

HIV-1 NEF: THERAPEUTIC STRATEGIES AND VIROLOGICAL  
SYNAPSE-MEDIATED INFECTION

Linden Ann Green

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

December 2010

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

Doctoral Committee  
October 19, 2010

---

Johnny J. He, Ph.D., Chair

---

Janice S. Blum, Ph.D.

---

Millie M. Georgiadis, Ph.D.

---

Ann Roman, Ph.D.

## ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Johnny He, for allowing me to work in his laboratory for my thesis dissertation, and for his advice and support during that time. I have learned a great deal over the last several years, and will keep the lessons I have learned with me for the rest of my life.

I also thank my committee members, Dr. Janice Blum, Dr. Millie Georgiadis, and Dr. Ann Roman, who offered much-appreciated comments, advice, and criticisms. This work would have been much poorer if not for them.

I thank the past and present members of Dr. He's laboratory for their friendship, support, and advice, especially Dr. In-Woo Park, Dr. Khalid Timani, Dr. Jorge Henao-Mejia and Yan Fan, who helped me with my experiments.

I would like to thank my entire family for their love, encouragement, and support during this time. I would especially like to thank my twin Linnea and my wonderful husband Alexander. I would not have been able to do this without them.

Finally, I give thanks to God, who makes all things possible.

## ABSTRACT

Linden Ann Green

### HIV-1 Nef: therapeutic strategies and virological synapse-mediated infection

HIV-1 infection is one of the greatest public health concerns today. The current HIV/AIDS therapy is effective in halting virus multiplication and has improved the outlook of AIDS; however, high cost, side effects, and the rise of drug-resistant viral strains have posed challenges for long-term treatment and management and mandate development of alternative anti-HIV therapies. Despite the fact that a great deal of progress has been made in our understanding of the infection over the last twenty-seven years, there are many unanswered basic scientific questions and no vaccines. In this study, we focused on two aspects related to the HIV-1 protein Nef: one is development of a Nef-based anti-HIV therapeutic strategy; the other is discovery of a novel mechanism that accounts for Nef-enhanced viral infectivity.

We first devised an anti-HIV therapeutic strategy that took advantage of the high virion incorporation of the Nef mutant Nef7 to deliver anti-HIV factors to the virion. We performed a series of proof-of-concept experiments, using the host anti-HIV cellular factor APOBEC3G (A3G). The Nef7.A3G fusion protein retains important properties of Nef7: higher virion incorporation efficiency, lack of PAK2 activation, and reduced CD4 and MHC I downregulation, as well the anti-HIV infectivity function of A3G. Moreover, virus-like particle (VLP)-mediated delivery of Nef7.A3G into infected CD4<sup>+</sup> T lymphocytes leads to inhibition of HIV-1 replication in these cells. These results support

the use of Nef7 as an anti-HIV therapeutic strategy for the delivery of therapeutic proteins into HIV-1 virions.

HIV-1 Nef protein has long been known to enhance viral infectivity. However, the underlying molecular mechanism remained elusive. Here we show that Nef is important for VS formation and VS-mediated virus transmission from cell to cell, especially in primary cells. Nef accomplishes this by inducing the clustering of VS components CD81 and ZAP70 and by inducing formation of actin protrusions, and these functions involve specific and distinct Nef domains. These findings not only yield new insights into the regulatory function of Nef in viral infectivity, but could also lead to development of more effective anti-HIV therapies that work equally well at blocking both VS-mediated and cell-free virus infection.

Johnny J. He, Ph.D., Chair

## TABLE OF CONTENTS

LIST OF TABLES .....	xii
LIST OF FIGURES .....	xiii
LIST OF ABBREVIATIONS.....	xvii
INTRODUCTION .....	1
<b>1. HIV-1 AND AIDS EPIDEMIOLOGY .....</b>	<b>1</b>
<b>1.1 HIV-1/AIDS epidemiology .....</b>	<b>1</b>
<b>1.2 Clinical aspects of HIV-1 infection.....</b>	<b>2</b>
<b>1.3 HIV-1.....</b>	<b>5</b>
<b>1.4 HIV-1 life cycle.....</b>	<b>6</b>
<i>1.4.1 Entry.....</i>	<i>6</i>
<i>1.4.2 Reverse transcription and integration .....</i>	<i>10</i>
<i>1.4.3 Transcription, splicing, and RNA export .....</i>	<i>14</i>
<i>1.4.4 Translation.....</i>	<i>15</i>
<i>1.4.5 Virus assembly and budding .....</i>	<i>17</i>
<b>1.5 Current HIV-1 treatment.....</b>	<b>18</b>
<i>1.5.1 HIV vaccines .....</i>	<i>18</i>
<i>1.5.2 HAART.....</i>	<i>18</i>
<b>2. CELLULAR COFACTORS OF THE HIV LIFE CYCLE .....</b>	<b>21</b>
<b>2.1 Positive cofactors.....</b>	<b>21</b>
<b>2.2 Negative cofactors .....</b>	<b>24</b>
<b>3. APOBEC3G.....</b>	<b>25</b>

<b>3.1 APOBEC family</b> .....	25
<b>3.2 APOBEC3G</b> .....	26
<b>4. NEF</b> .....	29
<b>4.1 Nef expression and structure</b> .....	29
<b>4.2 Nef importance during HIV infection</b> .....	32
<b>4.3 Nef functions during HIV infection</b> .....	33
<i>4.3.1 Alteration of surface receptors</i> .....	33
<i>4.3.2 Alteration of intracellular signaling pathways</i> .....	33
<i>4.3.3 Enhancement of infectivity</i> .....	34
<b>4.4 Nef7</b> .....	35
<b>5. TRANSIENT T CELL INTERACTIONS</b> .....	37
<b>5.1 Immunological synapses</b> .....	37
<i>5.1.1 Structure and function of immunological synapses</i> .....	37
<i>5.1.2 Nef involvement in the immunological synapse</i> .....	38
<b>5.2 Virological synapses</b> .....	42
<i>5.2.1 Types of virological synapses</i> .....	42
<i>5.2.2 HIV transfer through T cell virological synapses</i> .....	43
<b>6. LIPID RAFTS</b> .....	47
<b>6.1 Composition and function</b> .....	47
<b>6.2 Lipid rafts during HIV infection</b> .....	48
<b>Summary of the background and our hypothesis</b> .....	50
<b>MATERIALS AND METHODS</b> .....	52
<b>MATERIALS</b> .....	52

<b>Media and supplements</b> .....	52
<b>Antibodies</b> .....	52
<b>Reagents</b> .....	53
<b>Biotechnology systems</b> .....	54
<b>METHODS</b> .....	54
<b>Cells and cell cultures</b> .....	54
<i>Cell lines</i> .....	54
<i>Competent cells for cloning</i> .....	55
<i>Cell cultures</i> .....	55
<i>Isolation and culture of peripheral blood mononuclear cells (PBMC)</i> .....	55
<b>Plasmids</b> .....	56
<i>Reporter viruses</i> .....	56
<i>Nef mutants</i> .....	56
<i>HIV proviruses</i> .....	57
<i>Other plasmids</i> .....	58
<b>Bacterial transformation</b> .....	58
<b>Cell transfections</b> .....	59
<b>Preparation of viruses and virus-like particles (VLP)</b> .....	59
<b>Luciferase assays</b> .....	60
<b>Virus replication assays</b> .....	60
<i>VLP treatment</i> .....	60
<i>Virological synapse replication kinetics</i> .....	61
<b>Immunoblotting</b> .....	61



<i>Whole cell lysates</i> .....	61
<i>Membrane and cytosolic fractionation</i> .....	62
<i>Viruses</i> .....	62
<i>Immunoprecipitation and in vitro kinase assays (IVKA)</i> .....	63
<b>Raft floatation assays</b> .....	63
<b>Oil Red O (ORO) staining</b> .....	64
<b>Immunofluorescence staining</b> .....	64
<i>Stable cell lines</i> .....	64
<i>Virological synapse formation and Nef transfer</i> .....	65
<b>Flow cytometry analysis</b> .....	65
<i>MHC I and CD4 downregulation</i> .....	65
<i>Analysis of Nef-GFP intracellular delivery</i> .....	66
<i>GMI/CD81 polarization</i> .....	66
<i>Gag transfer</i> .....	67
<b>Data acquisition and statistical analysis</b> .....	67
RESULTS.....	68
<b>PART 1: NOVEL ANTI-HIV THERAPIES USING A NEF-MUTANT</b>	
<b>FUSION PROTEIN</b> .....	68
<b>1.1 Comparison of Nef and Nef7 mutants</b> .....	68
<b>1.2 Enhanced virion incorporation of Nef7 as compared to WT Nef</b> .....	71
<b>1.3 Efficient delivery of virion Nef7 into target cells</b> .....	76
<b>1.4 Higher virion incorporation of the Nef7.A3G fusion protein</b> .....	79
<b>1.5 No PAK2 activation by the Nef7.A3G fusion protein</b> .....	79

<b>1.6 Downregulation of CD4 and MHC I by Nef7.A3G</b> .....	87
<b>1.7 Impaired HIV-1 infectivity in 293T cells by Nef7.A3G virion incorporation</b> .....	92
<b>1.8 Impaired HIV-1 infectivity in U87 cells by Nef7.A3G virion incorporation</b> .....	98
<b>1.9 Block of HIV-1 replication by Nef7.A3G-containing VLP</b> .....	101
<b>PART 2: NEF ENHANCES THE FORMATION OF HIV-1</b>	
<b>VIROLOGICAL SYNAPSES</b> .....	109
<b>2.1 Nef increases the formation of VS</b> .....	109
<b>2.2 Piceatannol titration</b> .....	109
<b>2.3 Nef increases Gag transfer to target cells</b> .....	112
<b>2.4 Nef increases replication kinetics in conditions favoring VS formation</b> .....	115
<b>2.5 Nef induces polarization of VS components</b> .....	120
<i>2.5.1 Polarization in transfected 293T cells</i> .....	120
<i>2.5.2 Surface localization of VS components</i> .....	123
<i>2.5.3 M<math>\beta</math>CD titration</i> .....	130
<i>2.5.4 Polarization of VS components in Jurkat T cells</i> .....	133
<i>2.5.5 Polarization of ZAP70</i> .....	148
<b>2.6 Nef induces formation of actin protrusions</b> .....	148
<b>2.7 VS formation using Nef mutant viruses</b> .....	157
<b>2.8 VS formation in PBMC</b> .....	166
<b>DISCUSSION</b> .....	174

<b>Summary of the results</b> .....	174
<b>Virion incorporation of Nef7 and Nef7 fusion proteins</b> .....	176
<b>Pathogenicity of Nef7 fusion proteins</b> .....	178
<b>Inhibition of HIV-1 infectivity using Nef7.A3G</b> .....	179
<b>Effect of Nef on HIV-1 virological synapse formation</b> .....	181
<b>Nef-induced polarization of virological synapse components</b> .....	182
<b>Nef residues involved in clustering of virological synapse components</b> .....	184
<b>Nef-induced protrusions as an indication of viral transfer</b> .....	185
<b>ZAP70 involvement in Nef enhancement of HIV-1 virological synapses</b> .....	186
<b>Virological synapse formation in primary CD4<sup>+</sup> T lymphocytes</b> .....	187
<b>PERSPECTIVES</b> .....	191
<b>REFERENCES</b> .....	200
<b>CURRICULUM VITAE</b>	

## LIST OF TABLES

<b>Table 1.</b> FDA-approved anti-HIV drugs.....	20
<b>Table 2.</b> Major host factors involved in HIV-1 replication.....	22

## LIST OF FIGURES

<b>Figure 1.</b> Clinical course of HIV-1 infection.....	3
<b>Figure 2.</b> General overview of HIV-1 life cycle.....	7
<b>Figure 3.</b> Overview of HIV-1 reverse transcription.....	11
<b>Figure 4.</b> APOBEC3G function during HIV-1 replication.....	27
<b>Figure 5.</b> Functional domains and important residues of HIV-1 Nef .....	30
<b>Figure 6.</b> Schematic of the immunological synapse.....	39
<b>Figure 7.</b> Schematic of the known components of the HIV-1 virological synapse.....	45
<b>Figure 8.</b> Cytosolic and membrane expression of Myc-tagged Nef and Nef mutants....	69
<b>Figure 9.</b> Virus production and cytotoxicity in Nef7-expressing cells.....	72
<b>Figure 10.</b> Virion incorporation of Nef7.....	74
<b>Figure 11.</b> Delivery of virion WT Nef and Nef7 into cells.....	77
<b>Figure 12.</b> Expression of HA-tagged Nef, Nef7, A3G, and Nef7.A3G genes.....	80
<b>Figure 13.</b> Virion incorporation of Nef7.A3G.....	82
<b>Figure 14.</b> Effects of Nef, Nef7 and Nef7.A3G on PAK2 activation.....	85
<b>Figure 15.</b> Effects of Nef, Nef7 and Nef7.A3G on cell surface expression of CD4.....	88
<b>Figure 16.</b> Effects of Nef, Nef7 and Nef7.A3G on cell surface expression of MHC I.....	90
<b>Figure 17.</b> Expression and virion incorporation of Nef7.A3G and its derivatives.....	94

<b>Figure 18.</b> Effects of virion incorporation of Nef and its derivatives on the infectivity of VSG-pseudotyped HIV-Luc reporter viruses.....	96
<b>Figure 19.</b> Effects of virion incorporation of Nef and its derivatives on the infectivity of HXB2.env- and YU2.env-pseudotyped HIV-Luc reporter viruses.....	99
<b>Figure 20.</b> Effects of virion incorporation of Nef and its derivatives on the infectivity of VSV-G-pseudotyped HIV-Luc reporter viruses in U87 cells.....	102
<b>Figure 21.</b> VLP incorporation of Nef7.A3G.....	104
<b>Figure 22.</b> Effects of Nef-, Nef7-, Nef7.A3G- and A3G-containing VLP on HIV-1 replication.....	107
<b>Figure 23.</b> Nef effect on virological synapse formation in HIV-infected Jurkat cells.....	110
<b>Figure 24.</b> Piceatannol titration in HIV-infected Jurkat cells.....	113
<b>Figure 25.</b> Nef effect on virological synapse-mediated Gag transfer.....	116
<b>Figure 26.</b> Nef effect on HIV replication kinetics in conditions favoring VS formation.....	118
<b>Figure 27.</b> Effect of Nef on distribution of virological synapse components as shown by confocal microscopy.....	121
<b>Figure 28.</b> Effect of Nef on the membrane distribution of CD81 as measured by flow cytometry.....	124
<b>Figure 29.</b> Effect of Nef on the membrane distribution of GM1 as measured by flow cytometry.....	126
<b>Figure 30.</b> Effect of Nef on the total cellular and membrane expression of CD81 or GM1.....	128

<b>Figure 31.</b> M $\beta$ CD titration in 293T and Jurkat cells.....	131
<b>Figure 32.</b> Nef effect on the distribution of GM1 in Jurkat cells as shown by confocal microscopy.....	134
<b>Figure 33.</b> Nef effect on the distribution of CD81 in Jurkat cells as shown by confocal microscopy.....	136
<b>Figure 34.</b> Effect of Nef on the membrane distribution of GM1 and CD81 in Jurkat cells as measured by flow cytometry.....	139
<b>Figure 35.</b> Effect of piceatannol on Nef-induced alteration of GM1 membrane distribution .....	141
<b>Figure 36.</b> Membrane fractionation in the presence and absence of Nef.....	144
<b>Figure 37.</b> CD81 localization during membrane fractionation in the presence of Nef.....	146
<b>Figure 38.</b> Nef effect on the localization of pTyr to virological synapses.....	149
<b>Figure 39.</b> Nef effect on the localization of ZAP70 to virological synapses.....	151
<b>Figure 40.</b> Nef effect on actin organization .....	154
<b>Figure 41.</b> Nef transfer to neighboring cells.....	158
<b>Figure 42.</b> Replication-competent Nef mutant viruses.....	160
<b>Figure 43.</b> Nef residues important for VS formation.....	162
<b>Figure 44.</b> Nef residues important for VS-dependent virus transfer.....	164
<b>Figure 45.</b> Nef residues necessary for GM1 polarization.....	167
<b>Figure 46.</b> Relationship between Nef-induced activation of ZAP70 and VS-dependent transfer .....	169
<b>Figure 47.</b> Nef effect on VS formation in PBMC.....	172

**Figure 48.** Proposed working model for Nef-mediated enhancement of HIV

virological synapses.....189



## LIST OF ABBREVIATIONS

aa	amino acid
AID	activation-induced deaminase
AIDS	acquired immune deficiency syndrome
Amp	ampicillin
AP-1, 2, 3	adaptor protein-1, 2, 3
AP2	activating protein 2
APC	antigen presenting cell
APOBEC3G/A3G	apolipoproteinB mRNA editing enzyme, catalytic polypeptide-like
ATCC	American Tissue Culture Collection
BSA	bovine serum albumin
CA	HIV capsid protein
CCR5	CC chemokine receptor 5
CD	cluster of differentiation
CDK9	cyclin-dependent kinase 9
CFSE	carboxyfluorescein succinimidyl ester
CIP	calf intestinal phosphatase
COP-I	coat protein complex-I
cpm	counts per minute
CRM1	chromosome maintenance gene 1
cSMAC	central supramolecular activation complex

CXCR4	CXC chemokine receptor 4
DAPI	4',6'-diamidino-2-phenylindole
DC-SIGN	dendritic cell-specific ICAM-3-grabbing non-integrin
DDX3	DEAD box protein 3
DIC	Differential interference contrast
Dlg	Drosophila tumor suppressor protein
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic acid
EGTA	ethylene-glycol-tetra-acetic acid
Env	HIV envelope protein
ER	endoplasmic reticulum
Erk	extracellular signal-regulated protein kinase
ESCRT	endosomal sorting complex required for transport
FBS	fetal bovine serum
FC	flow cytometry
FDA	Federal Drug Administration
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue

GDP	guanosine diphosphate
GFP	green fluorescent protein
GI	gastrointestinal
GM1	monosialotetrahexosylganglioside
gp41	HIV-1 glycoprotein 41
gp120	HIV-1 glycoprotein 120
GTP	guanosine diphosphate
HAART	highly active anti-retroviral therapy
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	human immunodeficiency virus type 1
hRIP	human Rev-interacting protein
HRP	horseradish peroxidase
HTLV-1	human T-cell leukemia virus type 1
ICAM	intercellular adhesion molecule
IF	immunofluorescence
IL-2	interleukin 2
IN	HIV integrase
IP	immunoprecipitation
IRES	internal ribosome entry site
IS	immunological synapse
ITK	IL-2-inducible T cell kinase
JNK	c-Jun N-terminal kinase

kDa	kilodalton
LB	Luria broth
Lck	lymphocyte specific protein tyrosine kinase
LEDGF	lens epithelial derived growth factor
LFA-1	lymphocyte function-associated antigen 1
LTR	long terminal repeat
Luc	luciferase
M $\beta$ CD	methyl beta cyclodextrin
MA	HIV matrix protein
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MLV	murine leukemia virus
mRNA	messenger RNA
MVB	multivesicular body
NC	HIV nucleocapsid protein
Nef	HIV negative factor
NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institutes of Health
NP40	Nonidet P-40
N-Wasp	Wiskott-Aldrich Syndrome Protein
O/N	overnight
ORF	open reading frame

ORO	oil red O
PACS-1, 2	phosphofurin acidic cluster sorting protein-1, 2
PAK2	p21-activated kinase 2
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PFA	paraformaldehyde
PHA	phytohemagglutinin
PIC	pre-integration complex
PKC $\theta$	protein kinase C theta
PLC $\gamma$ -1	phospholipase C gamma 1
PMSF	phenylmethanesulphonylfluoride
PR	HIV-1 protease
pSMAC	peripheral supramolecular activation complex
REF-1	restriction factor 1
Rev	HIV-1 regulator of virion protein expression
Rlu	relative light units
RPMI	Roswell Park Memorial Institute
RNA	ribonucleic acid
RRE	Rev response element
RT	HIV-1 reverse transcriptase

Sam68	Src-associated protein in mitosis of 68 kDa
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH3	Src homology 3
SIV	simian immunodeficiency virus
ss	single-stranded
TAR	transactivation response element
Tat	HIV-1 transactivator of transcription
Tax	HTLV-1 transcriptional transactivator
TBST	Tris-buffered saline with 0.05% Tween-20
TCR	T cell receptor
tRNA	transfer RNA
TSG-101	tumor susceptibility gene 101
UTR	untranslated region
Vif	HIV-1 viral infectivity factor
VLP	virus-like particle
Vpr	HIV-1 viral protein R
Vpu	HIV-1 viral protein U
VS	virological synapse
VSV	vesicular stomatitis virus
WB	Western blot
ZAP70	zeta-chain (TCR)-associated kinase

## INTRODUCTION

### **1. HIV-1 AND AIDS EPIDEMIOLOGY**

#### **1.1 HIV-1/AIDS epidemiology**

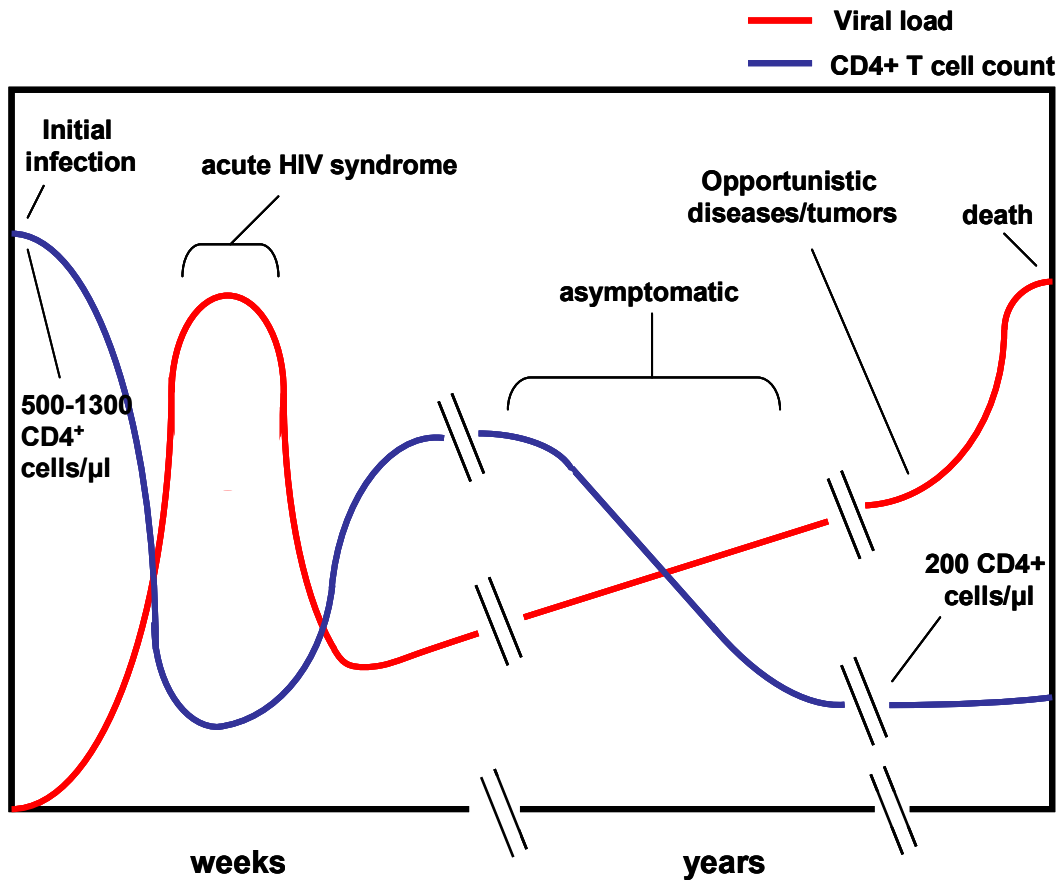
Human immunodeficiency virus type 1 (HIV-1) is the causative agent for acquired immune deficiency syndrome (AIDS), one of the greatest public health concerns in the world ([www.unaids.org](http://www.unaids.org)). HIV-1 has caused a global pandemic that has resulted in over 25 million deaths since it began in the early 1980s. The most recent worldwide statistics reveal that as of the end of 2008, there were 33.4 million people living with HIV worldwide and approximately 2 million deaths in 2008 alone due to AIDS. Of these, almost 75% were in sub-Saharan Africa where the availability of anti-retroviral drugs remains low, with less than 45% coverage of infected individuals. In recent years the incidence of infection has decreased slightly, with only 2.7 million new cases in 2008 as compared to approximately 3.5 million in 1996; nevertheless, the overall number of cases is still increasing steadily due to both newly acquired infections and the increase of life expectancy due to anti-HIV treatment ([www.who.int](http://www.who.int)). According to recent United Nations estimates, \$25 billion will be needed to provide HIV treatment in low- and middle-income countries in 2010: an estimate that is \$11.3 billion higher than what is currently available ([www.uaid.org](http://www.uaid.org)). Despite the large amount of effort put into resolving it, the HIV pandemic remains a huge concern.

## **1.2 Clinical aspects of HIV-1 infection**

HIV-infected individuals usually experience an acute illness 2-4 weeks post-infection, described as flu-like (Fig. 1). This typically lasts about 1-2 weeks and can involve a variety of symptoms such as fever, headache, gastrointestinal irritation, lymphadenopathy, weight loss, and myalgia (Hicks, Gay et al. 2007). During this time, the virus seeds lymphoid organs such as lymph nodes and gut-associated lymphoid tissue (GALT) to establish a persistent infection (Fauci, Pantaleo et al. 1996). The onset of symptoms corresponds with a high viral load, mainly produced from infected CD4<sup>+</sup>CCR5<sup>+</sup> T cells (Lyles, Munoz et al. 2000; Douek, Brenchley et al. 2002; Douek, Picker et al. 2003), and is accompanied by a sharp but transient decrease in the number of peripheral blood CD4<sup>+</sup> T cells as well as a largely permanent decrease in gastrointestinal mucosal CD4<sup>+</sup>CCR5<sup>+</sup> memory T cells (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004). After a week or two the acute phase of infection passes, with resolution of the flu-like symptoms, a drop in viral load, and an associated rise in peripheral blood CD4<sup>+</sup> T cells. At this point, a chronic infection is established.

The chronic phase is generally asymptomatic and is described as a period of clinical latency that lasts for an average of 10 years (Lyles, Munoz et al. 2000), although a small percentage of infected individuals known as long-term non-progressors exhibit no signs of disease progression even after 10 years or more, and without anti-retroviral treatment (Cao, Qin et al. 1995; Pantaleo, Menzo et al. 1995). During this time viral titers exhibit a slow but steady increase, while peripheral blood CD4<sup>+</sup> T cells decline steadily from the





**Figure 1. Clinical course of HIV-1 infection.** Clinical, virological, and immunological course of a typical HIV-1-infected patient, illustrated as changes in HIV-1 plasma virus load (red) and peripheral blood CD4<sup>+</sup> T cell counts (blue). The X axis represent the time after primary infection, while the Y axis represents viral RNA copies in the plasma or CD4<sup>+</sup> T cell counts. Major events in disease progression are indicated. Adapted from Grossman, Meier-Schellersheim et al. 2006.

small plateau reached after the partial recovery at the end of the acute illness (Blaak, de Wolf et al. 1997; Douek, Picker et al. 2003). The immune system becomes continuously activated during this period, resulting in T cell exhaustion and further depletion of T cells (Leng, Borkow et al. 2001; Brenchley, Price et al. 2006). Once the peripheral blood CD4<sup>+</sup> T cell count falls below 200 cells/ $\mu$ l, usually accompanied by a sharp increase in viral load, the infected individual is considered to have AIDS (Pantaleo, Graziosi et al. 1993). This stage is characterized by the onset of AIDS-related opportunistic infections or tumors not found in immuno-competent people, such as Kaposi sarcoma and *Toxoplasma Gondii* infections.

### **1.3 HIV-1**

HIV-1 is a complex retrovirus with a genome of about 10 kilobases in length. The virion itself is approximately 120 nm in diameter and is comprised of a conical core containing a diploid RNA genome, which is in turn encapsulated by a membrane bilayer derived from the host cell membrane. In addition to the *gag*, *pol*, and *env* genes that every retrovirus has, HIV-1 has 6 additional genes: *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*. These 9 open reading frames (ORF) result in fifteen proteins (Frankel and Young 1998).

