

TRANSFER OF INTRACELLULAR HIV NEF TO ENDOTHELIUM CAUSES
ENDOTHELIAL DYSFUNCTION

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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 Microbiology and Immunology,
Indiana University

May 2014

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

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ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my advisor Dr. Matthias Clauss, for allowing me to stay in his lab for my dissertation after my former advisor left Indiana University. His enthusiasm, encouragement and faith in me are greatly helpful on my path to this degree. He is always being very patient and supportive answering my questions and giving generously of his time and knowledge. Without his guidance and persistent help this dissertation would not have been possible.

I would like to thank my committee chair, Dr. Louis Pelus, for helping me go through my transition time and giving me advice on not only research but also for my future career. I would also thank my committee members, Dr. Alex Dent, Dr. Ron Wek and Dr. Andy Yu, who offered much appreciated comments, advice, and criticisms.

I thank former and present lab members in Dr. Clauss lab for help, advice and friendship: Dr. Linden Green, one of the first people I knew in IUSM, who helped me tremendously in my Ph.D study; Dr. Ru Yi and Maja Reinhard, who helped me with my experiments.

I thank all faculties and members in ICVBM (Indiana Center for Vascular Biology and Medicine) for the guidance, collaborations, and friendships, especially Dr. Keith March and Dr. Mike Murphy for their guidance; Dongni Feng, who always lend me experiment supplies and helped me with my experiments.

I would like to thank my entire family for their love, encouragement, and supports, especially my grandparents, who raised me up; my parents, who have been so open-minded and supportive for all my decisions.

Finally, I would like to give thanks to all my friends who I met during these years, who definitely made my life outside the lab easier and happier.

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With effective antiretroviral therapy (ART), cardiovascular diseases (CVD), are emerging as a major cause of morbidity and death in the aging population with HIV infection. Although this increase in CVD could be partially explained by the toxic effects of combined anti-retroviral therapy (ART), more recently, HIV infection has emerged as an independent risk factor for CVD. However, it is unclear how HIV can contribute to CVD in patients on ART, when viral titers are low or non-detectable. Here, we provide several lines of evidence that HIV-Nef, produced in infected cells even when virus production is halted by ART, can lead to endothelial activation and dysfunction, and thus may be involved in CVD. We demonstrate that HIV-infected T cell-induced endothelial cell activation requires direct contact as well as functional HIV-Nef. Nef protein from either HIV-infected or Nef-transfected T cells rapidly transfers to endothelial cells while inducing nanotube-like conduits connecting T cells to endothelial cells. This transfer or transfection of endothelial cells results in endothelial apoptosis, ROS generation and release of monocyte attractant protein-1 (MCP-1). A Nef SH3 binding site mutant abolishes Nef-induced apoptosis and ROS formation and reduces MCP-1 production in endothelial cells, suggesting that the Nef SH3 binding site is critical for Nef effects on endothelial cells. Nef induces apoptosis of endothelial cells

through both NADPH oxidase- and ROS-dependent mechanisms, while Nef-induced MCP-1 production is NF- κ B dependent. Importantly, Nef can be found in CD4 positive and bystander circulating blood cells in patients receiving virally suppressive ART, and in the endothelium of chimeric SIV-infected macaques. Together, these data indicate that Nef could exert pro-atherogenic effects on the endothelium even when HIV infection is controlled and that inhibition of Nef-associated pathways may be promising new therapeutic targets for reducing the risk for cardiovascular disease in the HIV-infected population.

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TABLE OF CONTENTS

LIST OF TABLES	xii
LIST OF FIGURES	xiii
LIST OF ABBREVIATIONS	xvi
INTRODUCTION	
1. HIV	
1.1 Epidemiology of HIV/AIDS	1
1.2 Clinical and virological aspects of HIV infection	2
1.3 HIV genome structure	5
1.4 HIV replication cycle	6
1.4.1 Entry	6
1.4.2 Replication and transcription	9
1.4.3 Assembly	10
1.5 HIV treatment	13
1.5.1 HIV vaccines	13
1.5.2 ART	13
2. HIV negative factor (Nef)	
2.1 Nef expression and structure	18
2.2 Nef protein function in HIV	18
2.2.1 Nef as a positive viral factor	18
2.2.2 Nef alleles show major differences in pathogenicity	22
2.2.3 Nef alters cell surface expression of critical proteins	22
2.2.4 Nef alters T cell receptors and cellular signaling pathways	23

3. Cardiovascular disease

3.1 Coronary artery disease (Atherosclerosis)	24
3.1.1 Cholesterol-dependent	24
3.1.2 Other risk factors: Cholesterol-independent	25
3.2 HIV-related cardiovascular diseases	26
3.2.1 ART in coronary artery disease	26
3.2.2 HIV viron-related cardiovascular disease	27
3.2.3 HIV proteins in cardiovascular disease	31

4. Summary of background and hypothesis of this study

36

METHODS AND MATERIALS

Materials	37
Medium and supplements	37
Antibodies	37
Reagents	38
Biotechnological systems	38
Animals	39
Cells	40
<i>Cell lines</i>	40
<i>Cell culture</i>	40
<i>Isolation PBMCs from HIV patients</i>	40
Plasmids and virus	41
<i>Virus</i>	41

<i>Nef and Nef mutant</i>	41
Methods.....	41
Bacterial transformation	41
Cell transfections.....	42
HIV replication assay.....	42
Tissue culture based assays	42
Detection of apoptosis by assessing DNA fragmentation.....	43
Confocal immunofluorescence microscopy	44
In Vitro Vascular Permeability Assay.....	44
Ex Vivo tissue sections staining.....	44
Detection of Reactive oxygen species (ROS) activity by Dihydroethidium (DHE)	45
NF- κ B p65 silencing in endothelial cells with small interfering RNA.....	45
Western Blot analysis	45
Monitoring mitochondrial function by JC-1 Dye-Mitochondrial Membrane	46
Detection of Nef in PBMCs	47
B cells isolation from PBMCs.....	47
Data acquisition and statistical analysis	47

RESULTS

PART I: HIV NEF CAUSES ENDOTHELIAL ACTIVATION AND

DYSFUCTION	49
1.1 HIV induces endothelial MCP-1 production and cell death.....	49
1.2 HIV Nef is necessary for endothelial activation and cell death.....	56
1.3 HIV Nef is sufficient to cause endothelial activation and cell death.....	65

PART II: NEF MEDIATES ITS OWN TRANSFER FROM T CELLS TO

ENDOTHELIAL CELLS	74
2.1 Nef enhances the formation of nanotubes from Jurkat cells to endothelial cell.....	74
2.2 Nef protein can be detected in the endothelium of in vivo HIV model.....	86
2.3 Nef protein can be detected in blood cells isolated from HIV positive individuals	93

PART III: SIGNAL TRANSDUCTION ANALYSIS OF NEF INDUCED

ENDOTHELIAL ACTIVATION AND DYSFUNCTION	109
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DISCUSSION

Summary of the results	121
Nef protein can be transferred to endothelial cells	122
Nef protein is detectable in HIV-patients with/without ART	124

Nef as the potential targets for HIV-related cardiovascular disease	125
Alternative ways to inhibit Nef expression	129
Future directions.....	133
To validate signal transduction target for therapeutical application	133
REFERENCES.....	136
CURRICULUM VITAE	

LIST OF TABLES

Table 1: FDA-approved antiretroviral drugs	16
Table 2: Nef is detectable in PBMCs from HIV patients with/ without ART	96

LIST OF FIGURES

Figure 1: Timecourse of HIV infection	3
Figure 2: The structure of HIV genome	7
Figure 3: HIV life cycle	11
Figure 4: Residues of HIV Nef protein.....	20
Figure 5: The process of atherosclerosis	28
Figure 6: A transwell system	50
Figure 7: HIV-infected Jurkat cells but not acellular HIV virus cause endothelial activation.....	52
Figure 8: HIV-infected Jurkat cells but not acellular HIV virus cause endothelial cell death.....	54
Figure 9: Virus titer determination of Δ Nef HIV and WT HIV in Jurkat cells.....	57
Figure 10: Nef is necessary for HIV-induced endothelial MCP-1 production and cell death	59
Figure 11: The demonstration of Nef transfections	61
Figure 12: Nef-Jurkat T cells induce endothelial MCP-1 production and cell death	63
Figure 13: Nef-monocytes THP1 cells induce endothelial MCP-1 production and cell death	66
Figure 14: Nef is sufficient to induce endothelial activation and dysfunction	68
Figure 15: Nef increases HCAEC permeability	70

Figure 16: Linear correlation between Nef protein concentration and endothelial apoptosis.....	72
Figure 17: Nef protein causes endothelial dysfunction.....	75
Figure 18: Correlation between intracellular Nef concentrations with endothelial MCP-1 production	77
Figure 19: Nef enhances live dye transfer between cells only when in direct contact	79
Figure 20: Nef protein induced nanotube formation between Jurkat cells to endothelial cell	82
Figure 21: Nef transfer to HCAEC	84
Figure 22: Nef induced GFP transferred from CD4+ cells to endothelial cells in Nef transgenic mice.....	87
Figure 23: More GFP were transferred from CD4+ primary cells to HCAEC with Nef present.....	89
Figure 24: Nef can be detected in SIV-hNef macaque models	91
Figure 25: Nef is detectable in PBMCs from HIV patients with/without ART	94
Figure 26: Nef distribution does not correlate with viral load.....	98
Figure 27: Validation of EH1 monoclonal antibody	100
Figure 28: Nef is expressed in blood cells of HIV patients not on antiviral therapy	102
Figure 29: Nef is prominent present in CD4- cells.....	105

Figure 30: Nef can be transferred from HIV-PBMCs to endothelial cells ex vivo	107
Figure 31: Nef induced endothelial MCP-1 production is NF-kB dependent	110
Figure 32: Optimize Trolox concentration in ROS inhibition.....	112
Figure 33: NADPH inhibitors dose determination to block Nef-induced intracellular ROS activation in HCAEC.....	114
Figure 34: Ikki inhibited Nef induced endothelial MCP-1 production.....	116
Figure 35. Nef induced endothelial cell death is ROS/NADPH dependent	118
Figure 36: Nef signaling pathways in HCAEC.....	130

LIST OF ABBREVIATIONS

aa	amino acid
AIDS	acquired immune deficiency syndrome
Amp	ampicillin
AP-1/2/3	adaptor protein-1/2/3
ART	antiretroviral therapy
ATCC	American Tissue Culture Collection
ATP	adenosine triphosphate
BrdU	bromodeoxyuridine
CA	capsid
CCR5	CC chemokine receptor 5
CD	clusters of differentiation
cmp	counts for minutes
CXCR4	CXC chemokine receptor 4
CVD	cardiovascular disease
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin
DHE	dihydroethidium
DMEM	Dulbecco's modification of Eagle's medium
DNA	deoxyribonucleic acid
EC	endothelial cell
EGM-2	endothelial cell growth media-2
ELISA	enzyme-linked immunosorbent assay
EMB-2	Eagle's basal medium-2

Env	envelope protein
FBS	fetal bovine serum
FGF	fibroblast growth factors
FITS	fluorescein isothiocyanate
FMD	flow-mediated dilation
GA-100	Gentamicin, Amphotecerin B
GFP	green fluorescent protein
HCAEC	human coronary artery endothelial cell
HIV	human immunodeficiency virus
HPF	High Power Field
HUVEC	human umbilical vein endothelial cell
ICAM-1	intercellular adhesion molecule-1
IGF-1	insulin-like growth factor 1
IKKi	I κ B kinase inhibitor
IL-2	interleukin 2
IMT	intima-media thickness
IN	integrase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LB	Luria broth
LTNP	long-term nonprogressors
LTR	long terminal repeats
MCP-1	monocytes attractant protein-1

MMP	matrix metalloproteinase enzymes
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NO	nitrogen oxide
NRTI	nucleoside analog reverse-transcriptase inhibitors
NNRTI	non nucleoside analog reverse-transcriptase inhibitors
PAK	p21-activated kinase
PBMC	peripheral blood monocyte cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PI	protease inhibitor
Rev	regulator of virion protein expression
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
SD	standard deviation
SH3	src-homology 3
TAR	trans-activation response
Tat	transactivator of transcription
TCR	T cell receptor

INTRODUCTION

1. HIV

1.1 Epidemiology of HIV/AIDS

Human immunodeficiency virus (HIV) is a single-stranded, positive-sense RNA lentivirus that causes acquired immunodeficiency syndrome (AIDS). HIV infection causes the progressive failure of the immune system, making patients vulnerable to life-threatening opportunistic infections and cancers (Sepkowitz 2001). HIV/AIDS is a global pandemic, with an average of 40,000 new HIV infections each year (Cohen, Hellmann et al. 2008). According to the 2011 world AIDS report (www.unaids.org), approximately 17.2 million men, 16.8 million women and 3.4 million children under 15 years old were infected with HIV. Of these, Sub-Saharan Africa is the region most affected: In 2010, an estimated 68% (22.9 million) of all HIV cases and 66% of all deaths (1.2 million). In the United States, the CDC estimates that 1.1 million persons are living with HIV infection, including 18.1% who are unaware of their infection. With the development of antiretroviral therapies, the deaths from AIDS decreased to 1.8 million in 2010 compared with 2.2 million in 2005. However, the deaths of persons with an AIDS diagnosis can be due to any cause: the death may or may not be related to AIDS (Cheung, Pantanowitz et al. 2005).

1.2 Clinical and virological aspects of HIV infection

There are three main phases of HIV infection: acute infection, clinical latency and AIDS (Bartlett 1990). Within 2-4 weeks after infection with HIV, some individuals experience “influenza-like illness” while others do not have any symptoms. The duration of the symptoms varies, but usually last one or two weeks. This is the period when HIV virus is rapidly produced in the patient’s body. The initial symptoms are followed by a stage called clinical latency, asymptomatic HIV, or chronic HIV. This stage can last from three years to twenty years without any treatments. During this phase, HIV reproduces at a very low level, with few or no symptoms at first. Towards the end of this phase, HIV-patients viral load begins to rise and the CD4 cell counts decrease (Figure 1). As this happens, many people experience fever, weight loss, gastrointestinal problems and muscle pains, and some of them may also develop persistent generalized lymphadenopathy. However, about 5% of HIV patients retain high CD4 cell counts without antiretroviral therapy (ART) for more than 5 years, and are considered as HIV long-term nonprogressors (LTNP) (Blankson 2010). Another small group of HIV-infected patients (0.03%) maintain a low or undetectable viral load without ART and are known as “elite controllers” or “elite suppressors”. Once the number of CD4 cells begins to fall below 200 cells per μL , these patients are diagnosed as AIDS patients (Alimonti, Ball et al. 2003). At this stage, people with AIDS have an increased risk of developing various viral induced cancers including: Kaposi's sarcoma, Burkitt's lymphoma, primary central

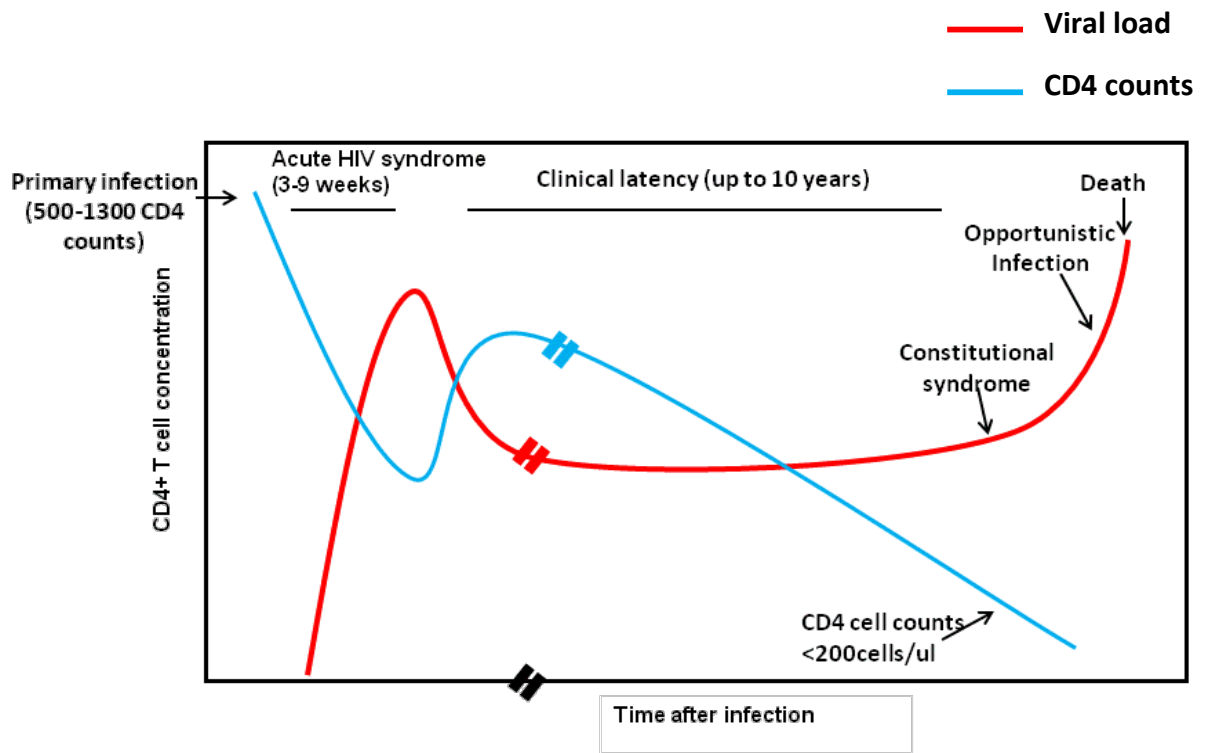


Figure 1: Timecourse of HIV infection

The Blue line represents CD4 cell counts while the red line represents viral load.

Major disease progressions were labeled in the figure. Adapted from Grossman,

Meier-Schellersheim et al. 2006.

nervous system lymphoma, and cervical cancer, which are less common in immuno-competent people.

1.3 HIV genome structure

HIV is a large virus with a diameter of about 120nm, composed of two copies of positive single-stranded RNA that codes for the virus's nine genes (*gag*, *pol*, and *env*, *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*). HIV is surrounded by the viral envelope (Lu, Heng et al. 2011). The protein, known as Env, consists of a cap made of three molecules called glycoprotein (gp) 120, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope. This protein is important to enable the virus to gain access into CD4+ cells to initiate the infectious cycle. *Env* together with other two genes: *Gag* and *Pol* are the structural genes for new virus particles. *Gag* encodes four structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (Poon, Wu et al. 1996; Cimarelli, Sandin et al. 2000; Wang and Aldovini 2002). *Pol* encodes the viral enzymes: protease, reverse transcriptase (RT), and integrase (IN) (Hill, Tachedjian et al. 2005). These enzymes are produced as a Gag-Pol precursor polyprotein by the viral protease, and are important for viral development. The six remaining viral genes, *Tat*, *Rev*, *Vpr*, *Vif*, and *Vpu* (or *Vpx*), *Nef*, play regulatory roles in controlling the ability of HIV to infect cells, produce new copies of virus, or cause disease. Of these, *Tat* is the transcriptional transactivators for HIV's landmark long terminal repeats (LTR) promoter acting by binding the trans-activation response (TAR) RNA element (Debaisieux, Rayne et al. 2012). *Rev*

provides nuclear export of non- or incompletely-spliced HIV mRNAs (Pollard and Malim 1998). Vpr is required for targeting the nuclear import of preintegration complexes, cell growth arrest, transactivation of cellular genes, and induction of cellular differentiation (Bukrinsky and Adzhubei 1999). Vif prevents the activity of a strongly anti-HIV host factor, APOBEC3G (Zhang, Pomerantz et al. 2000). Vpu increases the efficiency of viral budding from the host cell by down regulate CD4 in the endoplasmic reticulum (Bour and Strebel 1996). Nef is a multifunctional accessory protein (Das and Jameel 2005) (Figure 2) and will be discussed in subsequent sections.

1.4 HIV replication cycle

1.4.1 Entry

HIV virus entering host cells requires HIV gp120 molecules to bind to CD4 molecules on the host cell's surface. In addition to the main receptor CD4 and at least one of two chemokine receptors: CXCR4 and CCR5, is required for viral entry (Chan and Kim 1998). Following binding to CD4 protein, gp120 undergoes a structural change exposing the chemokine binding domains allowing them to interact with either CXCR4 (T-tropic isolates), CCR5 (M-tropic isolates) or both based on their tropism. This step helps the N-terminal fusion peptide gp41 to penetrate the cell membrane (Wyatt and Sodroski 1998). Of note, T-tropic isolates refer to the virus isolated from patients later in the infection in the symptomatic phase that infect T cells. On the other hand, M-tropic isolates are

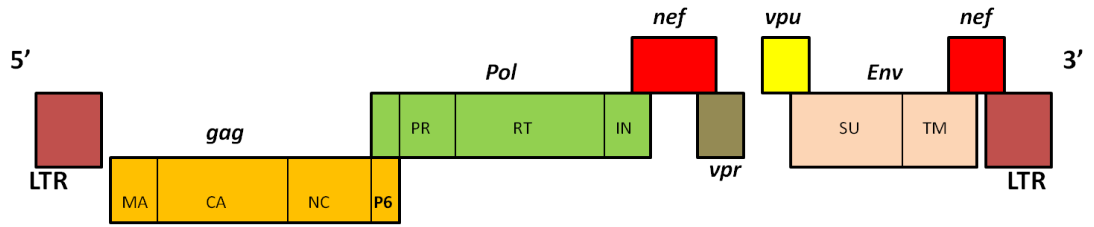


Figure 2: The structure of HIV genome.

The HIV genome consists of 9 genes, including the structure genes Gag, Pol and Env; the regulatory genes Tat and Rev; and the accessory genes Nef, Vif, Vpu and vpr. The HIV genome also has a "Long Terminal Repeat" (LTR) at each end of its genome.

the viruses isolated from individuals early in an infection during the asymptomatic phase, that typically infect macrophages, but not T cells. The mutation of co-receptors may reduce the ability of virus to infect target cells. Individuals with the CCR5-Δ32 mutation are resistant to infection with M-tropic isolates, as the mutation stops HIV from binding to the CCR5 co-receptor (Galvani and Slatkin 2003).

Besides the classical CD4/co-receptor mediated entry, a few non-standard receptors have been reported. For example, Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), a dendritic cell (DC)-specific HIV binding protein, enhances trans-infection of T cells. DC-SIGN does not function as a receptor for viral entry into DC but instead promotes efficient trans-infection in cells that express CD4 and chemokine receptors (Pope and Haase 2003; Haedicke, Brown et al. 2009). Similarly, for macrophages, mannose-specific macrophage endocytosis receptor interacting with gp120 also enhances viral entry.

