the presence of Atorvastatin.

Altogether, these data demonstrate that HIV-Nef expression promotes T cell adhesion to endothelial cells in a Rac 1-dependent fashion.

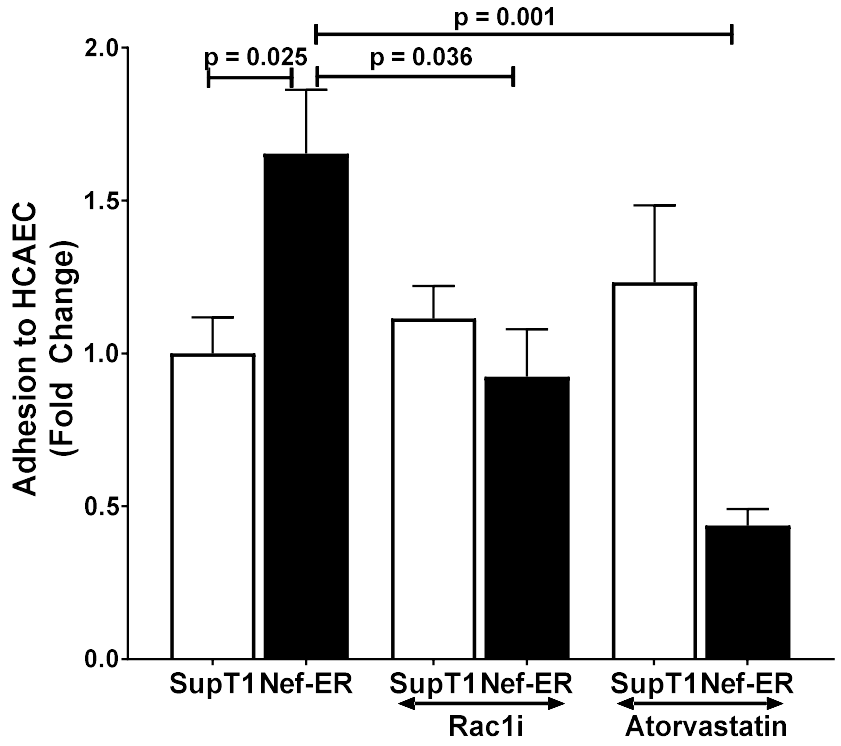

Figure 46: Compared to control SupT1 T cells (SupT1), Nef expressing T cells (Nef-ER) displayed increased adhesion to HCAEC under static conditions in the presence/absence of Rac1 inhibitor - NSC23766 or Atorvastatin.

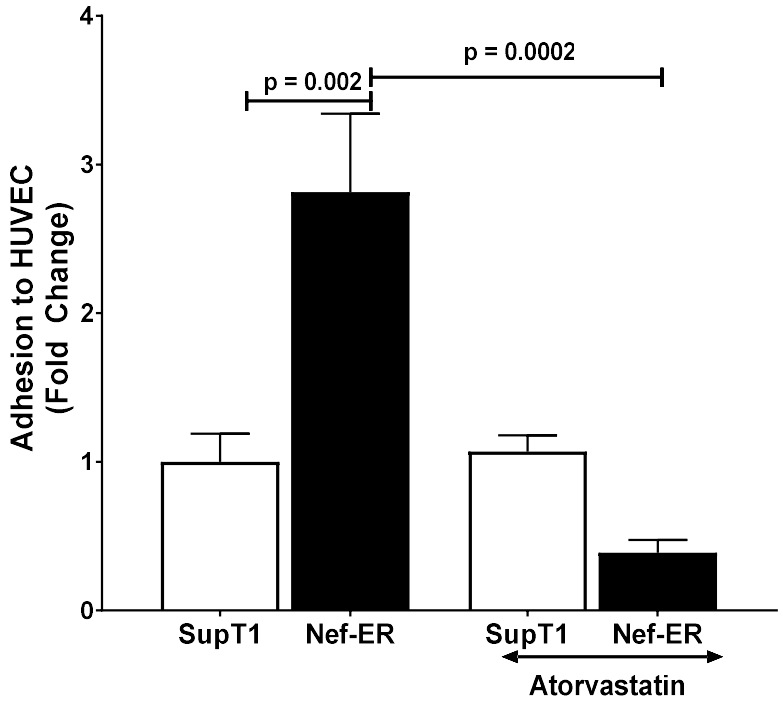

Figure 47: Compared to control SupT1 T cells (SupT1), Nef expressing T cells (Nef-ER) displayed increased adhesion to HCAEC under static conditions in the presence/absence of Atorvastatin.

2.3 HIV-Nef facilitates cytoplasmic transfer to endothelial cells

To further characterize how HIV-Nef affects T cell interaction with endothelial cells, we studied cytoplasmic transfer between HIV-Nef expressing T cells and HCAEC. Using microscopy, we quantified the amount of Cell Tracker Deep Red Dye transferred from T cells to endothelium.

Fig.48 and **Fig.49** show that cytoplasmic dye transfer to endothelial cells increased in a fashion dependent on the amount of added T cells; signal was invariably higher in monolayers interacting with Nef-expressing T cells when compared to control T cells. This HIV-Nef-induced increase of T cell cytoplasmic transfer was attenuated by both Rac1 inhibitor and Atorvastatin **(Fig.50)**. To confirm that HIV-Nef mediates its own transfer by facilitating cytoplasmic transfer from T cells, we assessed HIV-Nef levels in endothelium. As expected, HIV-Nef was detected in endothelium interacting with HIV-Nef-expressing T cells; Rac 1 inhibitor as well as Pitavastatin and Atorvastatin significantly attenuated levels of HIV-Nef transferred to endothelium **(Fig.51)**.

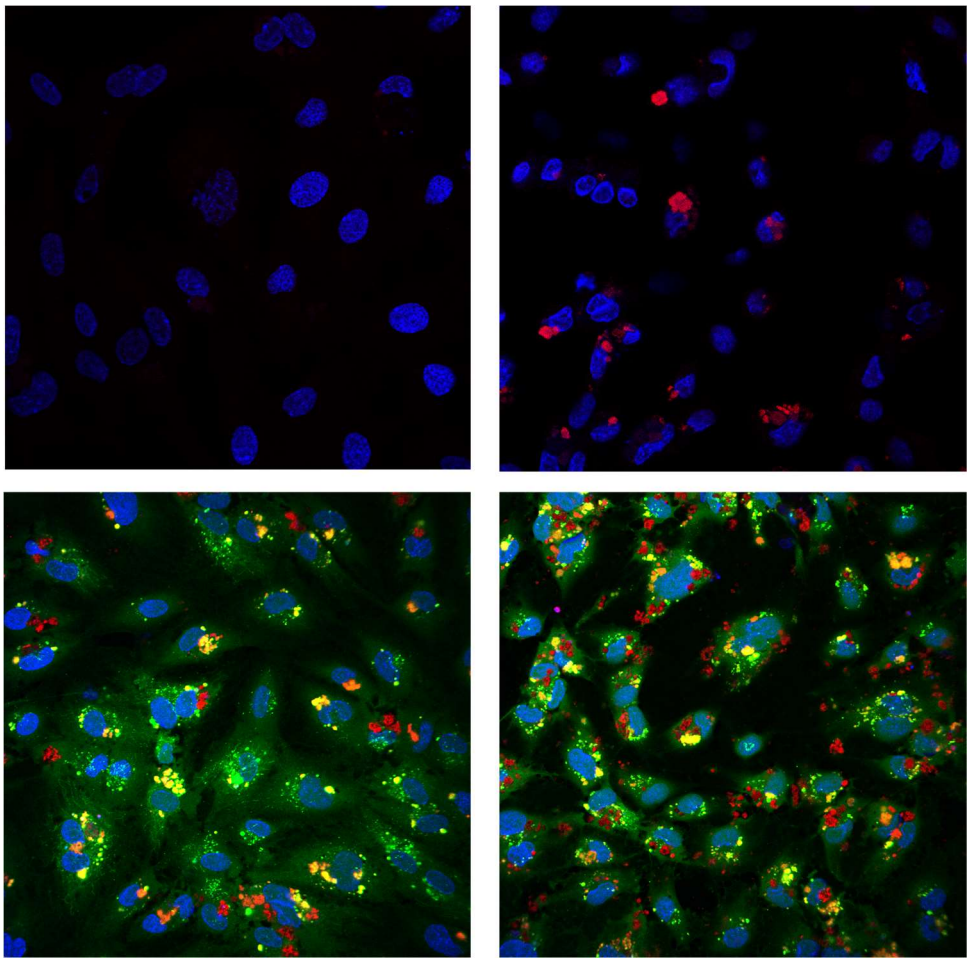

Figure 48: SupT1 (Left panels) and Nef-ER T cells (Right panels) were co-cultured with HCAEC. Nef protein transfer to HCAEC is identified using anti-Nef EH1 mAB (Top panels). Cytoplasmic transfer of Cell Tracker Deep Red Dye from T cells is measured in Calcein AM stained endothelial cells (bottom panel).

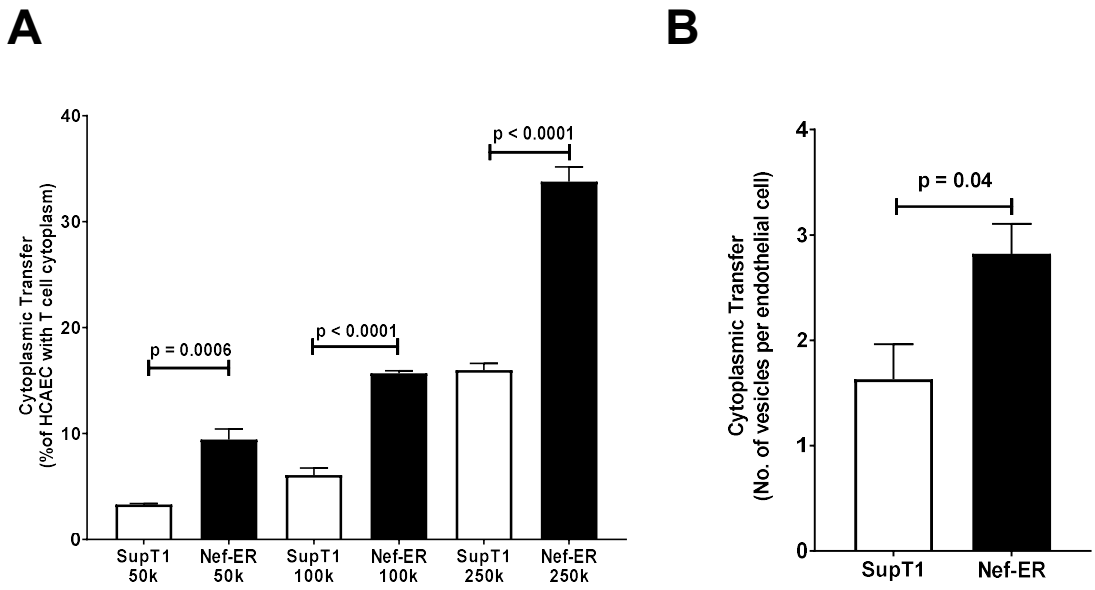

Figure 49: Nef expression enhanced cytoplasmic transfer from T cells to endothelial cells. Quantification was done using flow cytometry (A) expressed as percent of coronary artery endothelial cells double positive for Calcein AM and Cell Tracker Deep Red dye and confocal microscopy (B) followed by determination of no. of red vesicles per HCAEC cell using IMARIS imaging software.

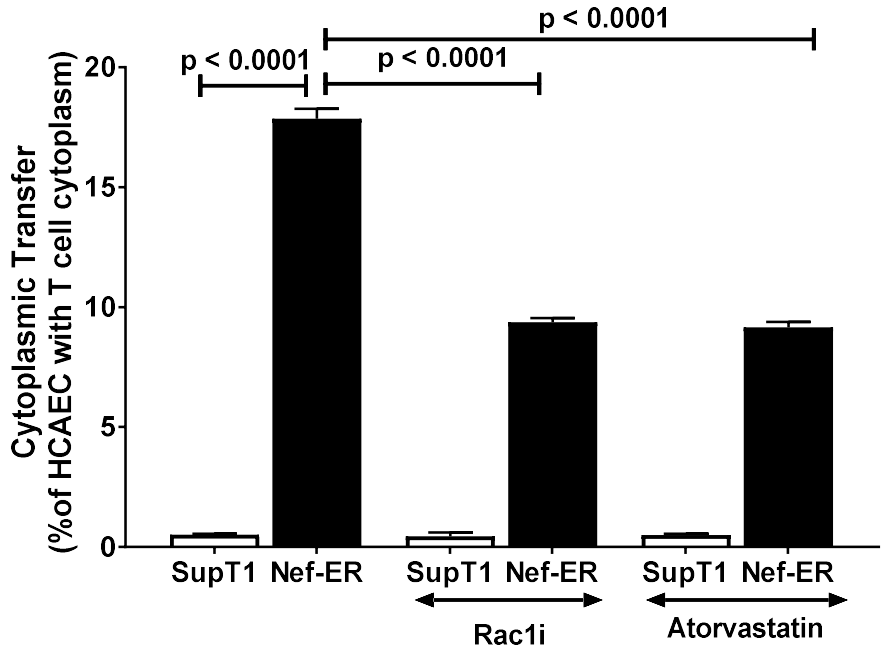

Figure 50: Nef expression enhanced cytoplasmic transfer from T cells to endothelial cells expressed as percent of coronary artery endothelial cells double positive for Calcein AM and Cell Tracker Deep Red dye indicating cytoplasmic transfer from T cells. Cells were treated with Rac1 inhibitor NSC23766 or Atorvastatin.