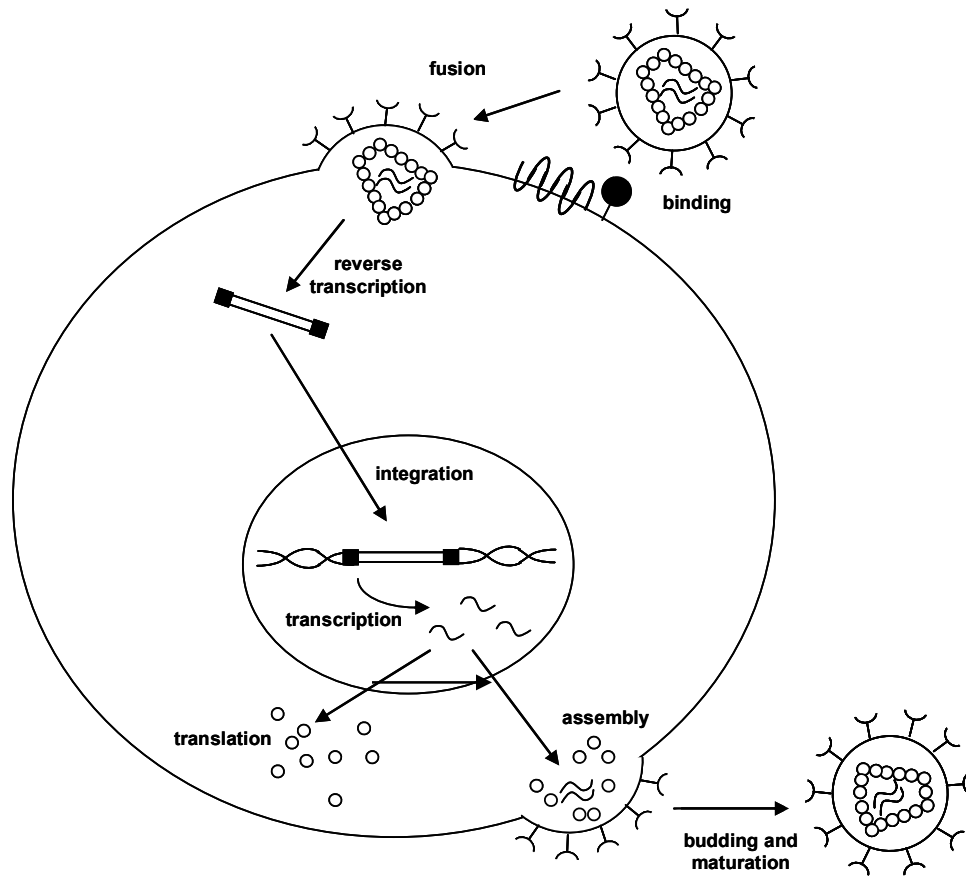
*Gag* encodes four structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC), and p6, which comprise the virion core. *Env* encodes one glycoprotein, gp160, which is then processed into gp120 and gp41 and is responsible for viral entry into the host cell (Hallenberger, Moulard et al. 1997). *Pol* encodes a long polyprotein that is cleaved into 3 essential enzymatic proteins, protease (PR), reverse transcriptase (RT), and integrase (IN), which are necessary for viral replication and are therefore encapsulated in the

virion. Vpr is necessary for importation of the provirus to the nucleus in non-dividing cells and also causes cell cycle arrest (He, Choe et al. 1995). Vpu increases the efficiency of viral budding from the host cell by inhibiting the activity of the host factor tetherin (Neil, Zang et al. 2008). Tat is the transactivator of HIV and is required for efficient transcription of HIV mRNA (Berkhout, Silverman et al. 1989; Laspia, Rice et al. 1989). Rev provides nuclear export of non- or incompletely-spliced HIV mRNAs (Malim, Bohnlein et al. 1989; Malim, Hauber et al. 1989). Vif abolishes the activity of a strongly anti-HIV host factor, APOBEC3G by inducing its ubiquitination (Mariani, Chen et al. 2003). The remaining accessory protein, Nef, has numerous effector functions and will be discussed at greater length in subsequent sections.

## **1.4 HIV-1 life cycle**

### *1.4.1 Entry*

The main cellular receptor of HIV-1 is CD4 (Sattentau and Weiss 1988). The virus also has two co-receptors, CCR5 and CXCR4, at least one of which is required in addition to CD4 for viral entry into a host cell (Alkhatib, Broder et al. 1996; Alkhatib, Combadiere et al. 1996; Feng, Broder et al. 1996). HIV-1 virions infect a susceptible cell by initially binding to CD4 via the HIV envelope glycoprotein gp120 (Fig. 2). Following binding to CD4, gp120 undergoes a conformational change that allows it to also bind to the co-receptors, either CXCR4 or CCR5. Depending on the tropism of the virion, gp120 binds preferentially to either CCR5 or CXCR4. Binding to the co-receptor induces an additional conformational change that exposes the gp41 glycoprotein that is needed for virus:cell fusion, thereby allowing the viral core to enter the cytoplasm



**Figure 2. General overview of HIV-1 life cycle.** Important steps in HIV-1 life cycle are highlighted: Binding of gp120 to CD4 and CCR5/CXCR4; fusion of the viral membrane with the cell membrane, resulting in virus penetration and uncoating of the core; reverse transcription of the ssRNA genome to dsDNA provirus; transport of the provirus to the nucleus and integration into the host chromosome; transcription of HIV-1 mRNA; translation of HIV proteins and viral assembly at the cell membrane; and finally, virion budding and maturation.

(Gallo, Finnegan et al. 2003). CD4 is expressed on most white blood cells, and defines the main restriction to viral entry. CCR5 is expressed on resting T lymphocytes with the memory and effector phenotypes, monocytes, macrophages, and immature dendritic cells, and also on a number of cells in the central nervous system including neurons, astrocytes, and microglia (Blanpain, Libert et al. 2002). CXCR4, on the other hand, is more widespread and expressed on a number of tissues, including hematopoietic and neuronal cells (Bleul, Wu et al. 1997; Rossi and Zlotnik 2000). The expression profile of these co-receptors reflects the tropism of HIV-1 during the course of infection. CCR5 tropic viruses are the major transmissible form of the virus; as such, the initial infection is primarily in macrophages, dendritic cells, and CD4<sup>+</sup> CCR5<sup>+</sup> memory T cells (Scarlati, Tresoldi et al. 1997). The preference for CCR5 tropic viruses as the major transmissible form may help to establish the infection, as the cells these viruses infect are common reservoirs of HIV-1 *in vivo*. As the infection progresses, the virus switches to a CCR5/CXCR4 tropic form, and finally to a CXCR4 tropic form (Clapham and McKnight 2001). The major cell target becomes T lymphocytes, resulting in an expanded tropism that reflects the more widespread expression of CXCR4 (Blaak, van't Wout et al. 2000; Douek, Picker et al. 2003).

In addition to the classical CD4/co-receptor mediated entry, there are a few non-standard receptors for HIV-1 that can enhance its binding to target cells although they do not mediate viral entry. For instance, DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin), which is expressed on dendritic cells, can bind tightly to gp120, and then subsequently enhance the *trans*-infection of HIV-1 to T

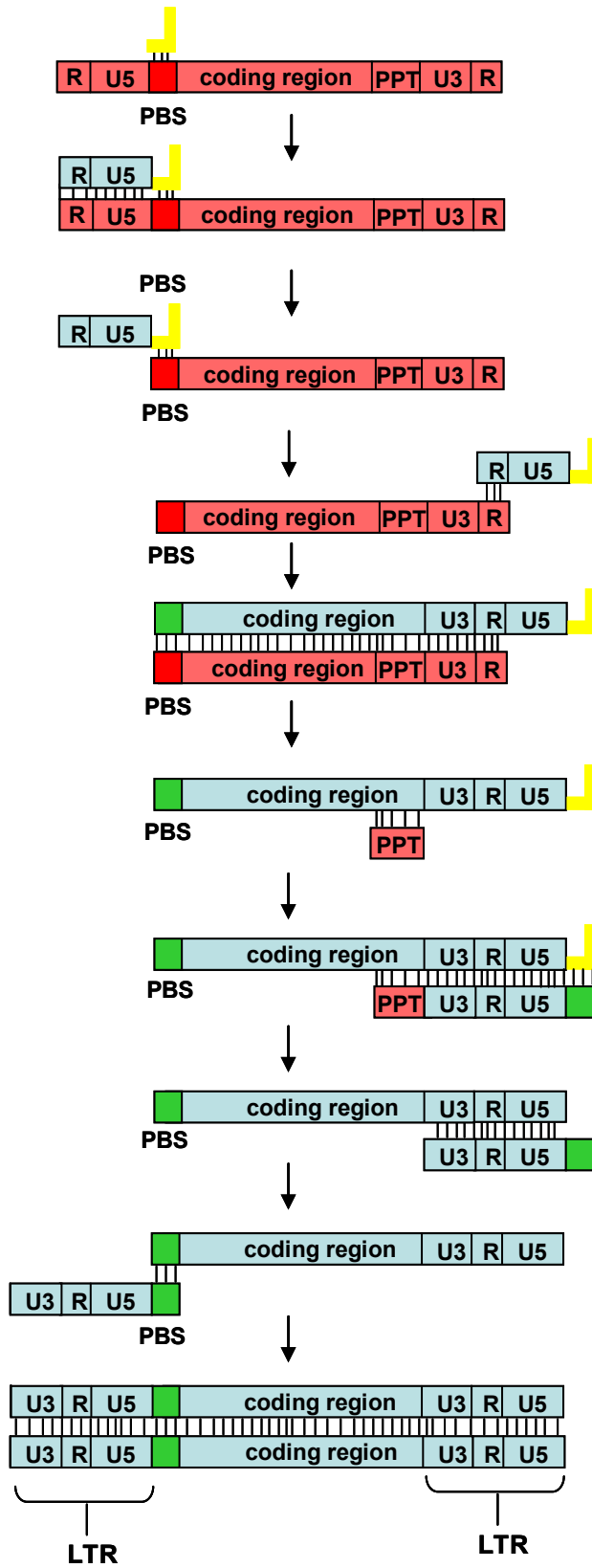
cells (Geijtenbeek, Kwon et al. 2000; Lozach, Amara et al. 2004). Likewise, the mannose-specific macrophage endocytosis receptor may enhance HIV-1 binding and entry into macrophages (Larkin, Childs et al. 1989; Hoffman, LaBranche et al. 1999; LaBranche, Hoffman et al. 1999; Liu, Liu et al. 2004). HIV-1 gp120 also binds to the glycolipid galactocerebroside (GalC) and its sulphated derivative sulphatide, which are expressed on macrophages, colonic epithelial cells, and on neuronal and glial cells in the brain (Fantini, Cook et al. 1993).

#### *1.4.2 Reverse transcription and integration*

Following viral entry into the host cell the virus core is released, exposing a nucleoprotein complex containing MA, RT, IN, Vpr, Vif, Nef, and the diploid RNA genome. The HIV-1 core also has additional incorporated proteins, including cellular proteins such as cyclophilin A and APOBEC3G when Vif is not expressed. The genomic single-stranded RNA then undergoes reverse transcription to create a dsDNA molecule by utilizing the HIV-1 enzyme reverse transcriptase (Fig. 3). RT is an extremely error-prone enzyme, which results in the ability of HIV to rapidly mutate. RT is comprised of three distinct enzymatic activities: an RNA-dependent DNA polymerase, an RNase H which degrades the DNA half of double-stranded DNA:RNA complexes, and a DNA-dependent DNA polymerase. Reverse transcription is initiated from a tRNA primer, which is packaged into the viral core and binds to the RNA genome at the primer binding site in its 5' end. Transcription commences using the RNA-dependent DNA polymerase activity until a DNA:RNA complex is formed. The RNA half is then degraded by the RNase H activity and the tRNA primer jumps to the 5' end of the newly synthesized



■ RNA  
■ DNA  
■ tRNA primer



**Figure 3. Overview of HIV-1 reverse transcription.** A general schematic describing the process of HIV-1 reverse transcription: 1) Synthesis of the first section of minus strand DNA; 2) Degradation of the RNA portion of the DNA:RNA complex and template switching of the remaining DNA fragment; 3) Synthesis of the coding region of the minus strand DNA; 4) Degradation of most of the remaining RNA; 5) Synthesis of a portion of the positive strand DNA and degradation of the remaining RNA; 6) Template switching of the positive strand DNA fragment; 7) Filling in of the remaining gaps to create the dsDNA provirus.

DNA molecule. Transcription then finishes, with the DNA-dependent DNA polymerase completing the construction of a double-stranded DNA provirus. Because of primer switching the ends of the RNA genome are duplicated, resulting in a flanking sequence known as the long-terminal repeat (LTR), which acts as the viral promoter (Basu, Song et al. 2008). The full-length linear cDNA, which is considered the direct precursor of the provirus, decays rapidly if not integrated (Zhou, Zhang et al. 2005).

The resulting dsDNA provirus forms a nucleic acid-protein complex known as the preintegration complex (PIC), which is comprised of the provirus, viral MA, Vpr, and IN. The PIC is imported to the nucleus via a nuclear localization sequence in IN, and is then incorporated into the host cell's genome in a process that is enhanced by cellular activation. Integration typically takes place in transcriptionally active regions of the host genome, particularly those having fewer GC-rich sequences (Lewinski, Yamashita et al. 2006). This may be in part due to the action of p75/LEDGF, a chromatin-associated protein that acts as a tethering factor for integrase (Vanegas, Llano et al. 2005; Llano, Vanegas et al. 2006). The 3'-processing reaction involves the removal of two nucleotides, adjacent to a highly conserved CA sequence, from the 3' end of both U3 and U5 viral LTR termini. Next, the cleaved DNA is used as a substrate for strand transfer, resulting in the covalent insertion of the provirus into the cellular genome (Esposito and Craigie 1998; Wu, Li et al. 2005).

### *1.4.3 Transcription, splicing, and RNA export*

Once integrated, the provirus serves as a template for virus replication and mRNA transcription. Initially, transcription proceeds at a basal level and is dependent upon the cellular transcription factors NF $\kappa$ B and AP2, which bind to the HIV LTR promoter (Harrich, Garcia et al. 1989; Perkins, Agranoff et al. 1994; Mallardo, Dragonetti et al. 1996). Viral transcripts produced at this point are short but contain the HIV-1 transactivator Tat. Tat interacts with the nascent viral mRNA through a stem-loop structure called TAR (Berkhout, Silverman et al. 1989) and recruits Cyclin T1 and CDK9 to the TAR-Tat complex, resulting in phosphorylation of the RNAPII C terminal domain and generation of full-length HIV RNA transcripts (Mancebo, Lee et al. 1997; Zhu, Pe'ery et al. 1997). The ability of HIV to replicate in peripheral blood lymphocytes is dependent upon induction of Cyclin T1 and CDK9 activity upon activation of the host cell, providing yet another reason why HIV requires host cell activation to replicate (Spina, Prince et al. 1997; Garriga, Peng et al. 1998; Flores, Lee et al. 1999).

There are three types of HIV transcripts: unspliced, partially spliced, and completely spliced. The completely spliced mRNAs include Tat, Rev, and Nef, and these are the first viral proteins made following HIV infection. Since eukaryotic cells cannot export incompletely spliced or unspliced mRNAs from the nucleus (Zenklusen and Stutz 2001), once translated the HIV-1 protein Rev returns to the nucleus and acts to transport these mRNAs to the cytoplasm. Rev first binds to the Rev-response element (RRE) present in unspliced mRNA transcripts (Malim, Bohnlein et al. 1989; Malim, Hauber et al. 1989). Rev then binds the chromosome maintenance gene 1 (CRM-1), which further recruits

Ran-GTP, resulting in the formation of a Rev-RRM-CRM-1-RanGFP complex. This complex is able to be exported from the nucleus through nuclear pores utilizing a mechanism that requires hydrolysis of Ran-GTP to Ran-GDP (Askjaer, Jensen et al. 1998).

There are many cellular factors involved in the Rev-RNA nuclear export process, among them Sam68, hRIP, and DDX3. DDX3 is a nuclear-cytoplasmic shuttling RNA helicase protein that localizes to nuclear pores (Yedavalli, Neuveut et al. 2004), while Rev-interacting protein (hRIP) is proposed to be involved in the release of HIV-1 mRNA from the perinuclear region (Sanchez-Velar, Udofia et al. 2004; Yu, Sanchez-Velar et al. 2005); both of these cellular factors are necessary for optimal HIV replication. SRC-associated protein during mitosis of 68kDa (Sam68) is absolutely required for Rev function (Li, Liu et al. 2002; Li, Liu et al. 2002; Modem, Badri et al. 2005). It binds to both the RRE and Rev, and its down-modulation results in extensive accumulation of Rev-CRM-1 complexes in the nucleus, significantly inhibiting HIV replication (Li, Liu et al. 2002; Modem, Badri et al. 2005).

#### *1.4.4 Translation*

Translation of HIV-1 mRNA remains one of the least understood areas of the HIV lifecycle. Due to mRNA splicing and processing, there are almost 30 distinct mRNA species produced from the HIV-1 provirus (Neumann, Harrison et al. 1994; Caputi, Freund et al. 2004). These mRNA are each capped and polyadenylated, and share the first 289 non-coding nucleotides. However, each mRNA species contains a unique 5'-

untranslated region (5'UTR) that varies in length and secondary structure (Parkin, Cohen et al. 1988). This may help regulate translation, but interestingly, the 5'UTR contains many highly conserved negative regulators of ribosomal scanning, such as TAR and the primer binding site (Geballe and Gray 1992; Berkhout 1996). Consequently, HIV-1 mRNA are not optimal substrates for cap-dependent translation. This has led to the theory that HIV translation could be controlled through an internal ribosome entry site (IRES) (Buck, Shen et al. 2001; Yilmaz, Bolinger et al. 2006). There have been conflicting reports in this area, with some studies showing an IRES in the 5'UTR and Gag coding sequences, and some studies that concluded there was no IRES present in unspliced HIV mRNA (Miele, Mouland et al. 1996; Brasey, Lopez-Lastra et al. 2003; Yilmaz, Bolinger et al. 2006). The wide array of secondary structures in HIV-1 mRNA could potentially result in differential translation regulation due to specific interactions between cellular or viral proteins and mRNA structures. For example, HIV-1 Gag inhibits its own translation at high concentrations by interacting with its 5'UTR (Anderson and Lever 2006). Likewise, the HIV-1 Rev protein can inhibit general translation of HIV-1 proteins at high concentrations (Groom, Anderson et al. 2009) and several host proteins can also affect translation of HIV-1 mRNA (Bolinger and Boris-Lawrie 2009).

HIV-1 utilizes a number of strategies to regulate translation of specific viral proteins. For example, the 5' UTR of both unspliced and spliced retroviral transcripts contain features that impede ribosomes scanning; this results in the ribosome skipping over a particular start codon and instead translating a different open reading frame (ORF) (Schwartz,

Felber et al. 1992; Anderson, Johnson et al. 2007). Additionally, mRNA containing a combination of repetitive sequences and a stable secondary structure can momentarily halt ribosome movement, leading to an mRNA reading frameshift (Jacks, Power et al. 1988; Gaudin, Mazauric et al. 2005).

#### *1.4.5 Virus assembly and budding*

The viral genomic RNA transcribed in the nucleus is exported to the cytoplasm. The Env protein is produced in the endoplasmic reticulum as gp160 and transits through the Golgi apparatus to localize to lipid rafts in the cell membrane, while Gag is synthesized on free cytosolic ribosomes. The Gag and Gag/Pol polyproteins once translated are transported to the intracellular face of lipid raft domains, possibly by utilizing the actin or microtubule network of the cell (Bieniasz 2009). Once there, immature nucleocapsids form by capsid proteins assembling a shell around two copies of the full length viral RNA genome. The immature virions bud from the cell surface, becoming encapsulated by a membrane derived from the lipid rafts, which contains the Env proteins as mentioned above (Ganser-Pornillos, Yeager et al. 2008; Ono 2009). During the budding process protease cleaves the matrix protein, causing the virion to undergo a morphological change and become mature and infectious (Frankel and Young 1998). In macrophages a similar process takes place, except the virions bud into large multivesicular bodies (MVB) inside the cell. The MVB then fuses with the plasma membrane, releasing the virions contained within (Benaroch, Billard et al.).

## **1.5 Current HIV-1 treatment**

### *1.5.1 HIV vaccines*

Initial efficacy trials on potential vaccines that attempted to produce antibody responses against gp120 failed, possibly due to the high variability in gp120 glycosylation patterns across different strains of HIV-1 (Derdeyn, Decker et al. 2004; Flynn, Forthal et al. 2005; Pitisuttithum, Gilbert et al. 2006). Since attempts to elicit broad antibody responses proved difficult, there occurred a shift in focus towards the development of non-protective vaccines that would elicit strong cellular immune responses. These would not prevent acquisition of the infection, but would instead delay disease progression. However, recent attempts to develop this approach were met with failure in clinical trials (Steinbrook 2007). Traditional ideas of vaccines do not appear to work in HIV infection; therefore, novel approaches must be conceived in order to create a workable HIV vaccine.

### *1.5.2 HAART*

Despite the difficulties faced in the creation of an HIV vaccine, an effective therapy in the form of highly active anti-retroviral therapy (HAART) has resulted in a dramatically improved outlook for HIV-positive patients. HAART has reduced the AIDS-associated mortality in developed countries by up to 80-90%, although the effect is much lower in developing countries where anti-retroviral drugs are cost-prohibitive and rare (Hammer, Eron et al. 2008; Ho and Bieniasz 2008). Proper HAART application can reduce plasma viral loads to undetectable levels, but it cannot eradicate the virus. Furthermore, replication can sometimes continue undetected in lymphoid tissues, and if treatment is



stopped for any reason viral loads become very high. A HAART regimen consists of a combination of at least three different anti-retroviral drugs from at least two different categories. These categories consist of reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors. Most drugs, however, are in the first two categories, with only one integrase inhibitor currently approved (Table 1).

Reverse transcriptase inhibitors can be nucleoside/nucleotide analogs or non-nucleoside analogs. Nucleoside/nucleotide analogs act by terminating reverse transcription when they are incorporated into the newly synthesized DNA. In contrast, non-nucleoside analogs block reverse transcriptase by binding to RT. They are not incorporated into the viral DNA but instead inhibit the movement of protein domains needed to carry out the process of DNA synthesis. Raltegravir, the lone integrase inhibitor, acts by inhibiting strand transfer during the integration process. Protease inhibitors usually act by inhibiting the HIV-1 protease-dependent cleavage of the MA protein in immature virions, making sure that these virions never become mature and infectious. In addition to these three types of anti-retroviral drugs, repeated attempts at developing entry and fusion inhibitors have been made (Deeks 2006). Currently there are two approved by the Food and Drug Administration (FDA): Enfuvirtide targets gp41 to prevent fusion of the viral envelope to the cell membrane and Maraviroc, a CCR5 antagonist, prevents binding of gp120 to CCR5 (Simon, Ho et al. 2006). These are not as universally effective as reverse transcriptase, integrase, and protease inhibitors, however, and are usually prescribed after the emergence of HIV-1 strains resistant to the more common treatments and administered in combination with other classes of antivirals (Rockstroh and Mauss 2004).

**Table 1. FDA-approved anti-HIV drugs**

<b>Brand name</b>	<b>Generic name</b>	<b>Category</b>
Epivir	Lamivudine	NRTI
Emtriva	Emtricitabine	NRTI
Retrovir	Zidovudine, azidotymidine	NRTI
Trizivir	Abacavir, zidovudine, lamivudine	NRTI
Zerit	Stavudine	NRTI
Ziagen	Abacavir sulfate	NRTI
Intelence	etravirine	NNRTI
Rescriptor	delavirdine	NNRTI
Sustive	efavirenz	NNRTI
Viramune	nevirapine	NNRTI
Agenerase	Amprenavir	Protease inhibitor
Crixivan	Indinavir	Protease inhibitor
Norvir	Ritonavir	Protease inhibitor
Prezista	Darunavir	Protease inhibitor
Reyataz	Atazanavir sulfate	Protease inhibitor
Fuzeon	Enfuvirtide (T-20)	Fusion inhibitor
Isentress	Raltegravir	Integrase inhibitor
Selzentry	Maraviroc	Binding inhibitor

NRTI = nucleoside reverse transcriptase inhibitor

NNRTI = non-nucleoside reverse transcriptase inhibitor

Data collected from FDA website [www.fda.gov/oashi/aids/virals](http://www.fda.gov/oashi/aids/virals)

All of these treatments, however, share the common potential to become ineffective due to the rise of resistant viruses. Unfortunately, the high cost of HAART and non-compliance issues due to toxicity often contribute to the rise of HAART-resistant HIV-1 strains. This has made the development of alternative anti-HIV therapies a priority. Additionally, none of the current anti-HIV treatments involve the HIV-1 accessory proteins. Since these proteins, especially Nef, can be expressed despite successful control of infection due to anti-retroviral therapy and contribute to disease progression, they make attractive targets for future therapy development (Fischer, Joos et al. 2004). Hence, this study focuses on the Nef protein.

## **2. CELLULAR COFACTORS OF THE HIV LIFE CYCLE**

### **2.1 Positive cofactors**

The majority of cellular factors involved in the HIV-1 life cycle are positive factors that HIV utilizes to facilitate its replication (Table 2). For example, TSG-101 is required for efficient virus budding (Hammarstedt and Garoff 2004). The HIV-1 Gag p6 protein binds to TSG-101 and helps recruit additional components of the ESCRT-I, -II, and -III complexes (Demirov, Orenstein et al. 2002). These complexes are part of the multivesicular body (MBV) pathway, which HIV-1 hijacks to bud from the cell. There are also several cellular factors that HIV-1 utilizes to facilitate its transcription and post-transcriptional processes. Sam68, as mentioned above, is required for Rev-dependent export of singly spliced and unspliced viral mRNAs from the nucleus through its interaction with the Rev/CRM-1 complex. Additionally, CyclinT1 is necessary for transcriptional elongation of HIV-1 transcripts from the LTR promoter (Price 2000)

**Table 2. Major host factors involved in HIV-1 replication**

<b>Host Factor</b>	<b>Effect on HIV</b>	<b>Step in life cycle affected</b>
APOBEC3G	-	reverse transcription
Tetherin	-	budding
REF-1	-	uncoating?
TSG-101	+	budding
CyclophilinA	+	uncoating
LEDGF/p75	+	integration
ITK	+	multiple steps
Cyclin T	+	transcription
Sam68	+	mRNA export
ESCRT complexes	+	virus assembly/budding

through its phosphorylation of the C-terminal domain of RNA polymerase II (Dahmus 1996; Dahmus 1996).

LEDGF/p75 stimulates nuclear localization and chromatin tethering for integrase, resulting in greater efficiency of proviral integration to the host chromosome (Llano, Vanegas et al. 2004; Llano, Vanegas et al. 2006). Interactions of LEDGF/p75 with components of the general transcription machinery and the transcription activation domain of certain proteins indicate participation in transcriptional regulation, possibly in the induction of stress response genes (Cherepanov, Maertens et al. 2003).

Inducible T cell kinase (ITK) is a Tec family tyrosine kinase that regulates T cell receptor-induced activation of PLC $\gamma$ -1, Ca<sup>2+</sup> mobilization and transcription factor activation, and actin rearrangement downstream of both TCR and chemokine receptors (Berg, Finkelstein et al. 2005). Recently, ITK has been found to be important for multiple steps of the HIV-1 life cycle: upon gp120 binding to CXCR4 it induces actin reorganizations that facilitate virus entry, upregulating transcription factors in response to ITK signaling resulting in increased transcription from the HIV-1 LTR, and increases budding through protein interactions and membrane recruitment (Readinger, Schiralli et al. 2008). The study of inhibitors for these and other pro-HIV cellular proteins is of growing interest, but care must be taken to ensure that inhibition of these proteins does not have a significant negative effect on the immune system or normal physiological function of the host.

## **2.2 Negative cofactors**

Because of the shortcomings of current anti-HIV therapies, there has recently been increased interest in exploiting cellular proteins involved in the HIV-1 lifecycle to develop new therapies. Currently, there have been few negative regulators of HIV identified; among them are APOBEC3G (Simon, Gaddis et al. 1998; Sheehy, Gaddis et al. 2002), tetherin (Neil, Zang et al. 2008), and REF-1 (Towers, Bock et al. 2000).

Tetherin is the term used to describe a cellular protein that prevents the release of certain enveloped virus particles and was identified to be the surface molecule CD317. When expressed, it results in the retention of virions in intracellular vacuoles and at the cell surface. The HIV-1 protein Vpu, however, downmodulates CD317/tetherin expression, allowing for the efficient release of viral particles (Neil, Zang et al. 2008).

Restriction factor 1 (REF-1) acts to restrict HIV-1 infection at a point between virus entry and reverse transcription, most likely during uncoating (Braaten, Franke et al. 1996). The mechanism REF-1 uses to do so is currently unknown, but seems to involve its interaction with the viral capsid. However, the positive factor cyclophilin A, through its incorporation into HIV-1 virions, inhibits the activity of REF-1 presumably through blocking the interaction between REF-1 and p24 (Towers, Hatziioannou et al. 2003).

The final anti-HIV cellular factor discovered so far is APOBEC3G, which will be covered in depth in the following section. These host factors appear to be part of an innate antiviral defense system which, if manipulated correctly, may be utilized in the

construction of novel anti-HIV therapies that have a lower risk of developing resistant viral strains than current treatments.

### **3. APOBEC3G**

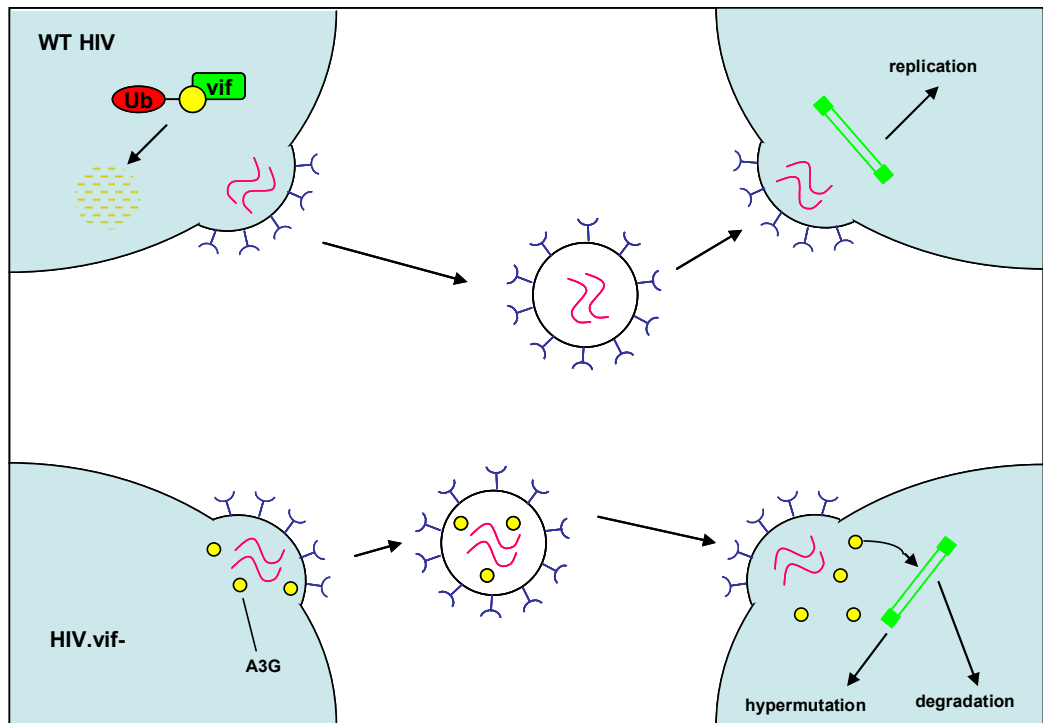
#### **3.1 APOBEC family**

The APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) superfamily is a large family of tissue-specific cytidine deaminases that display RNA-editing or DNA mutation abilities. It includes APOBEC1, APOBEC2, APOBEC3, and AID (Teng, Ochsner et al. 1999; Harris, Petersen-Mahrt et al. 2002; Jarmuz, Chester et al. 2002). APOBEC1, the prototypic enzyme of the family, is the key component of an RNA editing complex that deaminates cytosines in mammalian apolipoprotein B mRNA, resulting in a truncated form of apoB (Chester, Weinreb et al. 2004). AID (activation-induced cytidine deaminase) was the second family member to have its function identified and is the enzyme responsible for producing somatic hypermutations in immunoglobulins leading to antibody diversification during B cell development (Muramatsu, Kinoshita et al. 2000; Revy, Muto et al. 2000). APOBEC3 family members have been shown to have extensive anti-viral activities, suggesting that this family also plays an important role in innate immunity (Bishop, Holmes et al. 2004). There are a number of APOBEC3 proteins, which are expressed in a tissue-specific manner. For example, APOBEC3G, -3F, and -3C are expressed in spleen, ovary, testes, and peripheral blood lymphocytes, while APOBEC3B, -3C, and to a lesser extent -3A are prevalent in various cancer tissues (Jarmuz, Chester et al. 2002; Bishop, Holmes et al. 2004).