1.4.2 Replication and transcription

Following viral entry to the target cell, the virus's RNA and enzymes PI, RT, and IN enter the cytoplasm. RT allows the single-stranded RNA to be copied and double-stranded DNA (dsDNA) to be generated (Zheng, Lovsin et al. 2005). Because of the extremely error-prone process of RT, HIV is rapidly mutated and evades the body's immune system. Integrase enzyme then facilitates the integration of this viral DNA into the cellular chromosome. Provirus (HIV DNA) is

replicated along with the chromosome when the cell divides. The integration of provirus may be dormant in the latent stage of HIV infection that enables the virus to evade host responses so effectively. Next, the integrated provirus is transcribed into mRNA including Tat, Rev, and Nef, and these are the early viral proteins. These mRNA are spliced into smaller pieces, and exported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins. Rev binds to viral mRNAs to facilitate unspliced RNAs to leave the nucleus. Meanwhile, the structural proteins Gag and Env are produced from the full-length mRNA (Pollard and Malim 1998).

1.4.3 Assembly and release

Assembly of new HIV virions, Env protein (gp160) is localized to lipid rafts in cell membrane through the Golgi complex. Gp160 is cleaved into the two HIV envelope glycoproteins, gp41 and gp120 (Hallenberger, Bosch et al. 1992). They are transported to the plasma membrane of the host cell where gp41 anchors gp120 to the infected cell membrane. Once the Gag and Gag-Pol polyproteins associate with the inner surface of the plasma membrane, HIV genomic RNA begins to bud from the host cell. Gag protein is then cleaved into the actual matrix, capsid and nucleocapsid proteins by the packaged viral enzyme PI (Sundquist and Krausslich 2012). The virion now undergoes a morphological change and becomes mature and infectious (Figure 3).

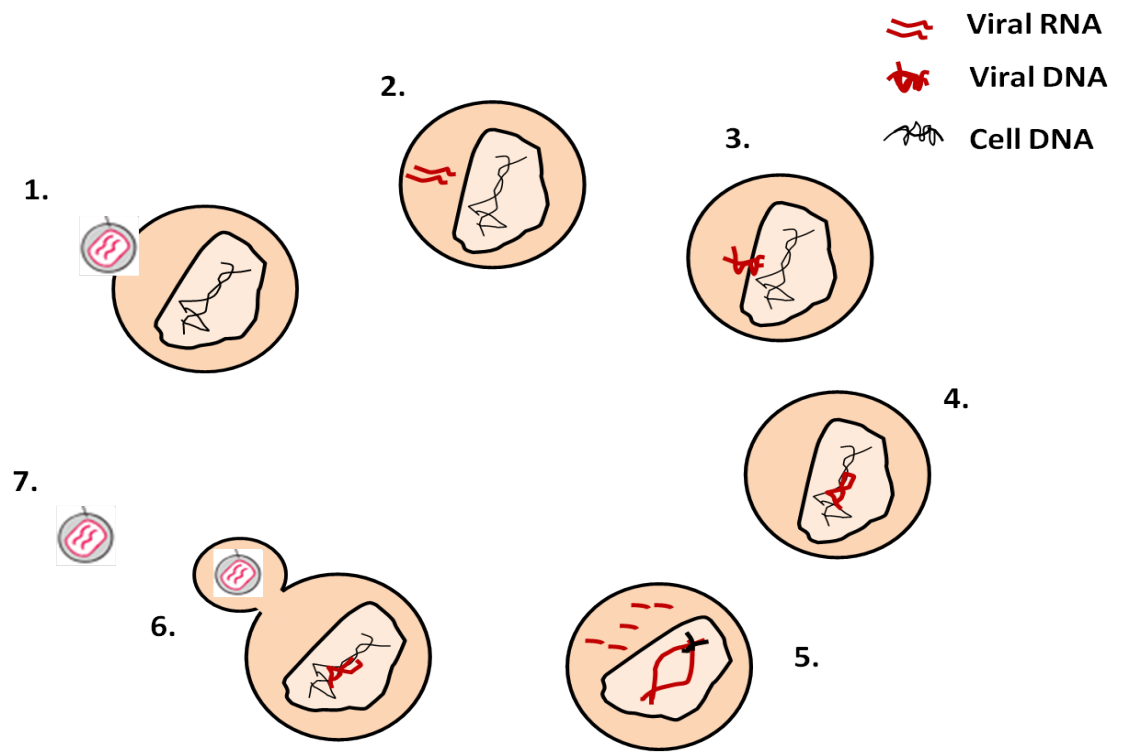


Figure 3: HIV life cycle

The important steps of HIV life cycle: 1. HIV gains the entrance to its target cell. 2. HIV releases RNA, the genetic code of the virus, into the cell. 3. HIV RNA is converted to DNA by reverse transcriptase. 4. The viral DNA enters the nucleus of its target cell followed by integration. 5. The DNA of the infected cell now produces RNA and proteins that are needed to assemble a new HIV. 6. HIV viral assembly at the cell membrane. 7. The new mature HIV virus.

1.5 HIV treatment

1.5.1 HIV vaccines

HIV surface proteins, especially gp120, are considered as targets for vaccines against HIV. However, the gp120 glycosylation patterns are highly variable in different HIV strains, which made producing gp120 antibodies difficult. In this decade, several research groups brought vaccines to clinical trials, including the STEP study and HVTN 505 trials. The STEP study was ceased in 2007, while HVTN 505 was stopped in 2013, both due to ineffectiveness in preventing HIV infections and lowering viral load among those participants who had become infected with HIV. In a recent report, a new vaccine has just finished FDA Phase I Clinical Trials and has shown promising results. This vaccine uses HIV that has been genetically engineered to make it non-infectious, which reduces the risk of causing HIV in the recipient compared to making a vaccine from whole, killed virus. HIV patients with vaccine intake had as much as 64 times more antibody against the p24 capsid antigen and up to eight times higher antibody against gp120 surface antigen than initial titers. And there were no major side effects or reactions to the vaccine reported. However, the Phase II and Phase III trials still have to be run, where most other vaccines had been deemed failures.

1.5.2 ART

Antiretroviral therapy dramatically improves the health and prolongs the lives of persons with HIV (Cooper, Nugent et al. 1996). ART efficiently decreases

patient's viral loads, maintains function of their immune system, and prevents opportunistic infections. Studies also showed that using ART can reduce the risk of sexually transmitting HIV by 90%. ART normally combines at least three antiretroviral drugs from at least two different categories. There are five categories of drugs which attack HIV in different ways, including nucleoside reverse transcriptase inhibitors (NRTI)/nucleotide reverse transcriptase inhibitors (NtRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PIs), viral entry inhibitors and integrase inhibitors (Table 1).

The first approved antiretroviral drugs (1987) NRTI and NtRTI are nucleoside and nucleotide analogues, lacking a 3'-hydroxyl group on the deoxyribose moiety, which inhibit reverse transcription. NRTIs need to be activated for viral DNA incorporation by addition of three phosphate groups to their deoxyribose moiety, to form NRTI triphosphates. In contrast, non-nucleoside analogs block reverse transcriptase by binding to RT. PIs block the viral protease enzyme, preventing cleavage of gag and gag/pol precursor proteins. Thus, the newly made virus particles in this case are defective and mostly non-infectious. The side effects of first generation PIs that have been reported include causing a syndrome of lipodystrophy, hyperlipidaemia, diabetes mellitus type 2 and kidney stones. New generation drugs have been developed that are less toxic and are effective against some HIV variants that are resistant to first generation PIs. Integrase inhibitors inhibit the enzyme integrase, which is responsible for integration of DNA from virus into the target cell. Currently, there are only two FDA approved integrase inhibitors: raltegravir (2007) and the latest one

Elvitegravir (2013), but several others are currently in clinical trials. Entry inhibitors interfere with virus binding, fusion and entry to the host cell by blocking its receptors. There are two FDA approved entry inhibitors: Maraviroc (targeting CCR5) and enfuvirtide (targeting CXCR4). However, using this class of drugs has a risk of tropism shift, which allows HIV to target an alternative co-receptor. Fuzeon (enfuvirtide), a peptide drug can act by interacting with the N-terminal heptad repeat of gp41 of HIV to form an inactive hetero six-helix bundle, therefore preventing infection of host cells.

Category	Brand Name	Generic Names
NRTIs	Epivir Emtriva Retrovir Trizivir Zerit Ziagen	Lamivudine Emtricitabine Zidovudine, Abacavir, Stavudine Abacavir sulfate
NNRTIs	Edurant Intelence Rescriptor Sustive Viramune	Rilpivirine Etravirine Delavirdine Efavirenz Nevirapine
PIs	Agenerase Crixivan Norvir Prezista Reyataz	Amprenavir Indinavir Ritonavir Darunavir Atazanavir sulfate
Entry Inhibitors	Fuzeon Selzentry	Enfuvirtide (T-20) Maraviroc
Integrase inhibitors	Isentress EVG	Raltegravir Elvitegravir

Table 1: FDA-approved antiretroviral drugs

Data collected from FDA website www.fda.gov/oashi/aids/virals.

2. HIV negative factor (Nef)

2.1 Nef expression and structure

Nef is a ~27 kDa accessory protein that is devoid of enzyme activity. It is found at the 3' end of the HIV genome, overlapping the 3'LTR (See Figure 2). Nef is N-terminal myristoylated, which is crucial for its membrane targeting. The Nef core domain structure resembles that of the helix-turn-helix (HTH) family of proteins involved in binding DNA. One of the most important motifs for Nef function is the proline-rich Src homology-3 (SH3) domain-binding sequence (PxxP), allowing Nef to bind to SH3 domain-containing proteins, such as Src, Lck, and Hck. This binding site is required for Nef to associate with a P21 activated kinase (PAK)-Related Kinase, and Nef-induced alterations of signal transduction in T cells. In addition, The Nef N-terminal stretch of basic residues can contribute to Nef membrane localization: an acidic region (62EEEE65) is responsible for Nef perinuclear localization, while three pairs of amino acid residues (W57/L58, L164/L165, and D174/D175) act to alter the surface expression of many cellular proteins, such as down regulating CD28 expression (Figure 4).

2.2 Nef protein function in HIV

2.2.1 Nef as a positive viral factor

Nef is an important HIV pathogenic factor, as demonstrated by *in vivo* studies. Studies in Rhesus monkeys have demonstrated that Nef is critical for attaining high virus loads and development of AIDS-like disease in SIV-infected

macaques. In parallel, studies on SCID-hu mice also demonstrated that nef-deleted HIV strains showed significantly lower levels of infectivity and pathogenicity. A recent study also demonstrated that transgenic mice expressing CD4-promoter driven Nef develop a spectrum of pathologies including AIDS-like disease. More importantly, in clinical studies, patients infected with a HIV Nef-deletion or Nef allele mutant strains showed lack of disease progression. However, Nef either has no effect or a positive effect on viral replication *in vitro* depending on cell types. Nef generally has no effect on viral replication in activated peripheral blood mononuclear cells (PBMCs) and activated CD4+ T cells, but plays a significant role in post infection-stimulated PBMCs or lymphoid cultures, immature dendritic cell-T cell co-cultures and in the ex vivo tonsil culture system.

Productive HIV infection is regulated by Nef-induced lymphocyte-stimulating factor release from macrophages. This provides an environment where Nef itself can stimulate viral replication by increasing the pool of lymphocytes. In addition, HIV Nef also facilitates the incorporation of Nef-associated cellular kinases that phosphorylate various substrates, including viral matrix protein necessary for generating fully infectious viral particles.

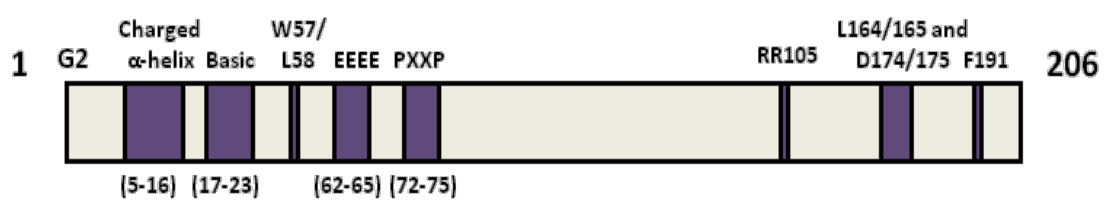


Figure 4: Residues of HIV Nef protein

Important Nef residues are labeled above the figure: The Nef N-terminal stretch of basic residues can contribute to Nef membrane localization; an acidic region (62EEEE65) is responsible for Nef perinuclear localization; the proline-rich Src homology-3 (SH3) domain-binding sequence (PxxP); the and three pairs of amino acid residues (W57/L58, L164/L165, and D174/D175) act to alter the surface expression of many cellular proteins.

2.2.2 Nef alleles show major differences in pathogenicity

Nef is a major determinant of pathogenicity in persons. Various Nef alleles were reported to show differences in pathogenicity, including SF2, JR-CSF, YU10x, and NL4-3 [T71R] Nef alleles, as well as some from long-term nonprogressors (AD-93, 032an, and 039nm alleles). Except YU10x, all these Nef alleles down regulated cell surface CD4 in human cells in vitro, and depleted of double-positive and single-positive thymocytes. A loss of peripheral CD4 T cells was observed with all alleles but was minimal in Nef YU10x Tg mice. Of these alleles, Nef032an and Nef SF2 are highly virulent; associated with severe T-cell loss. Moreover, all Nef alleles except the Nef YU10x and Nef NL4-3(T71R) alleles induced an enhanced activated memory (CD25(+) CD69(+) CD44(high) CD45RB(low) CD62L(low)) and apoptotic phenotype. Also, all could interact with and/or activate PAK2 except the Nef JR-CSF allele. Nef NL4-3(T71R), Nef 032an, Nef 039nm, and Nef SF2 Tg mice, but not Nef YU10x Tg and NefAD-93 mice, developed organ (lung and kidney) diseases (Priceputu, Hanna et al. 2007).

2.2.3 Nef alters cell surface expression of critical proteins

Nef protein was reported to down regulate several important proteins involved in the immune synapse, including CD4, MHC I, MHCII, CD28, NKG2D, CXCR4, and CD80(Le Gall, Erdtmann et al. 1998; Swigut, Shohdy et al. 2001; Blagoveshchenskaya, Thomas et al. 2002; Chaudhry, Das et al. 2005; Cerboni, Neri et al. 2007; Chaudhuri, Lindwasser et al. 2007). It is not fully understood

how Nef affects these proteins, but in most case, Nef down regulates these proteins by interacting with adaptor protein complexes AP-1, AP-2, AP-3, Clathrin, and PACS-1 (Foti, Mangasarian et al. 1997; Le Gall, Erdtmann et al. 1998; Lock, Greenberg et al. 1999).

2.2.4 Nef alters T cell receptors and cellular signaling pathways

Nef targets various signaling pathways, including the T cell receptor (TcR) signalling pathway (Luo and Peterlin 1997; Djordjevic, Schibeci et al. 2004), IL-2 receptor pathway (Greenway, Azad et al. 1995; Schragar and Marsh 1999), pathways in macrophages leading to chemokine production as well as the anti-apoptotic cascade. Nef binding to SH3 domains of the Src family of kinases p21 activated protein kinase (PAK2) is important for signal transduction alteration in T cells, such as inducing rapid and transient phosphorylation of the α and β subunits of the I κ B kinase complex and of JNK, ERK1/2 as well as p38 mitogen-activated protein kinase family members (Rudel, Zenke et al. 1998). In addition to the effects in infected cells, Nef has been shown to induce the formation of exosomes (Lenassi, Cagney et al. 2010) to cause cell death in bystander T cells and formation of nanotubes, which physically connect to bystander cells (Sowinski, Jolly et al. 2008). In infected monocytes, Nef induces nanotube-like conduits that can connect to B cells and mediate its own transfer to B cells where it inhibits Ig class switching (Qiao, He et al. 2006; Xu, Santini et al. 2009).

3. Cardiovascular diseases

3.1 Coronary artery disease (Atherosclerosis)

3.1.1 Cholesterol-dependent

Atherosclerosis refers to hardening of arteries that results from a chronic inflammatory response in the walls of arteries. It is caused by the accumulation of macrophages and white blood cells and promoted by low-density lipoproteins (LDL) (Ross 1999). Endothelial dysfunction is an early precursor to atherosclerosis and has been shown to predict future cardiovascular events in most population studies, which is defined as an imbalance between vasodilating and vasoconstricting substances produced by the endothelium (Deanfield, Donald et al. 2005). The normal arterial endothelium resists prolonged contact with leukocytes including the blood monocyte. Once endothelial cells undergo inflammatory activation, they attract monocytes by increasing adhesion molecule expression, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (Cybulsky, Iiyama et al. 2001). The activated endothelial cells secrete various chemokines, including monocyte chemoattractant protein-1 (MCP-1), which allow monocytes to penetrate into the intima, the innermost layer of the arterial wall (Aiello, Bourassa et al. 1999). After the monocyte acquires characteristics of the tissue macrophage, in the atheroma in particular, scavenger receptors on these macrophages bind to modified LDL by oxidation or glycation. These processes give rise to the arterial foam cell, a hallmark of the arterial lesion. The foam cell then secretes pro-inflammatory cytokines that amplify the local inflammatory response and reactive oxygen

species (ROS) production in the lesion (Mugge 1998). Matrix metalloproteinases (MMPs) production can degrade extracellular matrix, followed by the plaque ruptures, and eventually induce thrombus formation in the lumen (Figure 5).

3.1.2 Other risk factors: Cholesterol-independent

Smoking

Cigarette smoking (CS) is known to increase the incidence of myocardial infarction (MI) and fatal coronary artery disease (CAD), even for light smokers. Passive CS is associated with a 30% increased risk of CAD, compared with an 80% increase in active smokers (Jeremy, Mikhailidis et al. 1995; Ambrose and Barua 2004). Cigarette smoking impairs endothelium-dependent vasodilation through decreasing nitric oxide (NO) availability. NO is not only a vasoregulatory molecule, but it also helps regulate inflammation, leukocyte adhesion, platelet activation, and thrombosis (Napoli and Ignarro 2001). Thus, an alteration in NO biosynthesis could have both primary and secondary effects on the initiation and progression of atherosclerosis. Cigarette smoking also increases levels of inflammatory markers including C-reactive protein (Tracy, Psaty et al. 1997), interleukin-6 (IL6), and tumor necrosis factor alpha (TNF α) (Mendall, Patel et al. 1997). Furthermore, CS could promote atherosclerosis by its effects on lipid profile (Craig, Palomaki et al. 1989). Though the mechanism behind this was not fully understood, triglyceride/LDL abnormalities have been suggested to be related to insulin resistance. More importantly, CS increases oxidative modification of LDL that exposure to CS extract caused a modification of LDL

that promoted the formation of foam-cells, and it may also decrease the plasma activity of paraoxonase, an enzyme that protects against LDL oxidation (Yokode, Kita et al. 1988; Pech-Amsellem, Myara et al. 1996).

Diabetes

It is known that coronary heart disease is correlated with both type 1 and type 2 diabetes mellitus (Nathan, Lachin et al. 2003). These diabetic patients fail to achieve significant reduction in macrovascular events, which may be explained by diabetes-related metabolic abnormalities accelerating macrovascular complications. In some animal models, diabetes-induced hyperlipidemia occurs and may mask toxic effects of hyperglycemia that confer diabetes-specific risk. These effects may be caused by the development of increasing advanced glycation end products (AGEs) level, toxic intracellular reactions to glucose secondary to the production of increasing ROS level, and multiple effects on the production of matrix proteins and the biology of cells within the vessel wall (Brownlee 2001).

3.2 HIV-related cardiovascular diseases

3.2.1 ART in coronary artery disease

Acute coronary syndromes are observed with increasing frequency among HIV patients receiving therapy with protease inhibitors as part of ART regimens. Several studies reported that currently available HIV PIs are associated with increased levels of total cholesterol (36 [75%] of 48 studies), triglycerides (35 [73%] of 48 studies), and low-density lipoprotein (12 [100%] of 12 studies).

Protease inhibitor was also found to increase carotid intima thickness or atherosclerotic lesions (7 [88%] of 8 studies) (Rhew, Bernal et al. 2003). In particular, HIV PIs (ritonavir, indinavir, and atazanavir), caused the accumulation of free cholesterol in intracellular membranes, depleted endoplasmic reticulum calcium stores, activated caspase-12, increased apoptosis in macrophages and promoted atherosclerosis and cardiovascular disease in HIV-infected patients. The most substantial database has recently been provided by the D:A:D study group who demonstrated an increased incidence of myocardial infarction in HIV-infected persons on PIs or NtRTIs-containing therapy in a prospective observational cohort study. In a contrast, SMART (Strategies for Management of Anti-Retroviral Therapy) analysis revealed slightly elevated risk of cardiovascular disease in people who interrupt therapy, which implied that there are other factors may potentially increase HIV-cardiovascular diseases (McClay, David Fourteenth Conference on Retroviruses and Opportunistic Infections).

3.2.2 HIV viron-related cardiovascular disease

Controversy currently exists on whether people living with HIV and taking ART have greater cardiovascular risks than treatment-naive HIV-infected individuals. Untreated HIV infection has been found to associate with increased levels of IL6. Higher levels of IL-6 strongly predict cardiovascular events and overall mortality in antiretroviral-untreated and treated HIV infection (Kuller, Tracy et al. 2008).

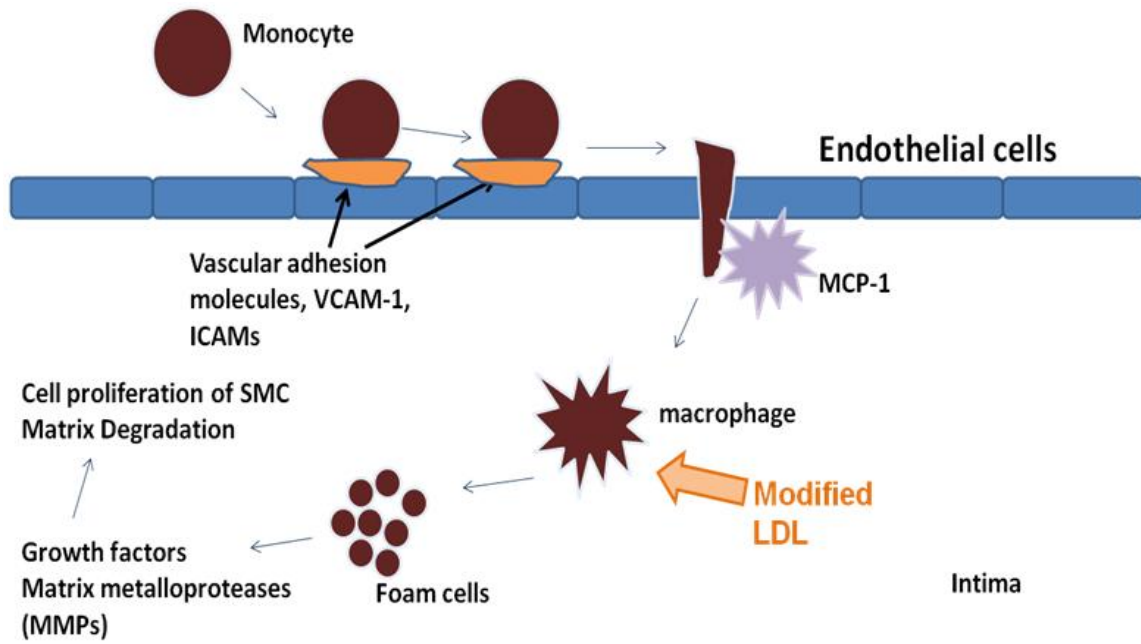


Figure 5: The process of atherosclerosis.