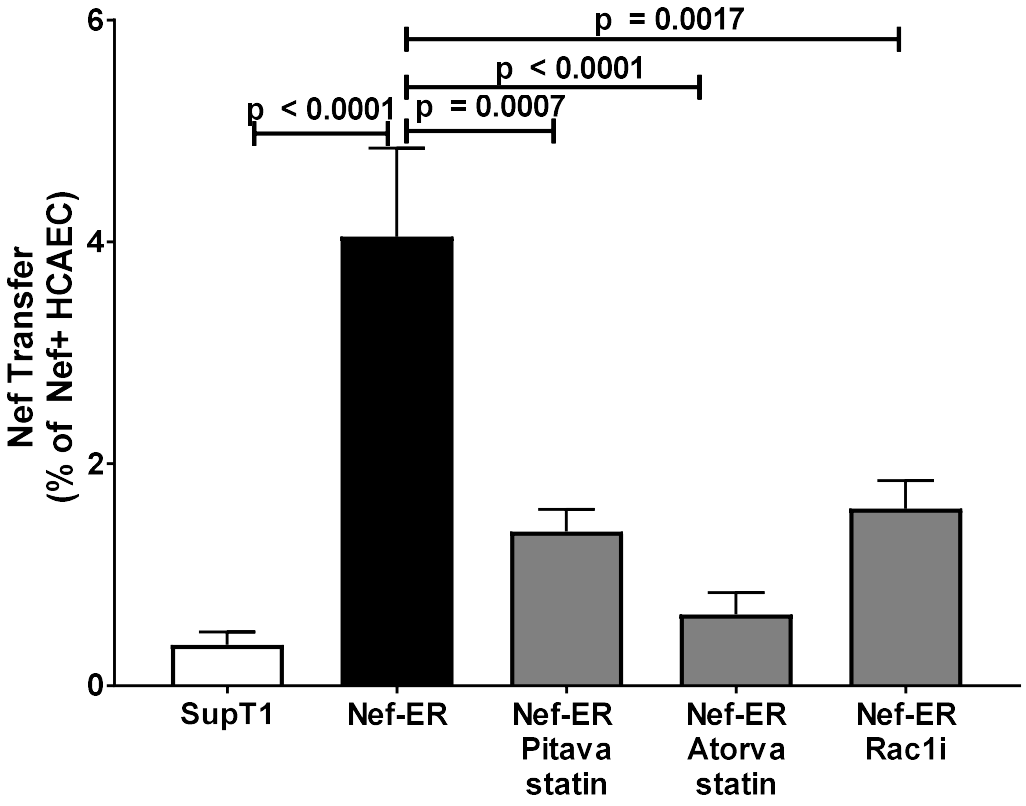

Figure 51: Nef transfer from Nef expressing T cells to endothelial cells expressed as percent of coronary artery endothelial cells containing Nef protein after 24h co-culture. Cells were treated with Rac1 inhibitor NSC23766, Pitavastatin or Atorvastatin.

2.4 HIV-Nef increase adhesion capacity of endothelial cells

We wanted to study whether exposure to HIV-Nef can influence the adhesive capacity of endothelial cells and enhance adhesion of lymphocytes to the endothelium. We found that addition of Nef EV **(Fig.52 A-D)** and co-culture with Nef-ER T cells **(Fig.53 A-D)** induces upregulation of endothelial adhesion markers - VCAM-1, ICAM, E-Selectin and P-Selectin. This HIV-Nef induced upregulation of adhesion markers subsequently enhanced adhesion of SupT1 T cells to Nef-EV treated HCAEC **(Fig.54)**. Interestingly, Rac1 inhibition with NSC23766 and Pitavastatin was capable of abrogating Nef induced endothelial cell activation and subsequently, T cell adhesion to endothelial monolayer. Nef EV pretreatment also enhanced adhesion of T cells to both microvascular (HMVEC) **(Fig.55)** and macrovascular pulmonary (Human Pulmonary Artery Endothelial Cells) **(Fig.56)** endothelial cells.

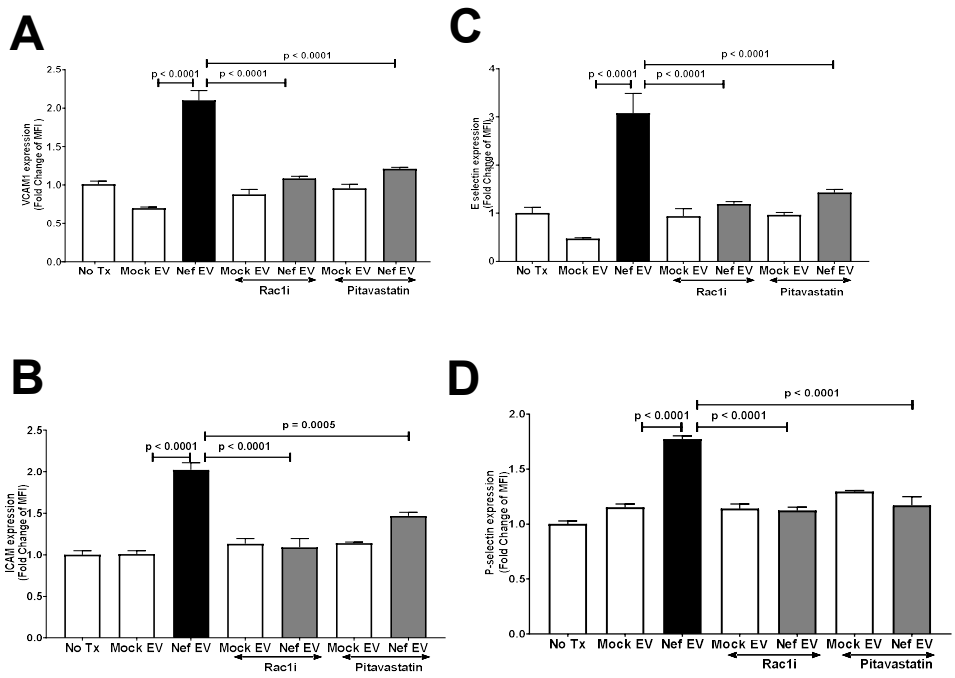

Figure 52: Addition of Nef EV to HCAEC upregulates surface expression of endothelial adhesion markers. VCAM1 (A), ICAM (B), E-Selectin (C) and P-Selectin (D) expression levels in HCAEC treated 24hr with 3µg/ml Nef EV in the presence of 5µmol/l NSC23766 (Rac1i) or 100nmol/l Pitavastatin.

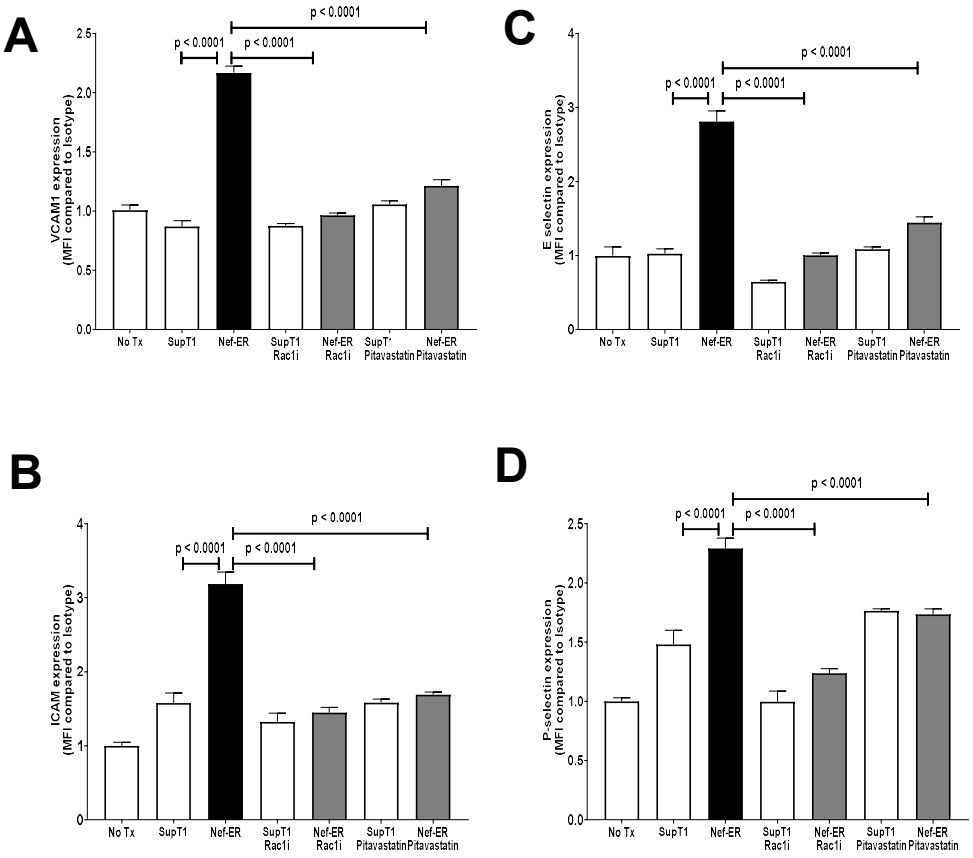

Figure 53: VCAM1 (A), ICAM (B), E-Selectin (C) and P-Selectin (D) expression levels in HCAEC co-cultured with Nef-ER T cells or control SupT1 T cells for 24h in the presence of 5µmol/l NSC23766 (Rac1i) or 100nmol/l Pitavastatin.

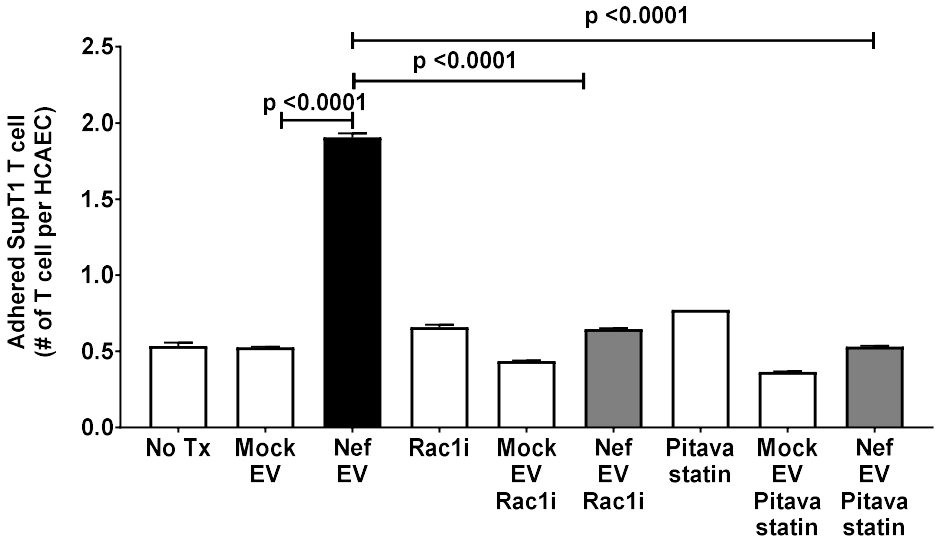

Figure 54: T cell adhesion to HCAEC pre-treated with 3µg/ml Mock/Nef EV in the presence of 5µmol/l NSC23766 (Rac1i) or 100nmol/l Pitavastatin.

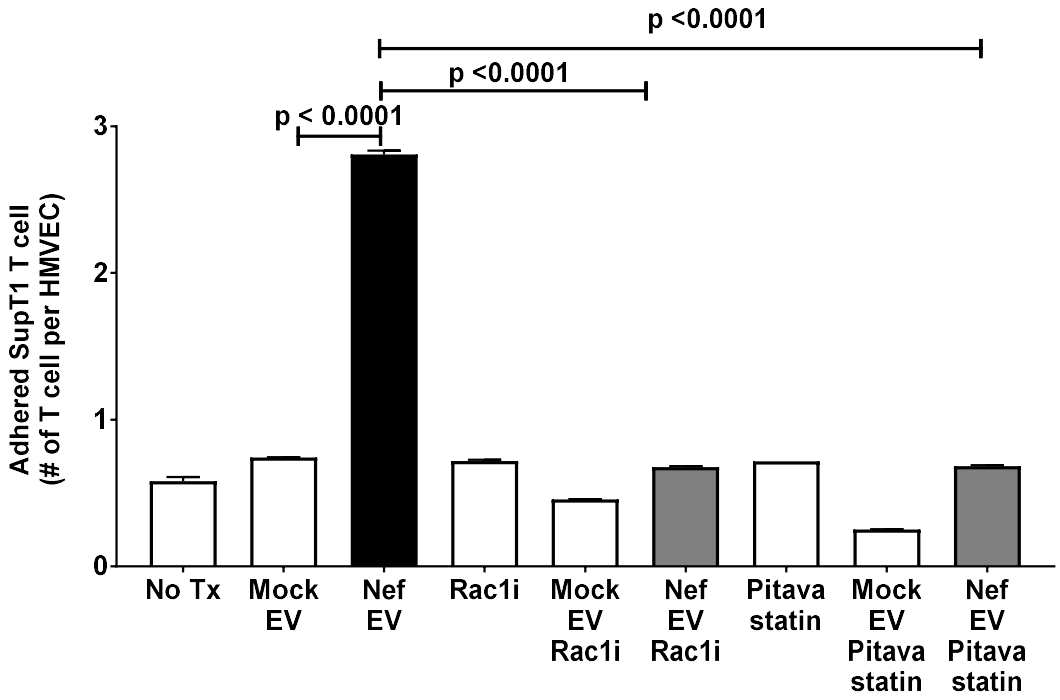

Figure 55: T cell adhesion to HMVEC pre-treated with 3µg/ml Mock/Nef EV in the presence of 5µmol/l NSC23766 (Rac1i) or 100nmol/l Pitavastatin.