### **3.2 APOBEC3G**

APOBEC3G (or A3G) is a cellular anti-HIV protein that restricts HIV replication at the stage of reverse transcription. It was discovered to be the factor that restricted HIV $\Delta$ vif replication in non-permissive cell lines (Simon, Gaddis et al. 1998; Sheehy, Gaddis et al. 2002). There is evidence that A3G may also restrict other viruses such as hepatitis B virus (HBV) as well as endogenous retroelements such as long terminal repeat (LTR) retrotransposons (Cullen 2006). A3G is a ss DNA cytidine deaminase of ~42 kDa that deaminates C $\rightarrow$ U on the proviral minus strand of HIV during reverse transcription, resulting in G $\rightarrow$ A hypermutations in the finished provirus (Harris, Bishop et al. 2003; Aguiar and Peterlin 2008). These mutations may have many consequences: 1) Cellular DNA repair enzymes such as uracil DNA glycosylase may attempt to repair the provirus, resulting in its degradation; 2) The hypermutation of the provirus may result in a non-functional LTR, thus impairing the ability of the provirus to integrate into the host genome and resulting in its degradation; or 3) The provirus manages to integrate into the host genome, but the large number of mutations results in a non-functional promoter or defective viral proteins (Fig. 4). Since the activity of A3G takes place during reverse transcription within the HIV-1 capsid, isolated from the cytoplasm and all other host cellular factors, it is essential for the function of A3G that it incorporates into virions. During HIV $\Delta$ vif replication A3G is incorporated into progeny virions via interactions with the HIV-1 nucleocapsid protein, thus allowing A3G to be present for reverse transcription. When those virions infect a second cell, A3G renders them non-replicative. However, A3G does not affect WT HIV replication because of the antagonistic effect of Vif. Vif binds to A3G and recruits the E3 ubiquitin ligase cullin 5-elongin B/C





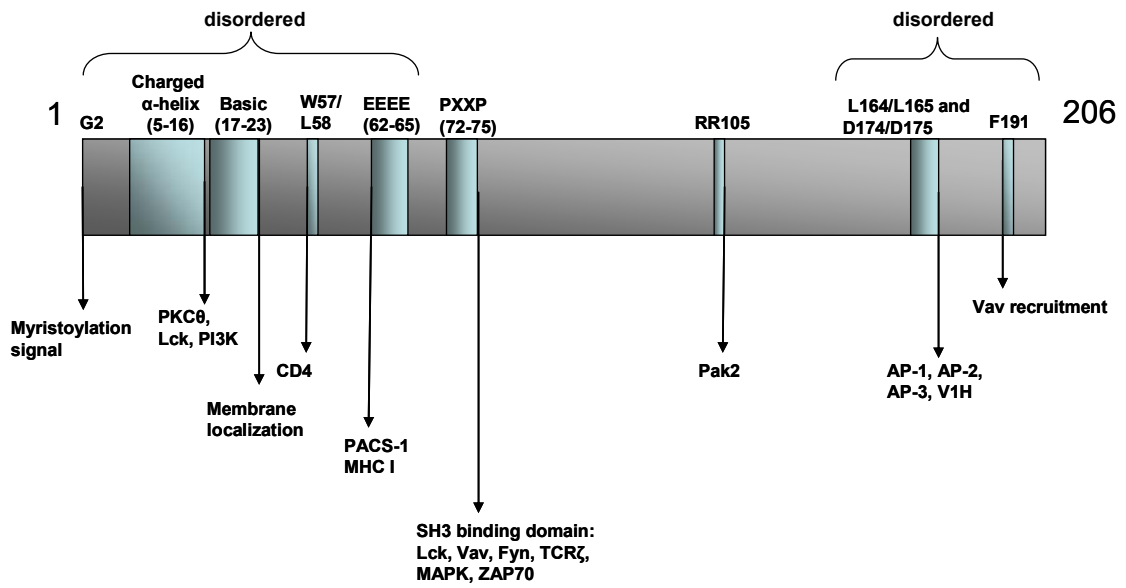
**Figure 4. APOBEC3G function during HIV-1 replication.** A schematic representation of the anti-viral function of APOBEC3G and its interaction with HIV-1 Vif. When Vif is not expressed, A3G is incorporated into progeny virions and is present in the secondary infection during reverse transcription where it induces deleterious hypermutations, preventing successful replication. When Vif is present, it induces ubiquitination of A3G, preventing its incorporation into progeny virions and allowing secondary infections to proceed unimpeded.

(Mehle, Goncalves et al. 2004). This targets A3G for ubiquitination and subsequent degradation by the proteasome, thus decreasing the available pool of A3G and limiting its incorporation into virions, and abolishing its ability to restrict HIV infection (Marin, Rose et al. 2003; Huthoff and Malim 2007).

## **4. NEF**

### **4.1 Nef expression and structure**

Nef is an accessory protein of ~27 kDa. It is post-translationally modified by phosphorylation and by the addition of a myristoyl moiety to its second amino acid (glycine), which aids in its membrane targeting and is required for most Nef functions (Fig. 5). The nef gene is found at the 3' end of the HIV genome, partially overlapping the 3' LTR (Foster and Garcia 2007; Raney, Shaw et al. 2007). While Nef primary sequences can vary, structurally Nef is well conserved with an N-terminal flexible disordered region, followed by a well-structured, globular core domain, and then a short C-terminal flexible domain (Arold and Baur 2001; Roeth and Collins 2006). One of the most important motifs for Nef function is the SH3 domain-binding sequence (PxxP) that is located in the globular core domain and is responsible for Nef binding to many SH3-domain-containing proteins, such as Src, Lck, and Hck and subsequent modulation of their kinase activities. Other important Nef residues include an N-terminal stretch of basic residues that can contribute to Nef membrane localization, an acidic region (<sup>62</sup>EEEE<sup>65</sup>) that is responsible for Nef perinuclear localization, and three pairs of amino acid residues (W<sup>57</sup>/L<sup>58</sup>, L<sup>164</sup>/L<sup>165</sup>, and D<sup>174</sup>/D<sup>175</sup>) that act to alter the surface expression of



**Figure 5. Functional domains and important residues of HIV-1 Nef.** Important Nef residues are labeled above the figure, with the corresponding function below.

many cellular proteins by serving as a bridge to protein sorting pathways (Geyer, Fackler et al. 2001).

#### **4.2 Nef importance during HIV infection**

While Nef is dispensable for *in vitro* replication of HIV-1, it is absolutely essential for efficient viral spread *in vivo*. It is the single most important HIV pathogenic factor, and is responsible for T cell activation and the establishment of a permissive replicative environment. It has been found that deletion of Nef significantly reduces the pathology observed in SIV-infected Rhesus Macaques (Kestler, Ringler et al. 1991). Additionally, HIV-1 isolates that contain defective Nef alleles have been found to correlate with long-term non-progressors, i.e. HIV-infected individuals who have had the virus for ten or more years without developing AIDS (Chowers, Spina et al. 1994; Spina, Kwok et al. 1994; Kirchhoff, Greenough et al. 1995; Salvi, Garbuglia et al. 1998).

Nef has no intrinsic enzymatic activity, but instead functions through interactions with cellular components. Over thirty putative Nef binding targets have been identified, and the number is still growing. It has been hypothesized that the great variety of Nef functions can be attributed to the large stretches of flexible, disordered regions mentioned above, as Nef could then potentially adopt different conformations depending on its binding partner, resulting in seemingly contradictory functions within the infected cell (Arold and Baur 2001). While Nef has numerous effector functions, they can be broadly separated into three categories: 1) Alteration of protein trafficking, especially in the

context of the downregulation of surface receptors; 2) Alterations in cell signaling cascades; and 3) Enhancement of HIV-1 infectivity.

### **4.3 Nef functions during HIV infection**

#### *4.3.1 Alteration of surface receptors*

The list of proteins affected by Nef has continued to grow over the past several years. To date, Nef has been reported to decrease the cell surface expression of CD4, MHC I, CD28, NKG2D, CXCR4, and CD80 among others (Garcia and Miller 1991; Anderson, Lenburg et al. 1994; Foster, Anderson et al. 1994; Schwartz, Marechal et al. 1996; Swigut, Shohdy et al. 2001; Keppler, Tibroni et al. 2006; Venzke, Michel et al. 2006). Additionally, Nef alters the subcellular localization of other proteins, such as sequestering recycling TCR-CD3 complexes and the Src kinase Lck with endosomes (Ehrlich, Ebert et al. 2002; Haller, Rauch et al. 2007). Not all of the mechanisms by which Nef affects these proteins have been fully elucidated, but in most cases Nef acts as a bridge between the targeted molecule and intracellular trafficking proteins. Nef is known to interact with many such proteins, including adaptor protein complexes AP-1, -2, and -3, PACS-1, and COP-I concatemers (Roeth and Collins 2006).

#### *4.3.2 Alteration of intracellular signaling pathways*

Nef accomplishes many of its pathogenic functions by interfering with cellular signaling cascades, such as PAK2-induced alteration of the actin cytoskeleton (Khan, Sawai et al. 1998; Manninen, Renkema et al. 2000; Renkema and Saksela 2000; Greenway, Holloway et al. 2003; Wei, Arora et al. 2005). For example, Nef can induce rapid and transient

phosphorylation of the  $\alpha$  and  $\beta$  subunits of the I $\kappa$ B kinase complex and of JNK, ERK1/2, and p38 mitogen-activated protein kinase family members among others (Mangino, Percario et al. 2007). Nef also modulates the TCR signaling cascade to provide a balance between the T cell activation required for efficient HIV-1 replication and the apoptosis that occurs if T cells are overly activated, which would limit the ability of infected cells to produce virus. It has been proposed that Nef accomplishes this by inducing endogenous activation of T cells while at the same time repressing exogenous activation (Fackler, Alcover et al. 2007). In addition to these effects in infected cells, Nef also has significant effects on bystander cells, such as inducing maturation of dendritic cells, apoptosis of uninfected T cells, and suppression of CD40-dependent immunoglobulin class switching in B cells (Qiao, He et al. 2006; Quaranta, Mattioli et al. 2006).

#### 4.3.3 *Enhancement of infectivity*

One of the most elusive activities of Nef is its ability to enhance the infectivity of HIV in primary cells and some T cell lines. Although there is generally no change in HIV-1 replication kinetics with or without Nef *in vitro*, Nef-defective HIV-1 exhibits greatly decreased replication in primary cells. The finding that Nef-containing viral particles were at least 10 times more infectious than Nef-deleted viruses in single-round infectivity assays partially explains this difference in replication (de Ronde, Klaver et al. 1992; Miller, Warmerdam et al. 1994; Spina, Kwoh et al. 1994). This Nef phenotype was observed over ten years ago, but the mechanism by which Nef achieves this enhancement has never been conclusively established. Enhancement of infectivity is dependent on the presence of Nef in the virus-producing cell, and is determined by virion incorporation of



Nef (Welker, Harris et al. 1998). Nef-containing viruses resemble Nef-defective viruses both structurally and biochemically, and they both fuse efficiently with cells (Tobiume, Lineberger et al. 2003; Cavrois, Neidleman et al. 2004; Day, Munk et al. 2004).

However, Nef-defective viruses exhibit decreased reverse transcription in target cells despite containing normal levels of viral genomic RNA and active reverse transcriptase (Chowers, Spina et al. 1994; Aiken and Trono 1995; Schwartz, Marechal et al. 1995).

An attractive recent hypothesis is that Nef acts to remodel the cortical actin barrier to allow the viral core to penetrate into the cytoplasm (Chazal, Singer et al. 2001). This hypothesis is supported by the finding that virions that are pseudotyped with pH-dependent envelope proteins that function by endocytosis of the virion, such as VSV-G, do not require Nef while pH-independent envelope proteins that fuse at the plasma membrane, such as that of HIV-1, do require Nef. In addition, disruption of the actin cytoskeleton in target cells compensates for the absence of Nef during infection only when virus enters the cell by membrane fusion but not when entry is by endocytosis (Campbell, Nunez et al. 2004). This theory may explain the increased infectivity of cell-free HIV-1 virions; however, increased cell-free viral infectivity may not completely explain the greatly increased replication kinetics of WT HIV over Nef-deleted viruses in primary cells.

#### **4.4 Nef7**

Recently a mutant of Nef known as Nef7 was characterized by the Federico laboratory in Italy. Nef7 is derived from the Nef allele of F12-HIV, a non-producing strain of HIV-1

that was cloned from Hut-78 cells previously infected with the supernatant of the peripheral blood lymphocytes (PBL) of an HIV seropositive patient (Carlini, Nicolini et al. 1996). In an attempt to discover the reasons behind the failure of F12-HIV to produce even aberrant HIV-1 particles they discovered that its *gag*, *vif*, and *nef* genes were all highly defective (D'Aloja, Olivetta et al. 1998). The F12-HIV*nef* allele contained three rare point mutations: <sup>E140</sup>G, <sup>V153</sup>L, and <sup>E177</sup>G, and blocked the replication of the highly productive NL4-3 HIV-1 strain at the level of virus assembly or release.

As part of an attempt to determine the importance of these mutations the Federico laboratory created a Nef mutant they termed Nef7 which retained two of the three point mutations: <sup>V153</sup>L, and <sup>E177</sup>G. Nef7 retained most of the properties of F12-HIV*nef* in that it was much less pathogenic than WT Nef. Nef7 was defective for many characteristic Nef functions, such as PAK2 activation, MHC I and CD4 down-regulation, and binding to the V1H regulatory subunit of the vacuolar ATPase (D'Aloja, Santarcangelo et al. 2001; Peretti, Schiavoni et al. 2005). It is important to note that Nef7 may be defective for other Nef properties, as these were the only phenotypes tested. Another property of Nef7 that distinguishes it from WT Nef is its incorporation into virions. Nef normally incorporates into progeny virions at the rate of <10 molecules per virion. Nef7, on the other hand, incorporates an estimate of up to 1100 Nef molecules per virion. This increased virion incorporation does not rely on HIV-specific components, as Nef7 also shows increased incorporation into murine leukemia virus (MLV) virions and virus-like particles (VLP). Nef7 incorporation into virions correlates well with its increased lipid raft localization (Peretti, Schiavoni et al. 2005). This likely explains why other viral

components are unnecessary; HIV and MLV both bud from lipid rafts, and the presence of increased levels of Nef7 in lipid rafts would allow more incorporation into virions.

One other important finding concerning Nef7 was that C-terminal fusion of proteins did not affect its virion incorporation. Both GFP and thymidine kinase (TK) C-terminal fusion proteins have been stably expressed, and their virion incorporation was not significantly affected (Peretti, Schiavoni et al. 2006). Because of this, and because of its less pathogenic properties, Nef7 shows great promise as a carrier protein for therapeutics that target components of the HIV virion or lipid rafts. Testing this potential is the focus of part of this thesis.

## **5. TRANSIENT T CELL INTERACTIONS**

### **5.1 Immunological synapses**

#### *5.1.1 Structure and function of immunological synapses*

The term “immunological synapse” (IS) refers to close cell-cell contacts between an antigen-presenting cell (APC) and T-lymphocytes that typically lead to the activation of the lymphocyte. Initial engagement of the T-cell receptor (TCR) on the lymphocyte with peptide-loaded MHC II molecules on the APC leads to rapid reorganization of plasma membrane lipids, surface receptors, and components associated with the inner leaflet of the plasma membrane. IS components are recruited to membrane microdomains, either from dispersed regions of the plasma membrane or from intracellular compartments, while TCR-inhibitory components are excluded (Huppa and Davis 2003; Friedl, den Boer et al. 2005; Saito and Yokosuka 2006). Immunological synapses form a structure that

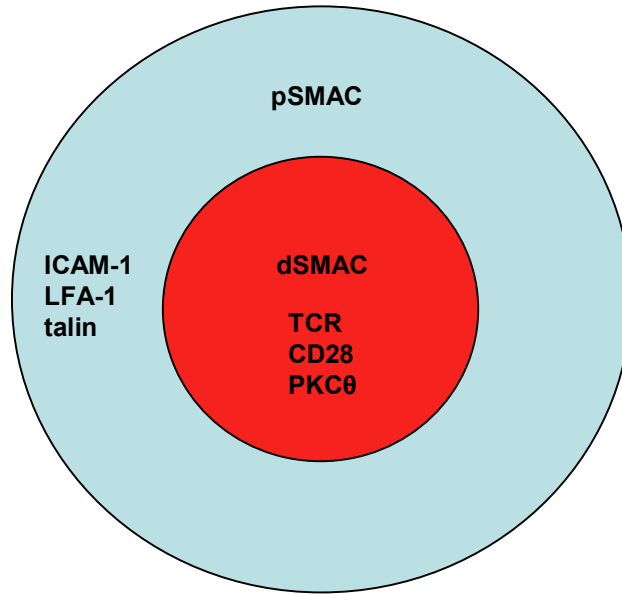
can be visualized microscopically, and generally resembles a bulls eye (Fig. 6). The central ring, or central supramolecular activation complex (cSMAC), consists of the TCR/CD3, CD28, and PKC $\theta$ . Surrounding the center is the peripheral supramolecular activation complex (pSMAC), which consists mainly of adhesion molecules such as ICAM-1 and LFA-1 that help to stabilize the interaction between the T-cell and APC (Monks, Freiberg et al. 1998; Grakoui, Bromley et al. 1999).

The formation of an IS allows for fast and potent mounting of a signaling response that includes a cascade of tyrosine phosphorylation, release of Ca<sup>2+</sup>, and activation of target genes, such as IL-2 (Bunnell, Hong et al. 2002; Barda-Saad, Braiman et al. 2005; Campi, Varma et al. 2005). Importantly, continuous activation of T cells via the TCR results in apoptotic cell death, creating a need for negative regulation of the IS. Consequently, following successful signal initiation the IS is down-modulated. This mainly occurs via the endocytosis of the TCR and associated proximal machinery, and results in the dissociation of IS components (von Essen, Bonefeld et al. 2004; Varma, Campi et al. 2006; Krammer, Arnold et al. 2007).

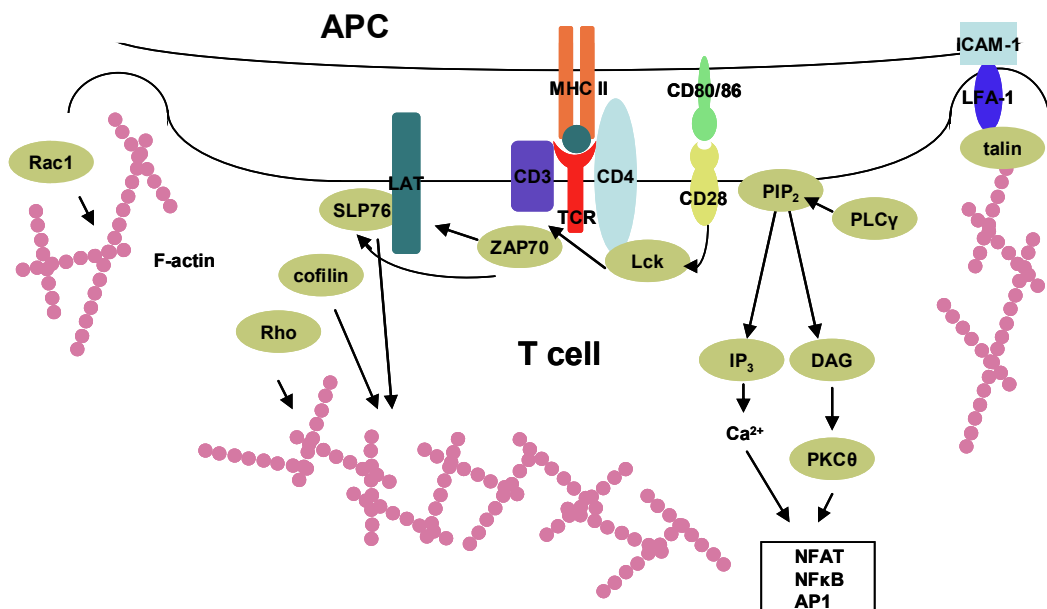
#### *5.1.2 Nef involvement in the immunological synapse*

HIV-1 replication in primary human CD4<sup>+</sup> T lymphocytes is strictly coupled to the activation state of these cells; while virus entry is unhampered in unactivated cells, subsequent steps of the viral life cycle are only supported during activation, as mentioned above (Stevenson 2003). However, while a minimal degree of activation is a prerequisite for efficient HIV-1 spread, full and sustained activation may shorten the life span

**A**



**B**



**Figure 6. Schematic of the immunological synapse.** The IS forms between an APC and a CD4<sup>+</sup> T cell. **A.** When viewed from above, the IS forms a bulls-eye structure with the cSMAC in the middle surrounded by the pSMAC. **B.** Components of the cSMAC, including TCR/CD3, CD28, and PKC $\theta$ , are recruited to the center of the IS in an actin-dependent manner. Adhesion molecules stabilize the connection utilizing a second actin-dependent pathway. Engagement of the TCR results in stimulation of the T cell, as shown by increased Ca<sup>2+</sup> levels and activation of transcription factors such as NFAT, NF $\kappa$ B, and AP1.

of an infected cell, thereby reducing the release of progeny viruses. Therefore, HIV-1 would ideally require a means to provide partial activation of T cells while preventing apoptosis due to excessive activation (Zhang, Schuler et al. 1999; Haase 2005).

Multiple effects of HIV-1 on IS organization and formation have been reported (Thoulouze, Sol-Foulon et al. 2006; Fackler, Alcover et al. 2007), many of which have been attributed to Nef. Firstly, Nef has profound effects on protein sorting at the IS. Once TCR/CD3 has been internalized following initial IS stimulation, Nef retains it in the recycling endosomes, thus preventing further activation. Nef also retains the proximal signaling kinase Lck in recycling endosomes, further preventing prolonged signaling through the IS (Haller, Rauch et al. 2007). Secondly, Nef interferes with maturation of the IS. Initial receptor engagement and signaling are unaffected in Nef-expressing cells, while later stages of IS signaling are inhibited. This is thought to occur by means of Nef preventing the translocation of the actin regulator N-Wasp to the cell periphery, thereby inhibiting cell spreading and late stage tyrosine phosphorylation (Haller, Rauch et al. 2006). In contrast to these negative effects on IS function, Nef enhances signaling cascades downstream of the IS (Fenard, Yonemoto et al. 2005). Late activation events, such as IL-2 production, NF-AT activation, and NF $\kappa$ B activation, are all enhanced in Nef-expressing cells resulting in increased T cell activation. In addition to inducing T cell activation, IL-2 is also pro-proliferative for T cells which could increase the number of target cells available for HIV-1 infection. This combination of Nef effects may confer an advantage to HIV-1 by allowing minimal stimulation while preventing apoptosis.

This process might also favor the development of quiescent, latently infected lymphocytes.

## **5.2 Virological synapses**

### *5.2.1 Types of virological synapses*

For decades cell-to-cell transmission of retroviruses has been intermittently described in the literature (Yamamoto, Okada et al. 1982; Popovic, Lange-Wantzin et al. 1983; Okochi, Sato et al. 1984; Sato, Orenstein et al. 1992; Johnson and Huber 2002). However, it was never fully explored until the description of an immunological synapse-like structure in HTLV-1 infected T lymphocytes that exhibited accumulation of the HTLV-1 core protein Gag and the HTLV-1 genome at the cell-cell junction (Igakura, Stinchcombe et al. 2003). The structure was termed a “virological synapse” due to its physical similarity to the immunological synapse and has since been described in HIV and murine leukemia virus (MLV) infections (Sherer, Lehmann et al. 2007). Much of what is known about cell-to-cell infection is inferred from microscopic image analysis; fluorescence microscopy shows accumulation of viral proteins at the cell-cell contact sites and electron microscopy shows virus particles localized between interacting cells (Igakura, Stinchcombe et al. 2003; Majorovits, Nejmeddine et al. 2008). In the case of HTLV-1, cytoskeletal reorganization at the VS is promoted by the viral protein Tax (Nejmeddine, Barnard et al. 2005; Nejmeddine, Negi et al. 2009); and utilizes the host scaffolding protein Dlg to stabilize contacts via interactions with the C-terminus of the cytoplasmic domain of HTLV-1 Gag (Blot, Delamarre et al. 2004).



In contrast with the strictly T cell-T cell VS used in HTLV-1 infections, HIV-1 can induce VS formation in a variety of cell types. Virological synapses have been reported between dendritic cells and T cells (McDonald, Wu et al. 2003) and macrophages and T cells (Carr, Hocking et al. 1999; Groot, Welsch et al. 2008) as well as between T cells alone. These differing cell types may or may not result in different requirement for VS formation and cell-cell transfer of virus.

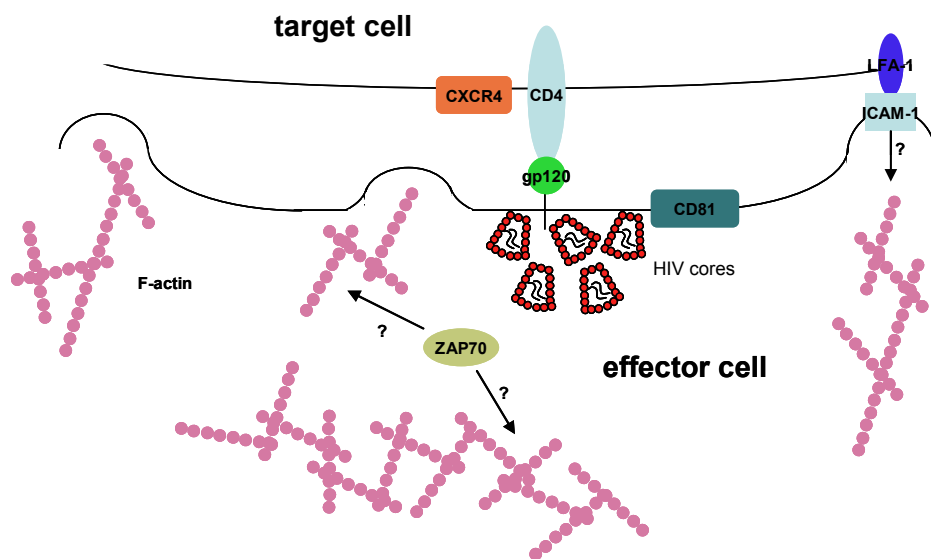
In addition to virological synapses, there exists a second means of direct cell-cell spread of HIV-1. These are more long-range, and are known as nanotubes or filopodial bridges (Sherer, Lehmann et al. 2007; Davis and Sowinski 2008; Sowinski, Jolly et al. 2008). It has been hypothesized that these protrusions are remnants of virological synapses, left after cell separation, or that they are the initial step in VS formation, establishing cell-cell connections that allow the infected and uninfected pair to come together more efficiently (Haller and Fackler 2008).

### *5.2.2 HIV transfer through T cell virological synapses*

Viral spread through virological synapses is now thought to be the main route of HIV-1 transmission, particularly in environments with close cellular contacts, such as peripheral lymph nodes and gut associated lymphoid tissue (GALT) (Phillips 1994; Haase 1999). Because viruses can pass directly between cells during VS formation instead of budding from infected cells and diffusing until a susceptible target cell is encountered, viral spread through VS is much quicker and more efficient than cell-free viral spread (Chen, Hubner et al. 2007; Sourisseau, Sol-Foulon et al. 2007). Indeed, one infected T cell can form

multiple synapses with target cells, thus ensuring an even faster rate of viral spread (Rudnicka, Feldmann et al. 2009). However, the HIV-1 VS has not been as well characterized as the HTLV-1 VS. Assembly of the VS requires engagement of the HIV Env surface subunit gp120 expressed on the effector cell, with its cellular receptors CD4 and CXCR4 on the target cell (Fais, Capobianchi et al. 1995; McDonald, Wu et al. 2003). Further recruitment of receptors and HIV-1 proteins to the conjugate interface is a cytoskeleton-dependent process in both target and effector T cells (Iyengar, Hildreth et al. 1998; Jolly, Mitar et al. 2007). Known components of the VS include leukocyte function-associated antigen 1 (LFA-1) and intercellular adhesion molecule 1 and 3 (ICAM-1/3) (Jolly, Mitar et al. 2007), along with ZAP-70 tyrosine kinase (Sol-Foulon, Sourisseau et al. 2007) (Fig. 7). Once the VS forms, HIV cores can be directly transferred to the target cell where they will undergo reverse transcription. Viral particles then specifically bud at the synapse and are taken up into target cells (Chen, Hubner et al. 2007; Bosch, Grigorov et al. 2008), which may be assisted by the formation of an F-actin-depleted zone in the target cell (Vasiliver-Shamis, Cho et al. 2009).