The schematic is the representation of atherosclerotic process. Monocytes recruit when adhesion molecules (e.g VCAM I and ICAM) express on endothelium. Once resident in the arterial intima, activated macrophages become phagocytically active. Followed by the macrophages take up enough cholesterol and become foam cells. Foam cells release growth factors that stimulate the growth of nearby smooth muscle cells and fibroblasts. Smooth muscle cells migrate from the tunica media into the intima. Once they deposit additional connective tissue, the abnormal region becomes a fibrous plaque. Ruptures of the fibrous cap expose thrombogenic material to the circulation and eventually induce thrombus formation.

HIV infection is reported to be associated with premature atherosclerosis. This occurs even in the absence of detectable viremia, overt immunodeficiency and exposure to ART and appears to be independent of traditional CD4+ T cell count. C-reactive protein was higher in HIV cardiac risk factors. They measured 401 HIV-seropositive participants by assessing carotid artery intima-media thickness (IMT) and found that IMT was strongly associated with the presence of HIV disease rather than viral load or controllers than HIV-seronegative persons. However, ART was also associated with higher IMT (Hsue, Hunt et al. 2009). Recent studies have shown that acute myocardial infarction (AMI) rates and cardiovascular risk factors were increased in HIV compared with non-HIV patients [11.13 (95% confidence interval [CI] 9.58-12.68) vs. 6.98 (95% CI 6.89-7.06)], particularly among women (Triant, Lee et al. 2007). Even though there were no reports showing that HIV-infected patients with the metabolic syndrome (MS) have a faster rate of progression of coronary atherosclerosis, Fitch et al reported that HIV-infected men with MS have higher cardiac risk factor - coronary artery calcium (CAC) score when compared to HIV-infected and non HIV-infected control groups with similar demographic parameters, age and smoking rates (Ullrich, Groopman et al. 2000). They also found that presence of plaque and numbers of noncalcified plaque segments were increased among both HIV-infected groups compared with HIV negative controls. Both of the evidence suggested that HIV patients without significant metabolic abnormalities may still develop noncalcified plaque and be at increased risk for coronary artery diseases.

3.2.3 HIV proteins in cardiovascular disease

Endothelial dysfunction injury is pivotal to the development of cardiovascular disease in HIV-infected patients (Chi, Henry et al. 2000). Endothelial dysfunction is defined as an imbalance between vasodilating and vasoconstricting substances produced by the endothelium (Deanfield, Donald et al. 2005). Endothelial dysfunction can result from or contribute to coronary artery disease, and other atherosclerotic diseases (Flammer, Anderson et al. 2012). The previous studies showed that endothelial “activation”, in the form of soluble adhesion molecules and procoagulant proteins, led to a pro-inflammatory, proliferative and prothrombotic state of the endothelium occurs. Proinflammatory cytokines (e.g IL-6, TNF alpha) and HIV proteins are contributory to endothelial dysfunction.

HIV envelope glycoprotein (HIV gp120)

HIV DNA or RNA was only found in inflammatory cells of heart tissues from HIV patients with or without HIV cardiomyopathy (HIVCM) but not endothelial cells or cardiomyocytes. Interestingly, HIV gp120 was exhibited in both of these cells (Fiala, Popik et al. 2004). Recombinant HIV gp120 protein has been shown to damage the endothelium by interaction with CXCR4 (Fiala, Murphy et al. 2004), and this induced endothelial apoptosis mediated by activating caspase 3,9 and by slightly enhancing expression of the pro-apoptotic molecule, Bax (Kanmogne, Primeaux et al. 2005). HIV gp120 was also reported to be able to reduce endothelial nitric oxide synthase (eNOS) expression in TNF- α -activated endothelial cells; HIV gp120 and TNF- α have synergistic effects on

inhibition of eNOS expression in endothelial cells (Jiang, Fu et al. 2010). Use of a primary human lymphatic endothelial cell model, HIV gp120 induced lymphatic hyperpermeability by disturbing the normal function of Robo4, a novel regulator of endothelial permeability. Moreover, pretreatment with an active N-terminus fragment of Slit2, a Robo4 agonist, protected lymphatic endothelial cells from HIV gp120-induced hyperpermeability by inhibiting c-Src kinase activation (Zhang, Yu et al. 2012).

HIV secreted protein: Tat protein

HIV Tat protein has been found to significantly decrease endothelium-dependent vasorelaxation and eNOS mRNA and protein expression in endothelial cells of porcine coronary arteries (Diaz, King et al. 2000). Tat is also known to cause apoptosis of primary microvascular endothelial cells of lung origin via a mechanism distinct from tumor necrosis factor secretion or the Fas pathway. On the other hand, Tat protein dissemination to extravascular tissue can activate human endothelial cells by expressing the endothelial-leukocyte adhesion molecules, E-selectin, critical for the initial binding of leukocytes to the blood vessel wall, and their increased synthesis of IL-6, a cytokine known to enhance endothelial cell permeability (Hofman, Wright et al. 1993). Tat protein is also found to play a role in HIV-related pulmonary hypertension through several different mechanisms: In the most recent study, the exposure of HUVECs to HIV Tat protein resulted in induced expression of cell adhesion molecule ICAM-1 through activation of mitogen-activated protein kinases and downstream transcription factor NF- κ B., leading to increased adhesion of monocytes to the

endothelium (Magalhaes, Greenberg et al. 2007); Three receptors: the integrins $\alpha v\beta 3$, $\alpha 5\beta 1$ and the vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) were reported to interact with Tat protein, and help Tat protein gain access to endothelium (Albini, Soldi et al. 1996; Barillari, Sgadari et al. 1999; Mitola, Soldi et al. 2000; Kline and Sutliff 2008). These findings further support the concept of how Tat protein causes aberrant cell signaling and leads to altered endothelial cell morphology, gene expression, and survival.

HIV Nef protein

There have been many reports of low-level transcription of HIV genes even after years of ART (Furtado, Callaway et al. 1999; Fischer, Gunthard et al. 2000; Gunthard, Havlir et al. 2001). Further analysis has shown that the multiply-spliced mRNAs, the “early” HIV genes Tat, Rev, and Nef, decrease to a lesser extent than other HIV gene products after initiation of treatment (Fischer, Joos et al. 2008) suggesting that these may play a role in mechanisms of cardiovascular damage even in patients on ART. In fact, intracellular mRNA encoding Nef showed the cellular viral rebound in PBMCs during therapy and was subsequently triggered by the plasma viremia that preceded the recurrence (Fischer, Joos et al. 2004). When Nef is expressed specifically in CD4⁺ cells in CD4.Nef Tg mice, Nef can cause development of AIDS-like phenotypes, cardiovascular disease and coronary vascular dysfunction (Fischer, Joos et al. 2004; Hanna, Priceputu et al. 2009; Jolicoeur 2011). Although no reported studies have directly assayed the effect of Nef on coronary arteries, Nef has been shown to be involved in pulmonary arterial dysfunction. Macaques infected

with a chimeric simian immunodeficiency virus (SIV) containing the HIV-nef gene (SHIV-Nef) exhibited vasculopathies similar to those in human pulmonary hypertension (PH) (Sehgal, Mukhopadhyay et al. 2009). Importantly, 10 polymorphisms in Nef were significantly more frequent in blood cells or lung tissue specimens from individuals with HIV and pulmonary hypertension compared to HIV-infected individuals without PH, suggesting the possibility of linking particular Nef sequences with specific pathogenic functions of Nef, i.e. ability to transfer to bystander cells.

HIV Nef protein has also been shown to block ATP-binding cassette transporter A1-dependent cholesterol efflux, resulting in the accumulation of lipids in macrophages, a condition previously shown to be highly atherogenic (Mujawar, Rose et al. 2006). There are at least two mechanisms involved for this: HIV infection and transfection with *Nef* induced post-transcriptional down regulation of ABCA1; and Nef mediated redistribution of ABCA1 to the plasma membrane and inhibited internalization of apolipoprotein A-I. Mutations in *ABCA1* cause Tangier disease, which is associated with low HDL cholesterol and accelerated atherosclerosis (Oram and Lawn 2001). Importantly, HDL cholesterol levels are reduced in untreated HIV infection, which is greater than the ART effect (Grunfeld, Pang et al. 1992; Shafran, Mashinter et al. 2005; Carr and Ory 2006; Johnson, Gathe et al. 2006).

Other HIV proteins

One group quantified the role of expression of HIV-1 proteins on the vascular function, biomechanics, and geometry of common carotid arteries and

aortas by accessing NL4-3Δ gag/pol transgenic mice (HIV-Tg). These HIV-Tg mice have been found to have impaired aortic endothelial function, increased c-IMT, and increased arterial stiffness; and decreased elastin content, increased cathepsin K and cathepsin S activity, and increased mechanical residual stress (Hansen, Parker et al. 2013).

4. Summary of background and the hypothesis of this study

Cardiovascular disease is emerging as a major cause of morbidity and mortality in the increasingly aging HIV-infected population. A hallmark of many of these diseases is evidence of chronic vascular inflammation. Although side effects of combined anti-retroviral therapy (ART) could be blamed for some increases in CVD, it is becoming evident that HIV infection is an independent risk factor for CVD. However, it remains a mystery how HIV can contribute to CVD in patients on ART, when viral titers are low or non-detectable. One possible explanation could be persistence of toxic viral products despite control of viral replication and viremia. In this regard, a myristoylated intracellular protein, Nef, has been shown to be produced in infected cells even after virus production is halted by ART. Endothelial cells, especially in developing atherosclerotic plaques, are in direct contact with circulating HIV-infected cells and in a prime position for Nef transfer. Therefore, my primary hypothesis is that Nef may also transfer to vascular endothelial cells and thus lead to endothelial activation, dysfunction and potentially progression to atherosclerosis. In this study, we propose a model in which Nef can mediate its transfer from Jurkat T cells to endothelial cells to trigger endothelial dysfunction. We further demonstrate that Nef contributes to endothelial dysfunction via two independent mechanisms, (1) apoptosis of endothelial cells through an NADPH oxidase-dependent mechanism and (2) MCP-1 production through the NF- κ B signaling pathway.

MATERIALS AND METHODS

Materials

Mediums and supplements

Roswell Park Memorial Institute 1640(RPMI-1640) medium, Eagle Basal medium-2 (EMB-2), penicillin, and endothelial cell growth bullet kit-2 (EGM-2MV) were purchased from Lonza (Walkersville, MD). EGM-2MV contains 5% FBS, 0.4% hydrocortisone, 0.4% hFGF, 0.1% VEGF, 0.1% IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-100 (Gentamicin, Amphotecerin B), and 0.1% heparin. Fetal bovine serum (FBS) was purchased from Hyclone sterile systems (Logan, UT). All bacterial culture materials were purchased from Becton Dickenson (Sparks, MA). The bacterial culture media were prepared in house, including Luria broth (LB) media containing 0.01 g/ml Bacto tryptone, 0.005 g/ml Bacto yeast extract, 0.005 g/ml NaCl, and 1 mM NaOH, with the addition of 15 g/L Bacto agar to make solid LB culture plates. Super optimal broth with catabolite repression (SOC) contained 0.02 g/ml Bacto tryptone, 0.005 g/ml Bacto yeast extract, 0.5 g/ml NaCl, 10mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose. Working concentrations of antibiotics were 100 µg/ml ampicillin and 50 µg/ml kanamycin, and they were purchased from Sigma-Aldrich (St Louis, MO).

Antibodies

Mouse anti-Nef EH1 antibody (IF 1:100; Flow 1:25) was obtained from the NIH AIDS Reagent Repository, Mouse anti-Nef 3D12 antibody (Flow 1:25), mouse anti-CD4 marker (Flow 1:50), phycoerythrin (PE)-conjugated anti-CD19

antibody and PE 650 donkey anti-sheep (IF 1:200) were purchased from Abcam (Cambridge, MA), Rabbit anti-p65 antibody (WB 1:1000) was purchased from Cell Signal Technologies (Danvers, MA). PE-conjugated phalloidin (IF 1:100), and AlexaFluor 488 goat anti-mouse (IF 1:200) were from Invitrogen (Carlsbad, CA). Normal mouse IgG, mouse anti-GAPDH (WB 1:5000), and anti-mouse HRP (WB 1:3000) were from Sigma-Aldrich.

Reagents

Live dye Vybrant DIO and JC-1 dye were obtained from Life Technologies (Grand Island, NY). The Lipofectamine LTX system was purchased from Invitrogen. Trolox, Apocynin, and I κ B kinase inhibitor (IKKi), were purchased from Abcam. P65 siRNA was purchased from Cell Signal Technologies. Western Blotting 4–20% bis-Tris polyacrylamide gels and ECL chemiluminescence reagents were purchased from Thermochemical (Waltham, MA). Protease inhibitor cocktail set, immunofluorescent mounting buffer, and collagen were purchased from Sigma-Aldrich. Anti-PE microbeads were purchased from Miltenyi Biotec (Auburn, CA). All other chemicals were purchased from Fisher scientific (Hampton, New Hampshire).

Biotechnological systems

Wizard® Plus Maxipreps DNA Purification System was purchased from Promega (Madison, WI). RT assay kit was purchased from Roche diagnostics (Indianapolis, IN). Nef enzyme-linked immunosorbent assay (ELISA) Kit was

purchased from ImmunoDX (Woburn, MA). MCP-1 Elisa kit was purchased from R&D Systems (Minneapolis, MN). In Vitro Vascular Permeability Assay kit was purchased from Fisher. Caspase 3 detection kit was purchased from Sigma-Aldrich. Apo-BrdU apoptosis detection kit and CPT™ Cell Preparation Tube with Sodium Citrate was purchased from BD biosciences (Franklin Lakes, NJ).

Animals

Macaque heart tissues were obtained from a cohort of male Indian rhesus macaques (*Macaca mulatta*) infected with SHIVnefSF33, at 1000 TCID₅₀ per animal [21]. The animals were housed at the California National Primate Research Center in accordance with the standards of the “Guide for the Care and Use of Laboratory Animals” and the American Association for Assessment and Accreditation of Laboratory Animal Care. Necropsies were performed between 37-62 weeks post-infection, when the animals showed signs of immunodeficiency. Mouse heart tissues were obtained from University of Montreal from mice age 3 to 6 month housed according to institutional biosafety/AWA regulations and protocols. These transgenic mice have Nef and GFP or GFP itself expressed under the regulatory sequences of the human CD4 gene promoter. The experiments described in this study used formalin-fixed, paraffin-embedded heart tissues from these animals.

Cells

Cell lines

Human coronary artery endothelial cells (HCAEC) and Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza. Jurkat cells and THP1 cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA).

Cell culture

HCAEC and HUVEC were maintained in culture medium consisting of Eagle's basal medium-2 (EMB-2), 5% FBS, 0.4% hydrocortisone, 0.4% hFGF, 0.1% VEGF, 0.1% IGF-1, 0.1% ascorbic acid, 0.1% hEGF, 0.1% GA-100 (Gentamicin, Amphotecerin B), and 0.1% heparin. Jurkat cells, THP1 cells and PBMCs were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin. All cell cultures were maintained at 37 °C in 5% CO₂ and 95% air. Culture medium was changed every two days.

Isolation of PBMCs from HIV patients blood

Peripheral blood from HIV patients was obtained from Indiana University hospital (Indianapolis, IN). Immediately after blood draw, CPT tubes with patients' blood were centrifuged for a minimum of 20 minutes at 1500-1800 with the brake on. The white layer of PBMCs was extracted, and then washed in 40

ml PBS three times by centrifugation at 440 x g for 5 min. This study was done under IRB approval.

Plasmids and viruses

Viruses

pNL4-3 and pNL4-3 Δ Nef were obtained from the NIH AIDS Reagent Repository.

Nef and Nef mutants

Nef.SF2 and Nef Δ SH3 mutant were obtained from the NIH AIDS Reagent Respository. Nef Δ SH3 mutant has a point mutation from Proline (PXXP) to Alanine (AXXA) in a conserved motif (aa72-75).

Methods

Bacterial transformation

GC5TM cells were mixed with 0.5 μ l DNA ligation reaction, and incubated on ice for 20 min. The cells were heat-shocked at 42 °C for 45 sec, followed by incubation on ice another 2 min with 1ml RT SOC medium. The cells were then incubated at 37 °C, for 1 h with shaking at 250 RPM. The cells were plated on LB plates with either ampicillin or kanamycin.

Cell transfections

HCAEC or Jurkat cells were transfected using the Lipofectamine LTX system (Invitrogen) according to the manufacturer's directions. pcDNA3 was used in the studies for normalization.

HIV replication assay

HIV NL4.3 or HIV NL4.3-Nef deficient virus was produced by transfection of 293T cells. Transfection was performed with Lipofectamine LTX reagent (Invitrogen), according to manufacturer's instructions. Viral supernatant was harvested at 48 h and centrifugated at 900 g for 10 min, to clarify the supernatant from remaining cells. Viral supernatant was used to infect Jurkat cells corresponding to a 10,000 cpm RT activity (equivalent to 1 ng/ml RT according to assay protocol (Roche). Virions in the supernatant were pelleted by centrifugation at 12,000 g for 1 hr and the RT activity was determined every other day for 9 days using the RT assay kit according to the manufacturer's instruction. Infected cells were cocultured with HCAEC when virus titers were the same in both infected cells.

Tissue culture based assays

For analysis using different pharmaceutical inhibitors, HCAEC were seeded in 6-well plates. Nef plasmid was transfected into HCAEC by Lipofectamine LTX reagent (Invitrogen). The transfection efficiency was ~70% as determined by FACS. After 6 h post transfection, culture medium was changed,

and inhibitors were added at optimized concentrations (200 nM Apocynin (Abcam), 200 nM Trolox (Abcam), and 100 nM Ikki (R&D Systems)). After a further 24 h, the supernatant was collected and analyzed for MCP-1 production assessed by sandwich ELISA (Quantikine, R&D Systems) and the cells were harvested for apoptosis analysis. For coculture working models, pcDNA only (mock control) or Nef-containing pcDNA plasmid was transfected into Jurkat cells by Lipofectamine LTX reagent. At 48 h post transfection, these Jurkat cells were cocultured with HCAEC in a 2:1 ratio (in some cases, inhibitors were added at optimized concentrations as described). After 24 h co-culture, the medium was centrifuged to remove Jurkat cells and the supernatant collected for analysis of MCP-1 production. Any remaining Jurkat cells were washed off with PBS and further gated from endothelial cells by FACS, based on forward scatter and side scatter profiles. HCAEC were harvested for apoptosis analysis.

Detection of apoptosis by assessing DNA fragmentation (TUNEL)

Apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling in endothelial cells was performed as previously described (Green, Petrusca et al. 2012). Detection of apoptosis by FACS via a modified TUNEL approach was carried out using a fluorescence labeling system to detect dUTP end nicks according to the manufacturer's instructions (APOBRDU kit, Becton Dickinson).

Confocal immunofluorescence microscopy

For *in vitro* studies, Nef and cDNA transfected Jurkat cells were stained with cytoplasm live dye Dyant DIO (Life Technologies) (5ul live dye/1 million cells at 37°C for 1 h), and cocultured with HCAEC for 24 h. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.01% Triton X-100 in PBS (20 min at 4°C) and stained with 10 uM phalloidin and in some cases, anti-Nef EH1 antibody. Visualization was achieved by confocal microscopy (Olympus FV1000-MPE) with the same fluorescence intensities during image acquisition. The derived images were analyzed using MetaMorph software.

In Vitro Vascular Permeability Assay

HCAEC were plated on transwell insert of 24 well Corning® Costar® cell culture plate with 0.5 ml EGM-2MV growth media. Nef was transfected to 80% confluent HCAEC for 24 h. One hundred fifty milliliters of FITC-Dextran working solution was added to each insert for 20 min at room temperature in the dark. One hundred milliliters of media was removed from each well for fluorescence measurement at 485 nm and 535 nm excitation and emission, respectively.

Ex vivo tissue sections staining

For staining mice and macaque heart sections, slides were de-paraffinized and processed as recently described (Marecki, Cool et al. 2006). Briefly, sections were stained with anti-GFP (for macaque), or anti-Nef (EH1) (for transgenic mice) antibody and anti-von Willebrand factor (vWF) prior to staining with

appropriate secondary fluorescent labeled antibodies. Visualization was achieved by confocal microscopy (Olympus FV1000-MPE) with the same fluorescence intensities during image acquisition.

Detection of Reactive oxygen species (ROS) activity by dihydroethidium (DHE)

ROS production stimulated by Nef was determined using DHE as a fluorescent probe. Confluent HCAEC on 96-well black-walled dishes were incubated in conditioned BME medium and 5 μ M DHE for 30 min to allow intracellular uptake. Cells were washed 3 times with PBS and media replaced with phenol-free RPMI. Fluorescence of the oxidized dye was subsequently determined at 520 nm (excitation), 605 nm (emission), with 590 nm cutoff on a Flex station microplate reader set for maximal detection.

NF- κ B p65 silencing in endothelial cells with small interfering RNA

For RNAi knockdown of the NF- κ B subunit p65 gene, we used Ambion's *Silencer*[®] Select Custom Designed siRNA against p65 was used as previously described (Rajashekhar, Traktuev et al. 2008). Briefly, cells were transfected with Lipofectamine 2000 transfection reagent (Invitrogen) and after incubation for 2 days at 37°C, total cell lysate was used to determine the knockdown of p65 in human endothelial cells by Western blotting.