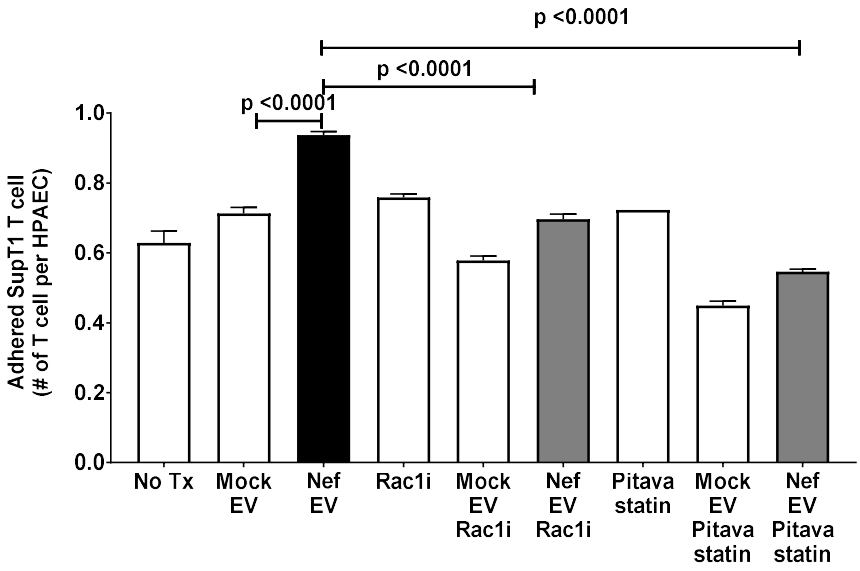

Figure 56: T cell adhesion to HPAEC pre-treated with 3µg/ml Mock/Nef EV in the presence of 5µmol/l NSC23766 (Rac1i) or 100nmol/l Pitavastatin

2.5 HIV-Nef activates Rac1-Pak2 signaling to induce NADPH Oxidase complex mediated production of Reactive Oxygen Species

Previously we have shown that HIV-Nef protein is necessary and sufficient to induce ROS production that leads to endothelial cell apoptosis. In order to further elucidate the underlying molecular mechanisms we analyzed the HIV-Nef induced Rac 1 activation previously shown to cause NADPH dependent ROS production in cells of the monocyte-macrophage lineage [108, 109]. HIV-Nef induced ROS production in HCAECs transfected with HIV-Nef expressing plasmid was mediated in a Rac 1-Pak2 pathway **(Fig.57)**. Furthermore, co-culture of Nef expressing SupT1 T cells **(Fig.58)** and addition of Nef EV **(Fig.59)** induced HCAEC ROS production which was reversed using Rac1 inhibitor NSC23766 as well dominant negative Rac T17N and Rac1 siRNA showing strong evidence for Rac1 activation being important for Nef induced endothelial stress. Nef EV utilizes the NADPH Oxidase complex for reactive oxygen species production since inhibition of the complex using GP91 inhibitory peptide, Gp91ds-tat **(Fig.60)**. Similarly, statin treatment was capable of inhibiting Nef mediated ROS production in all three models tested.

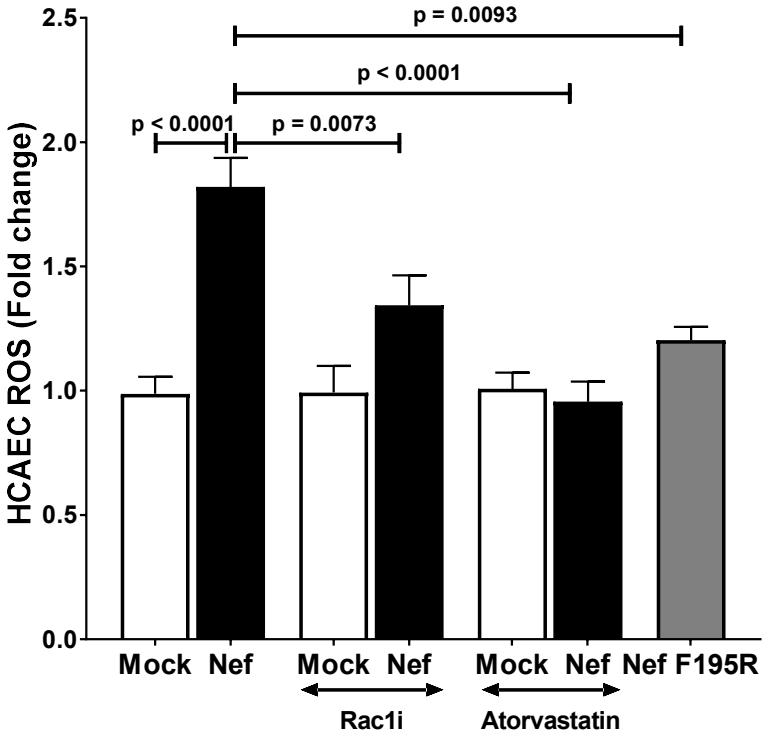

Figure 57: Transfection of HIV-Nef plasmid but not Pak2 activation mutant (Nef F195R) induced ROS production in HCAEC. Cells were cultured in the presence/absence of Rac1 inhibitor NSC23766 or Atorvastatin.

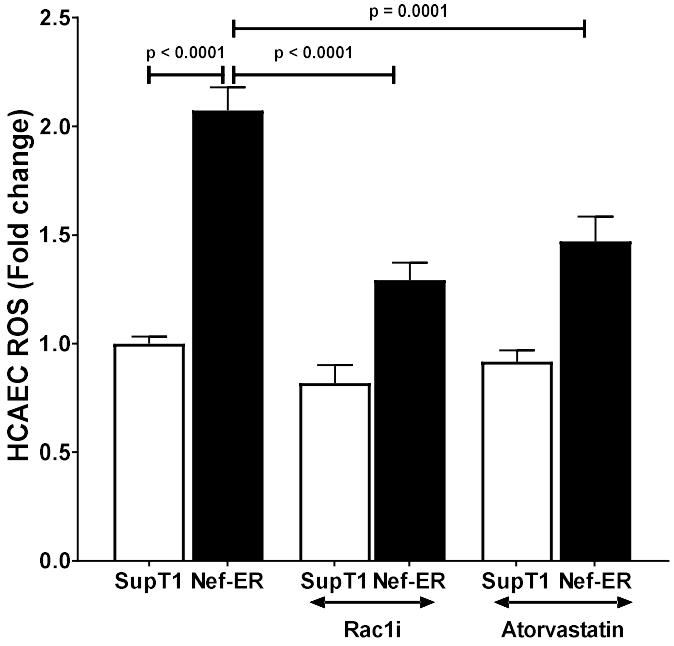

Figure 58: Co-culture of HIV-Nef expressing T cells induced ROS production in HCAEC. Cells were cultured in the presence/absence of Rac1 inhibitor NSC23766 or Atorvastatin.

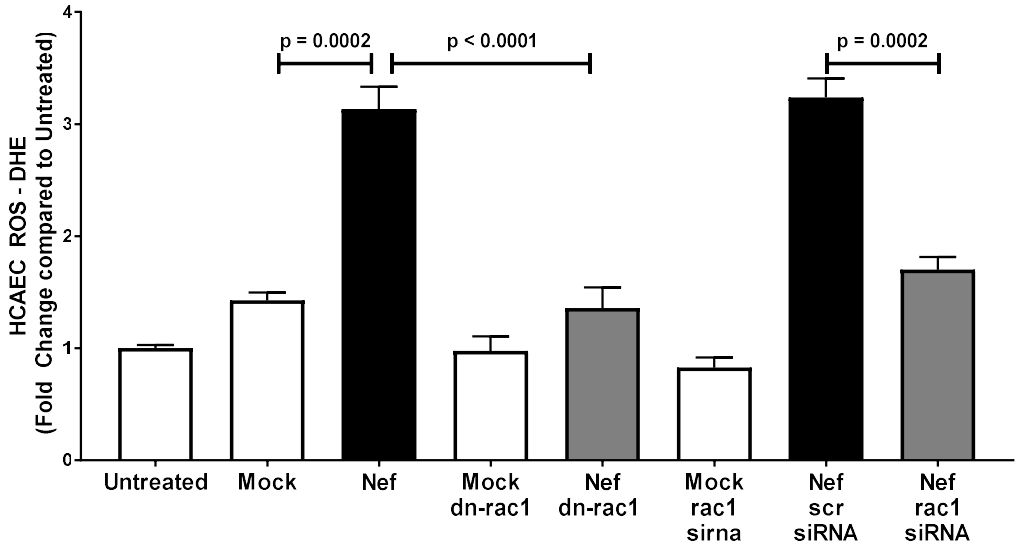

Figure 59: HCAEC ROS production after addition of Nef extracellular vesicles isolated from HEK 293T transfected with either mock, Nef cDNA or Nef cDNA plus RacT17N dominant negative. Rac1 silencing using siRNA in HCAEC concomitant with extracellular vesicle addition also blocked Nef induced ROS production.

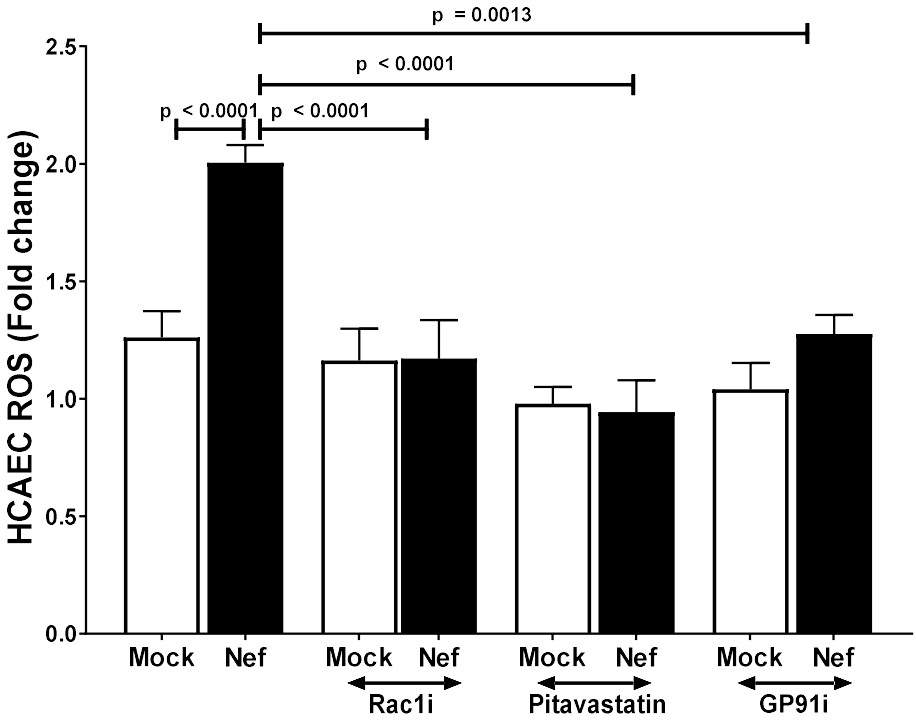

Figure 60: HCAEC ROS production after addition of Nef extracellular vesicles isolated from HEK 293T transfected with either mock or Nef cDNA. HCAEC were treated with Rac1 inhibitor-NSC23766, Pitavastatin and NADPH inhibitory peptide GP91ds-tat.

2.6 HIV-Nef protein induced endothelial cell apoptosis is dependent on Rac1 activation

As an extension to the previous finding that NADPH oxidase dependent ROS production plays an important role in Nef mediated endothelial cell apoptosis [177], we analyzed the role of Rac1 activation in mediating this effect. Using our co-culture model of Nef expressing T cells and HCAEC, we observed that Rac1 inhibition using 5μM NSC23766 and statin treatment potently blocked Nef induced HCAEC apoptosis measured using TUNEL **(Fig.61)**, active caspase 3 signaling **(Fig.62)** and JC-1 staining **(Fig.63)**. This effect was mediated by ROS production since inhibition of NADPH Oxidase complex using gp91 inhibitory peptide reversed this effect.