Microscopically, virological synapses can be identified by the clustering of VS components such as gp120, CD4, and p24 at the site of contact between an infected and an uninfected cell (Jolly, Kashefi et al. 2004; Jolly and Sattentau 2004). As compared to the immunological synapse, very little is known about the cellular or viral proteins that regulate the HIV-1 virological synapse. Aside from the requirement of gp120, nothing is known of the contribution of HIV-1 proteins to VS formation. Likewise, the only known cellular components of the VS are adhesion molecules, the HIV-1 receptors, and one



**Figure 7. Schematic of the known components of the HIV-1 virological synapse.**

The VS forms between an infected cell expressing gp120 and an uninfected target cell expressing CD4. Synapse components are recruited to the contact site in an actin-dependent manner. Adhesion molecules stabilize the connection, and provide some signaling to the actin pathways that promote VS formation. ZAP70 acts to increase the transfer of HIV virions to the target cell by an unknown mechanism, likely involving actin. Actin reorganization is required for recruitment of synapse components, viral transfer, and conjugate formation through adhesion molecules.

tyrosine kinase, ZAP70 (Sol-Foulon, Sourisseau et al. 2007). VS formation is dependent upon actin polymerization and requires lipid raft integrity in order to remain stabilized (Jolly and Sattentau 2005), but the regulators of both actin polymerization and lipid raft clustering are unknown. The mechanisms behind HIV-1 VS formation and viral transfer are important aspects of the HIV-1 life cycle. Furthermore, understanding of these mechanisms is useful in the development of new anti-HIV therapies particularly given the importance of VS-mediated HIV-1 transfer *in vivo*.

## **6. LIPID RAFTS**

### **6.1 Composition and function**

Lipid rafts are small detergent-resistant domains of the cell membrane, rich in sphingolipids and cholesterol in the outer membrane leaflet, connected to largely saturated phospholipids and cholesterol in the inner membrane leaflet. These assemblies are fluid, but more tightly packed and ordered than the surrounding bilayer due to the presence of a greater amount of saturated phospholipids as compared to the unsaturated lipids found in non-raft membranes (Brown and London 1998). Rafts are mainly present in apical plasma membrane domains, but are also present in lower concentrations in the basolateral plasma membrane and on Golgi and *trans*-Golgi membranes (Simons and Ikonen 1997; Brown and London 1998; Brown and London 2000). They play an essential role in signal transduction and cell activation. Many signaling proteins such as MHC I and II, CD3, and annexin localize to lipid rafts; these proteins are usually isolated in small, dynamic rafts. Upon stimulation, the small dynamic rafts cluster into larger platforms with sustained signal transduction (Harder, Scheiffele et al. 1998; Janes, Ley et

al. 1999). Aggregation of raft-associated proteins, either initiated by binding of ligands or antibody-mediated cross-linking, can recruit new proteins to raft regions to create a signaling complex; for example, the immunological synapse (Dykstra, Cherukuri et al. 2003; Gupta and DeFranco 2003; Horejsi 2003).

## **6.2 Lipid rafts during HIV infection**

HIV-1 utilizes lipid rafts for nearly all steps of its life cycle. Immediately following exposure the virus must pass through mucosal tissue in order to reach its cellular target; this epithelial transcytosis is dependent upon the formation of lipid rafts in the epithelial cells (Alfsen, Iniguez et al. 2001). Likewise, entry into permissive target CD4<sup>+</sup> T cells or macrophages requires clustering of lipid rafts, as the HIV-1 receptors localize to rafts and binding can be partially affected by the removal of cholesterol and thus destabilization of lipid rafts (Fantini, Hammache et al. 2000; Fantini, Maresca et al. 2000; Hammache, Pieroni et al. 2000; Manes, del Real et al. 2000). Finally, virus assembly and budding take place from lipid rafts (Nguyen and Hildreth 2000). This selective budding allows both the specific exclusion of host proteins that could negatively affect virus propagation or survival, and the inclusion of specific host membrane-associated proteins and lipids into the virion that are able to deregulate cellular and humoral immune responses (Frank, Stoiber et al. 1996; Cantin, Fortin et al. 1997; Peterlin and Trono 2003; Wilfingseder, Spruth et al. 2003).

The final, and extremely important, function of lipid rafts in the HIV-1 life cycle involves alteration of signaling mechanisms in the host cell for efficient replication and immune

evasion. The virus starts hijacking cellular signaling machinery immediately upon binding, when gp120 interaction with CD4-p56lck complexes in lipid rafts to activate a variety of signaling pathways, including PKC, Ras, ERK1/2, and MAPKs (Tamma, Chirmule et al. 1997; Briant, Robert-Hebmann et al. 1998). However, Nef exhibits by far the most interactions with raft-associated proteins. The highly conserved PxxP sequence binds to the SH3 domain of a variety of raft-associated molecules, including Src family kinases, PKC, PAK2, and Vav (Renkema and Saksela 2000). Furthermore, Nef can induce the activation of these molecules even in the absence of stimulation (Schrager, Der Minassian et al. 2002). However, it has been shown that upon T cell activation, 5-10% of the Nef cellular pool localizes to lipid rafts (Fenard, Yonemoto et al. 2005). Nef localization to lipid rafts is likely a key aspect of many of its functions. For instance, activation of PAK2 by Nef requires its localization to lipid rafts, where it interacts with Vav to activate PAK2 upstream regulators Cdc42 and Rac (Lu, Wu et al. 1996; Fackler, Luo et al. 1999; Rauch, Pulkkinen et al. 2008). Additionally, in Nef-expressing CD4<sup>+</sup> T cells UbcH7, an E2 ubiquitin-conjugating enzyme that acts as a negative regulator of signaling, is absent from lipid rafts. Exclusion of UbcH7 from rafts would result in accumulation of tyrosine-phosphorylated Vav and a subsequent increase in Cdc42 activity (Miura-Shimura, Duan et al. 2003; Simmons, Gangadharan et al. 2005). In addition to decreasing negative regulators of signaling, Nef can also increase the levels of signaling in lipid rafts by increasing the amount of signaling molecules within rafts as well as promoting the fusion of lipid rafts into the larger rafts required for sustained signaling (Djordjevic, Schibeci et al. 2004).

## **Summary of the background and our hypothesis**

Despite the overall success of HAART in reducing viral replication and prolonging the lifespan of HIV-infected individuals, multi-drug resistance, high cost, and toxicity remain a major concern. Therefore, the development of novel therapeutic strategies is a priority. Moreover, with the realization of the importance of virological synapses to the spread of HIV-1, the development of new therapies should take into account the differing requirements of cell-free and cell-cell infections.

The first part of this work addressed the search for novel anti-HIV therapies.

Collectively, the unique properties of Nef7 offer it as a potentially novel and non-toxic carrier platform to deliver therapeutic proteins into HIV-1 virions, inactivate virions, and subsequently block HIV-1 replication. Thus, we hypothesized that the high virion incorporation of Nef7 in the context of the Nef7.A3G fusion protein would override Vif-targeted A3G degradation and as a result, restore the anti-HIV phenotype of A3G.

The second part of this work addressed the formation of the HIV-1 virological synapse.

In order for new potential therapies to take into account the differing requirements of cell-free and cell-mediated HIV-1 infection, the mechanisms of virological synapse formation must be fully elucidated. Therefore, the involvement of HIV-1 Nef protein in VS formation was examined. Nef interacts with a vast array of cellular factors, resulting in the alteration of many functions in HIV-infected cells; in the context of the HIV-1 VS, we were especially interested in those involving cell signaling via lipid rafts and actin dynamics, due to the importance of these pathways in VS formation. We therefore



hypothesized that Nef could promote the formation of HIV-1 virological synapses. Since virological synapse-mediated viral spread is the preferred mode of transmission in primary cells, we also hypothesized that any enhancement of the virological synapse by Nef may explain the increased replication kinetics observed in primary cells in the presence of Nef.

## MATERIALS AND METHODS

### **MATERIALS**

#### **Media and supplements**

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI-1640) medium, and Hank's balanced salt solution (HBSS) were purchased from Lonza (Walkersville, MD). Penicillin-streptomycin-glutamine and 0.25% trypsin were purchased from Gibco (Grand Island, NY). Fetal bovine serum was purchased from Hyclone (Logan, UT). Ampicillin sodium salt and kanamycin sulfate were purchased from United States Biological (Swampscott, MD). The bacterial culture media described below were prepared in house, with materials purchased from Becton Dickenson (Sparks, MA). Luria broth (LB) media contained 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, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose. Working concentrations of antibiotics were 100 µg/ml ampicillin and 50 µg/ml kanamycin.

#### **Antibodies**

Mouse anti-JR-CSF Nef antibody (WB 1:1000, donated by Dr. K. Krohn and Dr. V. Ovod), Sim4 anti-CD4 antibody (IF 1:4, FC undil.), and p24 hybridoma supernatant (WB 1:3) were obtained from the National Institute of Health (NIH) AIDS Reference Reagent Program. W6/32 anti-MHC I hybridoma supernatant (FC undil.) was a gift from Dr. J.

Blum. Mouse anti-PAK2 (WB 1:1000), rabbit anti-Myc (WB 1:2000), mouse anti-HA (WB 1:2000), goat anti-Nef 4D10 (WB 1:2000, FC 1:100, IF 1:50), mouse anti-ZAP70 (WB 1:1000, IF 1:40), mouse anti-CD81 (WB 1:1000, IF 1:40), phycoerythrin (PE)-conjugated rabbit anti-mouse (1:50), and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (1:50) antibodies were obtained from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). KC57 PE-conjugated anti-p24 (IF 1:80, FC 1:40) antibody was obtained from Coulter (Brea, CA). PE- and horseradish peroxidase (HRP)-conjugated Cholera toxin subunit B (WB 1:5000), normal mouse IgG, and mouse anti- $\beta$ -actin (WB 1:2000) were from Sigma (St. Louis, MO). Anti-mouse-HRP and anti-rabbit-HRP (1:3000) were obtained from GE Healthcare (Buckinghamshire, UK). PE-conjugated phalloidin (IF 1:100), AlexaFluor 350 donkey anti-goat (1:50), AlexaFluor 488 donkey anti-goat (1:50), and AlexaFluor 488 goat anti-mouse (1:50) were from Invitrogen (Carlsbad, CA).

### **Reagents**

ECL chemiluminescence reagents for Western blot detection were made in house. Poly(A)<sub>x</sub>(dT) and protease inhibitor cocktail set V were purchased from Roche (Indianapolis, IN). N-acetyl T-20 was obtained from the National Institute of Health (NIH) AIDS Reference Reagent Program. G418 sulfate and hygromycin were purchased from Calbiochem (LaJolla, CA). The Lipofectamine 2000 system was purchased from Invitrogen (Carlsbad, CA). T4 DNA ligase, calf intestinal phosphatase (CIP), and all restriction endonucleases were from New England Biolabs (Beverly, MA). [Methyl-<sup>3</sup>H]-thymidine 5'-triphosphate and [ $\gamma$ -<sup>32</sup>P] ATP were from PerkinElmer (Boston, MA). Ficoll

was from Amersham Biosciences (Piscataway, NJ). Phytohemagglutinin (PHA), interleukin-2 (IL-2), poly-L-lysine, cytochalasin D, piceatannol, methyl- $\beta$ -cyclodextran, and 4',6'-diamidino-2-phenylindol (DAPI) were purchased from Sigma (St. Louis, MO). All other chemicals were from Fisher (LaGrange, KY).

### **Biotechnology systems**

The Expand High Fidelity PCR system was purchased from Roche (Indianapolis, IN). The QuickChange II Site-directed Mutagenesis kit and Strataclone PCR cloning kit were from Stratagene (Cedar Creek, TX). The firefly luciferase assay system and the Wizard SV Gel and PCR Clean-up kit were from Promega. The Bio-Rad DC Protein Assay kit was from Biorad Laboratories (Hercules, CA).

## **METHODS**

### **Cells and cell cultures**

#### *Cell lines*

Human embryonic kidney 293T cells, cervical carcinoma HeLa cells, and Jurkat lymphocytic cells were purchased from American Tissue Culture Collection (ATCC) (Manassas, VA). U87.CD4.CXCR4 and U87.CD4.CCR5 cells (donated by Dr. D. Littman) were obtained from the National Institute of Health (NIH) AIDS Reference Reagent Program. Stable cell lines were constructed by Dr. IW Park and have been previously described (Park and He 2009).

### *Competent cells for cloning*

GC5™ chemically competent *E. coli* were purchased from GeneChoice (Frederick, MD).

### *Cell cultures*

293T, U87.CD4.CXCR4, U87.CD4.CCR5, and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and streptomycin, and 2 mM glutamine at 37°C with 5% CO<sub>2</sub>. Jurkat cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and streptomycin, and 2 mM glutamine at 37°C with 5% CO<sub>2</sub>.

### *Isolation and culture of peripheral blood mononuclear cells (PBMC)*

Peripheral blood (PB) was obtained from the Indiana Blood Center (Indianapolis, IN). PBMCs were isolated on a Ficoll-Plaque density gradient by centrifugation. Briefly, buffy coats were diluted 1:2 in PBS and layered over 15 ml Ficoll in a 50 ml conical tube. The tubes were then centrifuged at 785 x g with no brake. 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. Cells were allowed to adhere to tissue culture dishes overnight, and non-adherent lymphocytes were collected. PBMCs were seeded at a density of 2.5 x 10<sup>6</sup> cells/ml in RPMI supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and streptomycin, and 2 mM glutamine, and stimulated with 3 µg/ml PHA for 48 hr followed by continued culture in RPMI containing 100 U/ml IL-2.

## **Plasmids**

### *Reporter viruses*

HIV-Luc plasmid was described elsewhere (He, Chen et al. 1997). HIV-Luc.*vif*- was constructed by first subcloning an EcoR1/Spe1 fragment of HIV-Luc containing the *vif* gene into pBluescript vector, followed by site-directed mutagenesis to introduce a stop codon in *vif* using the primer pairs 5'-GTA AAA CAC CAT TAG TAT ATT TCA AGG AAA GC-3' and 5'-GC TTT CCT TGA AAT ATA CTA ATG GTG TTT TAC-3'. The mutated *vif*- portion was then placed back in HIV-Luc to create HIV-Luc.*vif*-.

### *Nef mutants*

pNef.myc was constructed by first obtaining the *nef* gene from HIV-1 NL4-3 by PCR using the following primer pairs 5'-CCC AAG CTT ATG GGT GGC AAG TGG TCA-3' and 5'-CCG GGA TTC TCA AGC GTA ATC TGG AAC ATC GTA TGG GTA GCA GTT CTT GAA GTA CTC-3'. The resulting PCR product was digested with EcoRI/BspEI restriction enzymes. A YU2.*nef* plasmid was meanwhile digested with HindIII/BspEI and pcR3.1 with EcoRI/HindIII. The resulting three fragments were isolated and ligated to result in an NL4-3*nef* construct. *nef* point mutations (pNef153.myc, pNef177.myc, and Nef7.myc) were constructed in the context of pNef.Myc with primer pairs 5'-CCC AAG CTT ATG GGT GGC AAG TGG TCA-3' and 5'-CCG GAA TTC TCA AGA ACT TCA TGA GGC-3'; 5'-CCC AAG CTT CTT CTT CTC CGG TTA TTT CCT CTC TTG TGG-3' and 5'-CCC AAG CTT TAG ACC GAG TTG ACC ATG ATC GAA CAT-3'; 5'-C CTG CAT GGA ATG GAT GAC CCG GGG AGA GAA GTG TTA GAG TGG AG-3' and 5'-CT CCA CTC TAA CAC TTC

TCT CCC CGG GTC ATC CAT TCC ATG CAG G-3', respectively. For pNef.HA and pNef7.HA plasmids, the Myc tag in pNef.Myc and pNef7.Myc was replaced with primers 5'-CCC TTA CCA TAT GAT GTT CCA GAT TAC GCT TGA AGC CGA ATT CTG CAG ATA-3' and 5'-GG AAT TCC ATA TGG GTA CTC TGC GTT CTT GTA GTA CTC-3'. pNef.GFP and pNef7.GFP plasmids were constructed in the context of the pEGFP.N3 backbone (Clontech, Mountain View, CA) using pNef.Myc and pNef7.Myc as respective templates and primers 5'-CCG GAA TTC ATG GGT GGC AAG TGG TCA-3' and 5'-CCG ACT AGT GCA GTT CTT GAA GTA CTC-3'. pNef7.A3G fusion vector was constructed by first mutating the stop codon of pNef.Myc with oligonucleotides 5'-CCG GAA TTC GTT CTT GTA GTA CTC CGG ATG-3' and 5'-CAT CCG GAG TAC TAC AAG AAC GAA TTC CGG-3' introducing an EcoRI site, and then cloning the APOBEC3G ORF at the EcoRI site of Nef. pNef7.A3G/D128K (D128K) and pNef7.A3G/E259Q (E259Q) plasmids were constructed in the context of pNef7.A3G with primer pairs 5'-CTC TAC TAC TTC TGG AAG CCA GAT TAC CAG GAG GCG-3' and 5'-CGC CTC CTG GTA ATC TGG CTT CCA GAA GTA GTA GAG-3'; and 5'-GT TTC TTG AAG GCC GCC ATG CAC AGC TGT GCT TCC TG-3' and 5'-CA GGA AGC ACA GCT GTG CAT GGC GGC CTT CAA GAA AC-3', respectively.

### *HIV proviruses*

HIV.*env*<sup>vif</sup> plasmid has been previously described (Lewis, Hensel et al. 1992). pNL4-3 was obtained from NIH AIDS Reagent Program. HIV.*env*.*nef* plasmid was generously provided by Dr. M. Emmerman. HIV $\Delta$ Nef, HIV.*nef*-F191A, HIV.*nef*-AxxA, and

HIV.nef-62AAAA65 were constructed by first cloning an XhoI/NaeI NL4-3 Nef fragment into the pBlueScript KS+ vector, followed by site-directed mutagenesis using the Stratagene kit and the following primers. F191A: 5'-TTT GAC AGC CGC CTA GCA GCT CAT CAC GTG G-3' and 5'-CCA CGT GAT GAG CTG CTA GGC GGC TGT CAA A'3'; AxxA: 5'-TTT TCC AGT CAC AGC CCA GGT AGC TTT AAG ACC AAT GAC TTA CAA GG-3' and 5'-CCT TGT AAG TCA TTG GTC TTA AAG CTA CCT GGG CTG TGA CTG GAA AA-3'; 62AAAA65: CTT GTG CCT GGC TAG AAG CAC AAG CGG CGG CAG CGG TGG GTT TTC CAG TC-3' and 5'-GAC TGG AAA ACC CAC CGC TGC CGC CGC TTG TGC TTC TAG CCA GGC ACA AG-3'; nef-: 5'-TCT CGA GAC CTA TGA AAA CAT GGA GCA ATC ACA AG -3' and 5'-CT TGT GAT TGC TCC ATG TTT TCA TAG GTC TCG AGA-3'. The mutated Nef fragments were then cloned back to pNL4-3 using XhoI/NaeI.

#### *Other plasmids*

pcDNA3.1-APOBEC3G-HA and psPAX2 plasmids were obtained from NIH AIDS Research Reagent Program and were donated by Dr. W. C. Greene and Dr. D. Trono, respectively. pHLA-A2 plasmid was a kind gift from Dr. C. Toloukian. VSV-G, HXB2.env, YU2.env, pc.CXCR4, and pc.CD4 plasmids were described elsewhere (He, Chen et al. 1997).

#### **Bacterial transformation**

GC5™ cells were mixed with 0.5-1 µl DNA ligation reaction, and incubated on ice 30 min. The cells were heat-shocked at 42°C for 45 sec, followed by incubation on ice another 2 min, and addition of 250 µl of RT SOC medium. The cells were then incubated



at 37°C for 1 hr with shaking of 240 RPM, and plated on LB plates containing the appropriate antibiotics.

### **Cell transfections**

293T cells were transfected by the standard calcium phosphate precipitation method.

HeLa cells were transfected using the Lipofectamine 2000 system (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. pcDNA3 was used throughout the studies to normalize the amounts of DNA for all transfections.

### **Preparation of viruses and virus-like particles (VLP)**

Replication-competent HIV-1 and HIV viruses pseudotyped with different envelope proteins were prepared as previously described (He, Chen et al. 1997). Briefly, 293T cells were transfected with proviral plasmids; HIV-Luc or HIV-luc.*vif*<sup>-</sup>; VSV-G, HXB2.env, or YU2.env; and Nef or each of Nef derivatives. For inverse fusion VLP, 293T cells were transfected with psPAX2 packaging vector (Dull, Zufferey et al. 1998), pc.CXCR4, pc.CD4, and Nef, Nef7, Nef7.A3G, or A3G. Cell culture supernatants were collected 72 hr after transfection, filtered, and saved as progeny viruses. Progeny viruses were then assayed for RT activity (He, Chen et al. 1997). Briefly, filtered supernatant was centrifuged at 4°C for 1 hr at 14,000 RPM to pellet the progeny virus. The virus pellet was resuspended in 10 µl of dissociation buffer (0.25% Triton-X-100, 20% glycerol, 0.05 M Tris-HCl pH 7.5, 1 mM DTT, and 0.25 M KCl) and subjected to three freeze/thaw cycles. RT assay buffer (0.083 M Tris-HCl pH 7.5, 0.008 M DTT, 0.0125 M MgCl<sub>2</sub>, and 0.083% Triton-X-100), 1 µl [<sup>3</sup>H]-dTTP, and 5 µl of 5 U/ml poly (A)x(dT)

was added to the dissociated virus and incubated at 37°C for 1 hr. The reaction was then spotted onto DE81 filters (Whatman, England), washed three times with 2X SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7.0), and the radioactivity on the filter was determined in a Beckman LS6000IS scintillation counter. Virus titer was expressed as cpm/ml, and normalized for the number of cells in cultures.

### **Luciferase assays**

Firefly luciferase activity was measured using the luciferase assay system from Promega according to the manufacturer's directions. Briefly, cells were transduced with the appropriate amount of pseudotyped HIV-Luc virus at 37°C for 2 hr then washed and cultured for an additional 48 hr in fresh medium. In order to acquire approximately similar readings, the amount of VSV-G-pseudotyped viruses used was only one tenth that of YU2.env and HXB2.env-pseudotyped viruses. Cells were washed with ice-cold PBS and counted, and equal numbers of cells were lysed in 1X firefly luciferase lysis buffer. Lysates were centrifuged briefly to pellet debris, 20 µl of supernatant was mixed with firefly luciferase substrate, and luciferase activity was determined using an Opticom Luminometer (MGM Instruments, Hamden, CT).

### **Virus replication assays**

#### *VLP treatment*

VLP pseudotyped with CD4 and CXCR4 and containing Nef, Nef7, Nef7.A3G, or A3G were produced as described above. Jurkat cells were transduced with replication-competent HIV-1 HXB2 viruses of an RT activity of 10,000 cpm at 37°C for 3 hr, and

washed to remove the remaining viruses. Infected cells were then transduced twice with appropriate VLP; 50,000 cpm following HIV-1 infection and another 50,000 cpm the next day. One third volume of cell culture supernatants was collected every other day and assayed for the RT activity, with fresh medium was added to the cultures to maintain culture volume. The RT activity was determined as described and normalized to the cell counts.

#### *Virological synapse replication kinetics*

Jurkat cells were infected with replication-competent WT HIV or HIV $\Delta$ Nef and allowed to reach close to 100% infection as determined by intracellular p24 staining. Infected cells were then mixed with uninfected cells in a 1:3 ratio, with  $1 \times 10^6$  cells/ml in 24-well tissue culture plates. Infections were cultured with or without 100  $\mu$ g/ml T-20 and monitored for the RT activity daily as described.

#### **Immunoblotting**

##### *Whole cell lysates*

Cells were washed twice with ice-cold PBS. Cells were collected and lysed in RIPA buffer (10 mM NaHPO<sub>4</sub>, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% sodium azide, 0.004% sodium fluoride, 1 mM sodium orthovanadate in PBS) and 1x protease inhibitor cocktail for 30 min on ice, and cell lysates were obtained by centrifugation to remove cell debris. Protein concentration was determined using a Bio-Rad DC protein assay kit. Equal amounts of protein were separated by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and

transferred to Hybond ECL membranes (Amersham). The membranes were blocked for 1 hr in 5% milk or 3% BSA in TBST buffer, and then probed with specific primary antibodies and appropriate HRP-conjugated secondary antibodies. Chemiluminescence visualization was performed using a homemade ECL system. Relative levels of protein were determined by densitometric scanning of the blots and normalized using appropriate loading controls.

#### *Membrane and cytosolic fractionation*

Cells were washed twice with ice-cold PBS, then lysed in hypotonic buffer (10 mM Tris-HCl pH 8.0, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, protease inhibitors) for 20 min on ice. Cells were homogenized by passing through a 27-gauge needle 20 times, and then pelleted at 1500 x g for 10 min to pellet nuclei. The supernatant was then centrifuged at 14,000 rpm for an additional 45 min. This supernatant was saved as the cytosolic fraction, while the pellet was resuspended in RIPA buffer and saved as the membrane fraction.

#### *Viruses*

Viruses with an equal level of RT activity were pelleted by centrifugation at 14,000 RPM for 1 hr, then lysed in RIPA buffer and separated on a 12% SDS-PAGE gel. Relative levels of protein incorporation into virions were determined by densitometric scanning of the blots and calculated using HIV-1 p24 as an internal standard.

### *Immunoprecipitation and in vitro kinase assays (IVKA)*

For immunoprecipitation/Western blot analysis, cells were lysed in whole cell extraction buffer (WEB) (50 mM Tris-HCl pH8.0, 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1X protease inhibitor, 1mM sodium orthovanadate, 160 mM NaF, 10 mM pyrophosphate). Lysates containing equal amounts of protein were pre-cleared by incubation at 4°C for 30 min with ProteinA-agarose beads. Pre-cleared lysates were then incubated with appropriate antibodies, using normal mouse or rabbit IgG as a control, at 4°C for 16 hr with constant rocking. ProteinA-agarose beads were added, and incubated for an additional 2 hr. Beads were pelleted and washed twice with IP washing buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 0.25% NP-40, 4 mM NaF, 1 mM sodium orthovanadate, 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol) and resuspended in 25 µl SDS-PAGE loading buffer, followed by SDS-PAGE and Western blotting as described. For in vitro kinase assays, the immunoprecipitates were suspended in 30 µl kinase assay buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 5 mM EDTA, 10 mM MgCl<sub>2</sub>, 0.02% Triton X-100) and 10 µCi [ $\gamma$ -<sup>32</sup>P]-ATP (3000 Ci/mmol) was added to the reaction. After incubation at room temperature for 5 min, the reaction was separated by SDS-PAGE and the gel was dried for 2 hr using a vacuum gel drier (Pharacia Biotech) and exposed to X-ray film.

### **Raft floatation assays**

Transfected 293T cells or stable GFP- and Nef-GFP-expressing Jurkat cells were cultured at the same density for 48 hr, with some samples being treated with 8 mM methyl- $\beta$ -cyclodextran for 1 hr before harvesting. Cells were then incubated on ice for 30 min,

washed, and lysed in lipid raft lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, and 1X protease inhibitors) on ice for 60 min. Lysates were adjusted to 40% sucrose, and loaded into 5.2 ml ultracentrifuge tubes, then carefully layered with 2.2 ml 30% sucrose in PBS and 2.2 ml 5% sucrose in PBS. The gradients were then centrifuged at 200,000 x g, 4°C for 18 hr with no brake. Ten 0.5 ml aliquots were taken from the top and applied to Hybond ECL membranes via a slot blot manifold (Schleicher and Schuell, Keene, NH), then analyzed using the appropriate antibodies as described.

### **Oil Red O (ORO) staining**

Oil Red O stock solution was made by dissolving 0.5 g powder in 100 ml isopropanol. ORO stock solution was diluted 3:2 in dH<sub>2</sub>O and incubated at RT for 10 min, then filtered to remove precipitates. Diluted ORO was added to cells and stained for 15 min at RT, then cells were rinsed three times with 60% isopropanol and once with PBS.

### **Immunofluorescence staining**

#### *Stable cell lines*

Coverslips were coated with 0.1% poly-L-lysine for 5 min, then washed and dried for at least 2 hr. Stable GFP- or Nef mutant-GFP expressing Jurkat cells were seeded onto coated coverslips at a density of  $1 \times 10^6$  in 200  $\mu$ l RPMI, and incubated at 37°C for 1.5 hr. The cells were then fixed in 4% paraformaldehyde (PFA) at RT for 15 min, permeabilized in 0.1% Triton-X-100 at 4°C for 20 min and blocked in 5% FBS/1% BSA for 30 min. Cells were then stained for CD81 with anti-mouse-PE secondary antibody,

CD3 with anti-mouse PE secondary antibody, GM1-PE, or F-actin (phalloidin-PE), with extensive washing with HBSS between each step. A set of cells stained for GM1 were treated with 8 mM methyl- $\beta$ -cyclodextran for 1 hr before fixation as a negative control, and a set of cells stained for F-actin were treated with 30  $\mu$ g/ml piceatannol for 2 hr prior to staining. Approximately 100-150 cells were examined for each condition.

#### *Virological synapse formation and Nef transfer*

For quantification of VS formation, uninfected target Jurkat cells were stained with 2.5  $\mu$ M CFSE at 37°C for 10 min then mixed with infected cells in a 1:1 ratio and loaded onto poly-L-lysine-coated coverslips at a density of  $1 \times 10^6$  cells in 200  $\mu$ l RPMI.