Western Blot analysis

Proteins were isolated from HCAEC using a cell lysis buffer consisting of 2.5 mM EDTA, 20 mM Tris pH 7.4, 100 mM NaCl, 1 mM Na₃VO₄, 1% Triton X-

100, 10 mM NaF, 1% sodium deoxycholate, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, and 1 tablet/10 ml EDTA-free complete protease inhibitor mixture (Sigma Aldrich). Whole-cell extracts prepared from HCAEC were resolved in 4–20% bis-Tris polyacrylamide gels (Thermoscientific), followed by transfer to nitrocellulose membranes. Membranes were probed with primary Abs at suggested concentrations. Proteins were visualized by incubation with peroxidase-coupled secondary Abs in the presence of LumiGlo reagent while exposing in a Bio-Rad Chemidoc XRS/HQ (Bio-Rad, Hercules, CA).

Monitoring mitochondrial function by JC-1 Dye-Mitochondrial Membrane Potential

Probe

HCAEC were stained with 2 μ g/ml JC-1 dye (Life Technologies) at 24 h post Nef transfection. Briefly, JC-1 was dissolved in DMSO and Nef-transfected HCAECs were stained for 30 min at 37°C. The medium was replaced with PBS before the plate reading. Red fluorescence was determined at 455 nm (excitation), 590 nm (emission), with 590 nm cutoff on a Flex station set for maximal detection, whereas green fluorescence was determined at 485 nm (excitation), 538 nm (emission), with 530 nm cutoff. The ratio of red/green was determined as a measurement of mitochondrial depolarization.

Detection of Nef in PBMCs

PBMC were isolated from peripheral blood of HIV patients and uninfected controls by standard Ficoll purification. Cells were fixed with 4% PFA for 5 minutes at RT and permeabilized with 0.01% Triton in PBS for 20 min at 4°C. Cells were stained with Nef or IgG controls (in some cases, cells were co-stained with CD4 T cell marker). The percentage of Nef positive cells was determined based on IgG controls.

B cells isolation from PBMCs

Ten million PBMCs were stained with 10ul PE-conjugated CD19 B cell marker, followed by incubating with anti-PE microbeads (Miltenyi). The cells were then transferred to the column for magnetic separation step. After washing with rinsing buffer, the cells bound in the column represented CD19 positive cells, while the elute contained CD19 negative cells. The purity of selected cells from this assay was up to 95%.

Data acquisition and statistical analysis

MCP-1 production and apoptosis data were both expressed as fold increase normalized to controls (mock-transfected cells etc). Data are expressed as mean \pm SD for each group performed in triplicate and repeated at least three times. Shapiro–Wilk test was used to test for normality distribution (n=6). With normal distribution, all comparisons were made using parametric ANOVA and student's t test. On some occasions, Welch's correction was used when the

variances were different. With abnormal distribution, Kruskal-Wallis non-parametric ANOVA was used. A p value < 0.05 (marked as *) was considered statistically significant, and p < 0.01 (marked as **) highly significant.

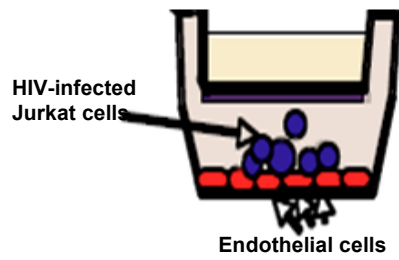
RESULTS

Part I: HIV Nef causes endothelial activation and dysfunction

1.1 HIV induces endothelial MCP-1 production and cell death

In viremic HIV patients, HIV envelope protein gp120 and transcription activator Tat are believed to mediate activation of vascular endothelium leading to endothelial dysfunction resulting in pulmonary hypertension and cardiovascular diseases. However, the role of intracellular proteins such as Nef, has been not addressed in cardiovascular diseases. To distinguish between effects of cellular Nef and specifically, soluble Tat or HIV-induced cytokine release, I asked whether direct contact between T cells and endothelial cells is required for endothelial activation. To address this question, I used a transwell filter system, which allowed comparison of direct versus cellular contact with indirect contact between HIV-infected Jurkat T cells and endothelial cells (Figure 6A and 6B). In this system, the bottom wells with human coronary artery endothelial cells (HCAEC) were either in direct contact with HIV-infected/uninfected Jurkat cells or separated by a semi-permeable membrane. As shown in Figures 7 and 8, HIV-infected Jurkat cells significantly increased endothelial MCP-1 production and apoptosis when in direct contact, whereas when HIV-infected Jurkat cells separated from the endothelial monolayer exhibited minimal effect. We detected only very minor MCP-1 release from HIV-infected Jurkat cells alone (Figure 7), suggesting that the observed increased MCP-1 production occurred from endothelial cells but not Jurkat cells. As HIV

A.



B.

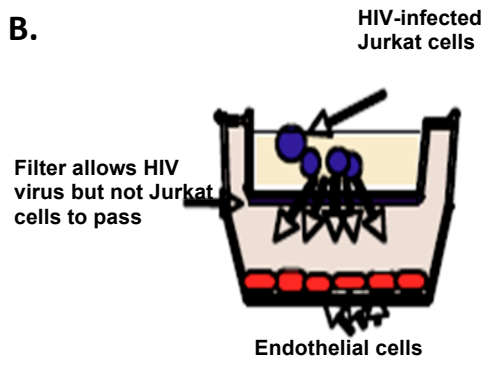


Figure 6: A transwell system

A transwell filter system was used to determine the effects of uninfected or HIV infected Jurkat T cells on endothelial MCP-1 release and apoptosis either in direct cellular contact (**A**) or separated by the semi-permeable membrane filter (**B**).

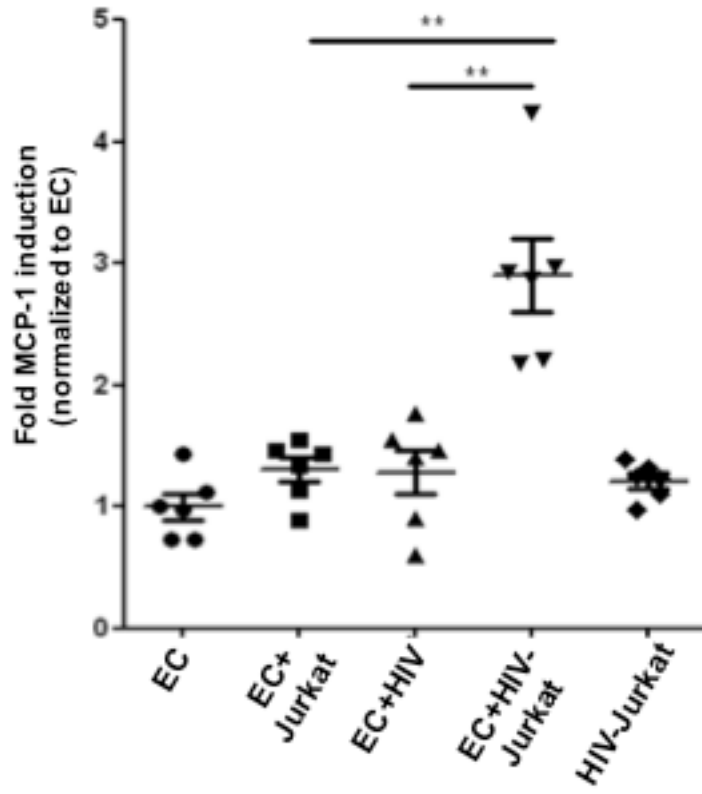
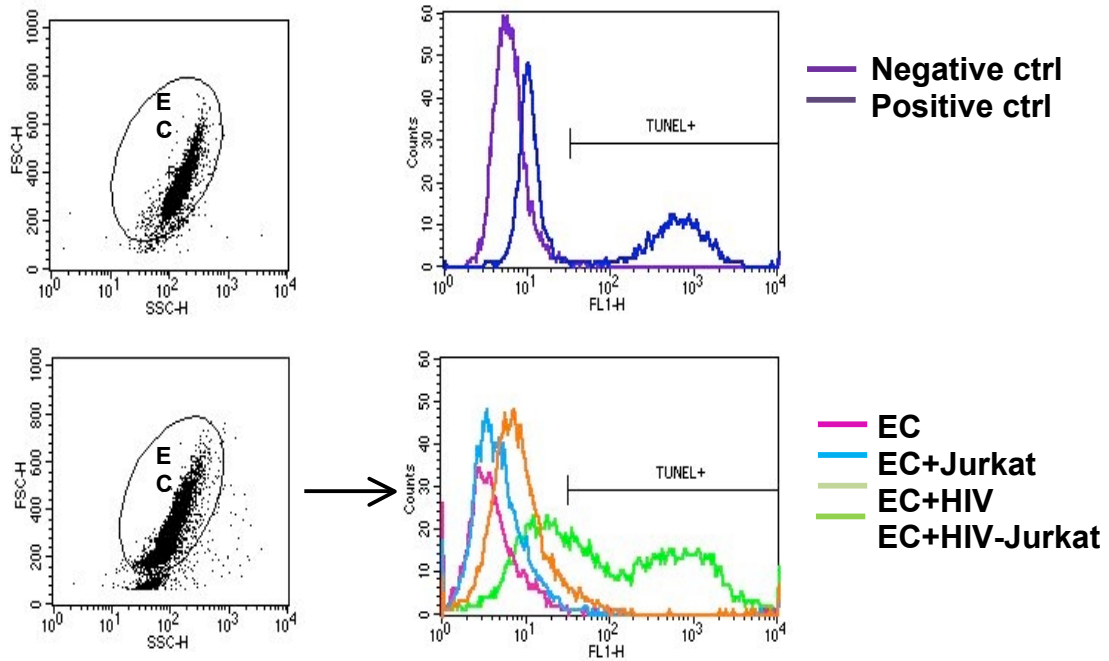


Figure 7: HIV-infected Jurkat cells but not acellular HIV virus cause endothelial activation

Endothelial cells were co-cultured in direct or indirect contact with HIV-infected Jurkat cells with HCAEC alone (EC), HCAEC in direct contact with uninfected Jurkat cells (EC + Jurkat), EC in indirect contact with infected Jurkat cells (EC + HIV) or HCAEC in direct contact with infected Jurkat cells (EC + HIV-Jurkat). MCP-1 release from HIV infected Jurkat cells was also included (HIV-Jurkat). MCP-1 release was analyzed by ELISA. Data were expressed as fold increase in MCP-1 production, normalized to mock controls (N=6. * $P < 0.05$, and ** $P < 0.01$).

A.



B.

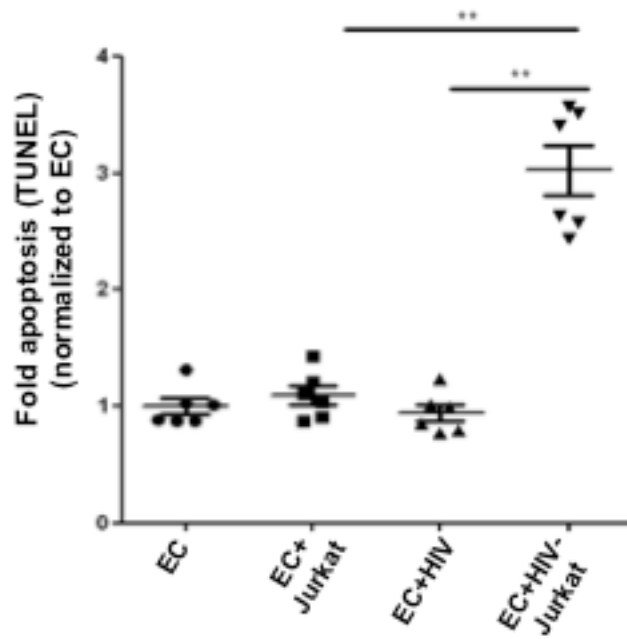


Figure 8: HIV-infected Jurkat cells but not acellular HIV virus cause endothelial cell death

A. HCAEC were co-cultured in direct or indirect contact with HIV-infected Jurkat cells (EC), in direct contact with uninfected Jurkat cells (EC + Jurkat), in indirect contact with infected Jurkat cells (EC + HIV) or in direct contact with infected Jurkat cells (EC + HIV-Jurkat); endothelial apoptosis was determined by TUNEL assay. The cells were then gated for endothelial cells depending on the cell sizes by FACS, and only endothelial cells were analyzed for apoptosis expression. The “TUNEL” gate was determined by controls from apoptosis kit. B. The relative endothelial apoptosis levels. Data were expressed as fold apoptosis, normalized to mock controls (N=6. * $P < 0.05$, and ** $P < 0.01$).

virions containing envelope protein gp120 and soluble Tat or cytokines released from infected T cells are able to pass the filter and move freely in the supernatant, this finding suggests that a cellular HIV protein in infected T cells or a protein induced by HIV causes endothelial MCP-1 production and cell death.

1.2 HIV Nef is necessary for endothelial activation and cell death

Since HIV gp120 and Tat protein were ruled out by our transwell system, HIV early protein, attention was drawn to Nef because of its traits. To specifically determine the involvement of Nef in these direct contact dependent effects, we employed the same virus titered wild type and Nef-deleted HIV-infected Jurkat cells to co-culture with HCAEC. Jurkat cells was infected with wild-type and Nef deleted HIV NL4.3 separately, and the virus titers were shown to be equivalent the same in both infected cells at day 9 (Figure 9). Jurkat cells infected with Nef deleted HIV induced a much weaker endothelial MCP-1 production (Figure 10A) and apoptosis (Figure 10B) in comparison to WT HIV, indicating that Nef protein is necessary for HIV-induced endothelial cell death and activation.

To further analyze whether Nef protein causes endothelial activation and dysfunction, we transfected Nef expressing plasmid into Jurkat cells and cocultured these cells with HCAEC for 24 h (Figure 11A). Indeed, Nef-Jurkat cells induced both endothelial MCP-1 production and cell death (Figure 12A and 12B). To confirm these Nef dependent activities also apply to monocytes, we transfected Nef plasmid into monocytic cell line THP1 cells. Similar to Jurkat cells

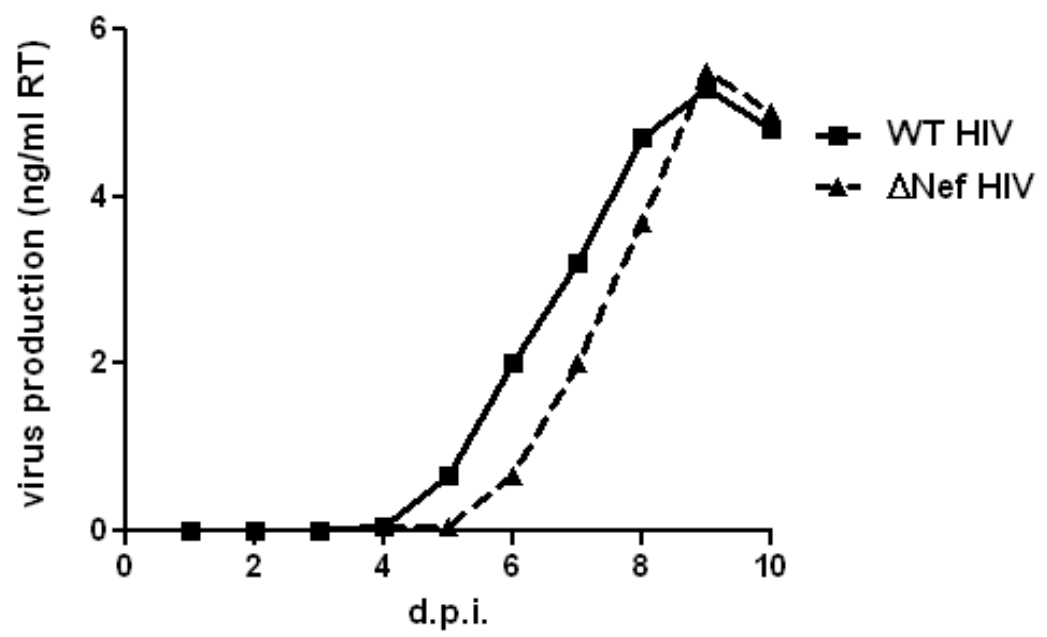


Figure 9: virus titer determination of Δ Nef HIV and WT HIV in Jurkat cells.

Both WT HIV and Δ Nef HIV production reach peak viral production on day 8 or 9 post infection (dpi) with Jurkat cells.

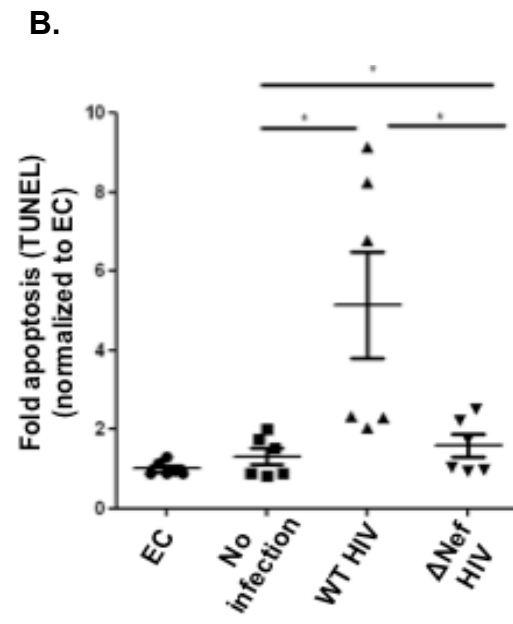
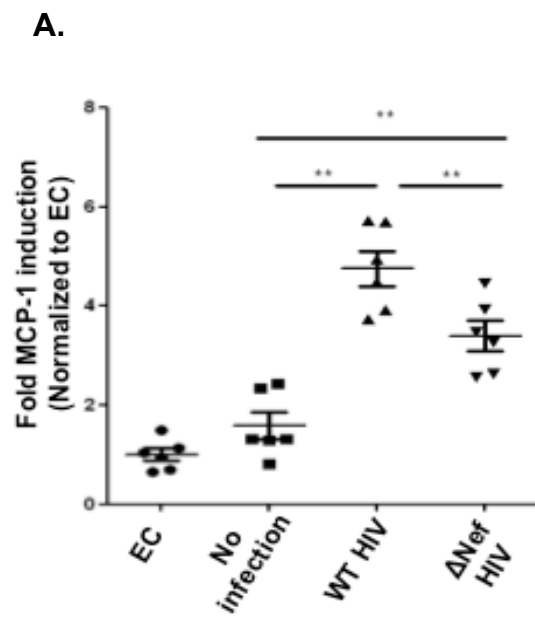
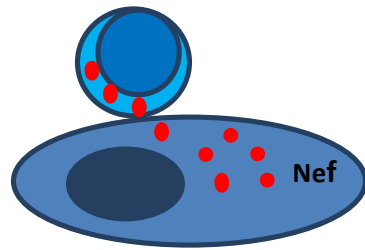


Figure 10: Nef is necessary for HIV-induced endothelial MCP-1 production and cell death

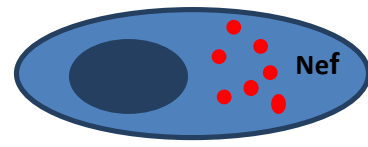
MCP-1 release (A) and apoptosis (B) in endothelial cells were determined after co-culture of endothelial cells with uninfected Jurkat cells (mock), HIV-infected Jurkat cells (WT HIV) or Nef deleted HIV-infected Jurkat cells (Δ Nef HIV). Data were expressed as fold MCP-1 production and apoptosis, normalized to mock controls (N=6. * P <0.05, and ** P <0.01).

Transfection of T cells/monocytes
and co-culture with EC



↓
Apoptosis/MCP-1 release

Direct transfection of EC



↓
Apoptosis/MCP-1 release

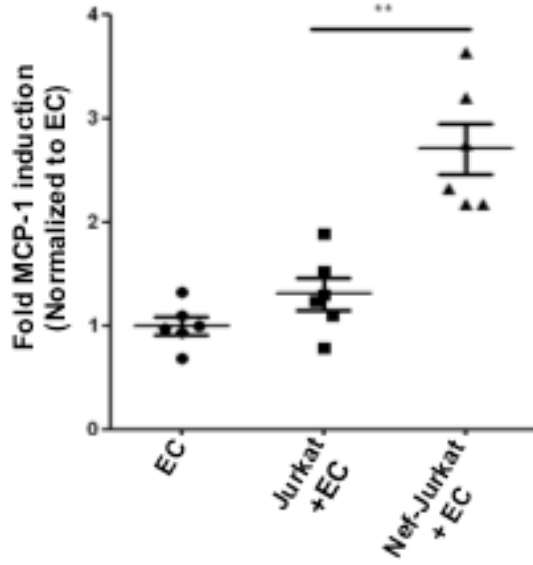
Figure 11: The demonstration of Nef transfections.

(A) Plasmid expressing Nef cDNA was either transfected into Jurkat cells/THP-1 cells and co-cultured with HCAEC to allow Nef protein transfers to HCAEC (A).

(B) Alternatively the Nef expressing plasmid was directly transfected into HCAEC. Endothelial cells were measured for or directed transfected to HCAEC

(B), followed by endothelial apoptosis, and MCP-1 release detection was assayed for the transfected cultures.

A.



B.

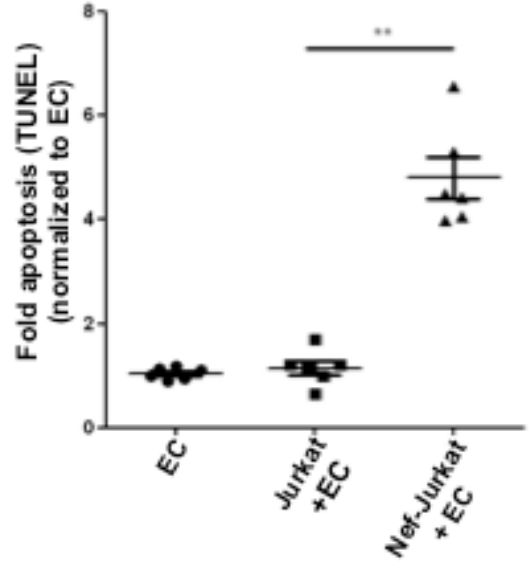


Figure 12: Nef-Jurkat T cells induce endothelial MCP-1 production and cell death

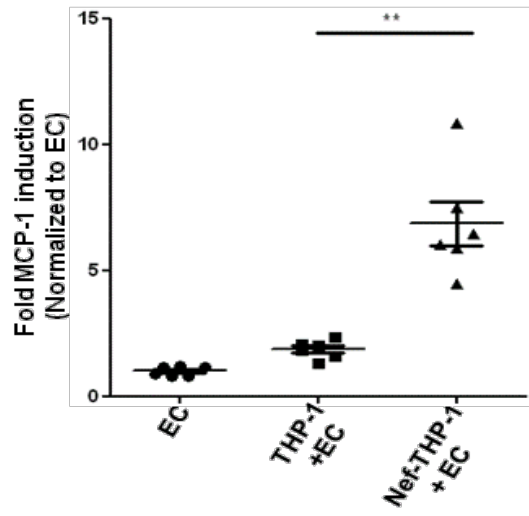
Endothelial MCP-1 production (A) and apoptosis (B) were determined in HCAEC alone (EC) or after 24 h coculture with cDNA (Jurkat + EC) or Nef transfected Jurkat cells (Jurkat+ Nef + EC). Data were expressed as fold MCP-1 production and apoptosis, normalized to mock controls (N=6. * $P < 0.05$, and ** $P < 0.01$).