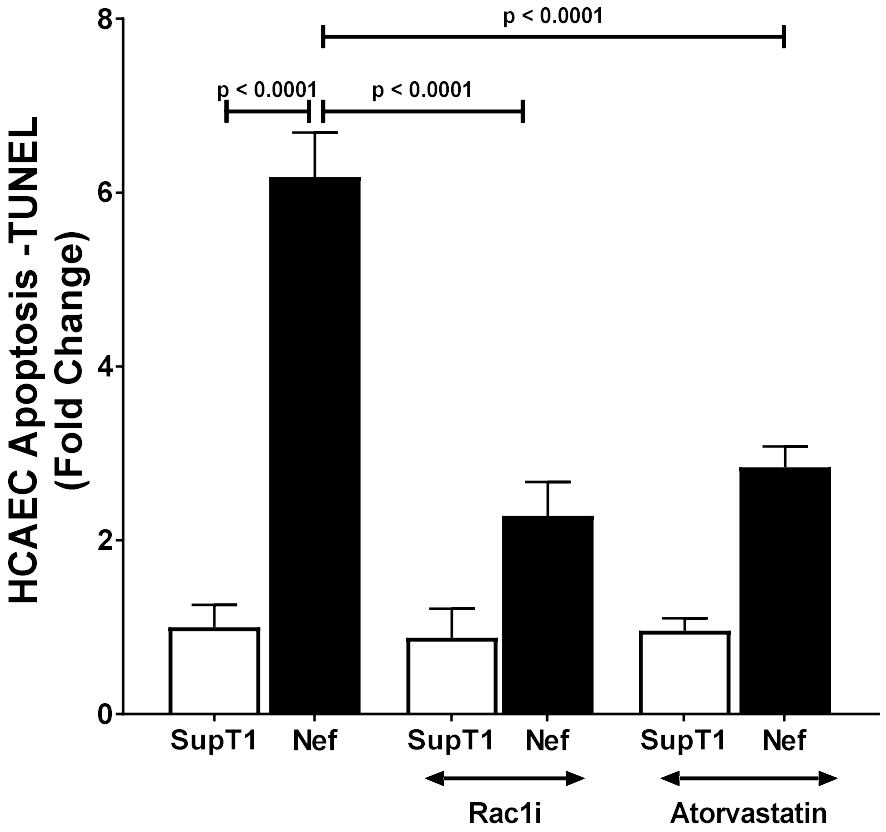

Figure 61: Co-culture of Nef expressing T cells induced HCAEC apoptosis measured using TUNEL staining. Co-culture was performed in the presence/absence of Rac1 inhibitor-NSC23766 and Atorvastatin.

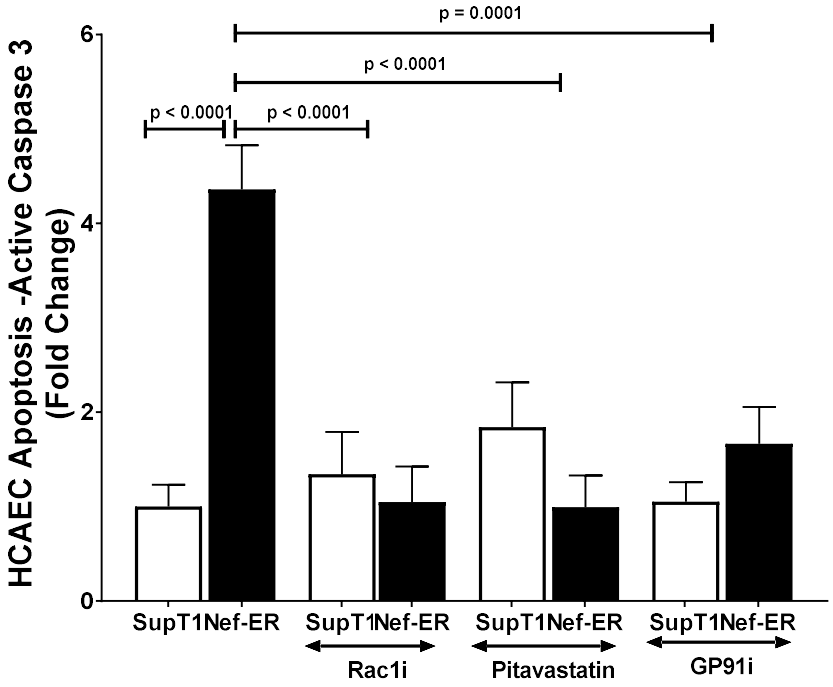

Figure 62: Co-culture of Nef expressing T cells induced HCAEC apoptosis measured using active caspase 3 signaling. Co-culture was performed in the presence/absence of Rac1 inhibitor-NSC23766, Pitavastatin and NADPH oxidase inhibition using Gp91 ds-tat.

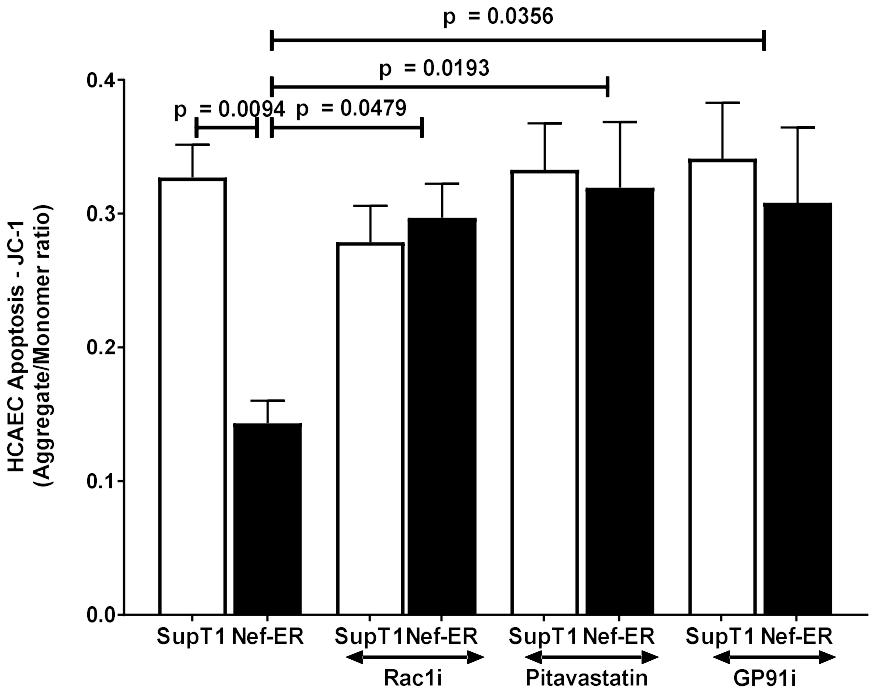

Figure 63: Co-culture of Nef expressing T cells induced HCAEC apoptosis measured using JC-1 staining to measure mitochondrial depolarization. Co-culture was performed in the presence/absence of Rac1 inhibitor-NSC23766, Pitavastatin and NADPH oxidase inhibition using Gp91 ds-tat.

2.7 Extracellular Vesicles containing Nef protein induces Rac1-Pak2 activation dependent endothelial cell apoptosis

Nef transfer via extracellular vesicles was shown to mediate this effect since addition of Nef EV also induced HCAEC apoptosis as measured using active caspase 3 signaling **(Fig.64 and 66)** and JC-1 staining **(Fig.65)**. However, extracellular vesicles isolated from HEK293T co-transfected with Nef and dominant negative Rac1, RacT17N, was incapable of inducing HCAEC apoptosis. Furthermore, Rac1 siRNA treatment **(Fig.64 and 65)** and small molecule wt inhibitor, NSC23766 **(Fig.66)** were able to ameliorate Nef EV induced HCAEC apoptosis. Similarly, Pak2 inhibition with 5μM FRAX 597 **(Fig.66)** was able to prevent Nef induced HCAEC apoptosis. Extracellular (vesicles isolated from HEK 293T transfected with Pak2 activation incapable mutant, Nef F195R was incapable of inducing HCAEC apoptosis **(Fig.67)**. Therefore, our data suggests that Nef EV uptake by HCAEC is followed by endothelial cell apoptosis. This Nef induced endothelial cell apoptosis is dependent on Nef-Rac1-Pak2 signaling axis.

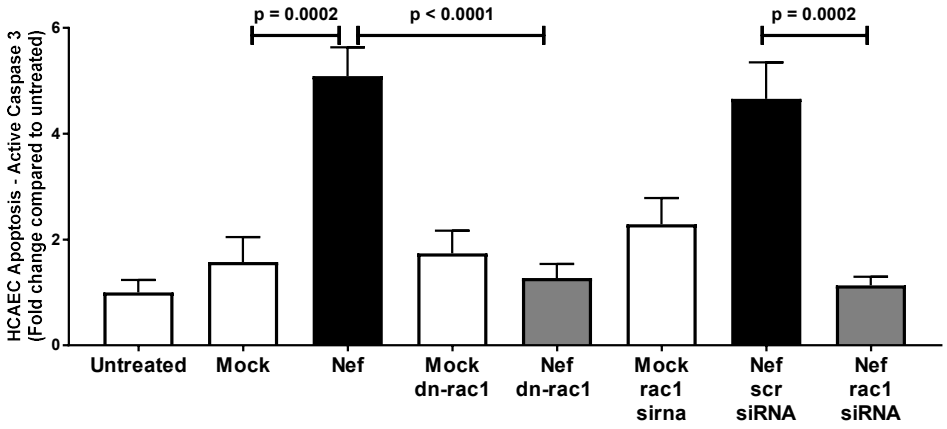

Figure 64: Active caspase 3 signaling in HCAEC after addition of Nef extracellular vesicles isolated from HEK 293T transfected with either mock, Nef cDNA or Nef cDNA plus RacT17N dominant negative. Rac1 silencing using siRNA in HCAEC concomitant with extracellular vesicle addition also blocked Nef induced apoptosis.

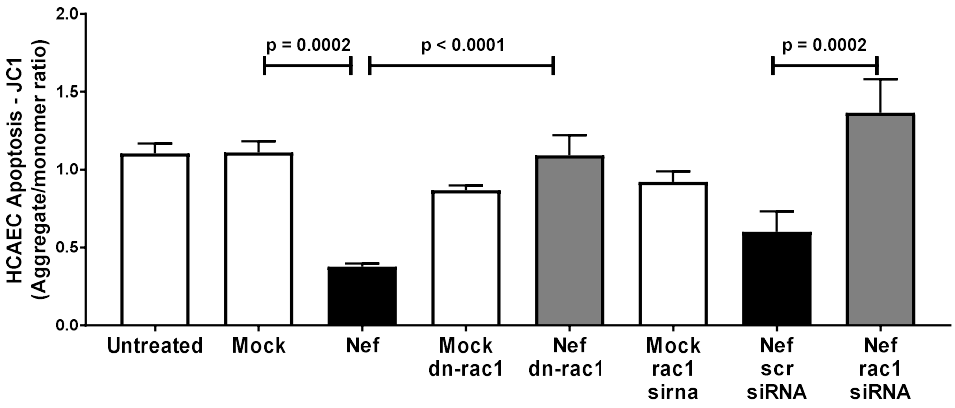

Figure 65: Mitochondrial depolarization quantified using JC-1 staining in HCAEC after addition of Nef extracellular vesicles isolated from HEK 293T transfected with either mock, Nef cDNA or Nef cDNA plus RacT17N dominant negative. Rac1 silencing using siRNA in HCAEC concomitant with extracellular vesicle addition also blocked Nef induced apoptosis.

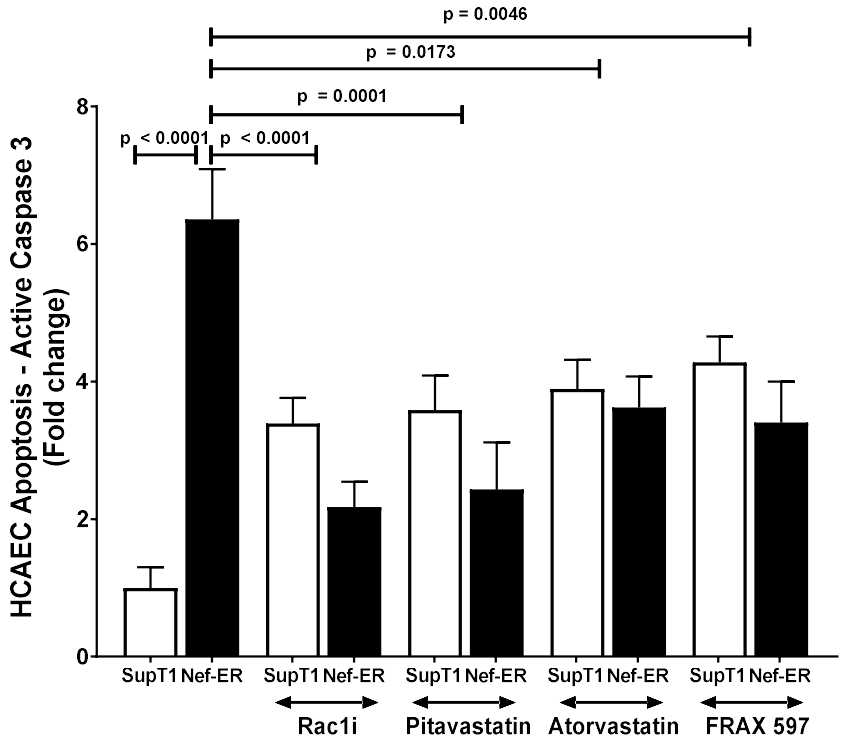

Figure 66: Apoptosis measured using active caspase 3 signaling in HCAEC after addition of Nef extracellular vesicles isolated from control T cells (SupT1) or Nef expressing T cells (Nef-ER). Nef EV induced apoptosis was blocked with Rac1 inhibition- NSC23766, Statins (Pitavastatin and Atorvastatin) and Pak2 inhibition using FRAX597.