Synapses were allowed to form for 1.5 hr, and then cells were fixed, permeablized, and stained as described above using p24-PE, CD4 with anti-mouse-PE secondary antibody, gp120 with anti-goat Alexa 350, ZAP70 with anti-mouse-FITC, or pTyr with anti-mouse-FITC. For Nef transfer staining, cells were fixed and permeablized as described and stained for Nef, then stained with 10  $\mu$ M DAPI at RT for 25 min. Confocal microscopy analysis was carried out on a Zeiss LM510, with red and green fluorescence acquired sequentially. Images were further processed to adjust brightness and contrast in Microsoft PowerPoint.

#### **Flow cytometry analysis**

##### *MHC I and CD4 downregulation*

Transfected HeLa cells were washed with cold PBS and detached from the tissue culture plate using 0.5 mM EDTA in PBS. Cells were then suspended in Sim4 (CD4) or W6/32

(MHC I) supernatant and incubated at 4°C for 1 hr, then PE-conjugated rabbit anti-mouse IgG for an additional 1 hr in the dark. Cells were washed three times with cold HBSS (1x Hank's Salt solution, 4 mM NaCHO<sub>3</sub>, 1% BSA and 0.02% NaN<sub>3</sub>) between all steps. The cells were then fixed in 1% paraformaldehyde at 4°C for 4 hr, then washed with PBS and analyzed for MHC I or CD4 expression using a FACScalibur II (Becton Dickinson) with CellQuest software.

#### *Analysis of Nef-GFP intracellular delivery*

To determine delivery of virion-incorporated Nef-GFP fusion proteins, VSV-G-pseudotyped HIV.*env*.*nef* virions containing Nef-GFP or Nef7-GFP were prepared as described above. The viruses were used to infect 293T cells via spinoculation as described (Peretti, Schiavoni et al. 2005). Briefly, 50,000 cpm of virus was allowed to adsorb onto cells by centrifugation at 150 x g at room temperature for 1 hr, followed by incubation at 37°C for 3 hr. Cells were then trypsinized and washed to remove cell surface-bound viruses and fixed, then analyzed for GFP-positive cells using a FACScalibur II.

#### *GM1/CD81 polarization*

Polarization of GFP- or Nef-transfected 293T cells was determined with or without treatment with 1 µM cytochalasin D for 1 hr and removed from the tissue culture plate using 0.5 mM EDTA in PBS. Polarization of stable Jurkat cell lines was determined with or without overnight treatment with 1 µM cytochalasin D or 30 µg/ml piceatannol. Cells were fixed and stained for GM1 or CD81 as described above, and analyzed for surface



intensity as a measurement of polarization using a FACScalibur II. For negative controls of GM1 distribution, cells were treated with 8 mM methyl- $\beta$ -cyclodextran for 1 hr prior to harvesting.

### *Gag transfer*

For Gag transfer analysis CFSE-labeled target Jurkat cells were incubated with infected cells in a 1:1 ratio in 96-well tissue culture plates, at a density of  $1 \times 10^6$  cells per 200  $\mu$ l RPMI. All samples contained 100  $\mu$ g/ml T-20, and 1  $\mu$ M cytochalasin D or 30  $\mu$ g/ml piceatannol were added to select samples. At the indicated timepoints cells were fixed, permeablized, and stained for p24 as described above. Flow cytometry samples were analyzed for the percent of Gag<sup>+</sup> target cells using a FacsCaliburII.

### **Data acquisition and statistical analysis**

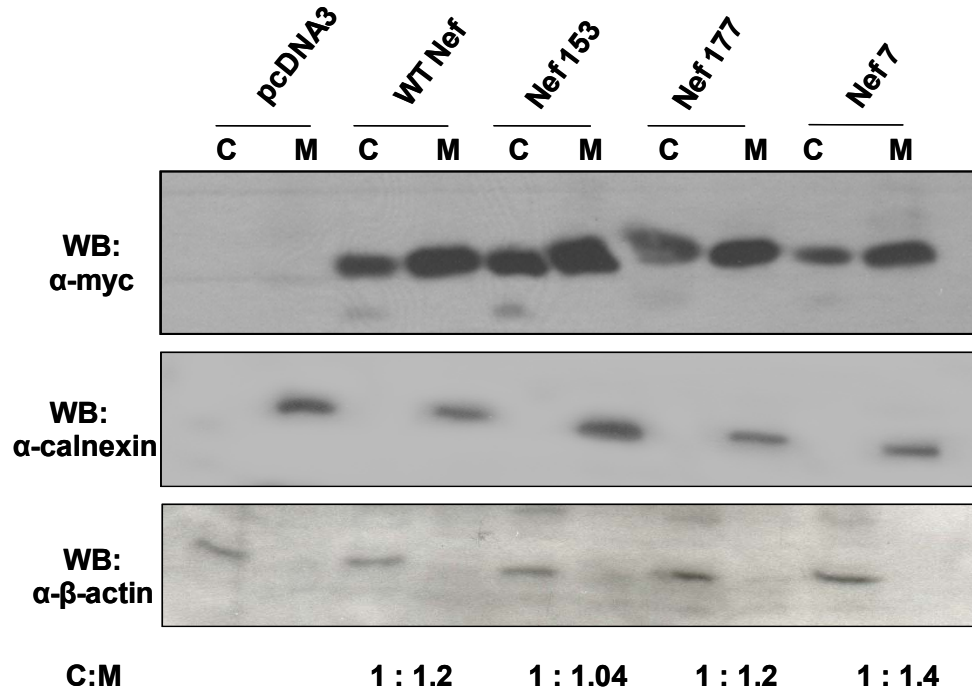
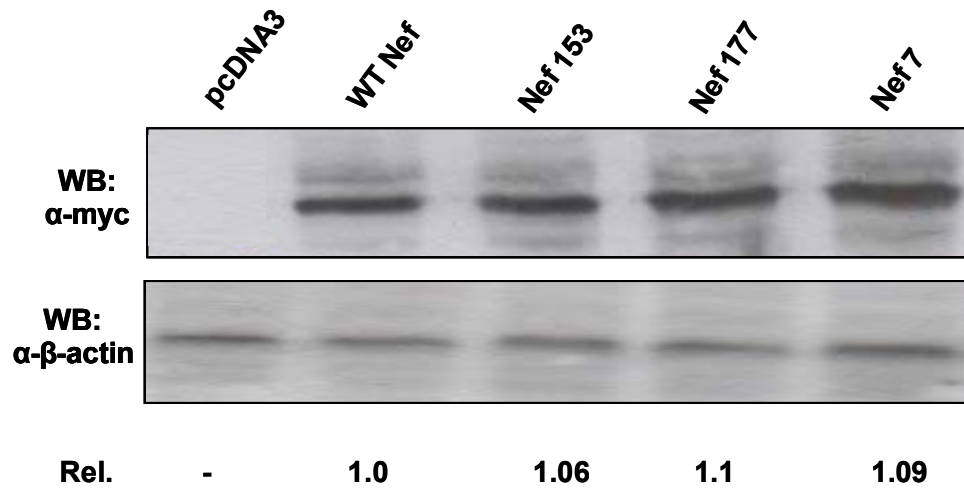
All values are expressed as mean  $\pm$  s.d. All comparisons were made using two-tailed Student's *t*-test. A *p* value of  $< 0.05$  was considered statistically significant (\*), and *p*  $< 0.01$  highly significant (\*\*).

## RESULTS

### **PART 1: NOVEL ANTI-HIV THERAPIES USING A NEF-MUTANT FUSION PROTEIN**

#### **1.1 Comparison of Nef and Nef7 mutants**

The properties of Nef7 render it uniquely suitable as a carrier platform for therapeutic proteins. Our strategy involved packaging of Nef7-fusion proteins into virus-like particles (VLP) that could then be targeted to HIV-infected cells through a process known as inverse fusion (Endres, Jaffer et al. 1997), wherein CD4 and CXCR4/CCR5 pseudotyped VLP bind to gp120 on the infected cell surface and allow delivery of VLP contents into the infected cell. We therefore constructed four Nef constructs: WT Nef, Nef<sup>V157L</sup>, Nef<sup>E177G</sup>, and Nef7, that contains both point mutations and analyzed them for their effects on the host cell. First, we transfected 293T cells with each of the Nef plasmids or the empty cDNA3 vector as a control and determined the cytosolic and membrane expression of each (Fig. 8A). Nef7 had been previously shown to localize to lipid rafts to a greater extent than WT Nef, but it was not shown whether this was due to an overall increase of Nef7 to the membrane or a more specific targeting of Nef7 to lipid rafts. Our data shows that while Nef7 does have a slight increase in membrane localization as compared to WT Nef, this is not a large difference and thus the increased lipid raft localization of Nef7 is likely not due to a general increase in membrane localization. Importantly, all four Nef constructs showed a similar level of overall cellular expression, indicating that they were stable (Fig. 8B). Next, we transfected 293T cells with HIV.*env*.*nef*, an HIV non-replication-competent provirus that is defective

**A****B**

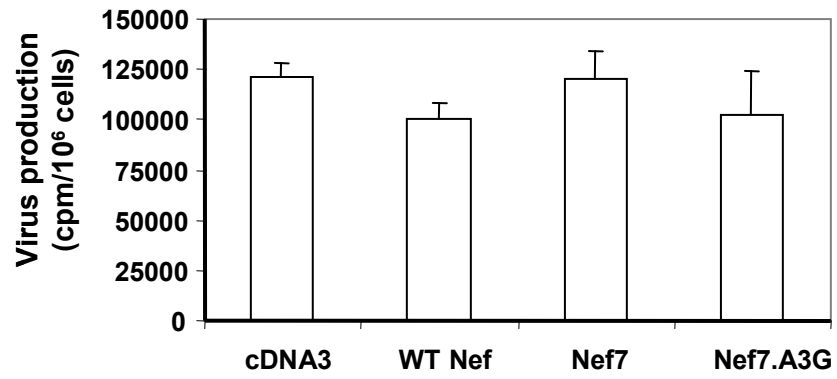
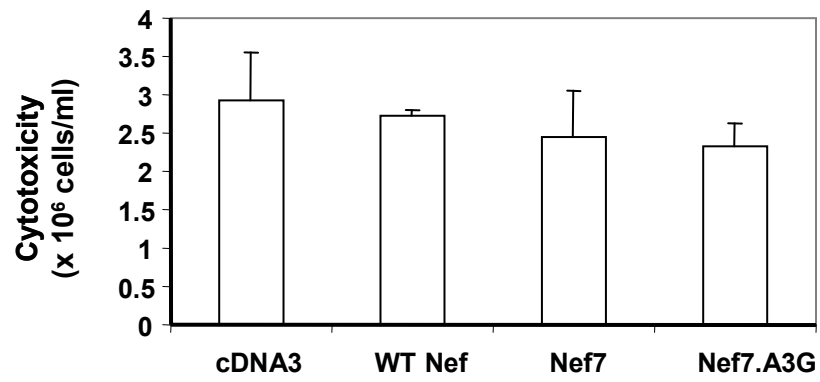
**Figure 8. Cytosolic and membrane expression of Myc-tagged Nef and Nef mutants.**

293T cells were transfected with WT Nef or each of the Nef mutants. Cells were harvested 72 hr post-transfection either as whole cell lysate or separated into cytosolic and membrane fractions, then separated by 12% SDS-PAGE. **A.** The ratio of cytosolic (C) to membrane (M) localization of Nef was determined by Western blot analysis using an anti-myc antibody, with membrane expression normalized to calnexin and cytosolic expression normalized to actin. **B.** Whole cell lysate was subjected to Western blot analysis using an anti-Myc antibody with antibodies against  $\beta$ -actin as a loading control. Data were representative of 3 independent experiments.

for Nef expression, in combination with each of the four Nef plasmids. We examined the virus production from transfected cells as well as the cytotoxicity of each of the Nef mutants and found that there was no significant difference in virus production (Fig. 9A) or cell death (Fig. 9B) among cells expressing these Nef proteins. Therefore, we concluded that at least in terms of its effect on the expressing cell, Nef7 had no properties that would disqualify it from being used as a protein carrier in our system.

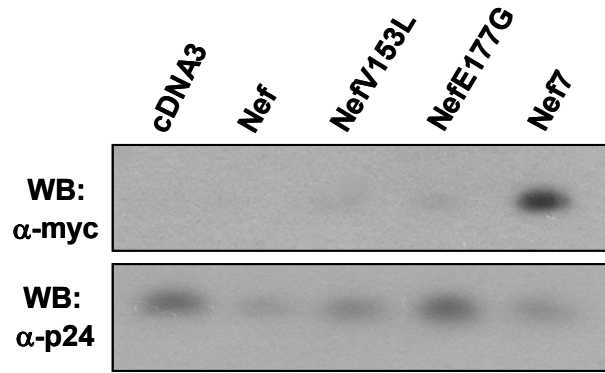
### **1.2 Enhanced virion incorporation of Nef7 as compared to WT Nef**

To test our hypothesis that A3G fusion to Nef7 (Nef7.3AG) can restore the virion incorporation of A3G and inactivate HIV-1 infectivity in the presence of HIV-1 Vif expression, we first ascertained the high virion incorporation property of Nef7. We utilized the constructed Nef and Nef7 mutants, in which <sup>V152<sup>L</sup></sup> and <sup>E177<sup>G</sup></sup> point mutations were introduced, to compare their ability to be incorporated into HIV-1 virions. We first transfected 293T cells with HIV.*env*.*nef* in combination with WT Nef or Nef 7. We also transfected plasmids containing each of the two single point Nef7 mutants <sup>V152<sup>L</sup></sup> and <sup>E177<sup>G</sup></sup> in these experiments. Viruses were collected 72 hr post transfection and assayed for RT activity. Next, supernatants containing equal levels of RT activity were centrifuged and the pellets lysed in RIPA buffer, then subjected to Western blot analysis using an antibody against a Myc epitope that was engineered to the C terminus of all Nef recombinant proteins and an anti-HIV p24 antibody that detected the HIV-1 capsid protein (Fig. 10A). The p24 levels detected by Western blot sometimes varied among samples, but as this was not consistently the case it is likely due to inconsistent pelleting of virus or accidental removal of a portion of the virus pellet when removing the

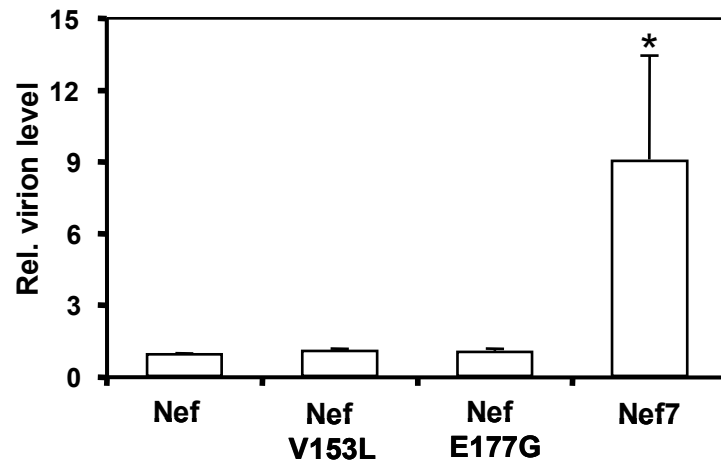
**A****B**

**Figure 9. Virus production and cytotoxicity in Nef7-expressing cells.** **A.** 293T cells were transfected with HIV.*env*.*nef* together with each of the Nef plasmids. Seventy-two hours post-transfection, cell supernatants were collected and virus production was determined as measured by reverse transcriptase activity, normalized to cell number. **B.** Transfected 293T cells were analyzed at 72 hr post-transfection for viability, with the number of live cells determined by trypan blue exclusion. Data were representative of 2 independent experiments.

**A**



**B**





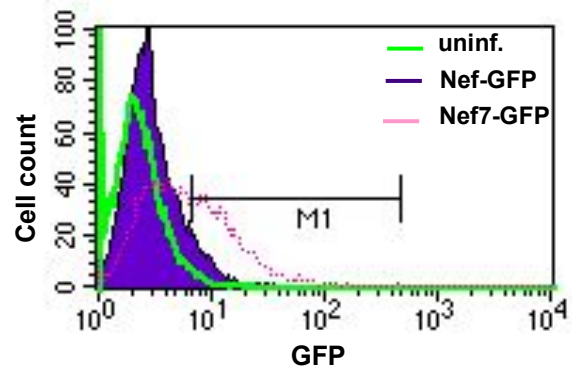
**Figure 10. Virion incorporation of Nef7.** **A.** 293T cells were transfected with HIV.*env*.*nef* and WT Nef, Nef<sup>V153L</sup>, Nef<sup>E177G</sup>, or Nef7 with pcDNA3 included as a control. Progeny viruses were collected 72 hr post transfection and quantified for their RT activity. Viruses with an equal level of RT activity were pelleted and analyzed for Western blot analysis using an antibody against the Myc epitope that was added to the C terminus of all Nefs and an antibody against HIV-1 p24 antigen. **B.** The relative (rel.) level of virion protein from three independent experiments was determined by densitometry and normalized to the virion p24 level. The WT Nef value was set as 1. \*: p<0.05 as compared to WT Nef. Data was mean ± s.d.

supernatant. Relative virion Nef incorporation was normalized to HIV-1 p24. We found that compared to WT Nef, Nef7 showed approximately 9-fold higher virion incorporation (Fig. 10B). There was little virion incorporation of WT Nef, <sup>V153L</sup>, and <sup>E177G</sup> over the cDNA background control. These results confirm that Nef7 is capable of being incorporated into HIV-1 virions at a higher level than WT Nef, and also show that both point mutations are required for increased virion incorporation.

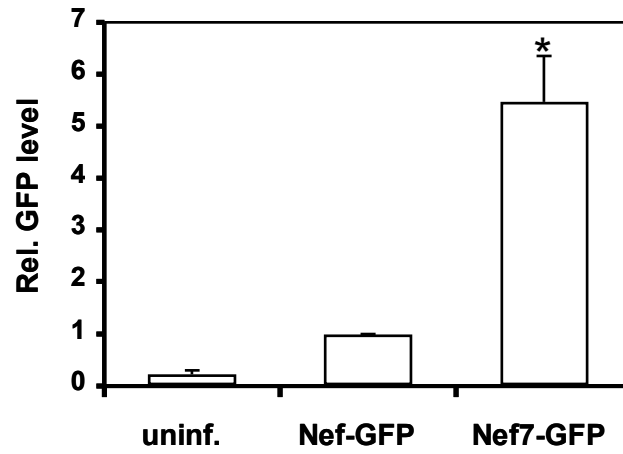
### **1.3 Efficient delivery of virion Nef7 into target cells**

We next determined whether virion incorporated Nef would be efficiently delivered into target cells. We constructed Nef-GFP and Nef7-GFP fusion plasmids and transfected them into 293T cells along with HIV.*env*.*nef* and vesicular stomatitis virus envelope (VSV-G) to produce GFP-labeled VSV-G-pseudotyped HIV-1 particles. GFP expression *in trans* and incorporation into HIV-1 virions would allow accurate quantitation of the delivery efficiency of virion proteins into host cells. Thus, we infected fresh 293T cells with these newly pseudotyped viruses containing an equal level of RT activity by spinoculation and analyzed the percentage of GFP<sup>+</sup> cells in each infection (Fig. 11A). In agreement with the virion incorporation, about 5-6 fold more GFP<sup>+</sup> cells were detected in the infections by viruses carrying Nef7-GFP than those of viruses carrying WT Nef-GFP (Fig. 11B), suggesting that virion Nef7 protein can be efficiently delivered into target cells through infection.

**A**



**B**



**Figure 11. Delivery of virion WT Nef and Nef7 into cells.** **A.** 293T cells were transfected with HIV.*env*.*nef* and VSV-G, and WT Nef-GFP or Nef7-GFP expression plasmids. Progeny viruses were collected and quantitated as stated above. Viruses with an equal level of RT activity were used to infect fresh 293T by spinoculation. Uninfected cells (uninf.) were used as a control. After 3 hr cells were washed to remove unbound viruses and then trypsinized to remove cell surface-bound virus. These cells were then analyzed for GFP-positive cells by FACS to detect intracellular delivery of virion Nef.

**B.** The relative level of GFP-positive cells from three independent experiments was calculated with the number of GFP-positive cells in WT Nef-GFP infection set to 1.

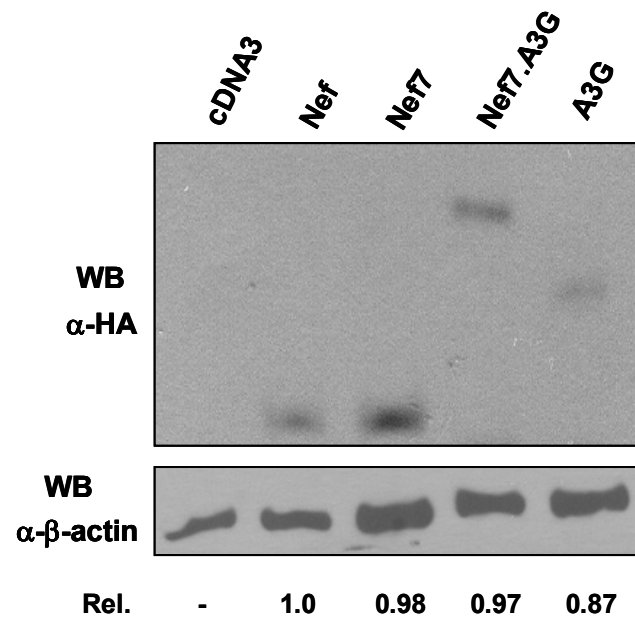
\*:  $p < 0.05$  as compared to uninfected cells. Data was mean  $\pm$  s.d.

#### **1.4 Higher virion incorporation of the Nef7.A3G fusion protein**

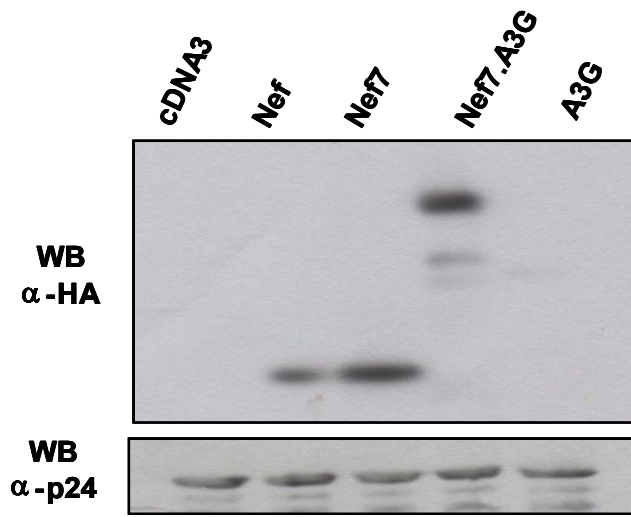
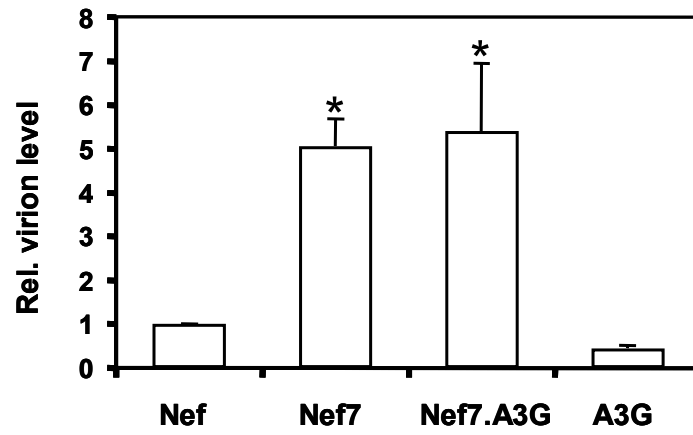
Next, we determined whether Nef7 in the form of fusion with A3G (Nef7.A3G) retained the higher virion incorporation property of Nef7. We therefore constructed the Nef7.A3G fusion expression plasmid, wherein the A3G without its start codon was cloned to the 3' end of the Nef7 gene before the termination signal. Since the A3G fusion construct contained an HA epitope tag and the Nef constructs all contained a Myc epitope tag, we also replaced the Myc tag on the Nef constructs with an HA tag in order to facilitate the comparison of expression levels of each of these constructs. We confirmed that the new HA tag was working and that all proteins were stably expressed by transfecting 293T cells with WT Nef, Nef7, Nef7.A3G, or A3G (Fig. 12). We then prepared HIV.*env*.*nef* viruses carrying WT Nef, Nef7, Nef7.A3G, or A3G, as described above and compared Nef7 and Nef7.A3G for their virion incorporation by Western blot analysis (Fig. 13A). We also included WT Nef and A3G as controls in these experiments. Quantitation of virion protein incorporation showed that Nef7 and Nef7.A3G had comparable levels in HIV-1 virions (Fig. 13B). As Nef is a virion protein, it was detected in the virus blot. On the other hand, Vif expression greatly diminishes encapsidation of A3G in the virus particles and as a result, only a very faint band of A3G appeared in the virus blot. These results indicate that Nef7.A3G fusion does not alter the higher incorporation of Nef7 into HIV-1 virions.

#### **1.5 No PAK2 activation by the Nef7.A3G fusion protein**

In order to utilize Nef7 as a therapeutic carrier molecule, it must not be inherently toxic. Previous work has shown that, while WT Nef is extremely pathogenic, Nef7 is defective



**Figure 12. Expression of HA-tagged Nef, Nef7, A3G, and Nef7.A3G genes.** 293T cells were transfected with WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids with pcDNA3 included as a control. Whole cell lysate was subjected to Western blot analysis using an antibody against an HA epitope that was added to the C terminus of all Nefs, Nef7.A3G and A3G and antibodies against  $\beta$ -actin as a loading control. Data were representative of 3 independent experiments.

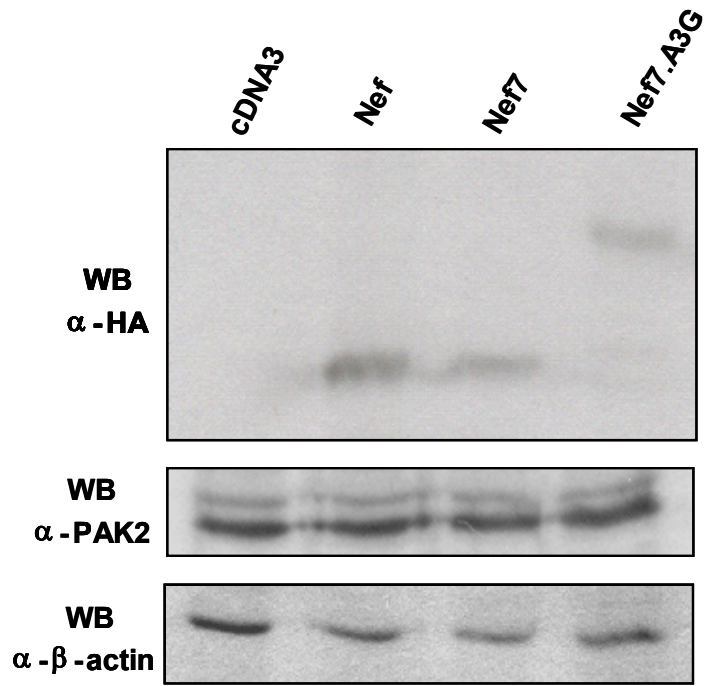
**A****B**



**Figure 13. Virion incorporation of Nef7.A3G.** **A.** 293T cells were transfected with HIV.*env*.*nef* and WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids with pcDNA3 included as a control. Progeny viruses were collected and subjected to Western blot analysis using an anti-HA antibody, with antibodies against HIV-1 p24 as a loading control. **B.** Virion protein levels were quantified from three independent experiments, as described above. \*:  $p < 0.05$ . Data was mean  $\pm$  s.d.

for a number of key Nef activities and is therefore much less toxic than WT Nef. Activation of p21-activated kinase-2 (PAK2) is an important mechanism whereby Nef exerts its pathogenic function on the host (Sawai, Khan et al. 1996). Nef7 does not activate PAK2 (D'Aloja, Santarcangelo et al. 2001). Therefore, we determined whether the Nef7.A3G fusion led to PAK2 reactivation. We transfected 293T cells with HIV.*env*.*nef* and WT Nef, Nef7, or Nef7.A3G. We first performed Western blot analysis for endogenous PAK2 expression in all transfections using an anti-PAK2 antibody. We also performed Western blot analysis using an anti-HA antibody to ensure a comparable level of Nef expression, using an anti- $\beta$ -actin antibody to ensure equal loading of the cell lysates. As expected, there was little change in endogenous PAK2 level among all transfections (Fig. 14A). We then performed immunoprecipitation of the cell lysates using an anti-HA antibody, followed by an in vitro kinase assay for PAK2 phosphorylation. PAK2 phosphorylation, an indicator of PAK2 activation, was only detected in cells that were transfected with WT Nef (Fig. 14B). There was no phosphorylation signal in cells that were transfected with Nef7, Nef7.A3G, or the pcDNA3 control. To ascertain whether the inability of Nef7 and Nef7.A3G to inactivate PAK2 was due to altered association between PAK2 and these Nef derivatives or an inability of associated Nef to activate PAK2, we performed immunoblotting of the immunoprecipitates using an anti-PAK2 antibody. Compared to the pcDNA3 control, Nef7 and Nef7.A3G exhibited a similar level of immunoprecipitated PAK2 to that of WT Nef (Fig. 14C). Taken together, these results show that like Nef7, Nef7.A3G does not activate PAK2 and that the failure of these two Nef derivatives to activate PAK2 is not due to changes in the level of endogenous PAK2 expression by these Nefs or to changes

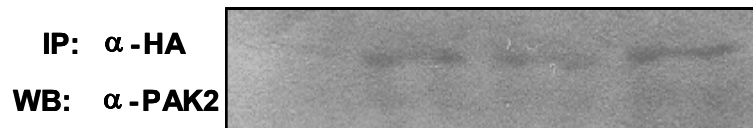
**A**



**B**



**C**

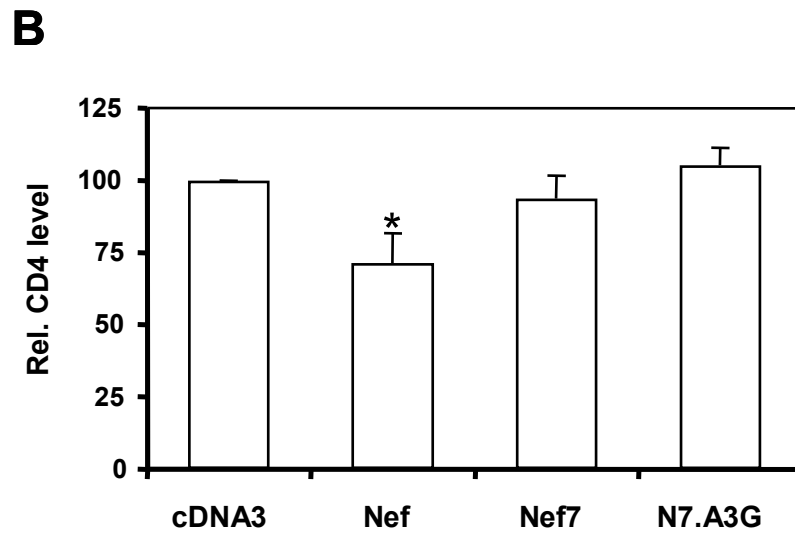
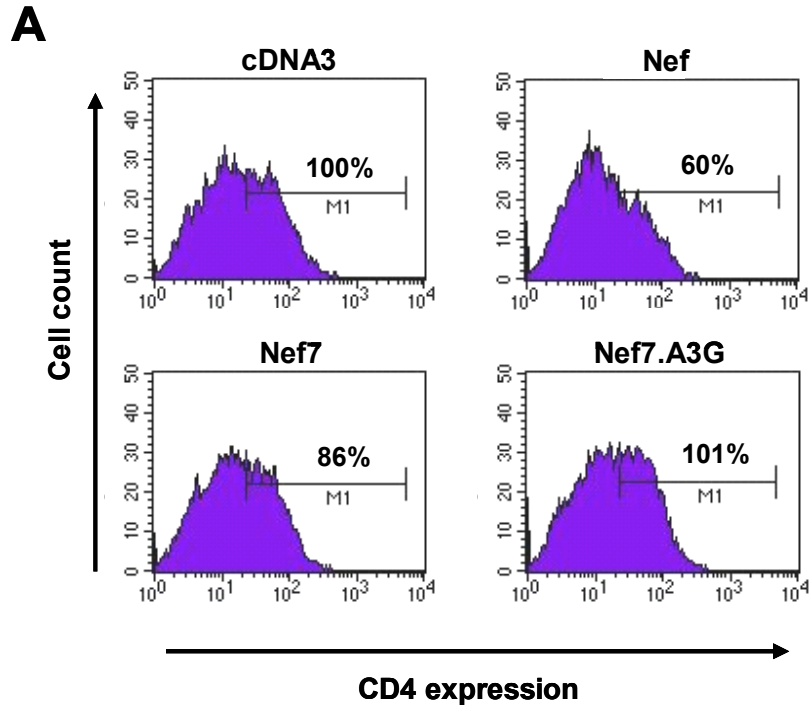


**Figure 14. Effects of Nef, Nef7 and Nef7.A3G on PAK2 activation.** **A.** 293T cells were transfected with HIV.*env*.*nef* and WT Nef, Nef7, or Nef7.A3G expression plasmids with pcDNA3 included as a control. Cells were harvested for whole cell lysates 72 hr after transfection for Western blot analysis against anti-HA and anti-PAK2, with  $\beta$ -actin used to ensure comparable loading of cell lysates. **B.** Cell lysates were also subjected to immunoprecipitation with anti-HA antibodies, followed by an in vitro kinase assay using  $\gamma$ -<sup>32</sup>P-ATP to detect PAK2 activation. **C.** Cell lysates were first immunoprecipitated with anti-Nef antibody and then Western blotted with anti-PAK2 antibody. Data were representative of 3 independent experiments.

in the binding affinity of these Nefs to PAK2 but instead due to an inability of Nef7 to induce autophosphorylation of PAK2.