Nef dependent increase in MCP-1 production and apoptosis were observed in THP-1 cells co-cultured with HCAEC (Figure 13A and 13B).

1.3 HIV Nef is sufficient to cause endothelial activation and cell death

Next, we questioned if Nef-Jurkat/THP-1 cells induced endothelial activation and dysfunction is due to Nef protein triggering cell membrane dysfunction or the outcome from the direct effect of Nef protein. We transfected Nef into HCAEC (Figure 11B) and measured endothelial MCP-1 release (Figure 14A), apoptosis (Figure 14B) and ROS formation (Figure 14C). Each was significantly increased after 24 h transfection with Nef expressing cells as compared to the pcDNA3 controls. Src homology-3 (SH3) domain binding site of Nef is essential for many functions in T cells (Manninen, Hiipakka et al. 1998; Foti, Cartier et al. 1999). To address the role of this binding site for Nef functions in endothelial cells, we also transfected HCAEC with SH3 domain mutated Nef. As shown in Fig. 13A-C, the SH3 binding site in Nef is essential for endothelial MCP-1 production, apoptosis induction and ROS formation. As ROS has been shown to cause vascular leakage, we employed an in vitro vascular permeability assay to detect if Nef could increase vascular permeability. Indeed, as shown in Figure 15A-C, Nef destroyed the endothelial monolayer and induced HCAEC permeability after 24 h post transfection (Figure 15D). In addition, endothelial cells transfected with increasing Nef-cDNA amounts display a Nef concentration-dependent increase (as determined by a Nef-specific ELISA, Immunodiagnosics) in apoptosis

A.



B.

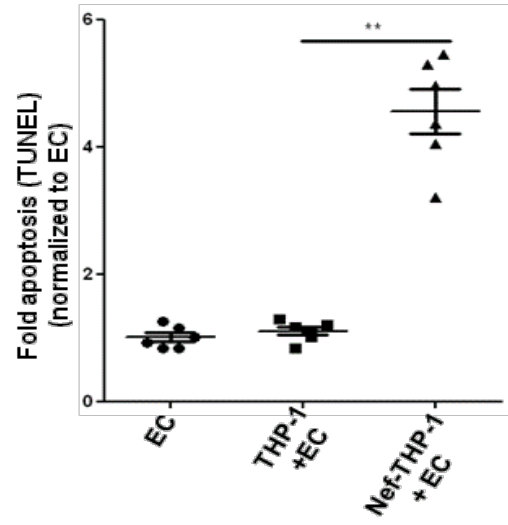


Figure 13: Nef-monocytes THP1 cells induce endothelial MCP-1 production and cell death

Endothelial MCP-1 production (A) and apoptosis (B) were determined in HCAEC alone (EC) or after 24 h coculture with cDNA (THP-1 + EC) or Nef transfected THP-1 cells (THP-1+ Nef + EC). Data were expressed as fold MCP-1 production and apoptosis, normalized to mock controls (N=6. * $P < 0.05$, and ** $P < 0.01$).

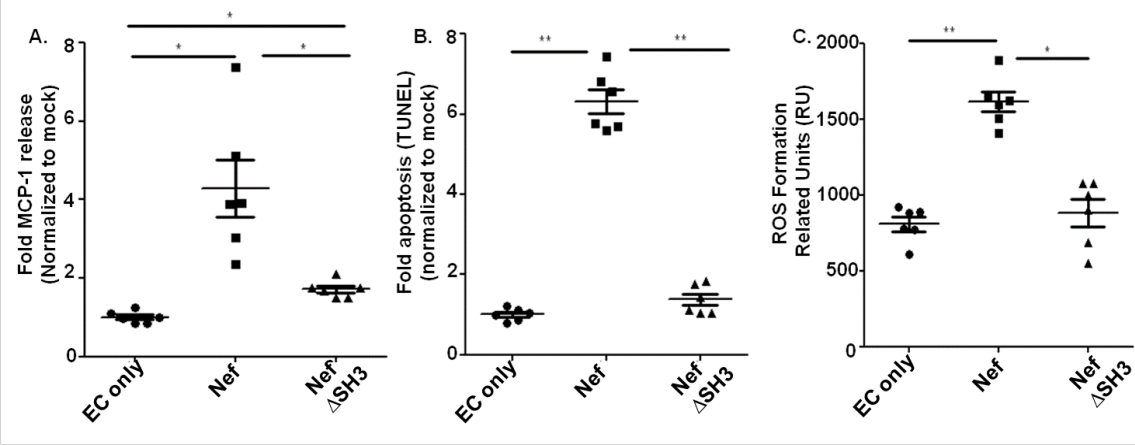


Figure 14. Nef is sufficient to induce endothelial activation and dysfunction.

A-C. Endothelial MCP-1 release (**A**), apoptosis (**B**) and ROS formation (**C**) were determined in endothelial cells transfected with cDNA (EC only), WT Nef (Nef) or SH3 binding site mutated Nef (Nef Δ SH3) after 24 h. Data were expressed as fold MCP-1 production and apoptosis, normalized to mock controls (N=6. * P <0.05, and ** P <0.01).

A. No growth factors B. pcDNA3 transfected C. Nef transfected

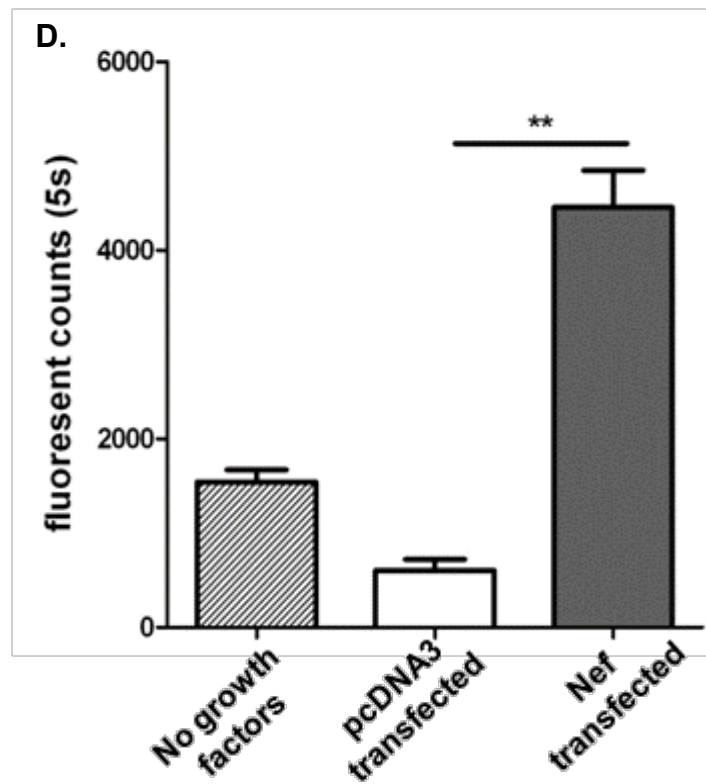
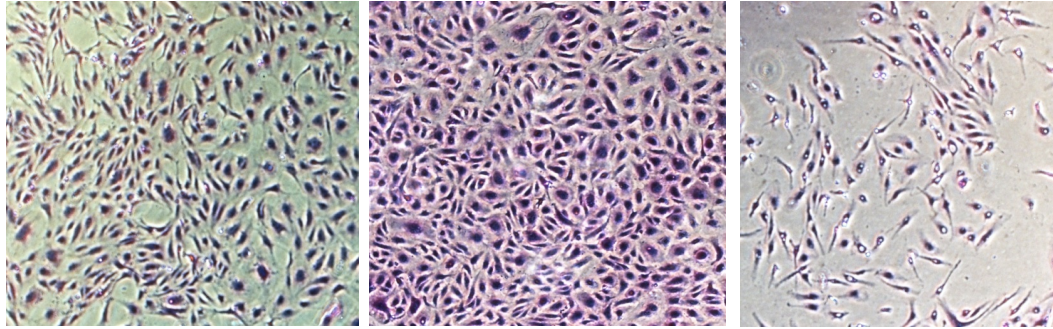


Figure 15: Nef increases HCAEC permeability.

A-C. Control cDNA or Nef was transfected to HCAEC monolayer in transwells. HCAEC vascular permeability was measure after 24 h post-transfection. Basal medium treated HCAEC was included as a positive control. **D.** HCAEC permeability was determined by flex station. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. *P<0.05, and **P<0.01.

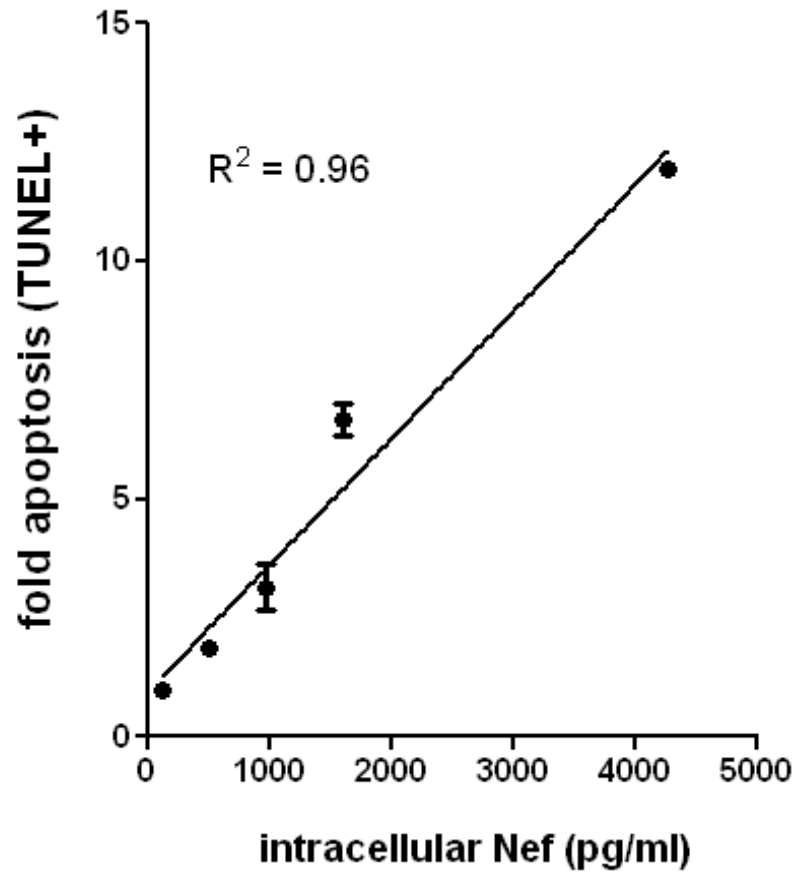


Figure 16: Linear correlation between Nef protein concentration and endothelial apoptosis

Increasing amounts of plasmid expressing HCAEC were transfected with Nef plasmid for 24 hrs and concentrations of Nef were measured by ELISA. The measured levels of Nef were then correlated with apoptosis as determined by TUNEL-FACS.

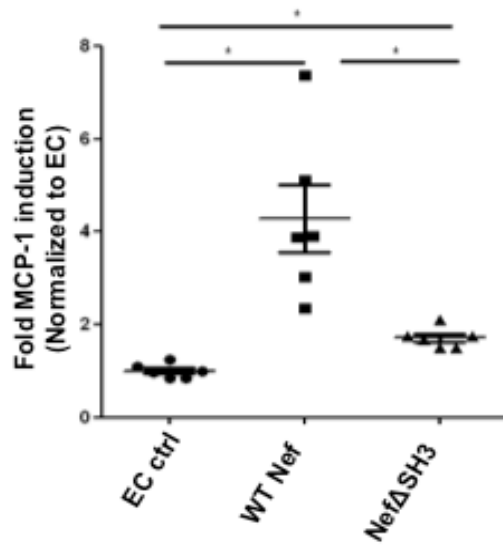
(Figure 16). In addition, Nef transfected HCAEC displayed increased mitochondrial dysfunction and elevated cleaved caspase 3 levels (Figure 17A and 17B). There was also a significant linear correlation between intracellular Nef concentrations and endothelial MCP-1 production ($P= 0.045$; Figure 18). These data demonstrate that Nef is necessary for HIV-infected T cells to elicit apoptosis in HCAEC and sufficient to cause HCAEC activation and increased permeability. Importantly, correlation between intracellular Nef concentrations with apoptosis is a first step in linking the presence of Nef with pathology.

Part II: Nef mediates its own transfer from T cells to endothelial cells

2.1 Nef enhances the formation of nanotubes from Jurkat cells to endothelial cells.

Nef protein, but not virus, has been shown to be transferred to HIV-uninfected bystander cells (Muratori, Cavallin et al. 2009); therefore, we tested whether Nef protein could be transferred to endothelial cells by direct contact. To achieve this, we labeled Nef or control cDNA transfected Jurkat cells with live dye (Vybrant DIO), then co-cultured them with HCAEC and determined live dye transfer from Jurkat cells to HCAEC. After 24 hours co-culture, Nef transfected Jurkat cells transferred significantly more dye into HCAEC (Figure 19A, predominately in perinuclear regions) in comparison to cDNA transfected Jurkat cells (Figure 19B). To determine how Nef induced live-dye transfer, we again employed the transwell filter system to distinguish between direct contact

A.



B.

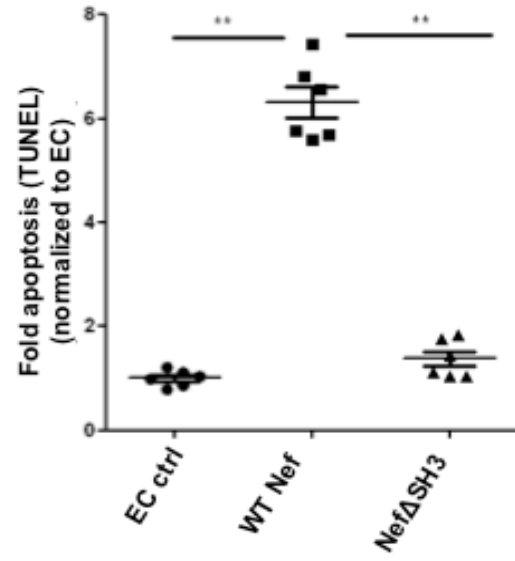


Figure 17: Nef protein causes endothelial dysfunction.

A. Mitochondria membrane potentials in endothelial were measured tested after 24 h post Nef transfection by the JC-1 staining kit according to the manufacturer's instructions. B. Caspase 3 activity in endothelial cells was tested at 24 h post Nef transfection using a caspase 3 kit according to the manufacturer's instructions. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. *P<0.05, and **P<0.01.

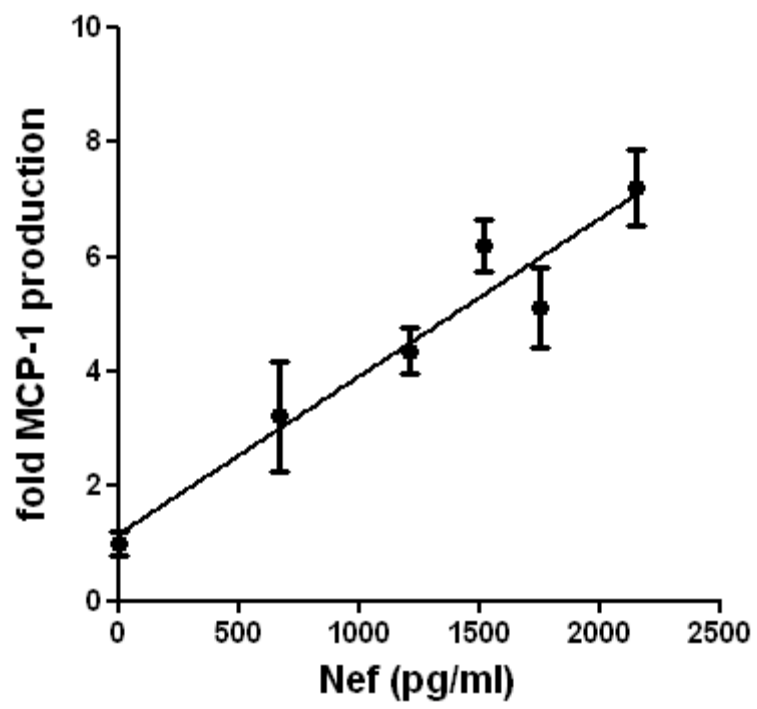


Figure 18: Correlation between intracellular Nef concentrations with endothelial MCP-1 production

HCAEC were transfected with Nef expressing plasmid. 24 hrs after transfection concentrations of Nef and correlated with endothelial MCP-1 production were measured by ELISA, which was also determined by ELISA assay.

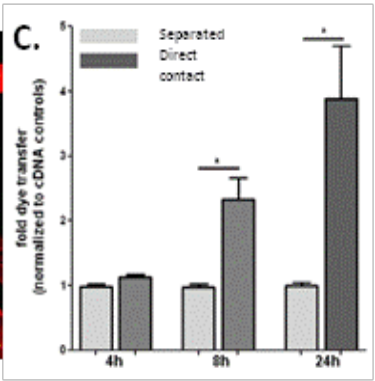
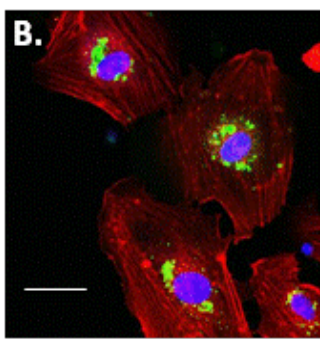
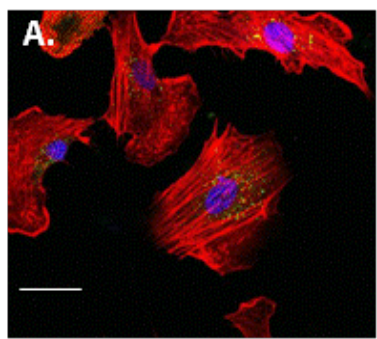


Figure 19: Nef enhances live dye transfer between cells only when in direct contact.

A-B. Live dye(green) transfer from cDNA transfected Jurkat cells (A) or Nef-transfected Jurkat cells (B) to endothelial cells after 24 h coculture. Endothelial cells (red) were stained by phalloidin. **C.** Live dye stained Nef or control vector transfected Jurkat cells were cocultured with HCAEC either in direct contact, or separated by transwell membranes for varying time points. Percentage of live dye transfer was determined and quantified by confocal microscopy. The fold dye transfer was normalized to cDNA controls for each condition. **B** is the representative figure from direct control experiments, which were quantified in **C**. Scale bars represent 10 μm .

(nanotube formation) mediated and indirect contact (exosome formation) transfer. Indeed, we observed that direct but not indirect contact mediated live dye transfer relatively early after 8 hours (Figure 19C). We also found that Nef-transfected Jurkat cells formed significantly more nanotubes to HCAEC compared to cDNA-transfected Jurkat cells (Figure 20A, arrow and Figure 20B), which also displayed perinuclear live dye (Figure 20A, arrowheads). These studies indicate that Nef can promote cytosol exchange and possibly propel its own transfer possibly through nanotubes.

The intracellular membrane bound Nef protein reportedly initiates its own transfer between blood cells in a process most likely mediated by nanotubes (Rudnicka, Feldmann et al. 2009; Rudnicka and Schwartz 2009). However, the mechanism of Nef transfer to target tissue cells including vascular endothelial cells has not been addressed yet. Therefore, we tested the ability of Nef to be transferred from Nef-transfected Jurkat T cells to endothelial cells. Transfected Jurkat cells were co-cultured with HCAEC prior to fixation/labeling with Nef mAB EH1. After 24 h of co-culture with Nef-transfected T cells, 19.4% of HCAEC were positive for Nef compared to only 2% for mock control via FACS analysis (Figure 21A), demonstrating the ability of Nef to be transferred from Jurkat cells to bystander endothelial cells. Furthermore, as shown in Figure 20B endothelial cells are positive for Nef after 24 hr exposure with Nef transfected Jurkat cells as determined by confocal microscopy.

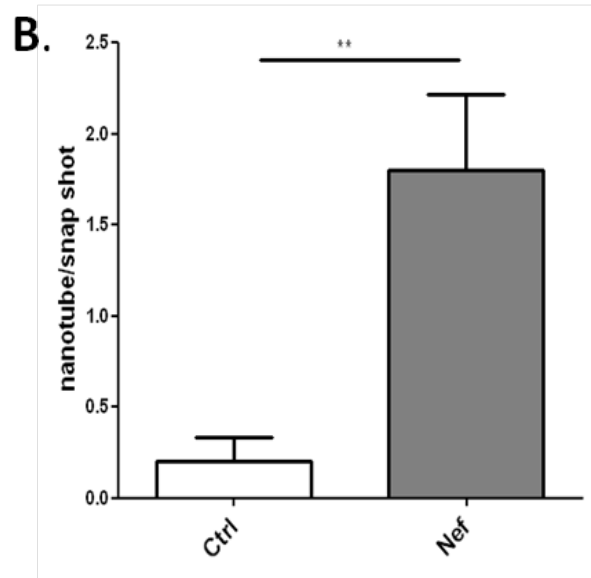
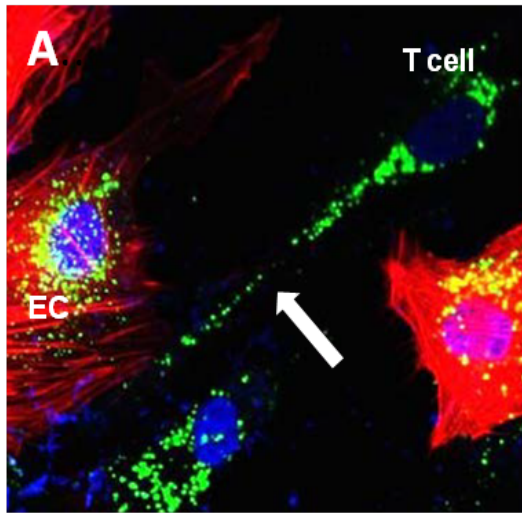


Figure 20: Nef protein induced nanotube formation between Jurkat cells to endothelial cells.