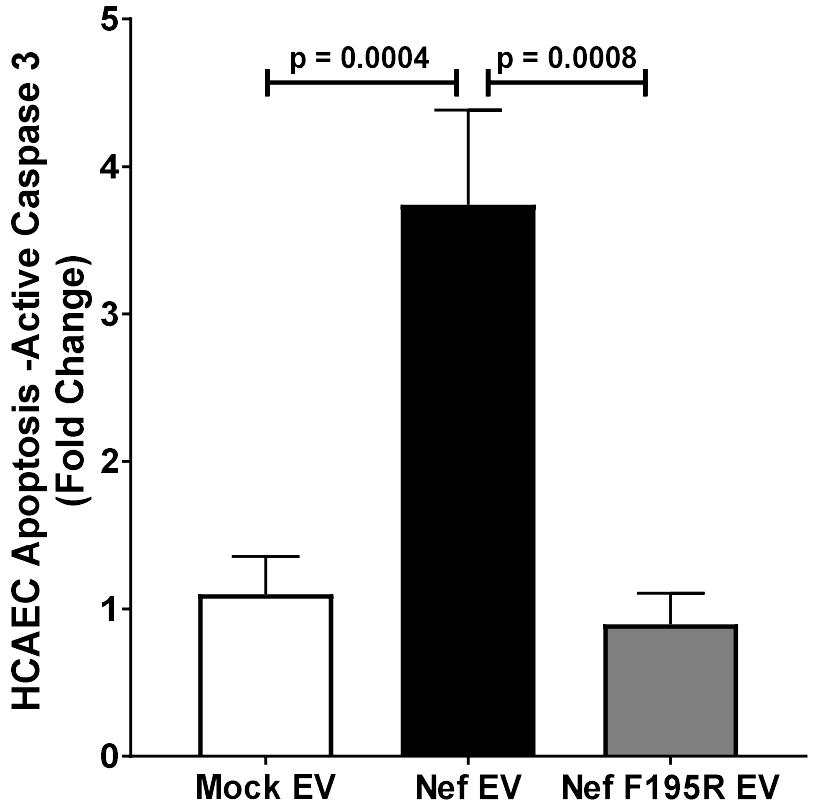

Figure 67: Apoptosis in HCAEC measured using active caspase 3 signaling in HCAEC after addition of Nef extracellular vesicles isolated from HEK 293T transfected with mock, wt Nef or Nef mutant incapable of Pak2 activation (Nef F195R).

2.8 Statin treatment alleviates HIV-Nef induced endothelial cell apoptosis

Since Nef induced ROS production and apoptosis in HCAEC was dependent on Rac1-Pak2 activation, we hypothesized that blocking Rac1 trafficking to the membrane with statin treatment could pleiotropically help protect HCAEC from Nef induced stress. In this regard, treatment with statin blocks Nef induced ROS production in co-culture models of HCAEC **(Fig.58)**. Similarly, statin treatment also blocked Nef EV induced HCAEC ROS production **(Fig.59)**. Subsequently, in both the co-culture model and Nef EV treatment model, statin treatment were comparably effective to Rac1 inhibition or NADPH oxidase complex inhibition in terms of protecting HCAEC from Nef induced endothelial cell apoptosis **(Fig.61, 62, 63 and 66).** These findings suggest potential therapeutic benefits of prophylactic prescription of statins to HIV patients on ART to prevent cardiovascular disease development.

Therefore, we demonstrate that HIV-Nef protein utilizes a Rac 1 mediated pathway to induce HCAEC apoptosis and that statin therapy can be used to protect HCAECs from HIV-Nef induced apoptosis.

2.9 Endothelial Nef expression leads to vascular pathologies in transgenic murine model

While HIV infection is limited to CD4 T cell and macrophages, we and others have shown that HIV-Nef protein is capable of mediating its own transfer to endothelial cells. To address the question whether endothelial presence of HIV-Nef protein is sufficient to cause vascular pathologies, we generated mice expressing HIV-Nef under control of VE-cadherin promoter.

To achieve that, transgenic mice expressing HIV-Nef under control of tetracycline responsive element were crossed with transgenic mice expressing tetracycline operator under control of VE-Cadherin promoter. The resultant double transgenic mice were not given tetracycline to ensure constitutive expression of HIV-Nef protein in endothelial cells.

Analysis of cardiac phenotype revealed no apparent changes in heart/body weight ratio at 4 month of age; however, at 5 month of age Nef+ mice demonstrated noticeable, although not significant decrease in heart mass (data not shown). In concert with in vitro data, we observed elevated level of endothelial cell apoptosis in the heart of HIV-Nef transgenic mice when compared to Nef- littermates **(Fig.68)** which could be reduced using anti-EMAPII neutralizing antibodies. Ultrasonic examination of Nef transgenic mice revealed significantly decreased diameter of left carotid coronary artery in HIV-Nef transgenic than in Nef-negative littermates **(Fig.69)**.

To assess endothelial-specific vascular function in Nef transgenic mice, we measured dilation of pre-constricted aortic rings in response to acetylcholine. Aortas from

3 month-old HIV-Nef transgenic animals showed dramatically impaired ability to dilate in endothelial-dependent manner. Importantly, aortas from Nef transgenic animals treated for three weeks with Atorvastatin showed normalized dilation in response to acetylcholine **(Fig.70)**. This phenotype of impaired endothelium mediated vasodilatory capacity was confirmed in another transgenic mouse model with HIV-Nef expression in CD4+ cells **(Fig.71).**

Analysis of pulmonary phenotype revealed no apparent changes in wet or dry lung to body weight ratio in Nef transgenic mice **(Fig.40)**. Nonetheless, we observed increased apoptosis in pulmonary endothelial cells of Nef transgenic animals **(Fig.39)**. Histological examination of Masson-3-Chrome and Verhoeff-Van Gieson-stained lung sections revealed marked changes in the appearance of small pulmonary arteries **(Fig.72)**. In particular, small arteries of HIV-Nef transgenic displayed elevated perivascular collagen deposition **(Fig.73)** and increased vessel thickness **(Fig.74)**. With these changes being consistent with the signs of developing pulmonary hypertension, we assessed PAT/PET ratio known to have inverted relationship with RVSP. PAT/PET was significantly decreased in Nef-transgenic mice compared to Nef-negative littermates **(Fig.75)**.

Taken together, our data strongly suggest that endothelial damage by HIV-Nef is sufficient to induce variety of vascular pathologies clinically relevant to those observed in HIV patients on ART.

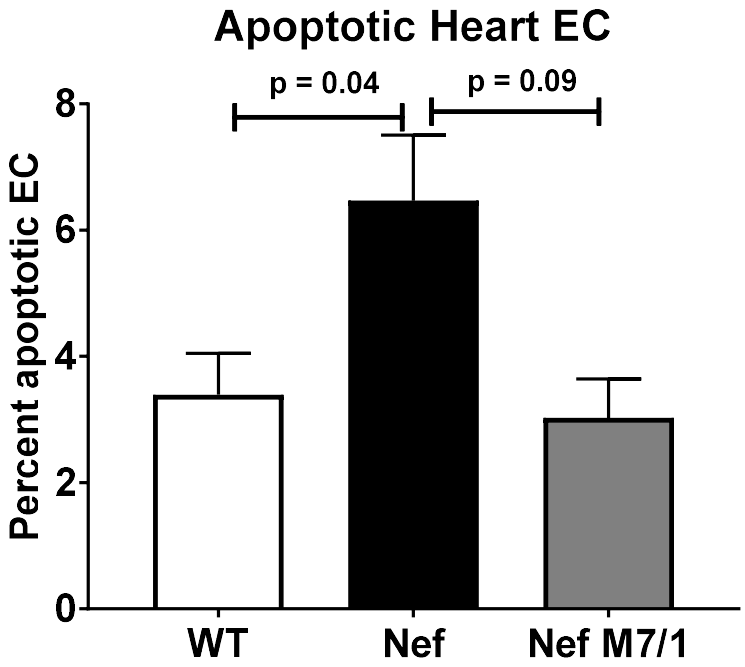

Figure 68: Cleaved caspase-3 staining in CD45-/CD326/CD31+ cells from the hearts in Nef transgenic mice with sham treatment or after 2x i.p injection of anti-EMAPII neutralizing antibody 72h and 24h prior to sacrifice.

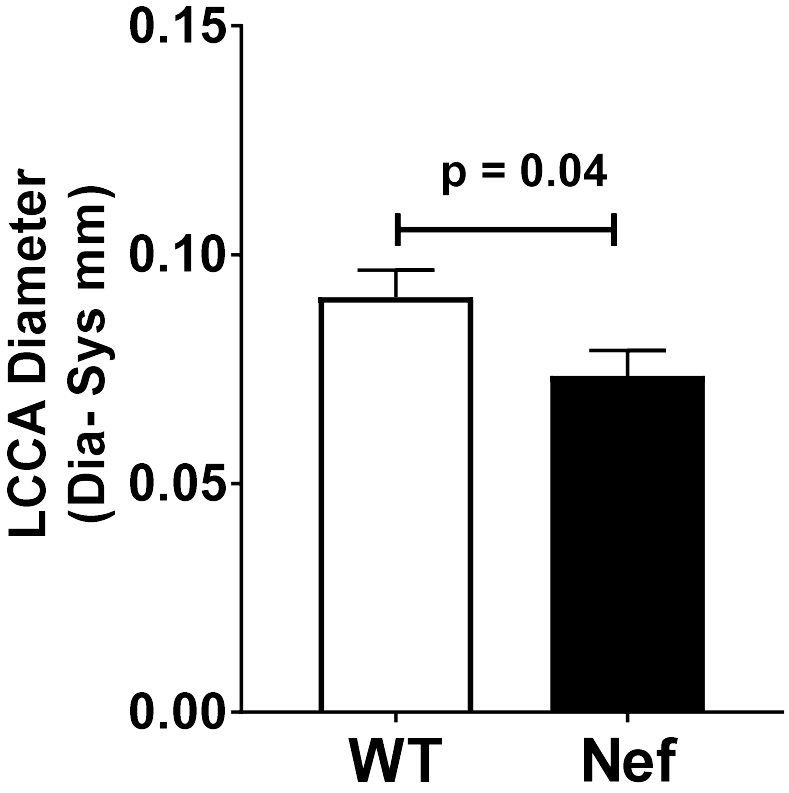

Figure 69: Echocardiography was used to evaluate LCCA diameter in 3mo old Nef Transgenic mice and their WT littermates

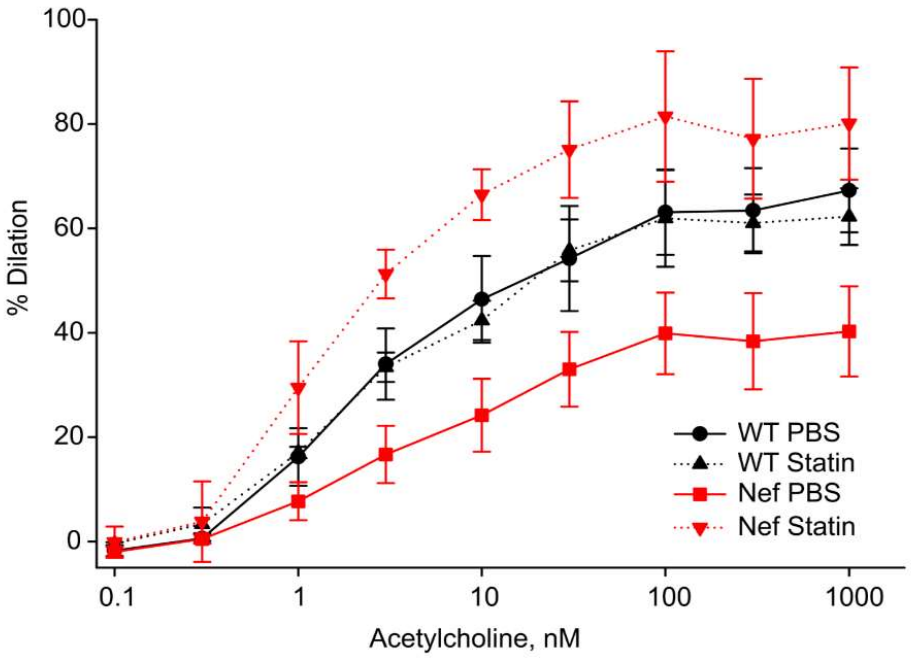

Figure 70: Aortic rings isolated from VE-Cadherin Nef expressing mice were preconstricted using phenylephrine. Vasodilation in response to increasing concentrations of acetylcholine were used to measure endothelium dependent vasodilation. Nef mice treated with 5mg/kg of Atorvastatin daily for 3 weeks show improved aortic vasodilation.