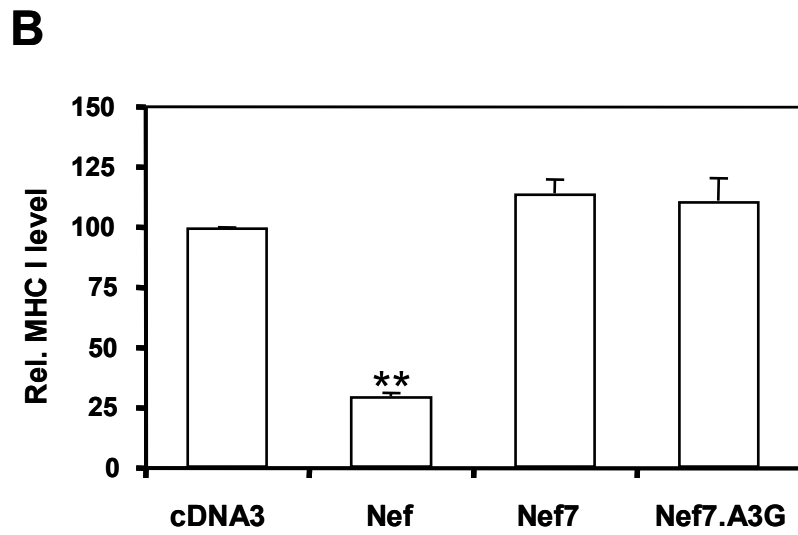
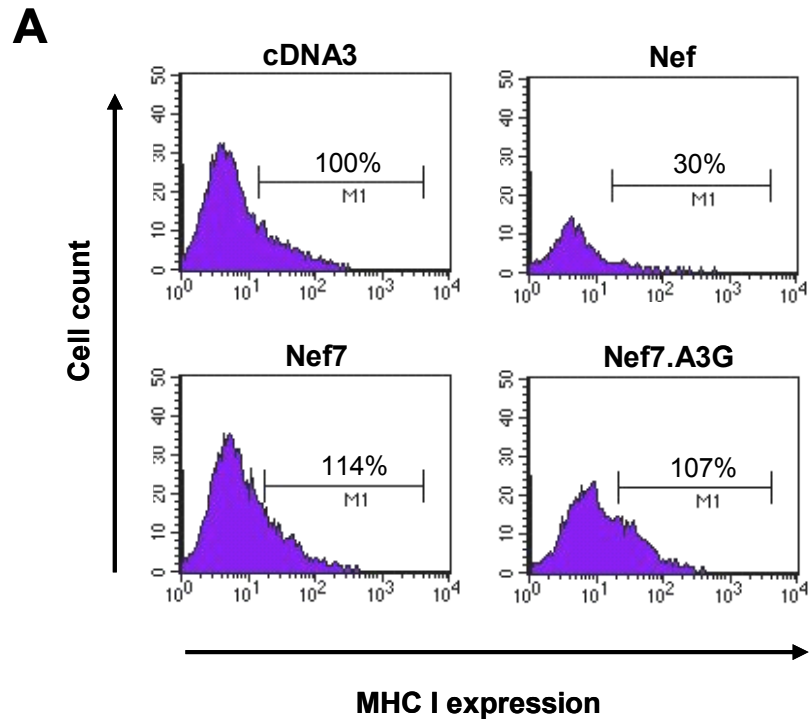
### **1.6 Downregulation of CD4 and MHC I by Nef7.A3G**

Another key function of Nef is the downregulation of cell surface expression of various membrane proteins, the most well-known being CD4 and MHC I (Roeth and Collins 2006). Nef7 is reported to be defective in both CD4 and MHC I downregulation (D'Aloja, Santarcangelo et al. 2001). We therefore attempted to confirm the diminished pathogenic phenotype of Nef7 and to determine whether this activity was present in the Nef7.A3G fusion protein by taking advantage of a system that was previously validated to study Nef-mediated MHC I downregulation (Lubben, Sahlender et al. 2007). We adapted this system to determine the relationship between cell surface expression of CD4 and MHC I and expression of Nef or its derivatives. We transfected HeLa cells with plasmids expressing CD4, GFP and each of the Nefs using Lipofectamine. After 24 hr we performed cell surface immunofluorescence staining using an anti-CD antibody and a phycoerythrin (PE)-conjugated secondary antibody followed by flow cytometry analysis. We first gated for GFP<sup>+</sup> cells, and then examined the levels of CD4 expression among all GFP<sup>+</sup> cells (Fig. 15). We also performed similar experiments with MHC I (Fig. 16). As expected, WT Nef downregulated both CD4 and MHC I expression. In contrast, Nef7 and Nef7.A3G showed no significant changes in the surface expression of these two receptors. These results show that neither Nef7 nor Nef7.A3G alter the cell surface expression of CD4 and MHC I.



**Figure 15. Effects of Nef, Nef7 and Nef7.A3G on cell surface expression of CD4.** **A.**

HeLa cells were transfected with CD4, GFP, and Nef, Nef7, or Nef7.A3G expression plasmids with pcDNA3 included as a control. At 24 hr post-transfection cells were harvested and stained with an anti-CD4 antibody, followed by a PE-conjugated secondary antibody. The cells were then gated for the GFP<sup>+</sup> cells by FACS and only the GFP<sup>+</sup> cells were analyzed for cell surface CD4 expression. The M1 gate was determined by staining with mouse IgG followed by anti-mouse-PE. **B.** The relative CD4 levels from three independent experiments were calculated with the CD4 level in pcDNA3-transfected cells set to 100%. \*: p<0.05. Data was mean ± s.d.





**Figure 16. Effects of Nef, Nef7 and Nef7.A3G on cell surface expression of MHC I.**

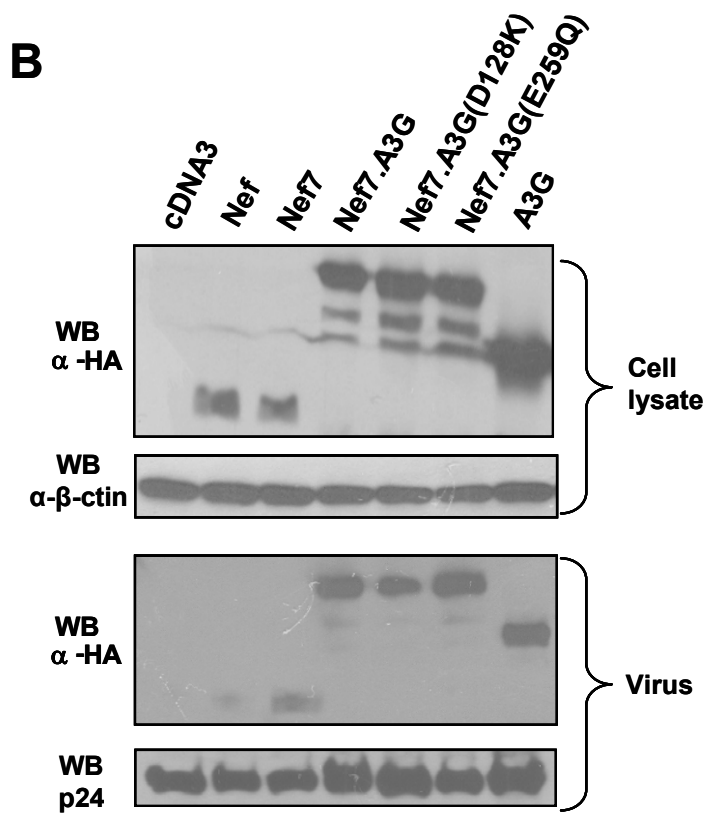
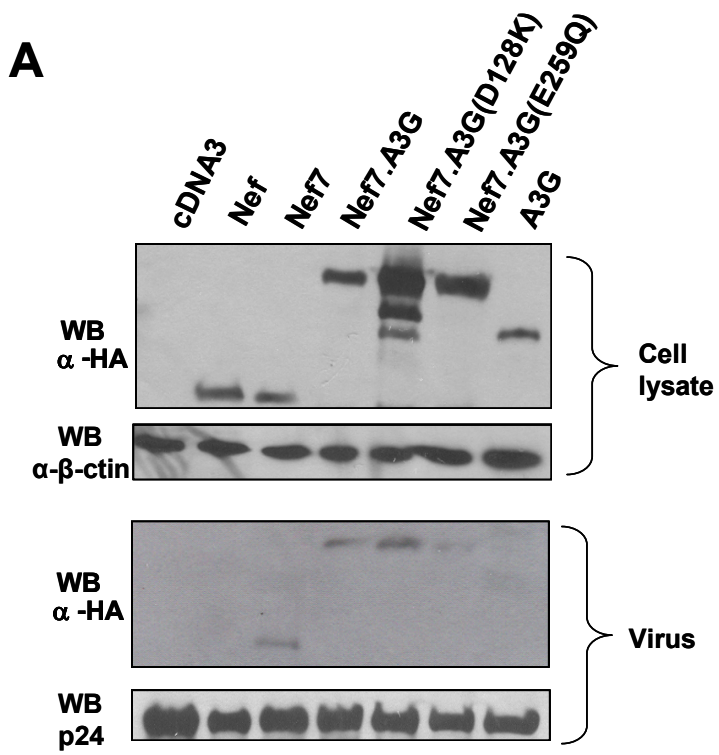
**A.** HeLa cells were transfected with GFP, and Nef, Nef7, or Nef7.A3G expression plasmids with pcDNA3 included as a control. At 24 hr post-transfection cells were harvested and stained with an anti-MHC I antibody, followed by a PE-conjugated secondary antibody. The cells were then gated for the GFP<sup>+</sup> cells by FACS and only the GFP<sup>+</sup> cells were analyzed for cell surface MHC I expression. The M1 gate was determined by staining with mouse IgG followed by anti-mouse-PE. **B.** The relative MHC I levels from three independent experiments were calculated with the MHC I level in pcDNA3-transfected cells set to 100%. \*\*: p<0.01. Data was mean ± s.d.

### **1.7 Impaired HIV-1 infectivity in 293T cells by Nef7.A3G virion incorporation**

Nef7.A3G was detected in HIV-1 virions in the context of HIV-1 Vif expression (Fig. 13). It did not activate PAK2 (Fig. 14) or downregulate CD4 and MHC I expression (Fig. 15, 16). Since we had determined that the Nef7.A3G fusion protein retained the characteristics of Nef7 and could therefore be utilized in our therapeutic strategy, we next determined whether it also retained the characteristics of A3G in the context of the fusion protein. Therefore we determined the effect of Nef7.A3G on the HIV-1 infectivity. We transfected 293T cells with HIV-Luc or HIV $\Delta$ vif, VSV-G and Nef or each of the Nef derivatives to produce VSV-G-pseudotyped HIV-Luc viruses carrying each of the Nefs. HIV $\Delta$ vif has no functional *env* or *vif* gene, while HIV-Luc contains no functional HIV-1 *env* or *nef* gene and has the Luc reporter gene inserted at the 5' end of the nonfunctional *nef* gene; it allows *in trans* complementation of other viral envelopes to determine the viral tropism and only single round infection for accurate determination of HIV-1 entry (He, Chen et al. 1997). VSV-G envelope was used to facilitate HIV-1 infection of cells that do not usually express HIV-1 receptor CD4 and chemokine receptors CXCR4 or CCR5. To ensure the specificity of Nef7.A3G, we also constructed and included two Nef7.A3G point mutants as controls: Nef7.A3G/D128K (D128K) and Nef7.A3G/E259Q (E259Q) in these experiments. Both point mutations were made in the A3G portion of the fusion protein. D128K mutation does not bind to HIV-1 Vif protein and renders the A3G protein Vif-resistant (Huthoff and Malim 2007; Zhang, Saadatmand et al. 2008), while E259Q mutation within the second deaminase domain leads to enzymatically inactive A3G (Schumacher, Hache et al. 2008). In addition, we also included A3G alone in the transfection as a control.

We collected viruses from transfected cells, harvested these transfected cells for cell lysates, and performed Western blot analysis using an anti-HA antibody on the cell lysate and virus of both the HIV-Luc (Fig. 17A) and HIV $\Delta$ vif (Fig. 17B) transfections. As expected, due to differential sensitivity to Vif-mediated degradation, A3G had the lowest level in the cells, while Nef7.A3G(D128K) had the highest level in the cells. The expression levels of both Nef7.A3G and Nef7.A3G(E259Q) were found to be between A3G and Nef7.A3G(D128K), suggesting that Nef fusion had made A3G less sensitive to Vif-mediated degradation. As a result, Nef7.A3G(D128K) was incorporated slightly more into viruses than Nef7, Nef7.A3G, or Nef7.A3G(E259Q). Compared to the above findings, lack of Vif expression (and therefore Vif-mediated degradation) resulted in a similar level of detection of A3G and the three A3G fusion proteins in both the cells and the viruses. On the other hand, lack of Vif expression did not alter the fact that Nef7 was always detected in virions much more than its wild-type counterpart Nef. Taken together, these results indicate that Nef7.A3G is partially Vif-resistant but not to the same extent as Nef7.A3G(D128K), possibly due to structural constraints on the binding of Vif to the fusion protein. However, the extent to which the levels of Nef7.A3G and Nef7.A3G(D128K) differ between cells and viruses suggests that the slight resistance of Nef7.A3G to Vif is not solely responsible for its higher virion incorporation.

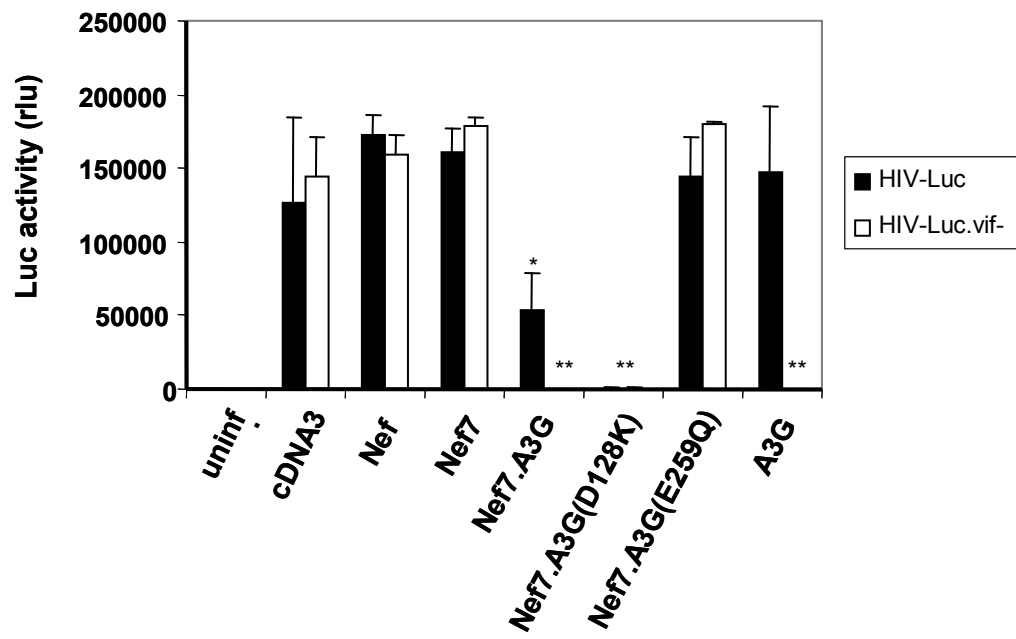
In parallel, we used virus collected from the HIV-Luc transfections to infect fresh 293T cells. As expected, viruses carrying WT Nef and Nef7 and HIV-Luc viruses collected from A3G-transfected cells showed a level of infectivity similar to that of pcDNA3 control viruses, as measured by the Luc activity (Fig. 18). Compared to viruses carrying



**Figure 17. Expression and virion incorporation of Nef7.A3G and its derivatives.** A.

293T cells were transfected with HIV-Luc and VSV-G, and WT Nef, Nef7, Nef7.A3G, Nef7.A3G(D128K), Nef7.A3G(E259Q), or A3G with pcDNA3 included as a control. At 72 hr post-transfection progeny viruses were collected and quantitated for RT activity. Whole cell lysates and progeny viruses were analyzed by Western blot using an anti-HA antibody, with a  $\beta$ -actin or p24 Western blot analysis to ensure comparable gel loading.

**B.** 293T cells were transfected with HIV-Luc.*vif*<sup>-</sup> and VSV-G, and WT Nef, Nef7, Nef7.A3G, Nef7.A3G(D128K), Nef7.A3G(E259Q), or A3G with pcDNA3 included as a control. At 72 hr post-transfection whole cell lysates and progeny viruses were analyzed for HA expression as described above. Data were representative of two independent experiments.



**Figure 18. Effects of virion incorporation of Nef and its derivatives on the infectivity of VSG-pseudotyped HIV-Luc reporter viruses.** 293T cells were transfected with HIV-Luc or HIV-Luc.*vif*<sup>-</sup>, VSV-G, and WT Nef, Nef7, Nef7.A3G, Nef7.A3G(D128K), Nef7.A3G(E259Q), or A3G with pcDNA3 included as a control. At 72 hr post-transfection progeny viruses were collected and quantitated for RT activity. HIV-Luc or HIV-Luc.*vif*<sup>-</sup> progeny viruses with an equal level of RT activity were then used to infect fresh 293T cells. Infected cells were harvested 48 hr post-infection and analyzed for luciferase activity. \*: p<0.05, \*\*: p<0.01. Data were mean ± s.d. and representative of 4 independent experiments.

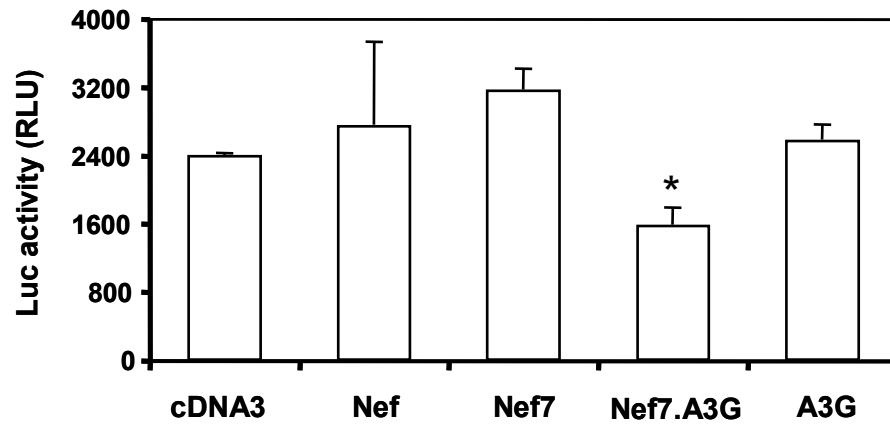
WT Nef or Nef7, HIV-Luc viruses carrying Nef7.A3G showed a marked decrease in infectivity. Moreover, the infectivity of the viruses carrying the Vif-resistant D128K mutant was further decreased over that of its counterpart Nef7.A3G, while the viruses carrying the nonfunctional A3G mutant E259Q showed little inhibition. To ensure the functionality of all the A3G derivatives and the non-functionality of the E259Q mutant, we constructed HIV-Luc.*vif*<sup>-</sup>, which contains a stop codon at the beginning of the *vif* gene, rendering it nonfunctional, and included it as a control in the infectivity experiments. The HIV-Luc.*vif*<sup>-</sup> viruses containing Nef7.A3G, Nef7.A3G(D128K) and A3G showed complete inhibition of infection, similar to that observed in the HIV-Luc viruses containing the Vif-resistant Nef7.A3G(D128K) mutant. On the other hand, the inactive Nef7.A3G(E259Q) mutant had little effect on infectivity, regardless of Vif expression. These results show that although Nef7.A3G virion incorporation does not restrict HIV-1 infection to the same extent as A3G does in the absence of Vif as demonstrated by the Nef7.A3G(D128K) mutation, it does significantly impair HIV-1 infectivity, even in the presence of Vif.

### **1.8 Impaired HIV-1 infectivity in U87 cells by Nef7.A3G virion incorporation**

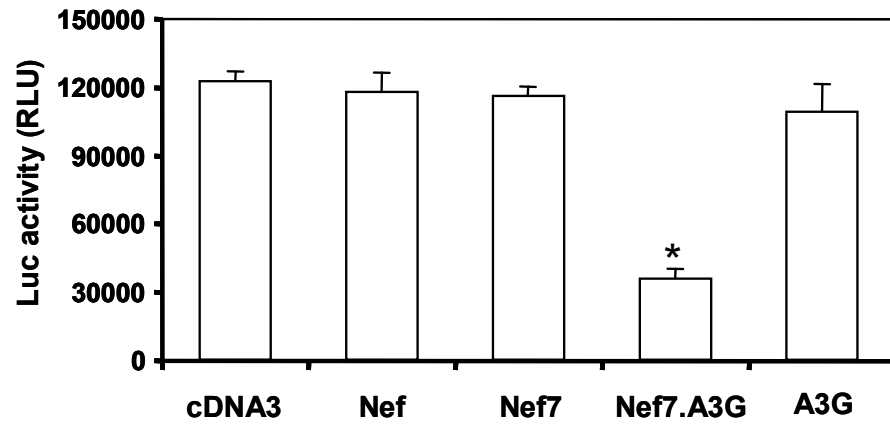
To further corroborate these findings and to ensure that Nef7.A3G also inhibited infectivity of HIV virions that enter the cell by utilizing the HIV-1 receptors instead of the pH-dependent VSV-G fusion, we prepared HXB2.env<sup>-</sup> and YU2.env<sup>-</sup> pseudotyped HIV-Luc viruses carrying WT Nef, Nef7, Nef7.A3G or A3G. We used HXB2.env<sup>-</sup> pseudotyped viruses to infect U87.CD4.CXCR4 cells (Fig. 19A) or YU2.env<sup>-</sup> pseudotyped viruses to infect U87.CD4.CCR5 cells (Fig. 19B), and determined the



**A**



**B**



**Figure 19. Effects of virion incorporation of Nef and its derivatives on the infectivity of HXB2.env- and YU2.env-pseudotyped HIV-Luc reporter viruses.** **A.**

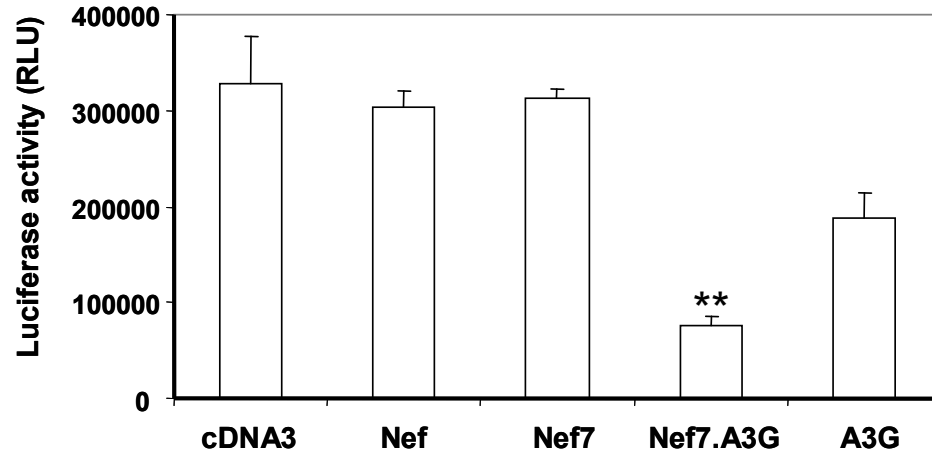
293T cells were transfected with HIV-Luc, HXB2.env, and WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids with pcDNA3 included as a control. At 72 hr post-transfection the viruses were collected and quantitated for RT activity. Viruses with an equal level of RT activity were used to infect U87.CD4.CXCR4 cells, and the infected cells were harvested for the Luc activity assay 48 hr post infection. **B.** Similarly, various YU2.env-pseudotyped HIV-Luc viruses carrying Nef and each of its derivatives were prepared and used to infect U87.CD4.CCR5 cells. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

infectivity. Similar to the results obtained using VSV-G-pseudotyped HIV-Luc viruses, Nef7.A3G-containing viruses showed a significantly lower level of viral infection than all others. Moreover, we also infected U87.CD4.CXCR4 and U87.CD4.CCR5 cells with VSV-G-pseudotyped viruses carrying the respective proteins and obtained similar results (Fig. 20). The inhibition is not complete, most likely because while fusion to Nef7 does partially restore virion incorporation of A3G, it cannot completely overcome the effect of Vif.

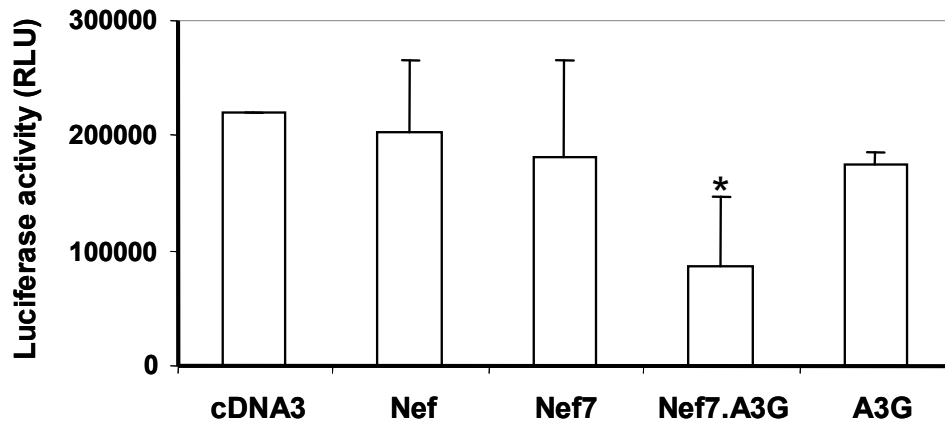
### **1.9 Block of HIV-1 replication by Nef7.A3G-containing VLP**

CD4<sup>+</sup> T lymphocytes are natural target cells for HIV-1 infection. Thus, we next determined the feasibility of using Nef7.A3G to target and inhibit HIV-1 replication in Jurkat cells, a CD4<sup>+</sup> human T lymphocyte cell line commonly used for HIV-1 infection. To ensure efficient delivery of Nef7.A3G into HIV-1 infected cells, we took advantage of the virus-like particles (VLP)-based inverse fusion strategy (Endres, Jaffer et al. 1997). We transfected 293T cells with an HIV-based packaging vector psPAX2 (Dull, Zufferey et al. 1998), Nef7.A3G, CD4, and CXCR4 and collected the cell culture supernatants as the VLP. We also included WT Nef, Nef7, and A3G to produce control VLP. CD4 and CXCR4 expression and presentation on the outer viral membrane allows recognition and binding to the gp120 that is expressed on the cell surface of HIV-1-infected cells, resulting in fusion of VLP with these cells and delivery of virion components within VLP into these cells (Endres, Jaffer et al. 1997). We determined the VLP production from transfected 293T cells as measured by reverse transcriptase activity and examined each of the VLP for incorporation of Nef or A3G by Western blot as described above (Fig. 21).