A. Live dye stained Nef-Jurkat cells formed more conduit-like nanotubes between Jurkat cells and endothelial cells compared to cDNA Jurkat cells. **B.**

Quantification of nanotubes formation in Nef Jurkat cells or cDNA Jurkat cells.

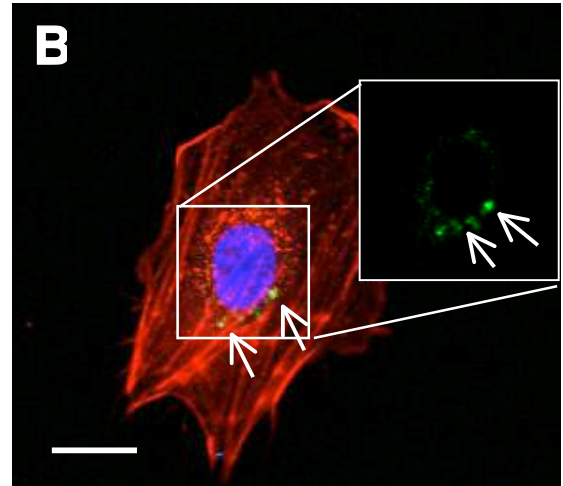
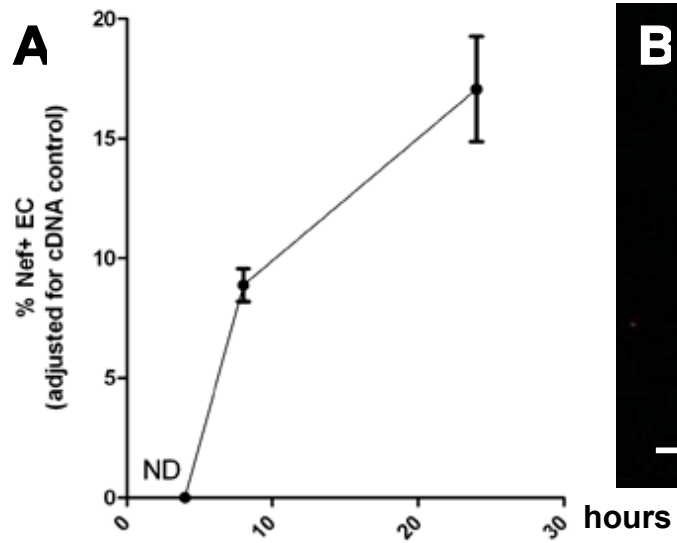


Figure 21. Nef transfer to HCAEC.

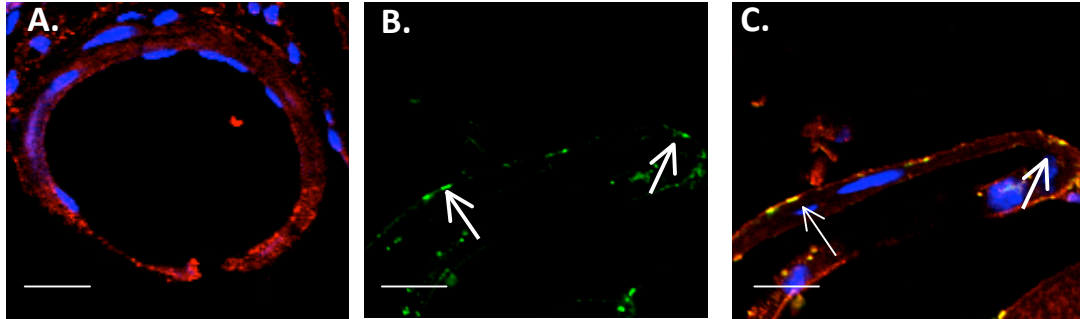
A: Nef transfected T cells were cocultured with HCAEC for varying time points, and HCAEC were stained for Nef expression to determine the timecourse of Nef transfer from T cells to endothelial cells. Endothelial cells were washed with PBS to ensure no adhesion of T cells. Any remaining T cells were gated from endothelial cells by FACS based on forward scatter and side scatter profiles. Percentage of Nef+EC was determined after subtraction of background cDNA signal. ND represents undetectable Nef **B:** Nef transfer to HCEAC after 24hr coculture. Endothelial cells were stained with phalloidin (red) and Nef (green). The right corner insert indicates Nef accumulation in HCAEC without an overlay. Original magnification, X60. Scale bars represent 10µm.

Together, these data suggest that Nef protein induces nanotube structure formation from Jurkat cells to endothelial cells, and these structures are central for Nef transfer between cells.

2.2 Nef protein can be detected in the endothelium of in vivo HIV models.

To access the potential relevance of Nef protein in coronary endothelial cells in in vivo models, I first used transgenic mice in which Nef-GFP was expressed under

the regulatory sequences of the human CD4 gene, thereby resulting in Nef expression in CD4+ T cells and monocytes. Using double staining with anti-Nef and endothelial specific antibodies, GFP signal can be demonstrated in coronary vessels of CD4-Nef-GFP transgenic mice by confocal microscopy (arrows, Figure 22B-22C) but not in single CD4-GFP Tg control mice (Figure 22A). We also confirmed that primary mouse T cells were isolated from either CD4-Nef-GFP or CD4-GFP transgenic mice cocultured with HCAEC for 24 h, demonstrated more GFP signal transfer from Nef-expressing primary T cells to HCAEC *ex vivo*, system by flow cytometry (17% v.s 3%; Figure 23). We then extended these results by using a chimeric simian immunodeficiency virus (SIV) expressing HIV Nef (SHIV Nef) macaque model. In this model, we detected Nef in coronary vessels (Figure 24B-24C); vWF co-staining confirmed the presence of Nef in the endothelial lining (Figure 24C, arrow, yellow overlay). Together these data provide in vivo evidence that Nef protein can target endothelial cells within the vascular system.



**Figure 22: Nef induced GFP transferred from CD4+ cells to endothelial cells
in Nef transgenic mice**

A-C. Heart sections of single CD4-GFP (**A**) and double CD4-Nef and GFP (**B, C**) transgenic mice (N=3; at least 4 pictures/slide) were double stained with GFP antibody (green, white arrows) and the endothelial marker vWF (red). Shown is Nef within the endothelial lining (arrows). Original magnification, X 60. Scale bars represent 100 μm .

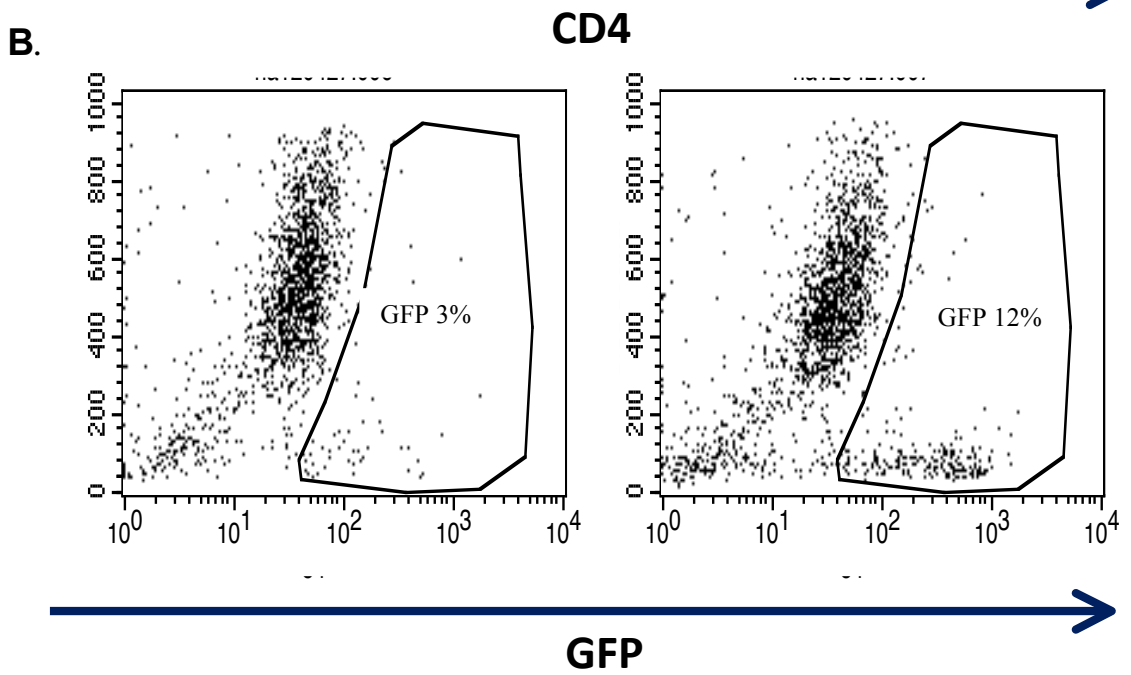
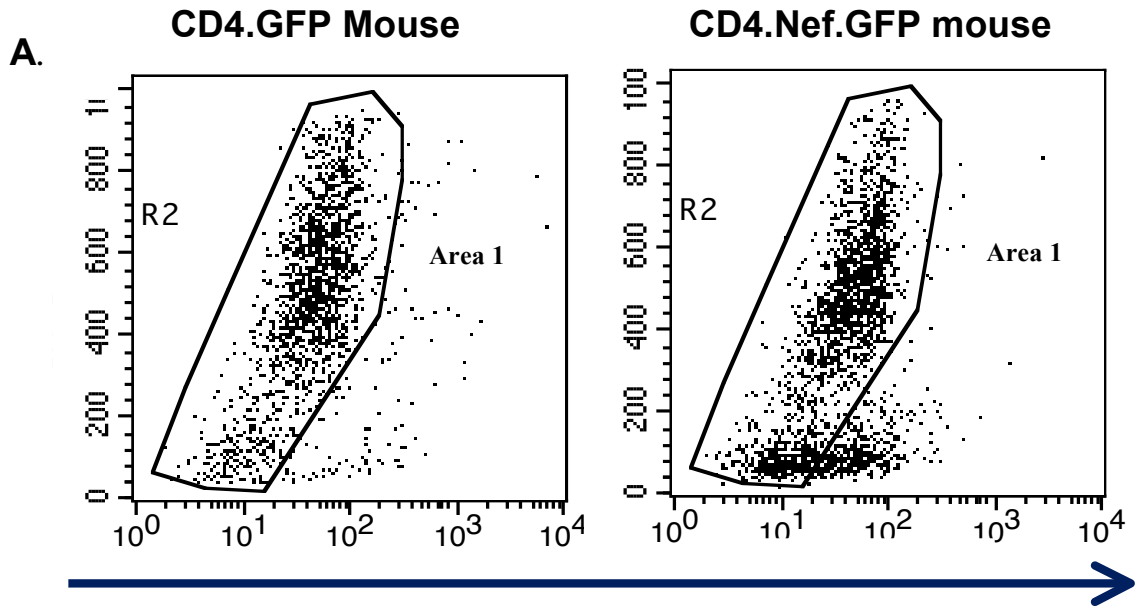


Figure 23: More GFP were transferred from CD4+ primary cells to HCAEC with Nef present.

CD4+ primary cells from either GFP mice or Nef.GFP mice were cocultured with HCAEC for 24 hours. CD4 negative HCAEC were sorted through flow cytometry (**A, area 1**), and then stained with anti-GFP antibody(**B**).

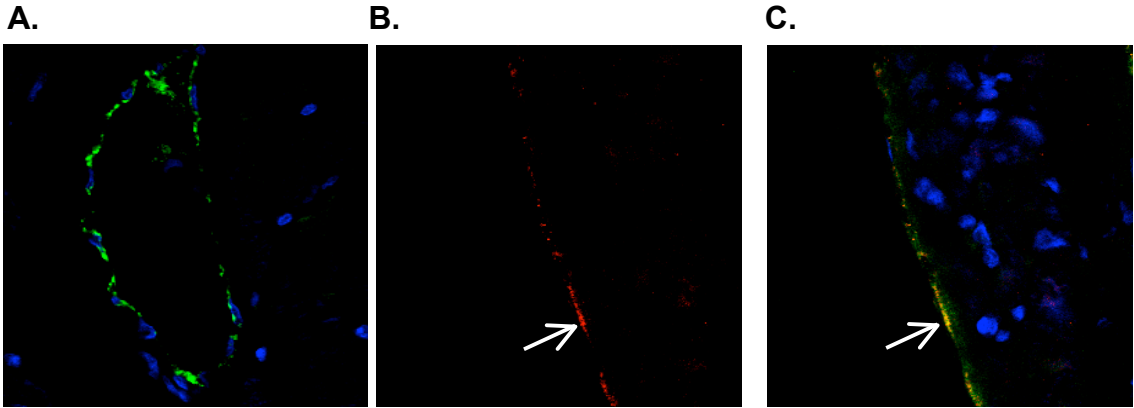


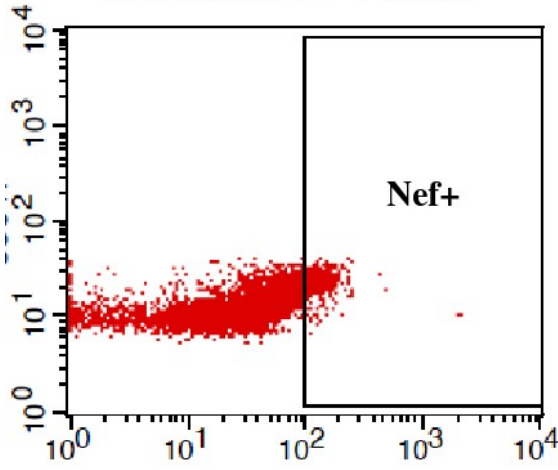
Figure 24: Nef can be detected in SIV-hNef macaque models.

Macaque heart sections (N=5; at least 4 pictures/slide) were double stained with IgG control (A) or Nef (B, C, red) and the endothelial marker vWF (green). Shown are cells double positive for Nef and vWF in coronary arteries (arrow). Original magnification, X 60. Scale bars represent 100 μ m.

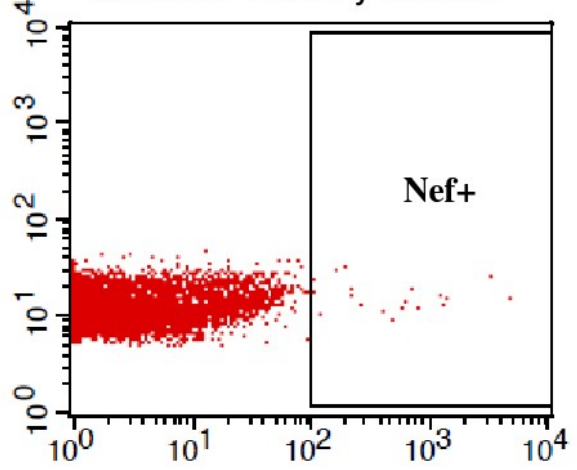
2.3 Nef protein can be detected in blood cells isolated from HIV positive individuals

Antiretroviral therapy reduces virion production but not Nef gene expression. It is possible that these adverse effects of Nef may persist even in patients receiving ART. If so, then the persistence of Nef-positive cells during ART might contribute to the higher risk of non-AIDS complications observed despite ART. We therefore performed a preliminary investigation to assess the detection of Nef-positive circulating cells from both ART-untreated and treated HIV-infected patients. PBMCs were isolated from HIV-infected patients and uninfected controls by standard Ficoll purification. We found a high mean (SD) number of PBMCs from HIV-infected untreated viremic patients that stained positive for Nef (Figure 25, Table 2). Of note, Nef dissemination did not correlate with viral titers ($r = 0.0677$; $p = 0.86$, Figure 26), and one of the HIV patients exhibited very low Nef dissemination in PBMC, even though viral titers were high (Table 2, patient 8). We confirmed these results in 4 of the samples using a commercially available Nef antibody (3D12, Abcam), which recognizes a different epitope, and observed similar Nef staining (EH1: $15.68 \pm 3.98\%$; 3D12: $14.35 \pm 5.139\%$; $p = 0.84$, $n = 4$) (Figure 27). In addition, we further detected not only Nef expression in patients PBMCs, but also nanotubes formation between cells by confocal microscopy (Figure 28). Surprisingly, Nef is also detectable in ART treated patients with undetectable viral loads (Table 2, patient 10-13). The unexpectedly high levels of Nef positive PBMCs cannot be explained by direct

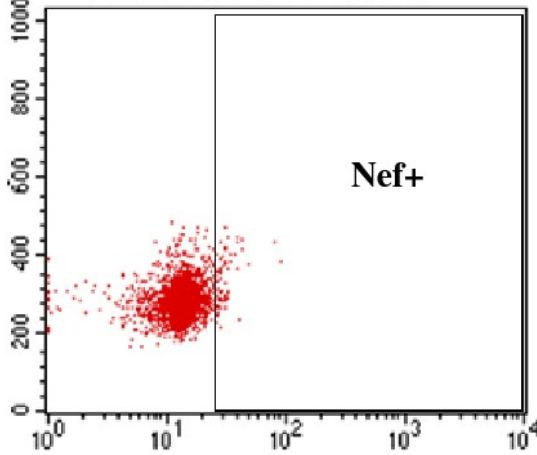
A. Untreated HIV-Patient



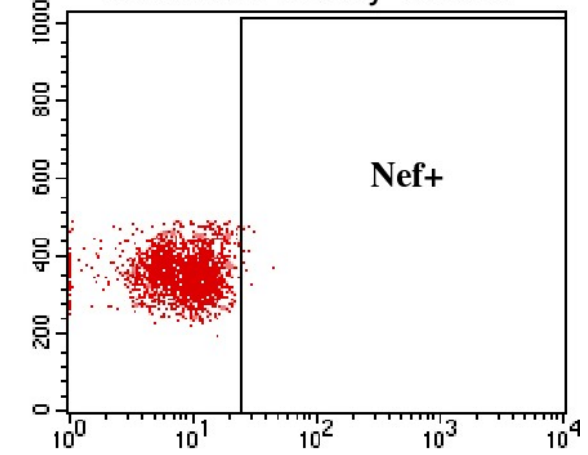
B. Matched healthy control



C. Treated HIV-Patient



D. Matched healthy control



→ Nef

Figure 25: Nef is detectable in PBMCs from HIV patients with/without ART.

FACS analysis of Nef expression in PBMC from untreated (A, frozen samples) and virologically suppressive ART treated (C, fresh samples) HIV patients. To assure specificity of Nef staining, we included PBMC from healthy donors and stained for Nef in parallel (“matched”) with patient derived PBMC (B and D).

	CD4+ count (cells/ul)	Viral load (RNA copies/ml)	Nef %(of total PBMC)	ART duration (weeks)
Pooled Uninfected ctrl	N/A	N/A	0.09	N/A
Patient 1	365	5700	7.78	N/A
Patient 2	797	8400	8.66	N/A
Patient 3	413	1490	12.84	N/A
Patient 4	913	47800	11.46	N/A
Patient 5	315	13000	8.04	N/A
Patient 6	386	5600	24.9	N/A
Patient 7	593	100000	25.72	N/A
Patient 8	321	85800	0.02	N/A
Patient 9	503	35,000	8.02	N/A
Patient 10	234	<50	1.09	32
Patient 11	254	<50	1.41	48
Patient 12	205	<50	1.23	16
Patient 13	855	<50	0.95	780

Table 2: Nef is detectable in PBMCs from HIV patients with/without ART.

Percentage of Nef+ PBMCs with clinical parameters in patients without and with (orange box) ART.

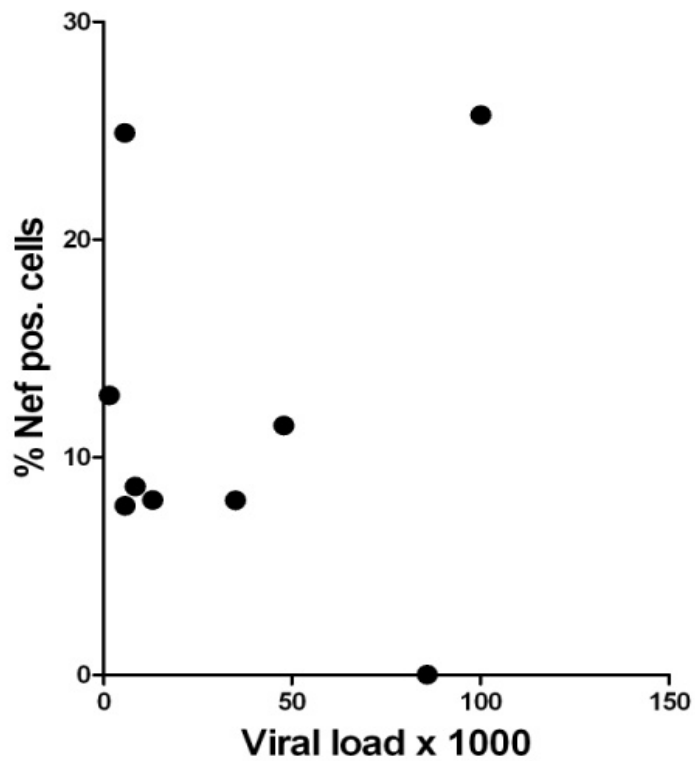


Figure 26: Nef distribution does not correlate with viral load.

Pearson correlation between viral load and Nef distribution in PBMCs.