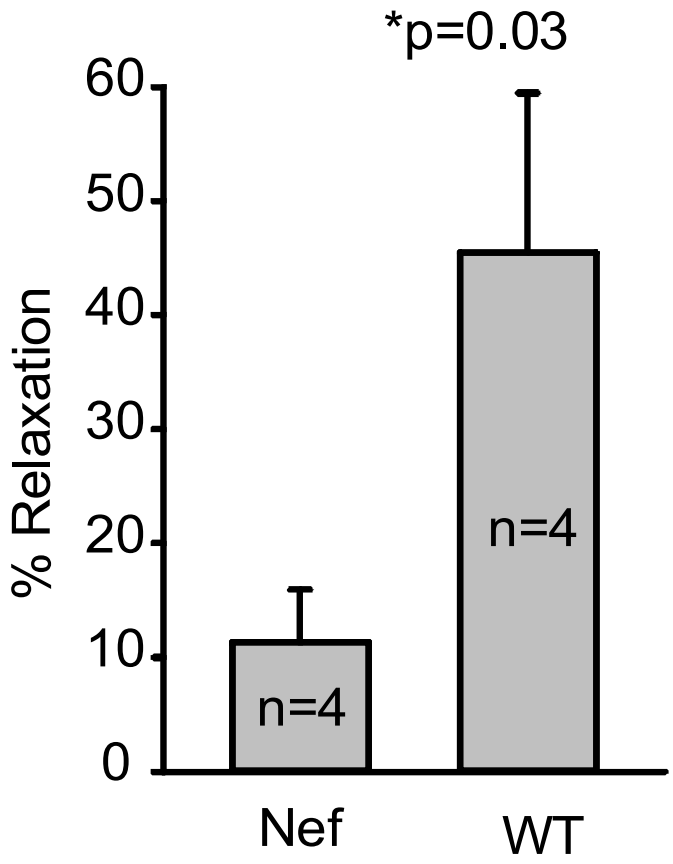

Figure 71: Aortic rings isolated from CD4c-Nef expressing mice were preconstricted using phenylephrine. Vasodilation in response to increasing concentrations of acetylcholine were used to measure endothelium dependent vasodilation

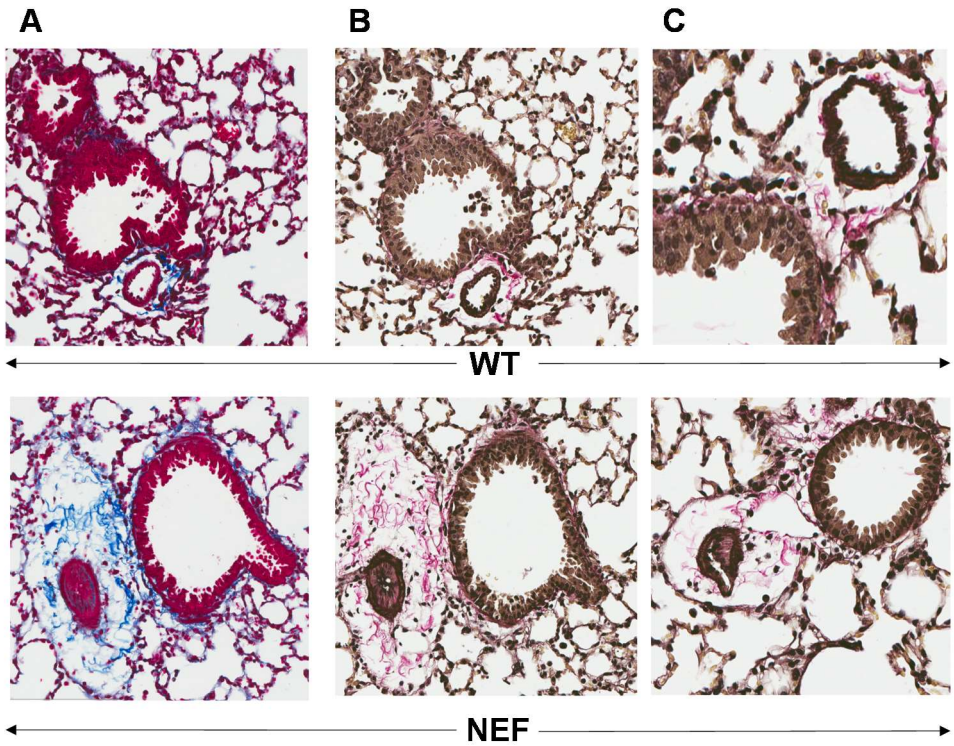

Figure 72: HIV-Nef transgenic mice display vascular remodeling of small pulmonary artery vessels. (A) Trichrome staining shows increased collagen deposition (B) VVG staining was used to measure smooth muscle remodeling in arteries <75μM in diameter in lung sections. (C) Evidence of elastin degradation.

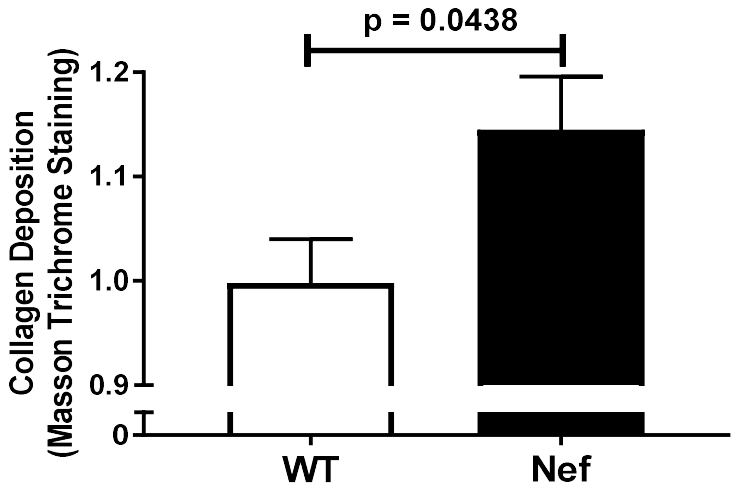

Figure 73: Increased perivascular collagen deposition in VE-Cadherin nef transgenic animals when compared to their WT littermates. Collagen deposition was quantified using Masson trichrome staining.

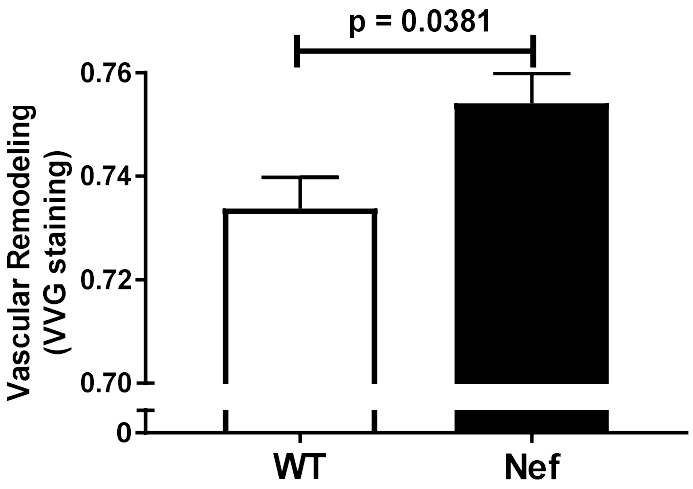

Figure 74: Increased vascular remodeling in pulmonary arteries with <75μM diameter in lung sections of VE-Cadherin Nef transgenic animals when compared to their WT littermates. Vascular remodeling was quantified using Verhoef- Von Gieson Staining.

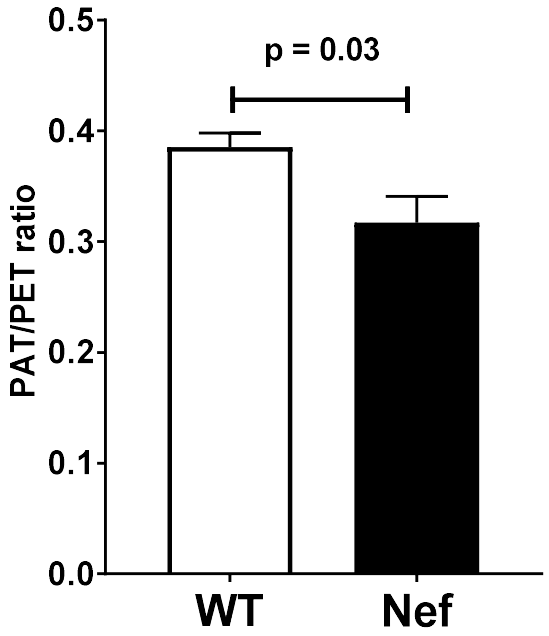

Figure 75: PAT/PET ratio was quantified using echocardiography is 3mo old Nef transgenic mice and their WT littermates.

2.10 Nef expression induces ECFC senescence

Endothelial colony forming cells are highly proliferative endothelial cells that play an important role in repair of damaged vasculature [180]. Here we addressed the effect of HIV viral proteins on endothelial colony forming cells.

Cord blood derived ECFCs were treated with HIV-Nef containing EVs **(Fig.76)** and HIV-Nef expressing SupT1 T cells **(Fig.77)**. Both intracellular and extracellular models of HIV-Nef persistence lead to increased senescence of ECFCs. Premature cellular senescence was quantified by calculating the percentage of ECFC which were positive of senescence associated β – galactosidase activity. This activity was measured using the fluorescent dye C12FDG via flow cytometry. Interestingly, treatment with Pitavastatin was able to protect ECFCs from HIV-Nef induced senescence. As previously observed [181, 182] , statin treatment was in general beneficial to ECFC since ECFC senescence was decreased upon Pitavastatin treatment.

Our results show that HIV-Nef protein is capable of inducing ECFC senescence which in turn could lead to the diminished capacity to repair vascular injury in HIV patients.

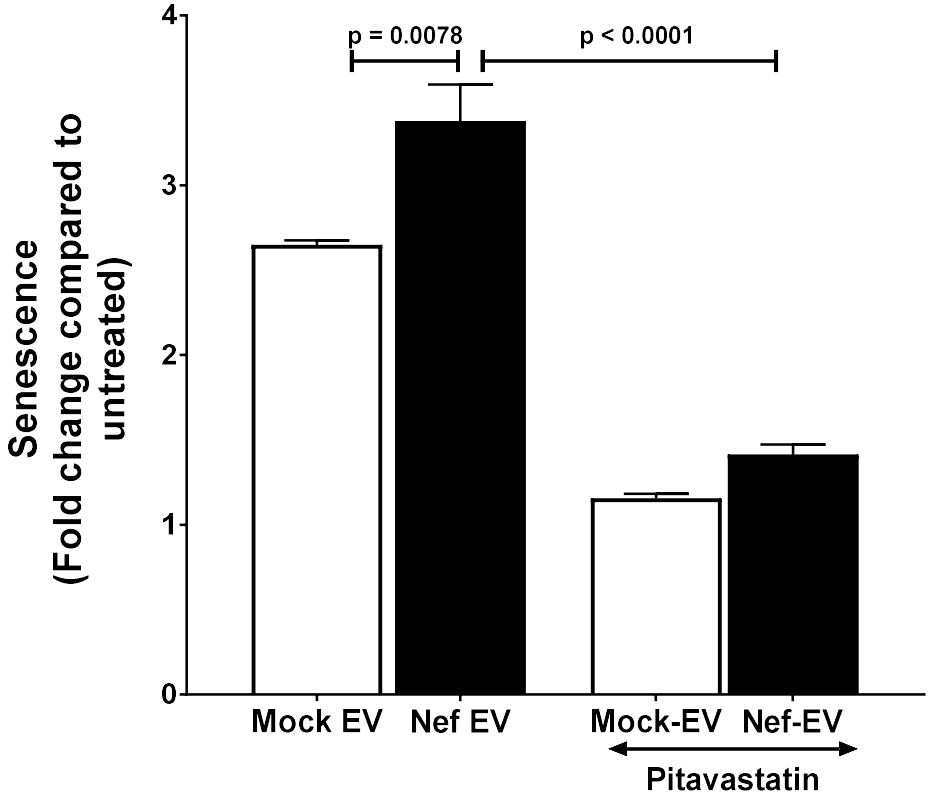

Figure 76: Addition of HIV-Nef containing EV induces senescence over 6 days (measured through senescence associated β Galactosidase activity) in ECFC which was reversed with Pitavastatin treatment.

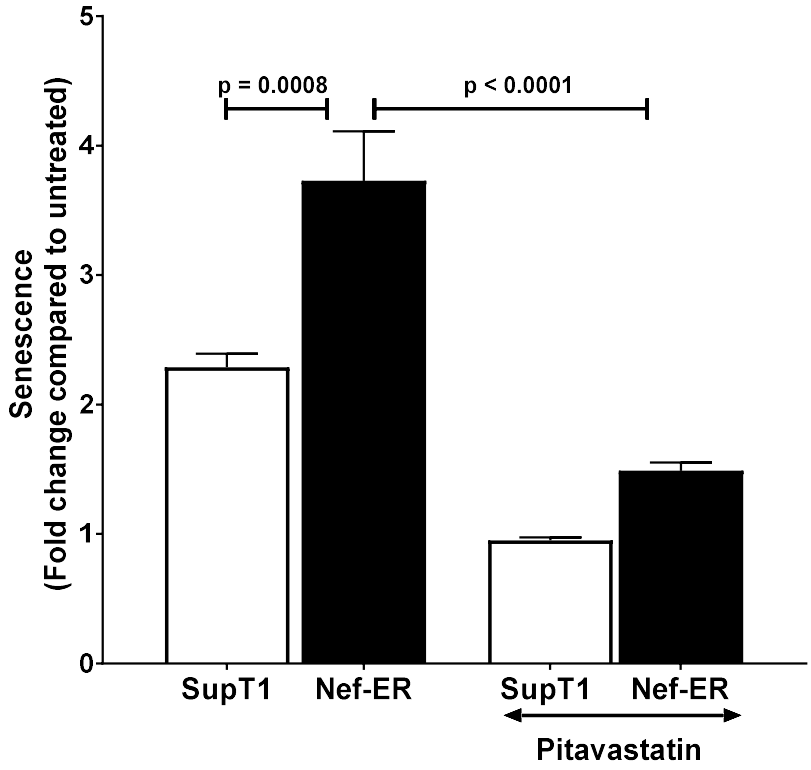

Figure 77: Co-culture of Nef-ER T cells induces senescence over 6 days (measured through senescence associated β Galactosidase activity) in ECFC which was reversed with Pitavastatin treatment.