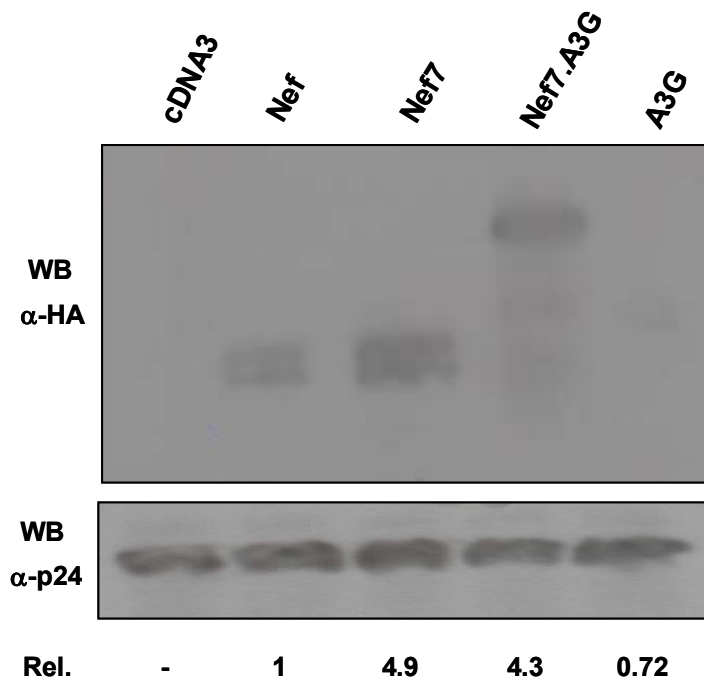
**A**



**B**



**Figure 20. Effects of virion incorporation of Nef and its derivatives on the infectivity of VSV-G-pseudotyped HIV-Luc reporter viruses in U87 cells.** **A.** 293T cells were transfected with HIV-Luc, VSV-G, and WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids with pcDNA3 included as a control. At 72 hr post-transfection the viruses were collected and quantitated for RT activity. Viruses with an equal level of RT activity were used to infect U87.CD4.CXCR4 cells, and the infected cells were harvested for the Luc activity assay 48 hr post infection. **B.** Similarly, HIV-Luc viruses carrying Nef and each of its derivatives were prepared and used to infect U87.CD4.CCR5 cells. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

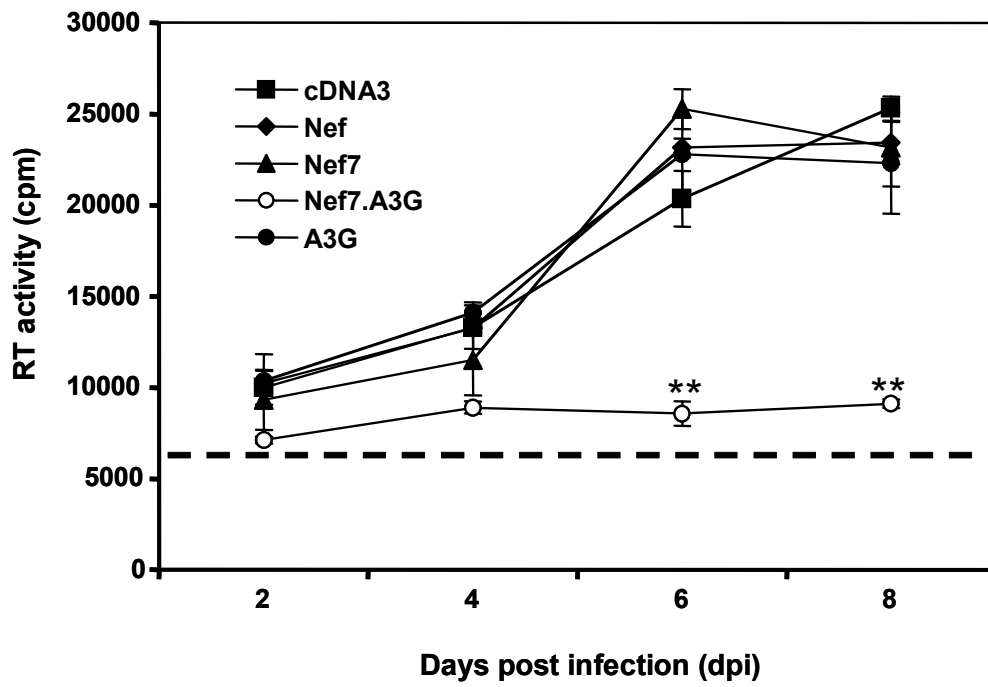


**Figure 21. VLP incorporation of Nef7.A3G.** 293T cells were transfected with psPAX2, CD4, CXCR4, and WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids with pcDNA3 included as a control. Progeny viruses were collected at 72 hr post-transfection quantified by reverse transcriptase assay, then subjected to Western blot analysis using an anti-HA antibody, with antibodies against HIV-1 p24 as a loading control. Data were representative of two independent experiments.

We found that even in the context of VLP, Nef7 and Nef7.A3G exhibited high virion incorporation.

We next infected Jurkat cells with replication-competent HIV-1 HXB2 viruses and exposed the infected cells to each of the VLP twice, once immediately following infection and once 24 hr later. We then monitored HIV-1 infection and replication in these cells. Notably, treatment of HIV-infected Jurkat cells with VLP that were derived from Nef7.A3G transfection gave rise to little viral replication (Fig. 22). In contrast, treatment of VLP derived from cells expressing WT Nef, Nef7, and A3G all showed similar viral replication kinetics to that of VLP from pcDNA3 control. The impact of Nef7.A3G is much higher in this context than in single-round infection assays (Fig. 18-20) because the effect was amplified over eight days instead of just 72 hr. To quantitatively compare the anti-HIV effects of these VLP, we stained the cells for HIV-1 p24 at day 8 and counted the total number of cells and the number of p24<sup>+</sup> cells. All samples had a similar number of viable cells, i.e. about  $1.2 \times 10^7$  cells. Nef7.A3G-treated cells had  $1.1 \pm 0.2\%$  p24<sup>+</sup> cells, while all others had  $92.5 \pm 4.2\%$  p24<sup>+</sup> cells at day 8 post-infection. Thus, the anti-HIV activity of the Nef7.A3G was calculated to be about  $10^7$  in this particular experimental setting. These results suggest that Nef7.A3G-containing VLP expressing CD4 and CXCR4 can successfully target HIV-infected cells and inhibit virus replication in HIV-1 infected Jurkat cells.





**Figure 22. Effects of Nef-, Nef7-, Nef7.A3G- and A3G-containing VLP on HIV-1**

**replication.** 293T cells were transfected with psPAX2, CD4, CXCR4, and WT Nef, Nef7, Nef7.A3G, or A3G expression plasmids. At 72 hr post-transfection culture supernatants containing VLP were collected, cleared of cell debris, and quantitated for the RT activity. CD4<sup>+</sup> Jurkat T lymphocytes were first infected with HIV-1 HXB2 strain. At 3 hr the remaining viruses were removed from the cells by repeated washes with fresh medium and then treated with each of the VLP, followed by a second treatment 24 hr after the initial infection. The cells were cultured for various lengths of time as indicated. At each time point, cell culture supernatant was collected and quantitated for RT activity. The RT activity of input VLP in the culture medium was marked as a dotted line. \*: p<0.05, \*\*: p<0.01. Data were mean ± s.d. and representative of 3 independent experiments.

## **PART 2: NEF ENHANCES THE FORMATION OF HIV-1 VIROLOGICAL SYNAPSES**

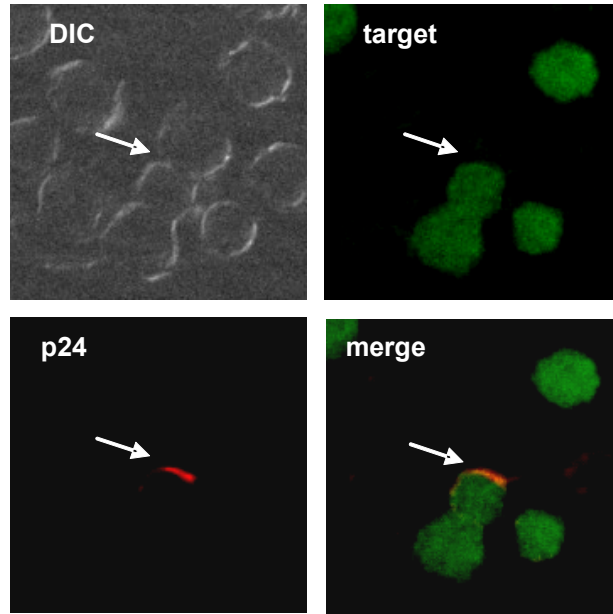
### **2.1 Nef increases the formation of VS**

To date, there has been no research done on the impact of HIV-1 accessory proteins on virological synapse formation. Currently, the only viral protein known to be involved in the HIV-1 virological synapse is gp120, which initiates the formation through its interaction with CD4. Because of its numerous important pathogenic functions in infected cells, many of which could potentially affect the VS, we investigated the effect of Nef on VS formation. First, Jurkat T cells were infected with either WT HIV or a Nef-deleted, replication-competent virus and cultured until close to 100% infection. Infected cells were mixed with CFSE-labeled target cells and allowed to form virological synapses on coverslips, and then conjugates were stained with either p24 or gp120 and CD4 antibodies. Formation of virological synapses was identified by polarization of p24 at the interface between an infected cell and a CFSE-labeled target cell (Fig. 23A), allowing for the percentage of infected cells engaged in a VS to be determined (Fig. 23B). The percentage of cells infected with HIV $\Delta$ Nef was significantly lower than that of cells infected with WT virus. We also performed experiments staining for co-localization of gp120 and CD4 and obtained similar results.

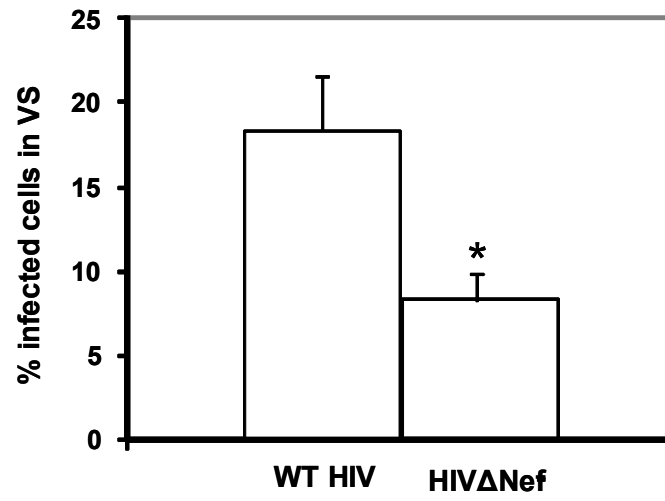
### **2.2 Piceatannol titration**

Next, we determined whether the impaired ability of Nef-defective HIV-1 viruses to form synapses translated to a decrease in their ability to transfer virions to target cells. However, we wished to use piceatannol, a Syk tyrosine kinase inhibitor that is a

**A**



**B**



**Figure 23. Nef effect on virological synapse formation in HIV-infected Jurkat cells.**

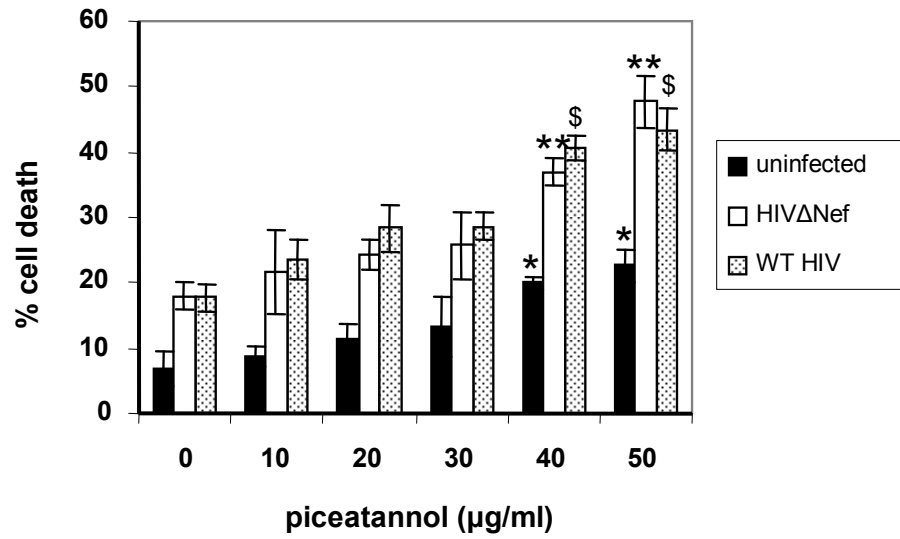
**A.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% of cells were infected. Uninfected cells were labeled with CFSE, and  $0.5 \times 10^6$  uninfected cells were incubated with  $0.5 \times 10^6$  infected cells for 1.5 hr on poly-L-lysine treated coverslips, in 200  $\mu$ l total RPMI to allow for VS formation. Cells were then fixed and stained for p24. A representative VS is shown. **B.** The percentage of infected cells involved in a VS was determined for each virus. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 4 independent experiments.

particularly effective inhibitor of the VS component ZAP70, as a negative control of VS-dependent virus transfer. Since piceatannol can be toxic at high concentrations and our experiment required its activity for 24 hr, we first determined the concentration of piceatannol that would inhibit ZAP70 phosphorylation at 24 hr with minimal cytotoxicity. We treated uninfected, WT HIV-infected, and HIV $\Delta$ Nef infected Jurkat cells with increasing concentrations of piceatannol for 24 hr. We then determined the percentage of dead cells by trypan blue staining and found that piceatannol seemed to have increased cytotoxicity in infected cells, possibly due to the additional stress these cells were already under (Fig. 24A). Cytotoxicity increases slightly at 10  $\mu$ g/ml piceatannol and stays approximately the same until 40  $\mu$ g/ml piceatannol where it increases dramatically. Lysis of the treated cells followed by immunoprecipitation for phospho-tyrosine and Western blot of ZAP70 showed that p-ZAP70 decreases steadily with increasing piceatannol treatment, with no detectable pZAP70 found in the 30  $\mu$ g/ml sample (Fig. 24B). Treatment with piceatannol did not affect overall ZAP70 expression. We therefore used 30  $\mu$ g/ml piceatannol in the following experiments as treatment at this concentration resulted in no detectable ZAP70 activation while minimizing cytotoxicity.

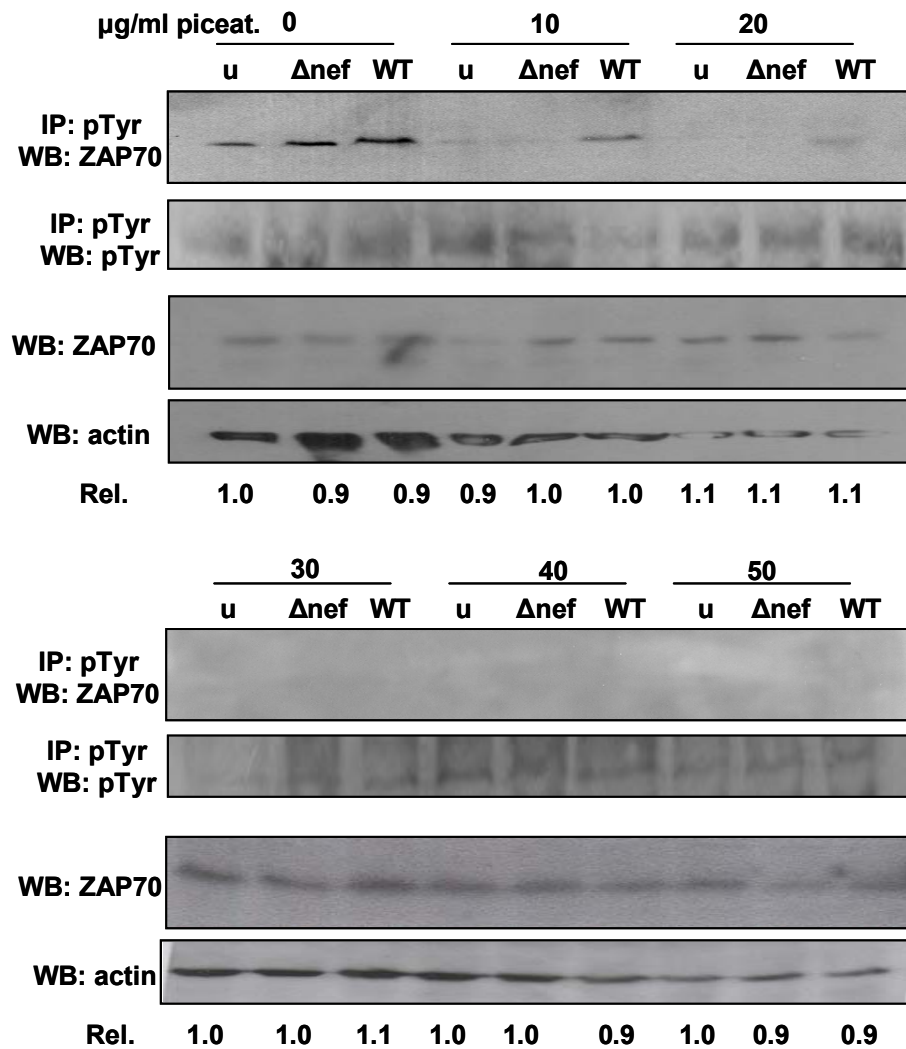
### **2.3 Nef increases Gag transfer to target cells**

Next, we analyzed the increase of gag expression in target cells over increasing periods of VS formation. We cultured Jurkat cells infected with WT HIV or HIV $\Delta$ Nef until they were close to 100% infected, and then mixed washed infected cells with CFSE-labeled target cells in a 1:1 ratio for increasing timepoints. After co-incubation, cells were fixed and stained for p24 to determine the percentage of p24<sup>+</sup> target cells (Fig. 25A). We

**A**



**B**



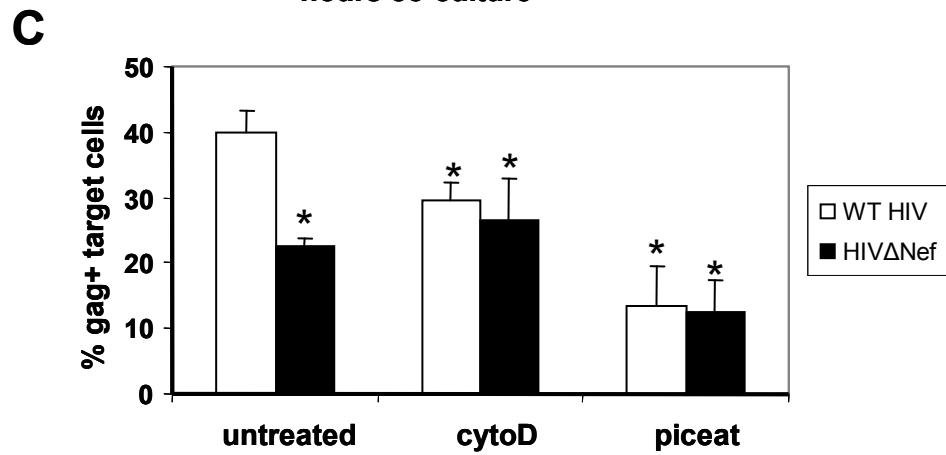
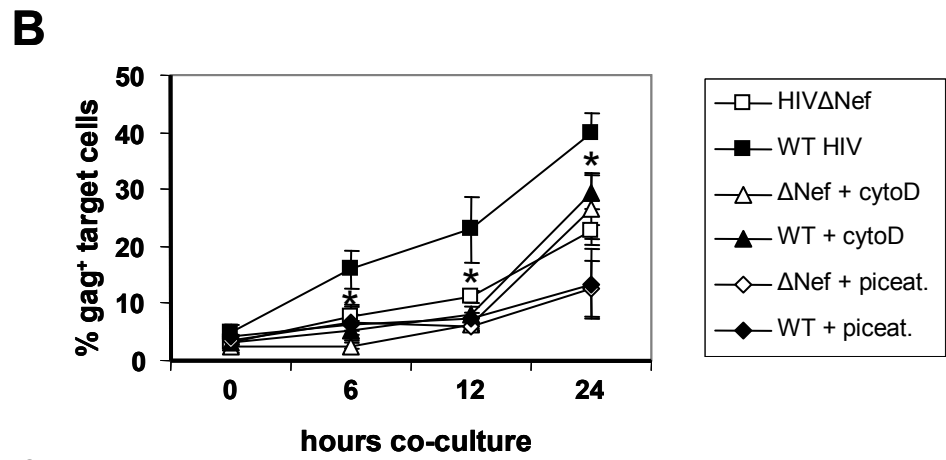
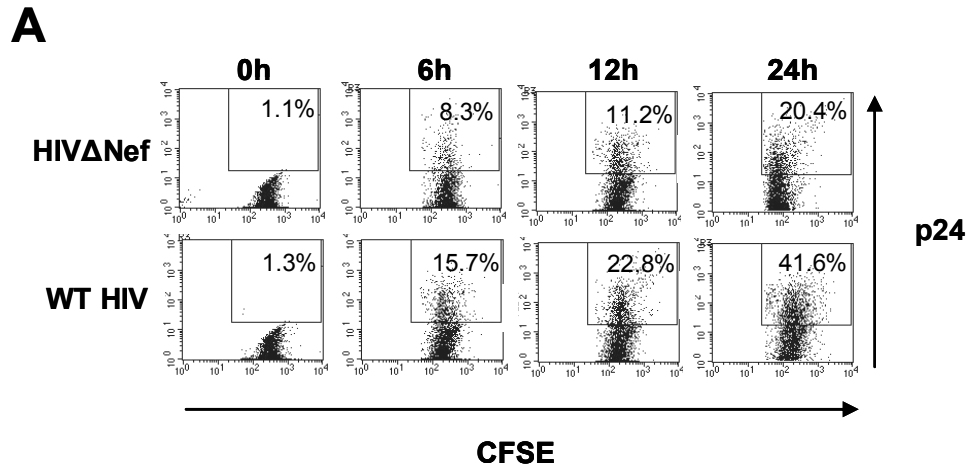
**Figure 24. Picetannol titration in HIV-infected Jurkat cells.** **A.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% infection, with uninfected cells included as a control.  $1 \times 10^6$  cells were then cultured in 96-well plates in the presence of increasing concentrations of picetannol. After 24 hr incubation, the percentage of dead cells was determined using trypan blue staining. **B.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% infection, with uninfected cells (U) included as a control.  $6 \times 10^6$  cells were then cultured in T25 flasks in the presence of increasing concentrations of picetannol. After 24 hr incubation, the cells were lysed and subjected to phosphotyrosine immunoprecipitation, followed by Western blot for ZAP70, and then pTyr. A phosphotyrosine band of ~55 kDa that was not affected by Nef expression was chosen as a loading control. Western blotting against ZAP70 was also performed and normalized to actin. \*:  $p < 0.05$  as compared to untreated uninfected sample; \*\*:  $p < 0.05$  as compared to untreated HIV $\Delta$ Nef-infected sample; \$:  $p < 0.05$  as compared to untreated WT HIV-infected sample. Data were mean  $\pm$  s.d. and representative of two independent experiments.



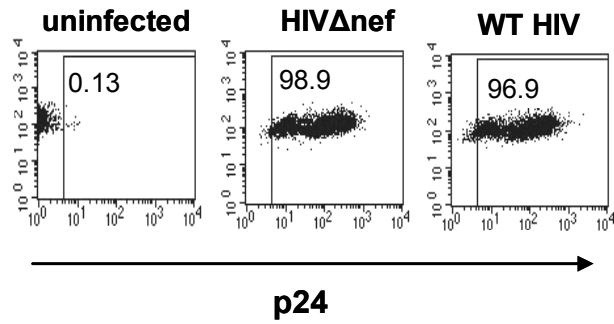
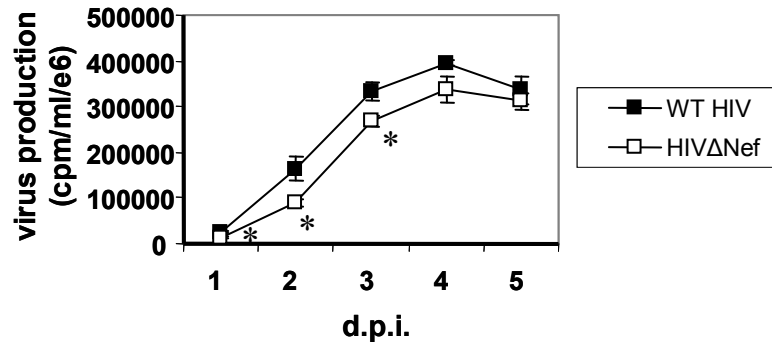
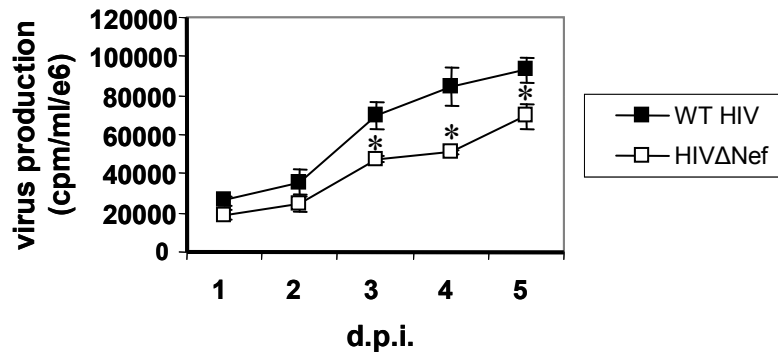
found that HIV $\Delta$ Nef infected cells transferred fewer virions to target cells during co-culture than cells infected with WT HIV, with increasing differences over time (Fig. 25B). The transfer efficiency of HIV $\Delta$ Nef-infected cells after 24 hr is equivalent to that of WT HIV in the presence of cytochalasin D, an actin depolymerizer that is known to inhibit VS transfer of viral particles (Jolly, Kashefi et al. 2004), but greater than WT HIV transfer in the presence of piceatannol, which greatly inhibits the activity of ZAP70, a necessary component of VS-dependent viral transfer (Fig. 25C).

#### **2.4 Nef increases replication kinetics in conditions favoring VS formation**

To see whether Nef's effect on virological synapse formation resulted in differences in viral spread over longer periods, we monitored replication kinetics in a situation that promoted virological synapse-dependent virus spread over cell-free virus spread:  $1 \times 10^6$  cells/ml resulting in an effective monolayer, with approximately 33% p24<sup>+</sup> (infected) cells at the start of the experiment (Fig. 26A). Because of the possibility that viral infection through virological synapses can be partially fusion-independent, the fusion inhibitor T-20 was also added to one set to eliminate any viral spread resulting from cell-free virus. We found that in these VS-favorable conditions there is a small but statistically significant lag in the replication kinetics of HIV $\Delta$ Nef infections as compared to WT infections (Fig. 26B). This lag becomes more pronounced and longer-lived when T-20 is included to eliminate any cell-free infections (Fig. 26C). Taken together, these results indicate that while not strictly necessary for virological synapse formation and synapse-dependent transfer of HIV-1 to target cells, Nef does indeed enhance the



**Figure 25. Nef effect on virological synapse-mediated Gag transfer.** **A.** Jurkat cells were infected with HIV $\Delta$ Nef or WT HIV viruses and cultured until close to 100% of cells were infected. Uninfected cells were labeled with CFSE, and  $0.5 \times 10^6$  uninfected cells were incubated with  $0.5 \times 10^6$  infected cells in 96-well plates, with 200  $\mu$ l total RPMI for the indicated timepoints, with or without cytochalasin D or piceatannol. Cells were then fixed, permeabilized, and stained for p24, then analyzed by flow cytometry to determine the transfer of HIV to target cells. Representative dot plots are shown gated for CFSE (target cells). **B.** The percentage of Gag<sup>+</sup> target cells was determined. **C.** A representation of the 24 hr timepoint is shown. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d and representative of 4 independent experiments.

**A****B****C**

**Figure 26. Nef effect on HIV replication kinetics in conditions favoring VS**

**formation.** **A.** Jurkat T cells were infected with either HIV $\Delta$ Nef or WT HIV viruses and cultured until close to 100% of cells were infected as determined by p24 staining. **B.**

Infected cells were washed and incubated with uninfected cells in a 1:3 ratio at a concentration of  $1 \times 10^6$  cells/ml in 24-well plates and virus production was monitored daily by RT assay of the supernatants. **C.** Infected cells were washed and incubated with uninfected cells as described above with the addition of 100  $\mu$ g/ml T-20. \*:  $p < 0.05$ .

Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

formation of virological synapses, and this enhancement leads to increased replication kinetics when cell-cell viral spread is the predominant mode of viral transmission.