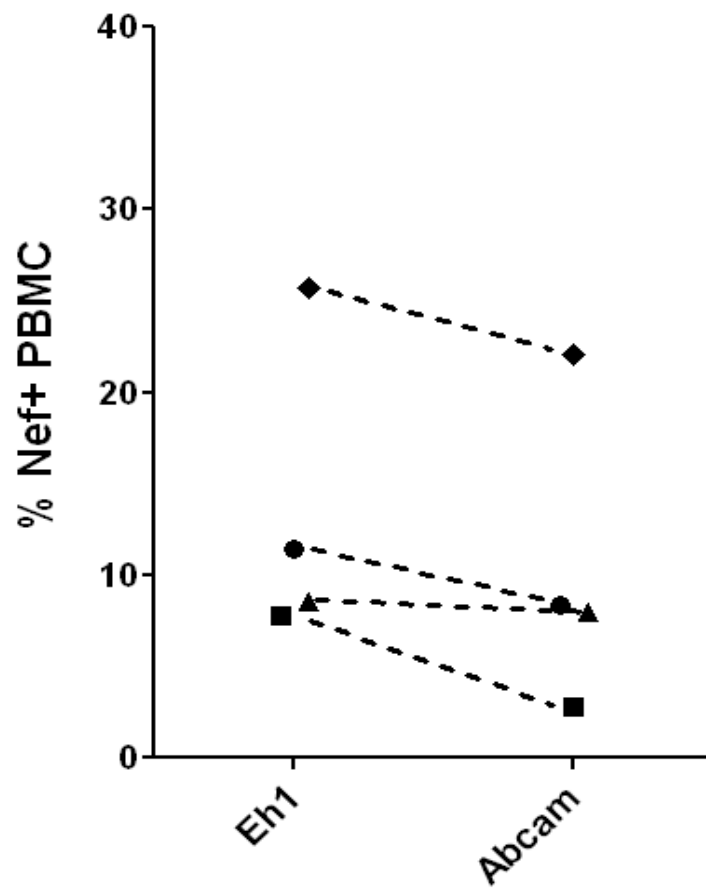


Figure 27: validation of EH1 monoclonal antibody

Comparison of 2 anti-NEF monoclonal antibodies (Eh1 and Abcam) which are directed against unrelated epitopes

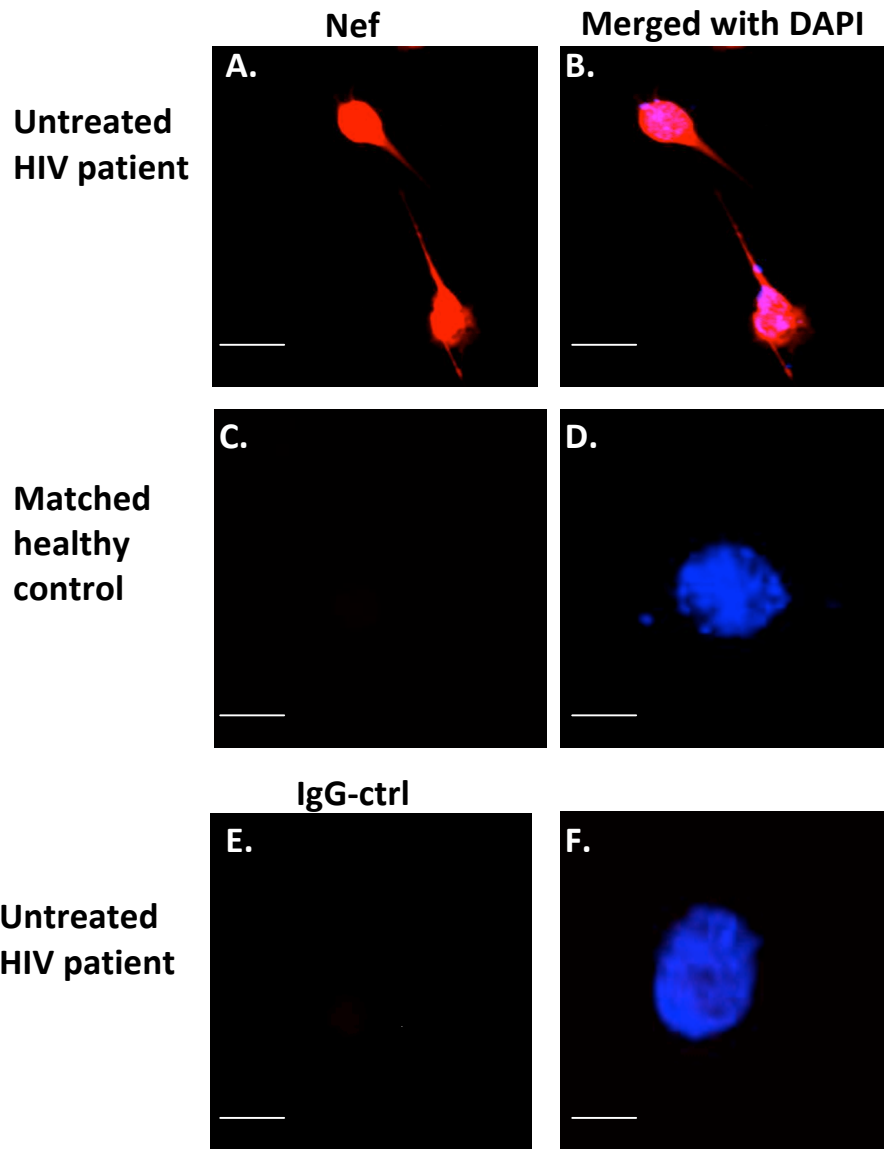


Figure 28: Nef protein is present in blood cells of HIV patients not on antiviral therapy.

A-B. PBMC from untreated HIV patients (N=3) were stained for Nef (red). Matched healthy control (**C** and **D**) and untreated patient with IgG control antibodies (**E** and **F**) were also included. Original magnification, X 60. Scale bars represent 5 μm in **A** and **B**. Scale bars in the inset of **C-F** represents 10 μm .

HIV infection of cells. In fact, Nef antibody staining was most prominent in non-CD4-positive cells (Figure 29A). Moreover, when we separated B cells from whole patient PBMCs using the Miltenyi anti-PE multisort kit and CD19 antibody and stained for Nef, almost 50% of Nef positive cells appear to be B cells (Figure 29B).

To test if Nef from blood derived mononuclear cells could also transfer to endothelial cells, we cocultured human umbilical cord vein endothelial cells (HUVEC) with PBMCs from viremic untreated HIV-infected patients for 24 hours. As shown in Figure 30, this experiment resulted in strongly (arrow) and less strongly (arrow head) Nef-positive endothelial cells, which is likely due to different levels of Nef transfer. Of note, these endothelial cells are not leukocytes based on characteristic cytoskeletal morphology as determined by staining with phalloidin-Cy5.

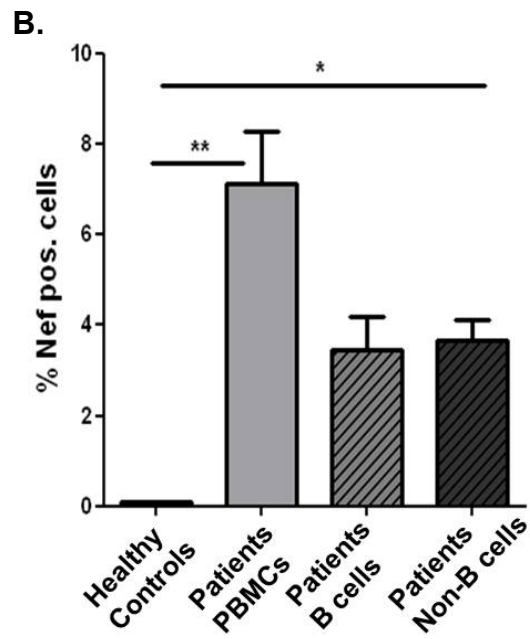
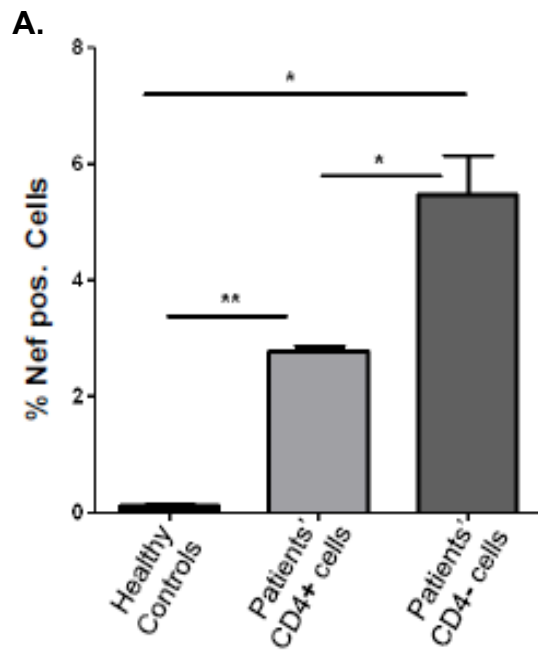
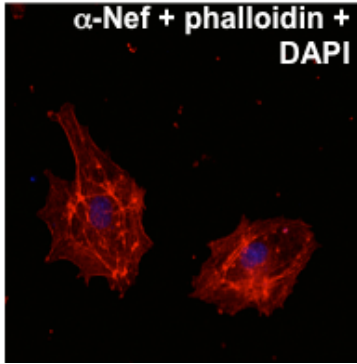


Figure 29: Nef is prominent present in CD4- cells.

A. Determination of CD4+ and CD4- Nef-positive PBMCs by double staining and FACS. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. **B.** Comparison of Nef-positive B cell versus non B cell populations after immune-magnetobead based separation. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate.

Healthy PBMC



Patient's PBMC

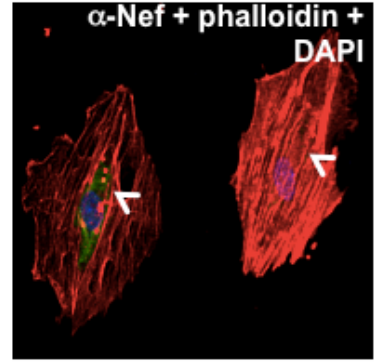
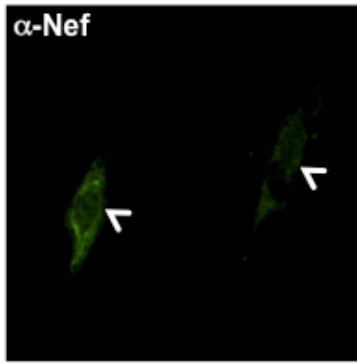


Figure 30: Nef can be transferred from HIV-PBMCs to endothelial cells ex vivo.

Nef staining of HUVEC after 24 hours in contact with PBMCs from HIV patients shown as Nef stain only (green) or as overlay with red phalloidin staining depicting endothelial-like cytoskeletal actin staining.

Part III: Signal transduction analysis of Nef induced endothelial activation and dysfunction.

We next aimed to specifically analyze the signal transduction pathway of Nef-induced HCAEC activation. First we tested involvement of *NF-κB signaling* for Nef-induced MCP-1 production. Using siRNA to knockdown the p65 subunit of NF-κB or the NF-κB inhibitor Ikki (Green, Kim et al. 2012), we addressed the role of NF-κB for Nef-induced chemokine production (Figure 31). Based on previous reports linking Nef to reactive oxygen species (ROS) formation (Olivetta, Pietraforte et al. 2005), we addressed the role of ROS in Nef-induced endothelial dysfunction. Whereas the ROS inhibitor vitamin E derivative Trolox (Figure 32) had no effect on Nef-induced MCP-1 production (Figure 34A), Nef-induced apoptosis in HCAEC was abolished by Trolox (Figure 35). We further examined if Nef-induced ROS production and apoptosis is derived from NADPH oxidase using the NADPH oxidase inhibitors apocynin and the gp91phox/Nox2 B-loop peptide (Nox2ds) (Figure 33). Both NADPH inhibitors abolished Nef-induced ROS production and endothelial apoptosis (Figure 35A). However, NF-κB inhibition (Ikki) did not reduce Nef-induced endothelial apoptosis, indicating that Nef-induced apoptosis does not require NF-κB activation (Figure 34). Furthermore, to rule out the possibility that Nef induced endothelial dysfunction is due to artificially high Nef concentrations caused by overexpression in endothelial cells, we used the Nef co-culture based transfer system as described above, in which Nef protein shuttles from Nef transfected Jurkat cells into

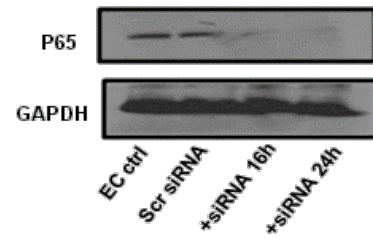
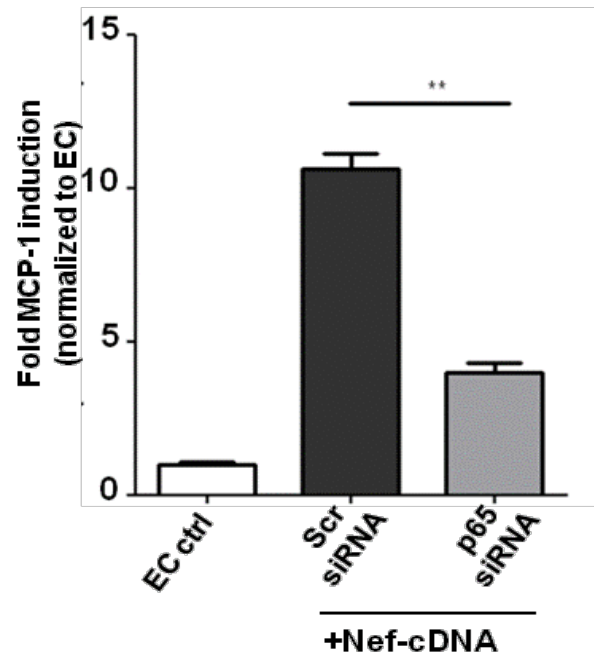


Figure 31. Nef induced endothelial MCP-1 production is NF- κ B dependent.

Endothelial cells were co-transfected with Nef cDNA and NF- κ B p65 siRNA. After 18 h and 24 h, knock-down of p65 was confirmed by Western blot (Inserts: Lane 1, control; Lane 2, scrambled siRNA; Lane 3, 18h p65siRNA knock-down; Lane 4, 24h p65siRNA knock-down). MCP-1 production was tested at 24 h post Nef transfection in the supernatant using ELISA. Data were expressed as fold MCP-1 production, normalized to mock controls. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. * P <0.05, and ** P <0.01.

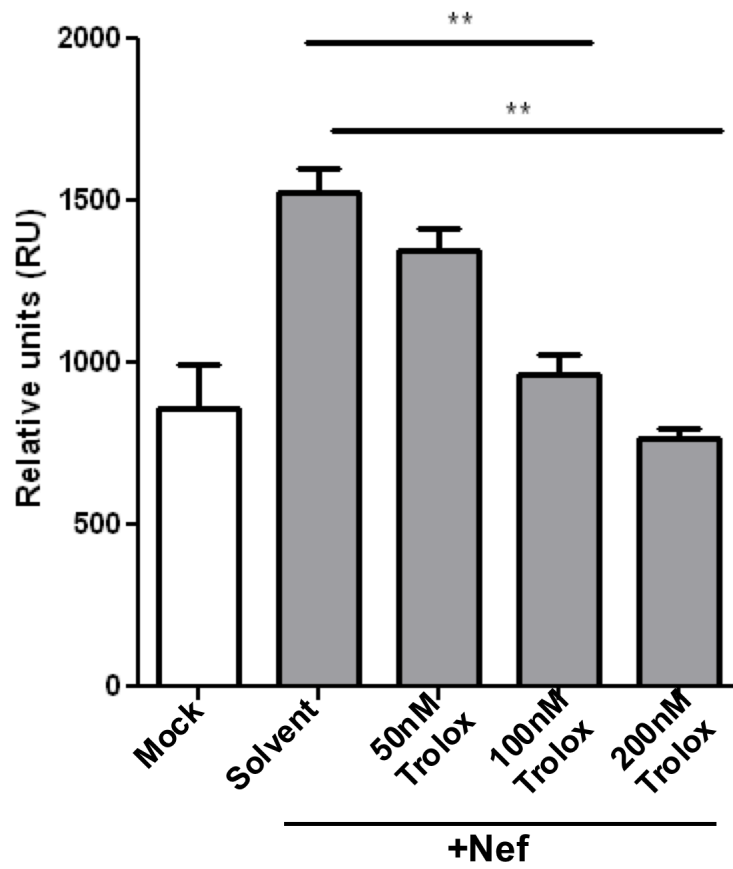


Figure 32: Optimize Trolox concentration in ROS inhibition.

Trolox with different dosage were separately added to Nef cDNA transfected HCAEC for 18 h incubation. Detection of intracellular ROS formation was assessed with DHE using a Flex station. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. *P<0.05, and **P<0.01.

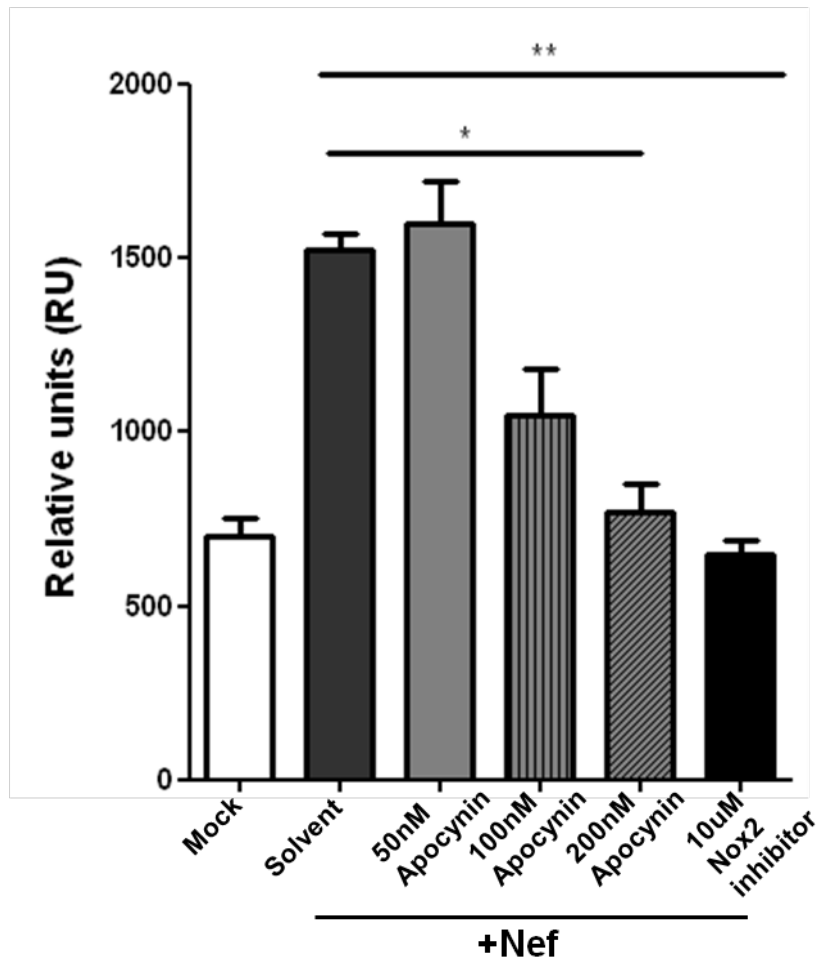
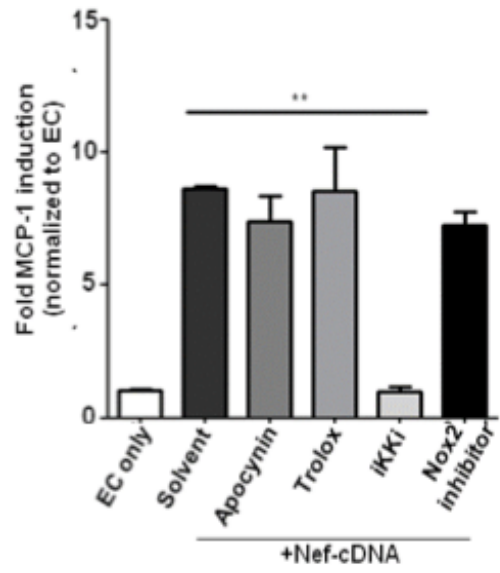


Figure 33: NADPH inhibitors dose determination to block Nef-induced intracellular ROS activation in HCAEC.

Apocynin with different dosage and optimized 10uM Nox2 inhibitor were separately added to Nef cDNA transfected HCAEC for 18 h incubation. Detection of intracellular ROS formation was assessed with DHE using a Flex station. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. *P<0.05, and **P<0.01.

A.



B.

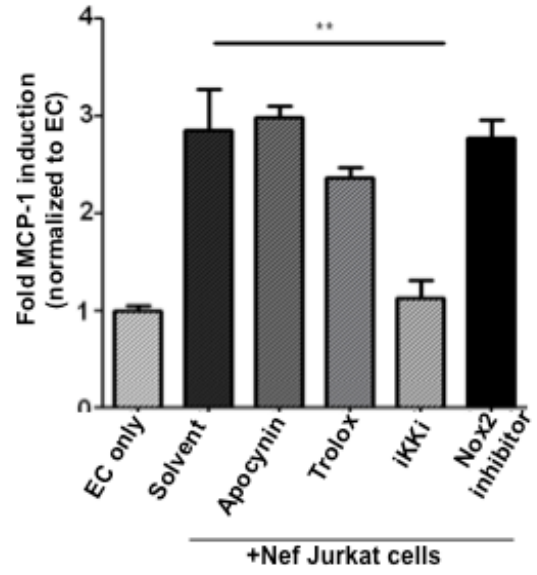


Figure 34. Ikki inhibited Nef induced endothelial MCP-1 production.

(A) Endothelial cells were transfected with Nef expressing plasmid, incubated for further 6 hours and treated with apocynin (200nM), trolox (200nM), or IKKi (100nM). After additional 18 hours supernatants were analyzed for Nef-induced MCP-1 production. **(B)** Endothelial cells were cocultured with Nef-transfected Jurkat cells for 24 h, and then treated with NADPH gp91 specific peptide Nox2 inhibitor (10uM), apocynin (200nM), trolox (200nM), or IKKi (100nM) and incubated an additional 12 h, then analyzed for Nef-induced MCP-1 production. Data were expressed as fold MCP-1 production, normalized to mock controls. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. * P <0.05, and ** P <0.01.

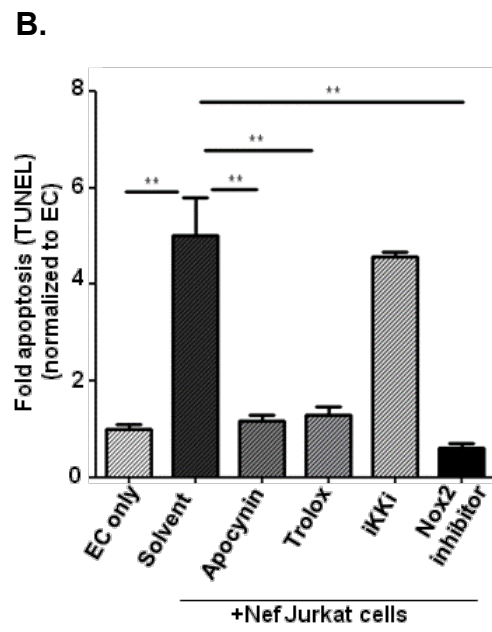
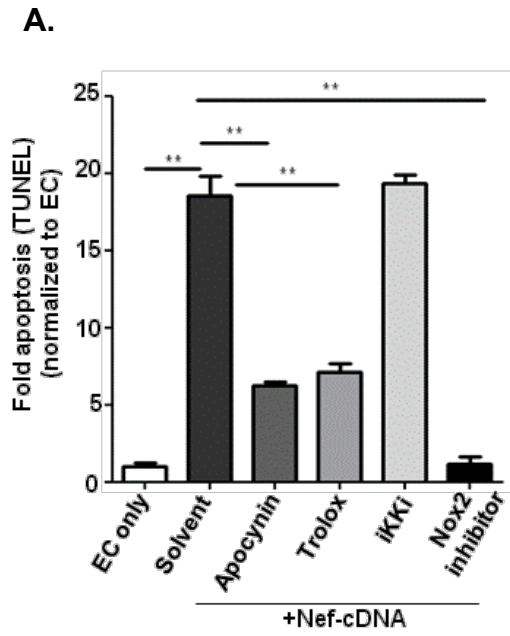


Figure 35. Nef induced endothelial cell death is ROS/NADPH dependent.