III Discussion

1 HIV-Nef persistence and endothelial cell apoptosis

In this study, we report for the first time that the virally encoded HIV-Nef protein persists in lungs of HIV patients on antiretroviral therapy (ART) in both cells and EV using three different techniques – flow cytometry to show intracellular Nef and ELISA and western blot to detect Nef persistence in extracellular vesicles. Our discovery highlights a likely source of cardiopulmonary vascular pathologies even in aviremic HIV patients.

Earlier, our lab had shown that Nef protein persists intracellularly even in patients on anti-retroviral therapy, albeit at very low levels. This could be attributed to two major challenges associated with detecting Nef protein in HIV patients on ART. The first concern is that since HIV has a high rate of mutation, using one antibody targeting a specific epitope leads to a scenario of under-reporting the frequency of Nef persistence. The second issue that commercially available ELISAs only work in acellular samples, thereby limiting the ability to accurately quantify the amount of protein that is present. However, the detection of HIV-Nef protein in patients on ART has the potential to become a biomarker for HIV reservoir activity and explain the inability of ART to revert the development of HIV associated cardio-pulmonary vascular diseases. Hence we used a strict validation approach deploying 3 monoclonal antibodies directed against 3 distinct epitopes (SN 20, 3D12, and EH1: see **Fig.2 and 5**) to meet challenges due to the high mutation rate of the HIV virus. We also conjugated our anti-nef abs using a novel UV-NBS conjugation system to enhance sensitivity and specificity of detection. Using this approach, we could demonstrate that HIV-Nef is disseminated equally in treated and

untreated (viremic) patients, indicating the significance of HIV-Nef for vascular complication in this aviremic HIV population. However, future studies accurately quantifying the amount of Nef protein present in HIV patients could be used to test if the amount of Nef protein predicts disease progression.

We also observed that HIV-Nef protein can be found in lung alveolar macrophages, CD4+ and CD8+ T cells in addition to BAL derived EV [66, 183]. Given that there is no cell type specificity of Nef protein expression and deep RNAsequencing did not reveal any HIV transcripts, we believe that the most likely explanation of disseminated expression of HIV-Nef in BAL cells is that it is a product of fusion with Nef containing EV. We backed up our ELISA quantification previously used to detect HIV-Nef in plasma EV [42] with WB using two different antibodies. In this regard, we found nearly half the patients to have Nef in extracellular vesicles in the BAL. Our hypothesis of HIV-Nef mediating its transfer through EV is in line with findings showing the persistence of HIV-Nef in plasma [42, 45] and our previous discovery of this protein in HIV-negative T cells and B cells in the blood [184]. While we do not have any aviremic HIV patients in the cohort from which we have BAL samples, Lee et.al [45] noted that Nef persists in plasma EV of both viremic and aviremic HIV patients. They also show that both groups have similar EV profiles as characterized by an increase in EV quantity and changes in miRNA and protein cargo when compared to non-infected individuals.

In the context of EV cargo and its biological significance, we demonstrated previously that Nef protein persistence in cells can directly lead to endothelial apoptosis. Here we show for the first time that HIV-Nef dissemination through PBMC leads to

endothelial cell apoptosis. In fact, PBMC from HIV patients on ART demonstrated caspase-3 activation and mitochondrial depolarization in cultivated human coronary arterial endothelial cells **(Fig.42 and 43).** The other fraction in which Nef persists, extracellular vesicles, is also capable of inducing endothelial cell apoptosis. These EV isolated from HIV patients, both from BAL and plasma were potently capable of inducing apoptosis in HMVEC **(Fig.13 and 14)** and HCAEC **(Fig.43 and 44)** respectively. While we only observed a moderate increase in apoptosis when HCAEC were cultured with whole plasma and even less with EV depleted plasma **(Fig.44 and 45)**, a 1:10 dilution of BAL fluid was sufficient to induce potent HMVEC apoptosis in the HIV patients who were found to have Nef persistence. While HIV patients without detectable Nef had less ability to induce endothelial apoptosis. Therefore, our ability to quantify Nef in EV isolated from BAL fluid to classify patients into Nef+ and Nef- **(Fig.1)** is of importance as it can be used to stratify patients. We did not have sufficient plasma to classify HIV patients into Nef+ and Nef- by analyzing EV isolated from plasma.

HIV-Nef is known to have the ability to interact with vesicular trafficking proteins and regulate EV secretion [185]. Similar to previous studies in plasma [44], we could not distinguish between micro-particles and exosomes and hence decided to use the term extracellular vesicles. To the best of our knowledge; this is the first report demonstrating Nef in EV isolated from HIV-infected patient's BAL fluid. This EV could be from latently HIV infected T cells and macrophages from HIV reservoirs in the lymphatic system as Nef containing EV were shown to be CD45+ [42]. Another possible

cellular source for Nef containing EV could be the liver based on miRNA and effector protein profiling of Nef containing EV isolated from plasma [47].

Furthermore, HIV-Nef persistence in the lung of patients corresponds to the increased presence of pro-inflammatory chemokines in the lung milieu **(Table 1 and 2)**. Although not significant given the relatively small number of patients analyzed, there was a trend towards higher chemokine levels in HIV-Nef positive group versus the HIV negative control and the HIV-Nef-negative groups, which was especially remarkable for IP-10. It would be interesting to address in longitudinal studies the relevance of these differences in the context of COPD development given that we have available PFT data (FEV1 and DLCO) and CT scans of the lung. It would be desirable to test whether blood samples also reflect the changes observed in the lung (BAL). However, considering the dilution factor of BAL, all of the chemokines except for RANTES are found in much higher concentration in the lung when compared to the blood. Furthermore, the Nef effects are more pronounced in the lung versus the blood compartment, indicating that the observed changes in chemokines and possibly other cytokines are restricted to the lung. The cohort from which all the PBMC and plasma were analyzed do not have the exquisite characterization of the INHALD cohort. Future studies could look at plasma and PBMC from patients in the INHALD cohort to extend our findings.

We discovered that HIV-Nef expressing T cells and PBMCs and HIV-Nef containing EV can induce HCAEC and ECFC stress. Combined with [42, 45] [50] previous observation of Nef containing EV in plasma of HIV patients on ART, our study highlights the possibility of HIV Nef containing, pro-apoptotic EV as inducers of endothelial dysfunction in HIV patients.

Specifically, this extracellular vesicle fraction from plasma and BAL was capable for inducing endothelial cell apoptosis. Although plasma in a 1:10 dilution with EGM2MV media only induced very low levels of HCAEC apoptosis, EV isolated from 50µl of either plasma or BAL dissolved in 100µl of EGM2MV media from HIV+ patients potently induced HCAEC and HMVEC apoptosis respectively. The EV depleted plasma fraction was incapable of inducing endothelial cell apoptosis. Therefore, the Nef persistent fractions of PBMC and extracellular vesicles from both BAL and plasma are capable of inducing endothelial cell apoptosis. Furthermore, treatment with EMAPII neutralizing antibodies was capable of ameliorating both BAL fluid as well as BAL fluid EV induced HMVEC apoptosis. This offers a potential therapeutic option to counter EMAPII mediated Nef EV induced apoptosis.

2 Inflammation and endothelial activation associated with Nef induced endothelial stress

The ability of HIV-Nef to alter the chemokine profile of alveolar macrophages is important since Nef could act as a major mediatory of HIV associated inflammatory profile in the lung. These changes contributing to T-cell dysfunction are essential for regulation of immunity and inflammation, and thus can influence pulmonary disease development. They are also believed to be one of the major mechanisms leading to a consistent imbalance of the pulmonary microbiome [186-188]. This imbalance contributes to both chronic inflammation in the lung and acts as a potential source for increasing the risk of lung cancer. Using alveolar macrophages, we can further elucidate mechanisms behind our finding that Nef stratification is meaningful and leads to

significant differences when for example tested in endothelial apoptosis assays. The ability of HIV-Nef containing EVs to promote an inflammatory phenotype can thus be studies in a lung-specific context. These findings reflect the ability of HIV-Nef containing EVs to promote activation of blood-derived monocytes and macrophages; a common feature in HIV associated end-organ diseases including neurocognitive disorders and atherosclerosis [43-47, 189]. On the other hand, the lung pathologies we characterized in our endothelial specific Nef-expressing mice can be attributed to increased endothelial cell apoptosis **(Fig.39)**. Future studies expressing Nef from other cellular sources like T cells and macrophages or with the injection of Nef EV could study the contribution of endothelial activation and inflammation in Nef-mediated endothelial damage. Furthermore, these models can be used to characterize the effect of Nef EV on inflammation mediated endothelial damage.

Pulmonary hypertension is another pulmonary vascular disease, in which HIV-Nef has already been associated with [176, 190]. Here we show that BAL fluid derived EV contain HIV-Nef and that HIV-Nef containing EV induce EMAPII surface expression in primary human pulmonary artery endothelial cells **(Fig.27)**. Furthermore, the addition of Nef EV to HPAEC and HMVEC also induced endothelial cell pro-inflammatory phenotype as described by an increased surface expression of VCAM-1, ICAM-1, P-Selectin and E-Selectin **(Fig.52 and 53)** followed by increased T cell adhesion **(Fig.55 and 56)**. It is possible that HIV-Nef containing EV from lung act as the initial insult that results in the loss of endothelium that is characteristic of pulmonary arterial hypertension (PAH). We addressed the transfer of Nef to coronary vascular endothelial cells on several levels; increased interaction between HIV-Nef positive T

cells and HCAEC, HIV-Nef transfer through EV as a long-range transport mechanism, and finally we elucidated the mechanism of how HIV-Nef causes cytosol and its own transfer to HCAEC.

Although nanotubes may in principle lead to transfer of HIV-Nef under static conditions [191, 192], in conditions of flow, extracellular vesicles maybe the preferred transport of cargo from latently infected HIV positive cells to the vasculature, as corroborated by a plethora of independent studies and investigators [40, 42, 43, 45, 48, 193-196]. Our study shows that HIV-Nef expression increases communication of toxic EV cargo including the Nef protein between T cells and endothelial cells. The ability of statins to block this enhanced interaction could be crucial in preventing Nef transfer and subsequent endothelial dysfunction.

The adhesion of lymphocytes to the vascular endothelium is a key driver in heart failure in both patients with non-ischemic heart failure and murine models of heart failure [197]. Specifically, the increased presence of endothelial adhesion proteins like ICAM1 [198, 199] suggests a strong role in immune-mediated endothelial cell activation leading to endothelial dysfunction in HIV patients. Secreted factors from CD8+/CD28- T lymphocytes of HIV patients and HIV proteins like GP120 [200] and HIV-Tat [201, 202] were shown to promote lymphocyte adhesion to endothelial monolayer via upregulation of ICAM-1. HIV-Nef protein in the form of EV is capable of increasing endothelial cell adhesion markers like ICAM-1 in an ERK signaling dependent fashion [203]. HIV-Nef containing EV becomes the leading candidate to induce lymphocyte adhesion the endothelium since it fits both criteria of being a persistent circulating factor in HIV patients on ART and a secreted factor from T cells. Again, statin treatment was capable

of abrogating Nef EV induced endothelial activation suggesting a potential mechanism for statin's ability to reduce vascular dysfunction in HIV patients.

In this model, we focused on the endothelium as the target tissue by expressing Nef through the endothelial cell specific promoter VE-Cadherin. , Given that Nef can lead to endothelial adhesion protein expression, the absence of inflammation in the lungs of these mice is surprising **(Fig.40)**. It is possible that endothelial cell-only expression levels of Nef are insufficient to induce adhesion protein but EMAPII surface expression, which is expected to cause little inflammation but apoptosis. Surprisingly, our in vitro transmigration experiments showed no significant increase in transmigration of THP-1 monocytes towards HMVEC treated with Nef-EV (data not shown) while alveolar macrophages treated with Nef-EV secrete pro-inflammatory cytokines **(Fig.20)**. It is possible that Nef-EV induced adhesion protein induction acts over a longer period than can be effectively tested in migration assays. On the other hand, it could also be related to Nef's inability to induce chemokines release from endothelial cells while a secondary cell type like macrophages might be needed to mediate this effect.

In our murine models, HIV-Nef-induced induction of endothelial apoptosis rather than inflammation since we did not observe a significant rise in inflammatory immune cells in the BAL edema and only a slight trend towards protein content in the BAL **(Fig.40)**. Hence, in this study, we focus on the contribution of endothelial cell apoptosis towards the development of pulmonary pathology. This observation is consistent with previous data showing that apoptosis of lung endothelial cells driven by VEGF receptor inhibitors is sufficient to cause emphysematous changes [204].

While we do observe an increase in surface EMAPII expression in lung endothelial cells of Nef transgenic mice **(Fig.28)**, we only detect a non-significant slight trend in the increase in secreted EMAPII in the BAL **(Fig.40D)**. Previously, we have shown that EMAPII, which is secreted from the lung epithelium induces an emphysema-like phenotype in mice with both increased apoptosis and inflammation in the lungs [142]. Future studies expressing HIV-Nef in other cell types including alveolar macrophages and injection of Nef containing extracellular vesicles can study whether HIV-Nef can also upregulate EMAPII secretion that in turn drives inflammation. These models are also required to evaluate the entire spectrum of immunological and pathological consequences of HIV-Nef-EV uptake by alveolar macrophages and other immune mediators.