(A) Endothelial cells were transfected with Nef cDNA, incubated for further 6 hours and treated with NADPH gp91 specific peptide Nox2 inhibitor (10uM), apocynin (200nM), trolox (200nM), or IKKi (100nM). After additional 18 hours cells, cells were collected or apoptosis TUNEL assay. **(B)** Endothelial cells were cocultured with Nef-transfected Jurkat cells for 24 h, and then treated with NADPH gp91 specific peptide Nox2 inhibitor (10uM), apocynin (200nM), trolox (200nM), or IKKi (100nM) and incubated an additional 18 h, then analyzed for Nef-induced endothelial apoptosis. Data were expressed as fold MCP-1 production, normalized to mock controls. Data represent mean \pm SD from 3 separate experiments in which measurements were made in triplicate. * P <0.05, and ** P <0.01.

cocultured HCAEC. We demonstrated the same signaling pathways for endothelial MCP-1 production and apoptosis in these HCAEC coculture with Nef-transfected Jurkat cells. We demonstrated the same signaling pathways for endothelial MCP-1 production and apoptosis in these HCAEC cocultured with Nef-transfected Jurkat cells (Figure 34B and 35B). Thus, Nef induces endothelial MCP-1 through NF-kB pathway while induces endothelial cell death through NADPH pathway.

DISCUSSION

Summary of the results

Nef protein induces its own transfer from T cells and monocytes to endothelial cells, which consequently and necessarily induces endothelial cell activation, dysfunction, and death. Nef induces apoptosis of endothelial cells through an NADPH oxidase- and ROS-dependent mechanism, while Nef-induced MCP-1 production is NF- κ B dependent. Our findings are in line with the widely accepted connection between endothelial dysfunction/oxidative stress and risk of cardiovascular events in patients with coronary artery disease in general (Heitzer, Schlinzig et al. 2001) and in HIV individuals specifically (Baliga, Chaves et al. 2005). Interestingly, Nef was already linked to pulmonary hypertension and endothelial dysfunction (Duffy, Wang et al. 2009; Almodovar, Hsue et al. 2011). Our finding of Nef-induced ROS production could explain Nef-induced decreased NO levels and ER dysfunctions in pulmonary arteries (Kojda and Harrison 1999; Duffy, Wang et al. 2009). Furthermore, our finding of Nef protein presence increasing endothelial MCP-1 production concurs with the important role of this chemokine in atherosclerosis. This finding is particularly noteworthy in light of a link between early atherosclerosis and MCP-1 levels in HIV patients (Gosling, Slaymaker et al. 1999; Alonso-Villaverde, Coll et al. 2004), as arterial inflammation in HIV patients is a common denominator and associated with a circulating marker of monocyte and macrophage activation (Subramanian, Tawakol et al. 2012).

The next aspect of Nef transfer we investigated was whether Nef protein could be also transferred *in vivo*. We demonstrated a great dissemination of Nef protein in PBMCs of HIV-infected individuals that most Nef positive cells were CD4- uninfected cells. We also found not only an unexpected high level of Nef expression in HIV-patients without ART, but also a significant levels of Nef-positive PBMCs in ART treated patients with undetectable HIV RNA viral loads. These *in vivo* findings suggest that Nef protein may be widely transferred from HIV-infected cells to uninfected cells, providing a means of pathogenic Nef activity even when virus replication is controlled.

Nef protein can be transferred to endothelial cells

In our tissue culture models, Nef transfection in uninfected Jurkat cells was sufficient to stimulate nanotube formation between Jurkat cells and endothelial cells. Quantification by FACS analysis revealed that 17% of HCAEC had Nef protein transferred to them from Jurkat cells/THP1 cells after 24 hours coculture. Our data suggests that the mechanism of Nef protein transfer between blood cells and vascular endothelial cells most likely involves nanotube formation. Reportedly, using IHC from human tissue many Nef protein expressing bystander cells can be found in lymphatic vessels, and under the low shear stress conditions in these vessels cellular transfer may be easily mediated by nanotube transfer from infected cells (Qiao, He et al. 2006). Under shear stress conditions, exosome-mediated transfer would be an alternative mechanism for transfer. However, despite previous reports of Nef transfer to

bystander cells by Nef-containing exosomes, we did not find evidence of Nef transfer through this mechanism. Importantly, we applied a membrane staining live dye (Vybrant Dio), which excludes the possibility of Nef transfer via gap junction. Nanotube transfer from blood cells to the endothelium is possible despite shear stress as T cells and monocytes are in constant close contact to vascular endothelium as part of immuno-surveillance. In addition, HIV infection itself may cause proinflammatory conditions leading to increased adhesion of infected and noninfected Nef carrying T cells and monocytes (Yang, Liu et al. 2010) and thus enhancement of nanotube formation and Nef transfer.

As a first proof of principle, we provided the evidence that Nef can be transferred into endothelial cells *in vivo*. We assessed a transgenic approach to address Nef protein transfer more specifically by using the regulatory sequences of the human CD4 gene to drive Nef expression in a Tg mouse model. Based on our *in vitro* finding that Nef protein can be readily transferred from infected or transfected Jurkat cells to endothelial cells, we anticipated that this model was of pathophysiological relevance. Indeed, demonstration of Nef protein in endothelial cells (Figure 20) provides further support for the hypothesis that Nef presence in endothelial cells can occur in the absence of viral infection. Although “leaky” CD4 regulatory elements cannot be totally excluded, our Nef staining occurs at a relatively high intensity, which is usually not seen in expression caused by leaky promoters. Importantly, a “leaky” promoter can be further ruled out by our established monkey model of HIV infection, in which macaques were infected with SHIV-Nef, an SIV construct containing HIV Nef alleles (Sehgal,

Mukhopadhyay et al. 2009; Almodovar, Hsue et al. 2011). In this model endothelial location of Nef has already been demonstrated in stenotic pulmonary arteries, indicating a role of HIV Nef in pulmonary hypertension. Unfortunately we could not include SHIV negative macaque hearts because they were not kept in the corresponding study. We looked for commercially available sections but the only provider we could identify had discontinued the provision of paraffin embedded coronary sections from apes. However, Nef staining in pulmonary arteries from SHIV but not healthy macaques had been demonstrated previously (Sehgal, Mukhopadhyay et al. 2009). In fact, this IF-based evidence of human Nef in HIV-Nef-SIV chimeric virus infected macaques is in confirmation of Nef being present in the vascular system. Further confirmation with human tissue samples should be addressed in future studies.

Nef protein is detectable in HIV-patients with/without ART

Unexpected high Nef levels were detected in most HIV naïve patients; however, one of the HIV patients exhibited very low Nef dissemination in PBMC, even though viral titers were high (Table 2, patient 8). The identification of mechanisms underlying this low spreading tendency of Nef, such as the possibility of a mutation impairing Nef protein transfer, would be interesting for therapeutic targeting of Nef. The unexpectedly high levels of Nef positive PBMCs cannot be explained by direct HIV infection of cells. In fact, Nef antibody staining was most prominent in non-CD4-positive cells, which is in line with the reported ability of Nef protein to be transferred in vitro from infected monocytes and T cells

to bystander cells (Figure 27A). Moreover, our finding that almost 50% of Nef positive cells appear to be B cells (Figure 27B) is in line with previous in vitro studies showing that Nef can be transferred from HIV infected monocytes to B cells (Qiao, He et al. 2006).

Nef is one of the 3 immediate early HIV genes, which are still transcribed in HIV-infected cells even in those receiving ART. Interestingly, we also found significant levels of Nef-positive PBMCs (mean (SD), $1.17 \pm 0.19\%$, $P=0.001$ compared to non-infected controls) in ART treated patients with HIV RNA viral loads < 50 copies/ml (Table 2, orange box). This finding could be explained by transfer of Nef from infected cells located in lymphatic tissues, a major HIV reservoir (Chiueh, Andoh et al. 2005; Hunt 2010). High endothelial venules enable lymphocyte circulation between blood and lymph nodes (Mackay, Marston et al. 1990) and are most likely in prolonged direct contact with Nef-containing mononuclear cells (Stolp, Imle et al. 2012).

Nef as the potential targets for HIV-related cardiovascular disease

To explore potential targets that could interfere with Nef-induced endothelial dysfunction we addressed the mechanism of Nef action. First, we employed the fact that Nef protein contains a conserved motif with the minimal consensus (PxxP) site for SH3-mediated protein-protein interactions. This SH3 domain was shown previously to be involved in many Nef activities (Foster and Garcia 2008). Further, our demonstration that the Nef SH3 binding site plays a key role in Nef-induced endothelial apoptosis is in line with previous reports that

Nef activates and induces NADPH oxidase (Vilhardt, Plastre et al. 2002; Salmen, Colmenares et al. 2010), which may also explain the known impairment of eNOS activity and reduced NO bioavailability in HIV-related vascular dysfunction (Kline, Kleinhenz et al. 2008; Duffy, Wang et al. 2009). Indeed, we observed that Nef induces apoptosis of endothelial cells through a NADPH oxidase- and ROS-dependent mechanism that is independent of NF- κ B activation. In this study, we included the ROS inhibitor Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E, as a scavenger of O_2^- (Arts, Haenen et al. 2004). Apocynin usually acts as an NADPH oxidase inhibitor, but recent results from Heumüller et al. (Heumüller, Wind et al. 2008) suggest that apocynin does not inhibit NADPH oxidase in endothelial cells because apocynin dimers cannot form without myeloperoxidase (MPO). These results, however, are based on cultured HEK293 cells that were transfected to overexpress NADPH oxidase isoforms. Other studies have shown the controversial results, that apocynin dimers are present in endothelial cells (Johnson, Schillinger et al. 2002) and MPO may be transferred into endothelial cells by a cytokeratin 1 pathway (Astern, Pendergraft et al. 2007). Other peroxidases in endothelial cells may also substitute for MPO. Schlüter et al. (Schluter, Steinbach et al. 2008) have shown that Apocynin does not inhibit vascular NADPH-oxidase-dependent superoxide formation but does inhibit Rho kinase activity. Recently, Steven J Miller et al found that mesenteric arteries in spontaneously hypertensive rats (SHR) (Zhou, Pyriochou et al. 2008) and retired breeder Wistar-Kyoto rats (WKY) have in vivo hydrogen peroxide concentrations in excess of what was necessary for apocynin-

mediated inhibition of superoxide production in the Schlüter study (Miller, Coppinger et al. 2010). In contrast, the Nox2ds peptide we used in this study specifically inhibits $O(2)(\bullet-)$ production by the vascular isoform of NADPH oxidase-Nox2 oxidase, but does not inhibit ROS production by either Nox1- or Nox4-oxidase (Csanyi, Cifuentes-Pagano et al. 2011). Because these NADPH oxidase- and ROS inhibitors were added early (6 hours) after transfection with Nef cDNA followed by determination of MCP-1 release, mitochondrial dysfunctions, ROS and apoptosis after 18 hours, we hypothesize that NADPH oxidase induces ROS formation, mitochondria dysfunction and apoptosis in endothelial cells. Although we could demonstrate mitochondrial dysfunction in Nef-induced endothelial cell apoptosis at later time points, early inhibition of NADPH oxidase using either pharmacological or biochemical peptide based inhibitors strongly suggests that NADPH activation is an essential early step for Nef-induced apoptosis by ROS-dependent mitochondrial dysfunction and subsequent release of cytochrome c and caspase activation, as was previously reported as a mechanism of ROS-dependent apoptosis. The cross talk between mitochondria and NADPH oxidases is bidirectional (Dikalov 2011; Dikalov and Ungvari 2013). Our proposed scheme suggests that Nef dysregulates endothelial mitochondria through inducing ROS and caspase activity. Increasing evidence shows that hypertension is associated with an increased mitochondria-derived production of ROS in various animal models, including mitochondria increased vascular ROS production by resistant mesenteric arteries and aorta (Viel, Benkirane et al. 2008; Dikalova, Bikineyeva et al. 2010). Mitochondria-derived

ROS is also critical for the central regulation of systemic cardiovascular function (Nautiyal, Arnold et al. 2013).

Given our finding that Nef-induced ROS production is necessary for Nef-induced endothelial cell death, anti-oxidant supplementation may be an exciting and novel means of reducing CVD in this population. In fact, in non-HIV infected patients, long-term administration of antioxidant vitamins C and E improved coronary and brachial artery endothelial function in patients with coronary artery disease, while multivitamin supplementation of HIV positive women during pregnancy reduced hypertension (Kinlay, Behrendt et al. 2004; Merchant, Msamanga et al. 2005). However, the beneficial effects of antioxidants for CVD in the HIV negative population are controversial (Hodis, Mack et al. 2002; Riccioni, Frigiola et al. 2012) and further post-data analyses or new studies within the HIV positive population would be interesting.

In previous studies, MCP-1-stimulated migration was described to require ROS production in certain cells (Lo, Shih et al. 2005; Habibzadegah-Tari, Byer et al. 2006). In our study, we clearly stated that the MCP-1 pathway is independent of ROS activation (Figure 36). We found that NF- κ B inhibition but not NADPH or ROS inhibition strongly reduced MCP-1 production. We chose MCP-1 production in endothelial cells as a readout for proinflammatory endothelial activation and dysfunction because it has been linked to cardiovascular diseases in a series of human and mouse studies (Ohman, Wright et al. 2010; Liu, Zhang et al. 2012) as well as with HIV infection (Alonso-Villaverde, Coll et al. 2004; Eugenin, Gaskill et al. 2009) . Recent studies have reported that pharmaceutical MCP-1 inhibitor L-

enantiomeric RNA oligonucleotide mNOX-E36 (a so-called Spiegelmer) successfully blocked MCP-1 during chronic liver inflammation and damage in an animal model (Baeck, Wehr et al. 2012). This could also be applied to future studies to test if this inhibitor can block Nef induced endothelial MCP-1 production. In this context, the myristoylation site of Nef itself could be a target for drugs which block protein farnesylation. Of note, statins, as HMG-CoA reductase inhibitors, are expected to have this function (Stein, Merwood et al. 2004; Hurlimann, Chenevard et al. 2006). It would be interesting to test whether statins are also able to reduce Nef activity, which would provide a rationale to apply an already existing cardiovascular protective regimen for HIV infected patients regardless of their cholesterol levels.

Alternative ways to inhibit Nef expression

Instead of inhibiting Nef-induced endothelium activation and dysfunction mechanism, an alternative could be the use Nef inhibitors. So far, only a few Nef inhibitors have been described, including chemical compounds capable of interfering Nef SH3 binding domains (Hiipakka, Huotari et al. 2001; Betzi, Restouin et al. 2007) and an Hck activation blocker (Emert-Sedlak, Kodama et al. 2009). Unfortunately, some inhibitors were either too cytotoxic or only showed activity in cellular and biochemistry-based assays. However, the new breakthrough monoclonal single-domain Nef neutralization antibody (anti-Nef sdAb) overcomes these problems and it inhibits the positive effect of Nef on virus replication in PBMCs (Bouchet, Basmaciogullari et al. 2011). This antibody is

composed of heavy chains only, with a single variable domain (VHH) capable of recognizing cognate antigens. The advantage of sdAb is having no requirement for disulfide bond formation when targeting of proteins found in reducing cell compartments. Therefore, it will be interesting to test if anti-Nef sdAb can block Nef-induced endothelial dysfunction in future studies.

In summary, the main significance of this study is that the HIV Nef protein can transfer to endothelial cells where it has dramatic effects, including the release of atherosclerotic chemokines, ROS formation, mitochondrial dysfunction and apoptosis. Pharmacologic interventional studies are now needed to determine the effects of Nef pathway inhibition, in addition to ART, to improve endothelial function and reduce the risk of cardiovascular disease in those infected with HIV.

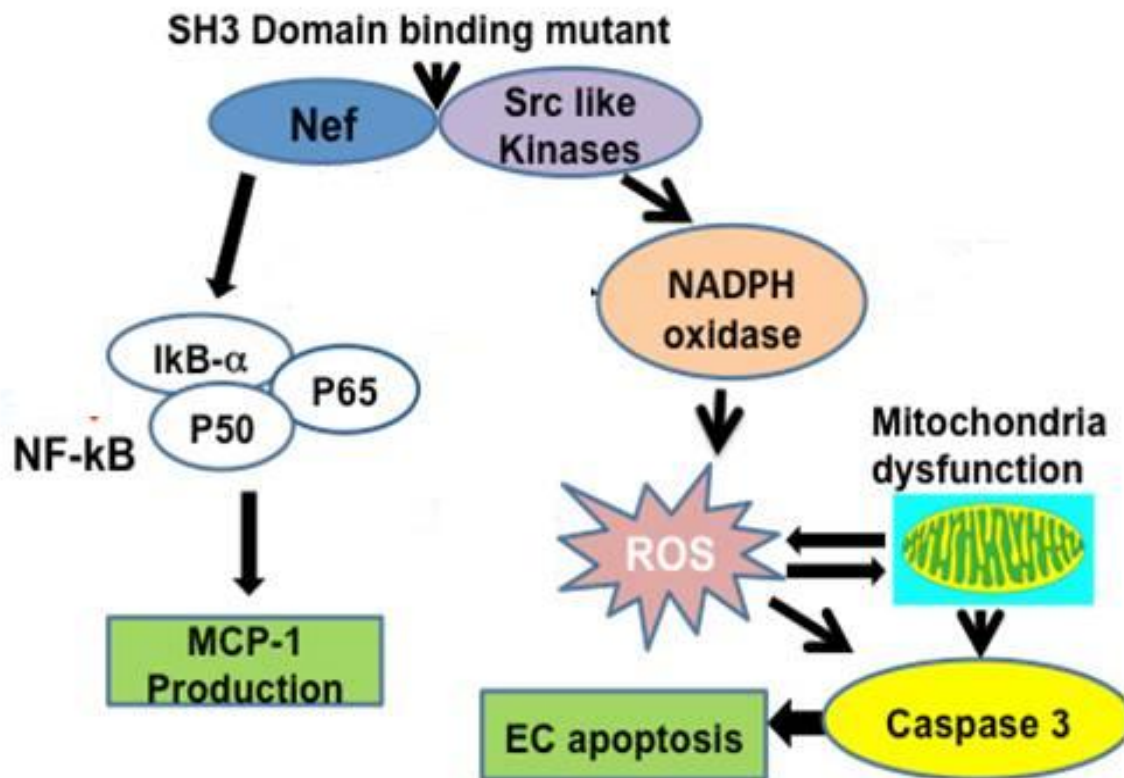


Figure 36: Nef signaling pathways in HCAEC

Nef induced endothelial MCP-1 production and apoptosis pathways are shown in the above scheme.

Future Directions:

To validate signal transduction target for therapeutical application

1. Transgenic Mice Models

Based on my finding of Nef presents in endothelium from CD4.Nef.GFP mice, we could further investigate these transgenic animals. Our collaborator (who provided the mice heart sections) had reported that using this transgenic approach tissue specific expression of Nef was sufficient to cause AIDS-like disease, B and T cell dysfunction, end organ diseases including lymphatic, kidney, and pulmonary, cardiac and coronary pathologies. Although coronary vascular dysfunction was compromised in these mice (Kay, Yue et al. 2002), the significance of these findings for atherosclerotic diseases was not addressed. Therefore, we could crossover Nef Tg mice with ApoE gene-deficient mice, an established model of atherosclerosis. Importantly, antiretroviral therapy is well known to cause hyperlipidemia (Carpentier, Patterson et al. 2005), which is best addressed in the widely used ApoE $-/-$ model (Hofker, van Vlijmen et al. 1998; Jawien, Nastalek et al. 2004; Koga, Kai et al. 2007). In this model we can further address the hypothesis that Nef in CD4 and possibly also in other PBMC as well as in arterial vascular endothelial cells combines Nef-induced mononuclear and endothelial cell dysfunction to cause and/or enhance atherosclerotic changes. In addition to more specifically addressing the role endothelial Nef in atherosclerosis, we can generate Tg mice expressing Nef only in endothelial cells, under a tetracycline regulated (tetracycline treatment to be stopped to turn expression on) endothelial specific tie2 and/or VE-cadherin promoters (Mukai,

Rikitake et al. 2006). Again we will backcross these endothelial Nef expressing mice into the apopE ^{-/-} background. Thus, these transgenic lines can be used for assessing targets of Nef signaling in order to intervene in HIV associated vascular diseases, starting these studies with a proof of principle Nef transgenic signaling mutant (delta SH3) as this mutant was described previously to lead to reduced AIDS-like symptoms in comparison with wild type Nef transgenic mice (Hanna, Weng et al. 2001).

2. Pilot Study

As ART reduces virion production but not Nef gene expression, it is possible that these adverse effects of Nef may persist even in patients receiving ART. If so, then the persistence of Nef-positive cells during ART might contribute to the higher risk of non-AIDS complications observed despite ART. In the previous experiments (Table 2), we have demonstrated the dissemination of Nef in PBMC of HIV patients and its ability to be transferred to endothelial cells (Figure 21). Thus, it is important to further quantify the cellular dissemination of Nef protein in the blood from HIV patients on ART and its ability to affect the vascular system. We can isolate PBMCs from up to 40 HIV-infected patients and uninfected volunteers to determine the amount of Nef protein in their PBMCs by using Nef ELISA kit. In the meantime, the Nef protein concentration in each patient will be compared to their malondialdehyde (MDA) level, a circulating biomarker of oxidative stress (Gerritsen, van Boven et al. 2006) and flow-mediated dilation (FMD) level of the brachial artery (Abbott, Harkness et al. 2002), a well-accepted measure of in vivo endothelial function and predictor of

CVD. In addition, my data showed that anti-oxidant could block HIV Nef-induced endothelial apoptosis. Thus, we could also assess the effects of the antioxidant agents N-acetylcysteine (NAC) and the ACE-inhibitor lisinopril on circulating MDA levels and FMD in HIV-infected persons receiving virologically suppressive ART.

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Educational Background

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Research Experience

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