In a murine model of chronic ecoHIV, a chimeric HIV, in which the gp120 in HIV-1/NDK was replaced with that of gp80 from ecotropic murine leukemia virus (MLV) to establish chronic infection in immunocompetent mice developed COPD upon exposure to cigarette smoke [205]. ecoHIV infected mice had significantly reduced forced expiratory flow 50%/forced vital capacity and enhanced distal airspace enlargement following cigarette smoke exposure. HIV infection was detected in alveolar macrophages and contributed lung IL-6, granulocyte-macrophage colony-stimulating factor, neutrophil elastase, cathepsin G, and matrix metalloproteinase-9 expression. Similarly, IκBα, ERK1/2, p38, and STAT3 phosphorylation and lung cell apoptosis were also seen in this mouse model. It would be interesting to perform similar studies in our endothelial Nef expression model using a second hit of pulmonary stress like cigarette smoke exposure. EMAPII neutralizing therapy was shown to prevent the progression of

emphysema in a cigarette smoke exposure model of emphysema [142]. Therefore EMAPII neutralizing antibodies and statin treatment could be tested, along or in combination using this model of ecoHIV/cigarette smoke exposure to study whether they can protect from HIV and cigarette smoke exposure induced emphysema.

3 Nef induced endothelial damage could prime the vasculature for disease

Our finding that persistence of HIV-Nef protein in latent infection is relevant for an end organ disease reflects findings in other viral infection; such as adenoviral protein E1A persists in latent infections and primes the lung for the development of COPD/emphysema [206]. Similarly, the risk of developing pulmonary diseases like COPD is heightened in HIV patients on ART. Here we show that persistence of HIV-Nef protein in end organs like the lung could induce secretion of pro-apoptotic EV and thus act as a precursor for the development of pulmonary pathologies. In our exquisitely characterized ART-treated HIV patient cohort **(Fig.1, 2 and Tables 1 and 3)**, we discovered a close correlation between HIV-Nef and EMAPII in BAL cells. This is in line with our previous demonstration that cigarette smoke also induces upregulation of EMAPII thus leading to endothelial apoptosis [142, 207]. Our findings showing that HIV-Nef can utilize an EMAPII mediated pathway to induce endothelial cell apoptosis is highly relevant to explaining lung pathologies in HIV patients since endothelial cell death has been suggested as an important mechanism for inducing lung emphysema in rodents [204, 208-210] and humans [211]. HIV-Nef induced cytotoxic lymphocyte activation and lung capillary endothelial cell apoptosis may explain the increased incidence of emphysema in HIV-infected patients [212]. Our results demonstrated that transgenic expression of HIV-Nef in the endothelium causes remodeling resembling

emphysematous changes **(Fig.37 and 38)** in the lungs of these mice. Given the recent proposition that endothelial cell apoptosis may be a common denominator for both emphysema and PAH development [213, 214], the mechanism here suggested about how HIV contributes to endothelial apoptosis should be of general interest. In this regard, our finding that Nef-induced EV release and biological activities to endothelial cells and alveolar macrophage is important. For example, targeting PAK2 activation dependent EMAPII pathways could be of therapeutic interest to combat HIV associated pulmonary vascular diseases.

Cardiovascular dysfunction [215-218], impaired endothelium-mediated vasodilation [219, 220] and arterial stiffening [221-223] are well established phenotypes in the HIV patient population. Similarly, endothelial dysfunction has been characterized in mammals including transgenic rats expressing HIV proteins [224], ex vivo porcine pulmonary arterial rings treated with HIV-Nef [225] and SHIV Nef infected macaques [226]. Our study sheds light on the ability of endothelial HIV-Nef protein to independently impair endothelium-mediated vasodilation and cause a mild vascular dysfunction phenotype even in the absence of other HIV associated confounding variables like ART, immune cell activation and viral replication.

Transgenic expression of all HIV viral accessory proteins in mice [224], rats [227] and SHIV-infected macaque [228] show a strong link between HIV and pulmonary vascular remodeling. Our observation of vascular remodeling in endothelium-specific HIV-Nef transgenic mice is the next piece of the puzzle in determining the source of vascular pathologies in HIV infected individuals on ART. Here we describe that HIV-Nef protein can potently induce endothelial cell apoptosis due to increased ROS production

by the NADPH Oxidase complex that is dependent on Rac 1 activation. This Nef-induced endothelial stress was ameliorated with statin treatment, both *in vitro* and *in vivo* showing the benefits of statin treatment even in the absence of dyslipidemia.

4 HIV-Nef persistence as a common denominator for HIV associated vascular co-morbidities

Our study highlights how HIV infection could link pulmonary and systemic vascular diseases since Nef persistence is capable of affecting the endothelium regardless of location. In a study evaluating emphysema and coronary artery disease found an association between emphysema and coronary artery scores (Odds Ratio 1.43) after correcting for age, sex, smoking status, pack-years of smoking, visceral adiposity and duration of HIV infection[229]. This association was found be even higher (Odds Ratio 1.52) when corrected for Framingham risk scores. This association could potentially be explained by using Nef persistence as a lens. Furthermore, comparison of Nef persistence in various compartments - blood, BAL and cerebrospinal fluid could be used to study the effect of HIV on end organ-specific vascular diseases.

The endothelial progenitor cell population is also affected in HIV patients as these cells have impaired proliferative capacity and angiogenic potential in HIV patients [230, 231]. Studies describing the frequency of circulating endothelial progenitor populations in HIV patients have conflicting reports depending on the classification of circulating endothelial cells [232] [233] [234] [231, 233, 235-237]. Here we show that HIV Nef-expressing T cells and EV can induce senescence in these highly proliferative progenitors that have an important role in tissue repair. Since Nef protein persists in EV and is disseminated throughout the body, Nef EV's capacity to induce ECFC senescence could

have longstanding implications on global circulations. HIV associated renal vascular disease [238] [239] and cerebrovascular [20, 240-244] disease are other end organ diseases prevalent in the HIV patient population. Therefore, future studies should explore Nef-induced damage to renal and cerebral vasculature to extend the findings from these studies. Furthermore, since ECFC were described to play an important role in not only vascular homeostasis but also for recovering from vascular injury. In this regard, the VE-Cadherin Nef transgenic mouse model could be used in vascular injury models like hind limb ischemia, kidney ischemia-reperfusion, coronary artery ligation, and hypoxia exposure. Delayed or impaired repair response in these models and reduced capacity of ECFC to heal vascular damage could further explain the increased propensity of these diseases in the HIV patient population.

5 Therapeutic strategies to counter Nef induced endothelial stress

Our findings here suggest that HIV-Nef uses a Rac 1 mediated pathway to induce endothelial cell stress which in turn leads to endothelial dysfunction. Downstream of Rac1 activation, we described that Pak2 kinase activation is important for mediating endothelial cell apoptosis since it leads to EMAPII surface expression that potently amplifies endothelial cell apoptosis. Targeting an HIV-induced pro-apoptotic pathway in endothelial cells via blocking EMAPII using neutralizing antibodies may be sufficient to treat HIV-associated pulmonary diseases. In support of this, short term treatment of mice with EMAPII neutralizing antibody was able to reduce the amount of apoptotic pulmonary endothelial cells. This suggests that long term treatment or treatment at the onset of Nef protein expression could reverse pulmonary vascular pathology development.

Furthermore, the Nef-Rac1-Pak2 signaling axis is important for both EMAPII mediated and ROS mediated endothelial cell stress. Treatment with SRC kinase inhibitors, PP2 and Nef-Hck signaling inhibitor, B9, did not block Nef-induced endothelial cell apoptosis (data not shown). EMAPII neutralizing antibodies and statin treatment provide promising workarounds to block downstream effects of Nef-induced Rac1-Pak2 activation. This is of special importance since Rac1 inhibitors could have side effects because of the important role of Rac1 in a wide variety of cell signaling pathways. On the other hand, Pak2 inhibitors like PF-03758309 have poor bioavailability (Clinical trial - NCT00932126). This makes targeting of Rac1-Pak2 signaling prophylactically in a global population of HIV patients a huge challenge. Hence, future ventures should be directed at unearthing specific inhibitors of Nef-Rac1 activation by targeting the PxxP motif – SH3 domain interaction site. Another strategy would be to test inhibitors that can block Nef-induced Pak2 activation by specifically blocking the 195 phenylalanine residue but does not interfere with Pak2 recruitment. Since only a small fraction of Pak2 is associated with Nef [89], inhibition of Nef-Pak2 activation should have minimal effects on essential Pak2 function but abrogate Nef-induced pathological Pak2 signaling.

Our study provides the context for combination therapy to protect vascular health in HIV patients. Upon diagnosis with HIV+, in addition to starting patients on antiretroviral therapy, an initial α-EMAPII neutralizing antibody treatment could help slow down EMAPII's apoptosis driven feed-forward loop that promotes endothelial stress.

Statins have been deployed in clinical trials like REPRIEVE to study their ability to prevent cardiovascular diseases in HIV patients [245]. Rosuvastatin, Atorvastatin, and

Pitavastatin have been shown to protect HIV patients from the development of CVD to a larger extent than can be attributed to a drop in LDL [246-252]. Specifically, Atorvastatin and Pitavastatin have minimal to no drug-drug interactions with ART, making it attractive to prescribe to HIV patients. [253-256]. Subsequently, similar to the REPRIEVE trial, HIV patients can be placed on statin therapy to target Nef-induced Rac1 activation which should reduce Nef EV release as well as ROS production and endothelial apoptosis that follows the uptake of Nef EV. Pitavastatin's ability to reverse HIV-Nef induced effects on ECFC is in line with other studies showing the benefits of statin treatment on endothelial progenitor population in murine models [257-260] and clinical trials of statin intervention [261-265].

6 Conclusion

HIV-Nef protein persists in HIV patients on ART at levels comparable to those not on therapy. Furthermore, Nef protein was identified in extracellular vesicles of bronchoalveolar lavage fluid as well CD4, CD8 T cells and Alveolar Macrophages isolated from bronchoalveolar lavage. These findings highlight the importance of studying Nef protein persistence and its effect on endothelial dysfunction in HIV patients. The ability of statin treatment to block HIV-Nef mediated Rac 1 signaling could help limit vascular dysfunction in HIV patients on ART and potentially delay the development of cardiopulmonary vascular diseases.

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Curriculum Vitae

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Master of Biotechnology GPA 3.85

August 2011- May 2013

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Work Experience:

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Tutor for General Biology, General Chemistry and College Physics I

August 2009 – May 2011

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Publications:

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Wang, Yi, R, Green, L. A, Chelvanambi, S., Seimetz, M., & Clauss, M.. "Increased cardiovascular disease risk in the HIV-positive population on ART: potential role of HIV-Nef and Tat." Cardiovasc Pathol. 2015

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Chelvanambi, S., Chen, X., Ellis, B., Bogatcheva, N., Jolicoeur, P., Zorlutuna, P.,., Obukhov, A., Gupta, S., Clauss, M. "HIV-Nef Protein Induced Cardiovascular Dysfunction Is Mediated by Rac 1 Activation: Implications for Statin Therapy in HIV"<Manuscript in submission>

Maier, B., Chelvanambi, S., Lam, N., Clauss, M. “HIV-Nef promotes Surface Downregulation and Degradation of S1P1 Receptor: Implication for HIV Reservoirs” <Manuscript in preparation>

Chelvanambi,S., Kuriakose, J., Colbert, T., Jolicoeur, P., Gupta,S., Bogatcheva,N., Clauss,M. “HIV-Nef induces senescence in Endothelial Colony Forming Cells via alteration of TNF Receptor 2 signaling.” < Manuscript in preparation>

Chelvanambi,S., Bogatcheva,N., Jolicouer,P., Twigg, H.L., Clauss,M. “EMAPII Neutralizing Monoclonal Antibody blocks HIV-Nef induced vascular damage in murine models of transgenic Nef expression.” < Manuscript in preparation>

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Chelvanambi, S., Maier,B., Zorlutuna,P.,Gupta,S. and Clauss,M . "Abstract 18229: HIV-NEF Modulates T-Cell Interaction With Endothelial Cells in a Rac-Dependent Pathway to Enhance Transfer of Nef Protein to Endothelium." Circulation 2016 134(Suppl 1): A18229.

Chelvanambi, S., Gupta, S., Dahl, N., Twigg, H., Clauss, M. "Abstract 20122: HIV Protein Negative Factor (Nef) - Induced Release of Endothelial Monocyte Activating Polypeptide II (EMAPII) Mediates Endothelial Dysfunction in HIV Patients. Circulation 2017 136(Suppl 1) A20122.

Chelvanambi S., Dhillon N.K., Agarwal, S., Bogatcheva, N.V., Twigg H.L., Clauss M. "HIV-Nef Protein Persists Inside Extracellular Vesicles in Lungs of Aviremic HIV+ Patients on ART and Induces Endothelial Cell Death Via Pro-Apoptotic miRNA Extracellular Vesicle Cargo" AJRCCM 2018 (pp.A7404)

Awards:

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Communication Skills:

1 national championship (2010) and 5 state championships (Ohio) in public speaking (2008-2011)

Semi-finalist in Interstate Oratory Competition in 2011.

1st place in 3 MinuteThesis competition at IUPUI's Preparing Future Faculty and Professionals Annual Pathway Conference in 2018.

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