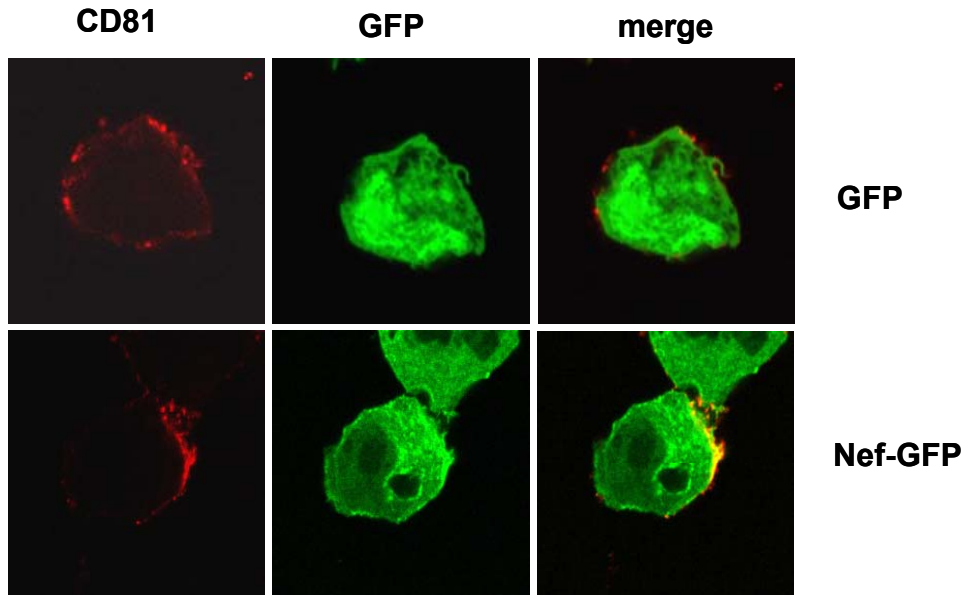
## **2.5 Nef induces polarization of VS components**

### *2.5.1 Polarization in transfected 293T cells*

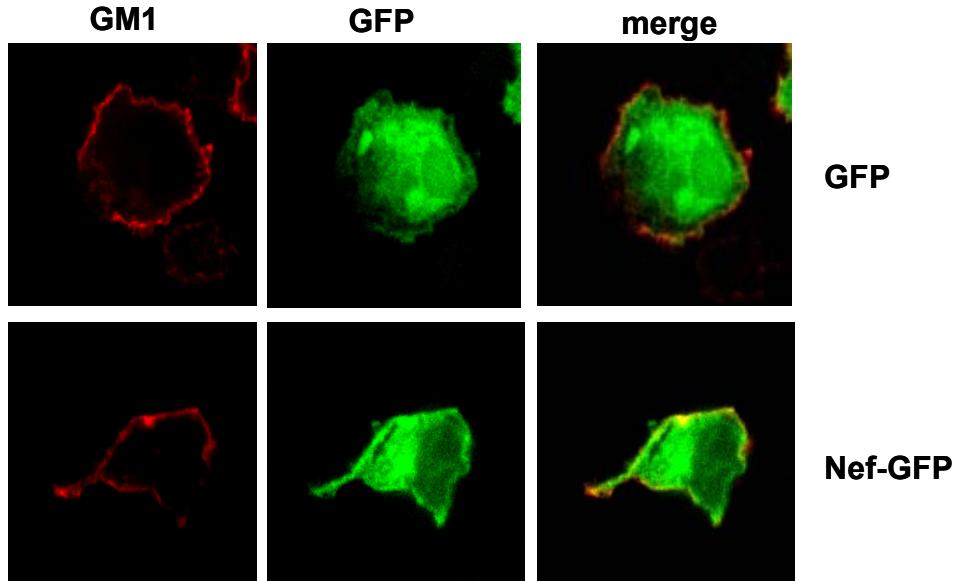
The next step was to investigate the differences between WT and HIV $\Delta$ Nef virological synapses that might account for the observed changes in VS formation and VS-dependent viral transfer. Because of the previously identified importance of GM1 and CD81 in both immunological and virological synapse formation, we first examined the effect of Nef on lipid raft and CD81 distribution. Thus, we transfected 293T cells with GFP or Nef-GFP and stained for either CD81 or GM1, a lipid raft marker. Confocal microscopy analysis of the transfected cells showed that while both CD81 and GM1 were evenly distributed around the cell in GFP-expressing cells, in the majority of Nef-expressing cells CD81 was polarized to one side of the cell (Fig. 27A) while GM1 tended to form one or two large clusters instead of many small, evenly distributed rafts as seen in GFP-expressing cells (Fig. 27B).

We next examined the membrane expression patterns of both CD81 and GM1 by flow cytometry. We reasoned that congregation of fluorophores exhibits brighter fluorescence intensity than evenly distributed fluorophores, and the resulting shift in fluorescence intensity detected by FACS would allow for a quantifiable measurement of changes of CD81 and GM1 expression patterns. We therefore transfected 293T cells with GFP or Nef-GFP, stained for CD81 or GM1, and measured the staining intensity of GFP<sup>+</sup> cells.

**A**



**B**



**Figure 27. Effect of Nef on distribution of virological synapse components as shown by confocal microscopy.** **A.** 293T cells were transfected with GFP or Nef-GFP. At 48 hr post-transfection,  $0.5 \times 10^6$  cells were seeded onto ploy-L-lysine coated coverslips and allowed to attach for 3 hr. The cells were then fixed and stained for CD81. **B.** GFP- and Nef-GFP-transfected cells were seeded onto coverslips and as above and stained for GM1. Data were representative of 3 independent experiments, with at least 75 cells examined for each condition.

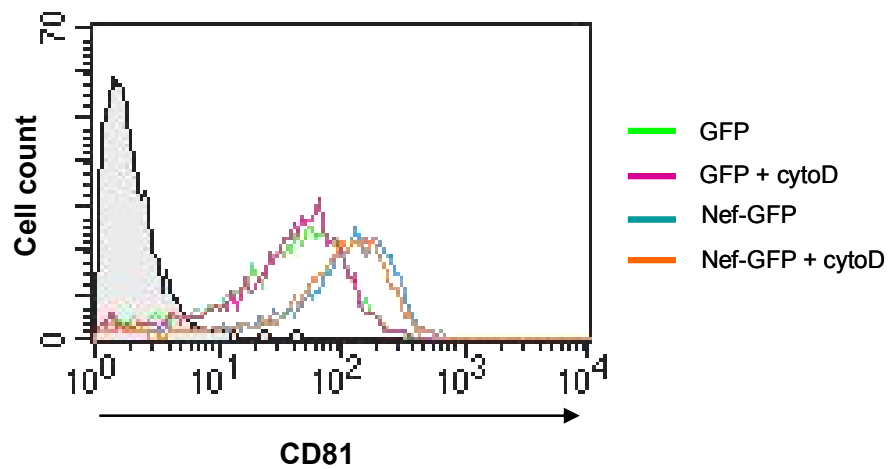


We found that expression of Nef led to an approximately 1.5-fold increase in the staining intensity of both CD81 (Fig. 28) and GM1 (Fig. 29). Since virological synapse formation is dependent upon cytoskeletal dynamics, we also treated a set of transfections with cytochalasin D for 1 hr before analysis. We found that cytochalasin D treatment did not affect polarization of either CD81 or GM1, indicating that retention of the polarized phenotype did not require actin remodeling, at least in short term. However, by the time cytochalasin D was added to these cells polarization had already been established, and 1 hr of treatment may not have been long enough for disassembly to occur. Longer treatment of transfected cells with cytochalasin D was not possible due to the cell type used in these experiments; adherent cells depend upon actin to remain attached to the plate and healthy, so longer treatment resulted in cell death. However, when we replicated these experiments in Jurkat cells later we were able to treat with cytochalasin D for 16 hr without adversely affecting the cells, and found that polarization was indeed actin-dependent.

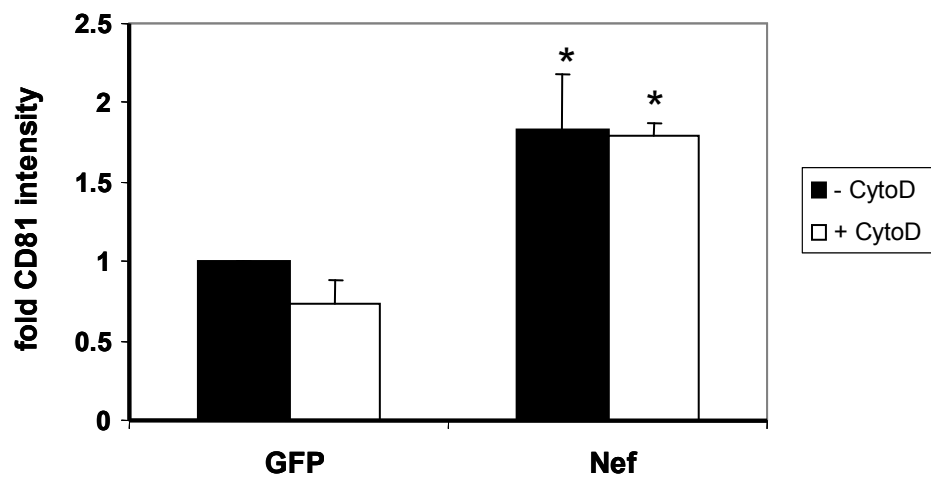
### *2.5.2 Surface localization of VS components*

Next, we needed to confirm that our flow cytometry polarization assay was an accurate measurement of polarization; namely, that Nef was not in fact altering the surface expression of either CD81 or GM1 and the increased intensity was indeed a result of polarization. To do this, we examined the total cellular expression of CD81 in GFP or Nef-GFP stable Jurkat cell lines and found that the expression of Nef does not affect the overall level of CD81 (Fig. 30A). We next examined just the membrane fraction of these cells to determine whether Nef affected the surface expression of CD81 or GM1, and

**A**

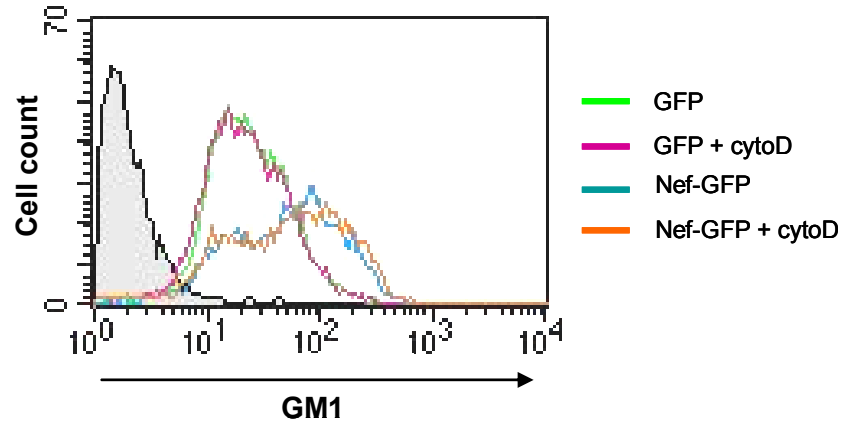


**B**

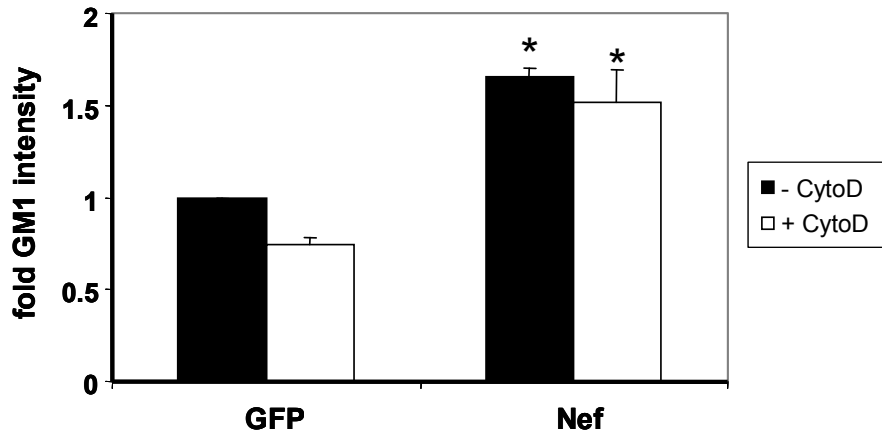


**Figure 28. Effect of Nef on the membrane distribution of CD81 as measured by flow cytometry.** **A.** 293T cells were transfected with GFP or Nef-GFP and harvested 48 hr later, with 1  $\mu$ M cytochalasin D added to one set of samples 1 hr prior to harvesting. Cells were fixed and stained for CD81, then gated for GFP<sup>+</sup> cells and analyzed by flow cytometry for CD81 intensity. **B.** The fold change in CD81 intensity was determined, with untreated GFP set to 1. \*: p<0.05. Data were mean  $\pm$  s.d and representative of 3 independent experiments.

**A**

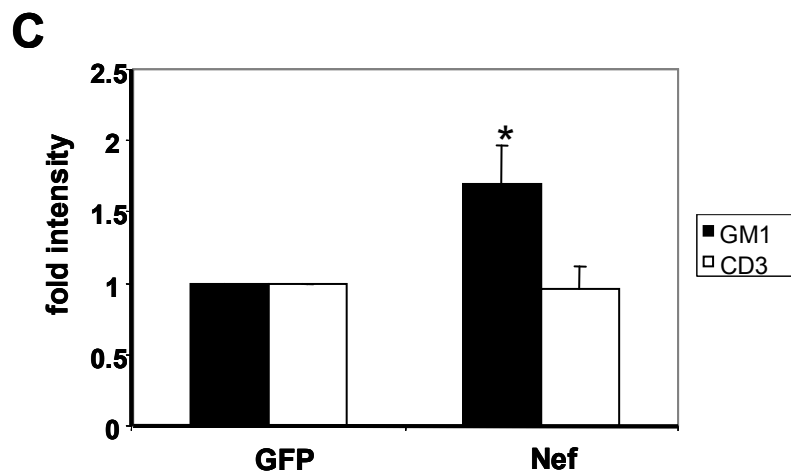
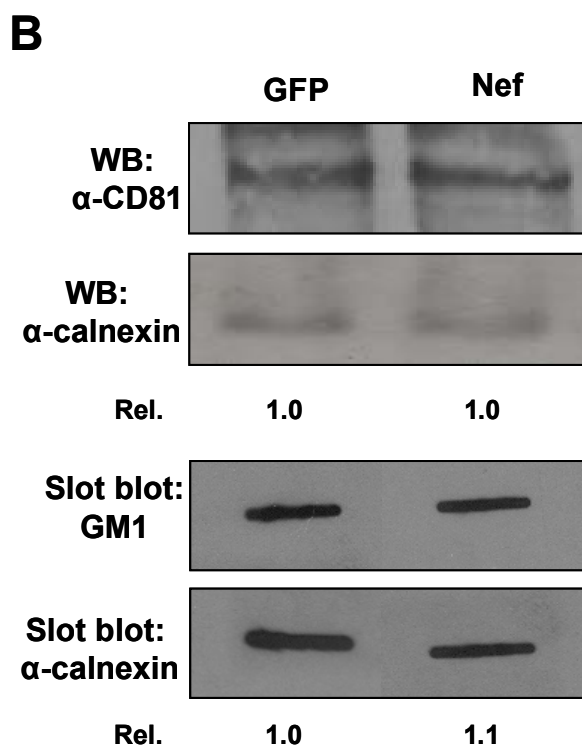
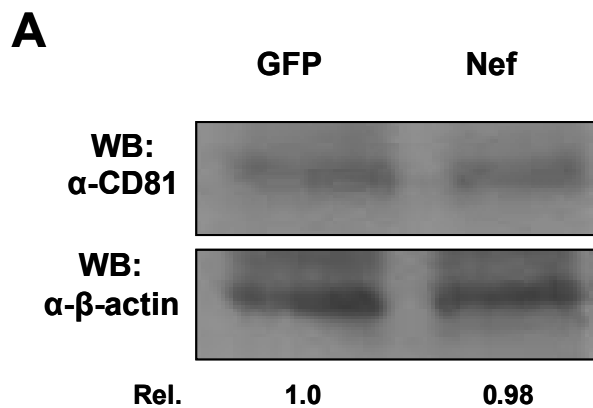


**B**



**Figure 29. Effect of Nef on the membrane distribution of GM1 as measured by flow**

**cytometry.** **A.** 293T cells were transfected with GFP or Nef-GFP and harvested 48 hr later, with 1  $\mu$ M cytochalasin D added to one set of samples 1 hr prior to harvesting. Cells were fixed and stained for GM1, then gated for GFP<sup>+</sup> cells and analyzed by flow cytometry for GM1 intensity. **B.** The fold change in GM1 intensity was determined, with untreated GFP set to 1. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.



**Figure 30. Effect of Nef on the total cellular and membrane expression of CD81 or**

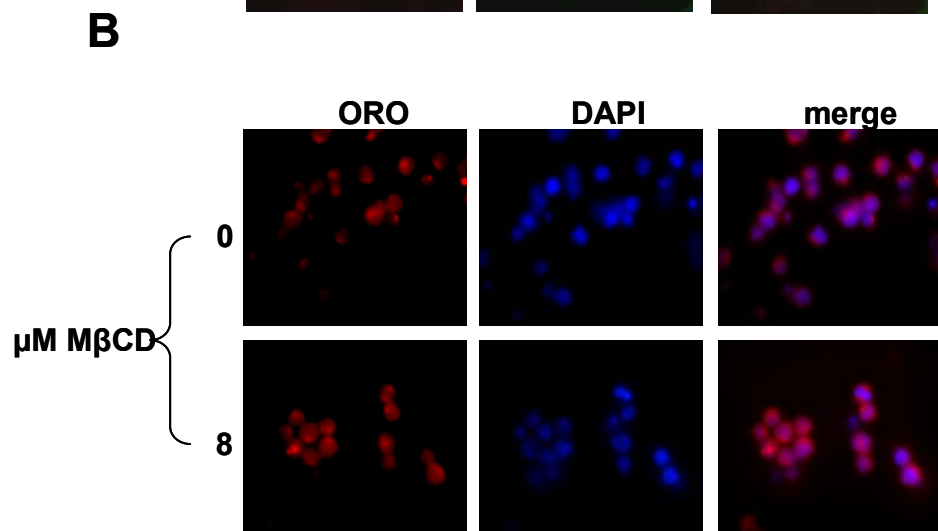
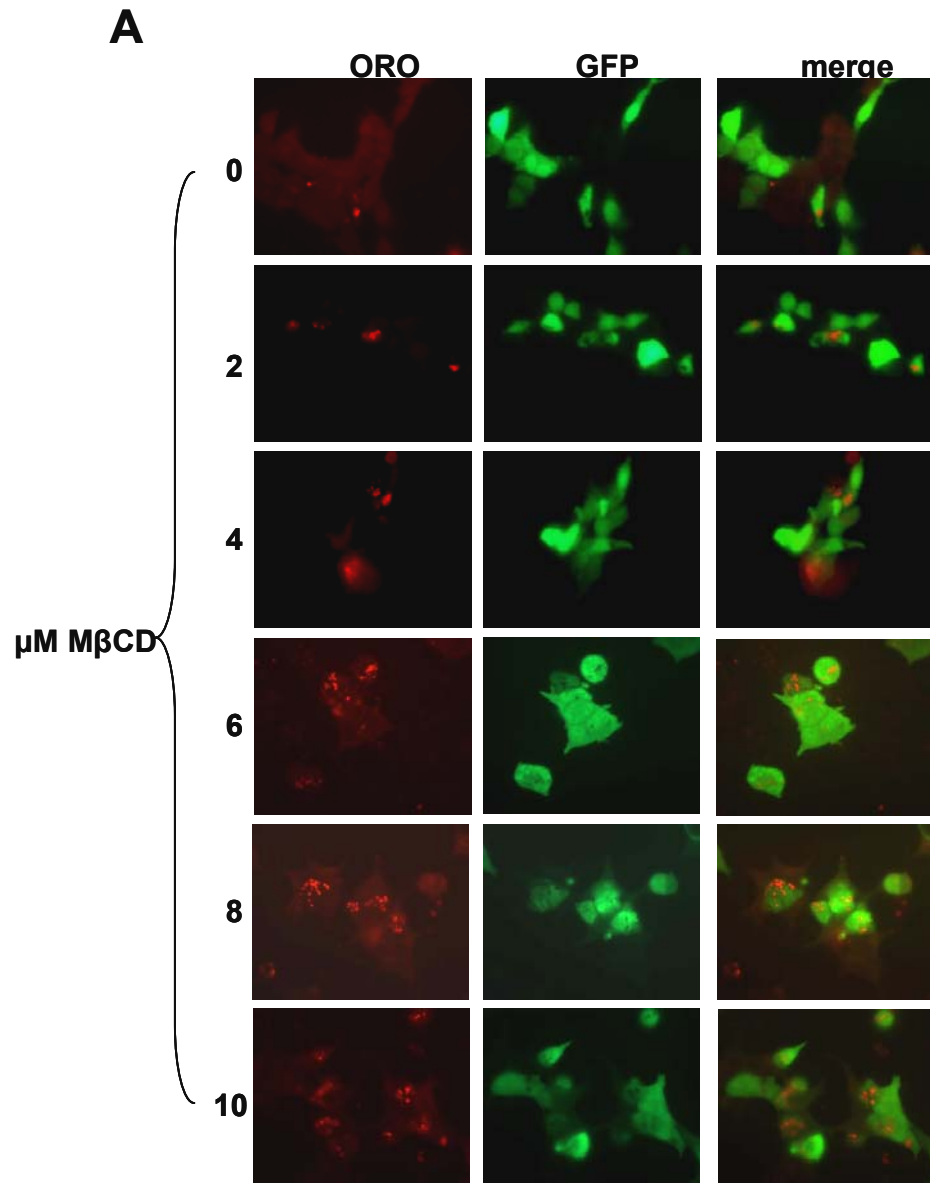
**GM1.** **A.** Jurkat cells stably expressing GFP or Nef-GFP were lysed and analyzed for CD81 expression, with  $\beta$ -actin used as a loading control. **B.** GFP and Nef-GFP stable cell lines were lysed in hypotonic buffer, and membrane fractions were obtained through further centrifugation of the supernatant. Membranes were analyzed for CD81 expression by Western blot and GM1 expression by slot blot, with calnexin used as a loading control. **C.** GFP and Nef-GFP stable cell lines were fixed and stained for GM1 or CD3 then gated for GFP<sup>+</sup> cells and analyzed by flow cytometry for GM1 or CD3 intensity. The fold change in GM1 or CD3 intensity was determined, with GFP set to 1. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 2 independent experiments.

found that there was again no detectable difference in either CD81 or GM1 surface expression (Fig. 30B). Additionally, we performed our GM1 polarization assay with CD3 as a control, and found that Nef expression did not increase CD3 intensity. Therefore, we concluded that our flow cytometry assay was indeed an accurate means of determining Nef-induced polarization of VS components and that the higher intensity was due to the increased brightness inherent in denser expression patterns as opposed to diffuse expression.

### *2.5.3 M $\beta$ CD titration*

In order to confirm our results indicating that Nef induces polarization of lipid rafts, we needed to confirm that the large clusters of GM1 staining observed were, in fact, lipid rafts and not staining artifacts. Methyl- $\beta$ -cyclodextrin M $\beta$ CD destabilizes lipid rafts by extracting the cholesterol from the membrane, and we utilized this as a negative control for GM1 staining. Once extracted from the membrane, lipids localize to cytosolic lipid droplets, so the effectiveness of M $\beta$ CD treatment can be determined by Oil Red O (ORO) staining (Subramaniam, Zhou et al. 2010). First we determined the appropriate concentration of (M $\beta$ CD) to use. Therefore, we seeded 293T cells onto poly-L-lysine treated coverslips, and treated them with increasing concentrations of M $\beta$ CD, followed by ORO staining (Fig. 31A). Lipid droplets started appearing at 6  $\mu$ M M $\beta$ CD, increased in number and size at 8  $\mu$ M M $\beta$ CD, and stayed about the same after that. However, at 10  $\mu$ M M $\beta$ CD there was noticeable cell death and unhealthy-looking cells, so we chose to use 8  $\mu$ M M $\beta$ CD in our experiments. We next treated Jurkat cells with 8  $\mu$ M M $\beta$ CD to confirm that this concentration also worked in these cells. Unfortunately, Jurkat cells





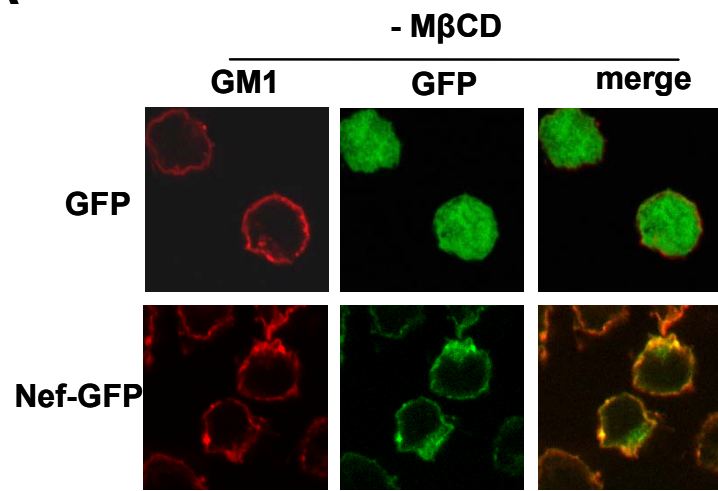
**Figure 31. M $\beta$ CD titration in 293T and Jurkat cells.** **A.** 293T cells were transfected with GFP. At 48 hr post-transfection,  $1 \times 10^6$  cells were seeded onto poly-L-lysine-coated coverslips and allowed to attach for 3 hr. Cells were treated with increasing concentrations of M $\beta$ CD for 1 hr, fixed, and stained by ORO. **B.**  $1 \times 10^6$  Jurkat cells were seeded onto poly-L-lysine-coated coverslips and treated with 8  $\mu$ M M $\beta$ CD for 1 hr, then fixed and stained by ORO, followed by DAPI. Data were representative of two independent experiments.

contain very little cytoplasm so lipid droplets were not easily detectable (Fig. 31B), but there was no cell death using this concentration of M $\beta$ CD.

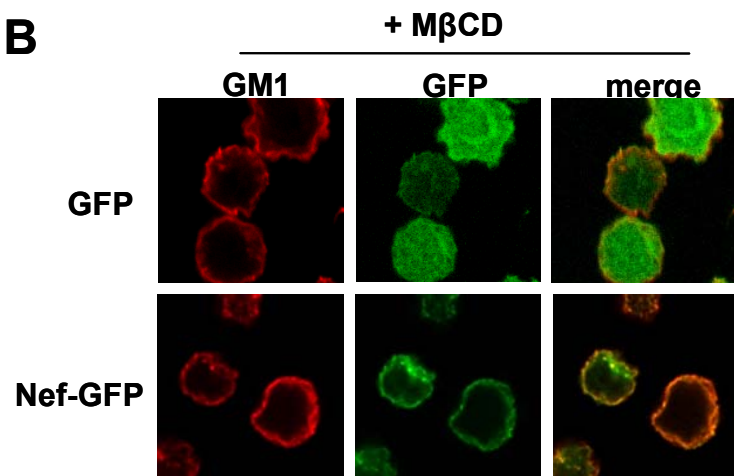
#### *2.5.4 Polarization of VS components in Jurkat T cells*

Since the biologically relevant cell type in HIV-1 infections are CD4<sup>+</sup> T lymphocytes, we confirmed that the Nef-induced polarization of CD81 and GM1 that we observed in transfected 293T cells was also present in Nef-expressing Jurkat cells. To confirm our GM1 staining in Jurkat cells, we took advantage of a previously constructed panel of Jurkat cell lines stably expressing Nef-mutant-GFP fusion proteins for further analysis of the effect of Nef on virological synapse components. We found that, as with the transfected cells, in the majority of Nef-expressing cells GM1 staining showed a large clustering of lipid rafts, whereas GFP-expressing cells exhibited a uniform distribution of small rafts along the cell surface (Fig. 32A). To ensure that these bright spots were indeed large clusters of lipid rafts, we also treated the cells with (M $\beta$ CD), which destabilizes lipid rafts by extracting the cholesterol from the membrane. In the M $\beta$ CD-treated cells GM1 was found to be uniformly distributed around the membrane of both GFP- and Nef-expressing cells, indicating that the large GM1 spots were actually rafts (Fig. 32B). Upon staining with CD81, confocal microscopy revealed that CD81 exhibits polarization similar to that of GM1 in Nef-expressing cells (Fig. 33A). To ensure that this phenomenon was specific to virological synapse components and not a general polarization of cell surface proteins, we also stained for surface CD3, which has been shown to be absent from virological synapses and is not downregulated by Nef under

**A**

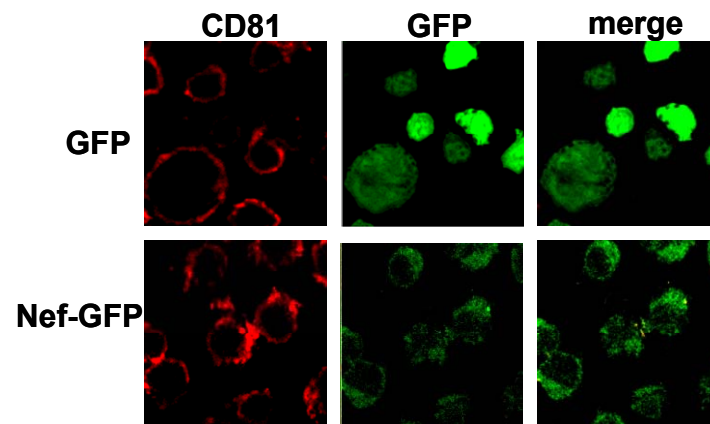


**B**

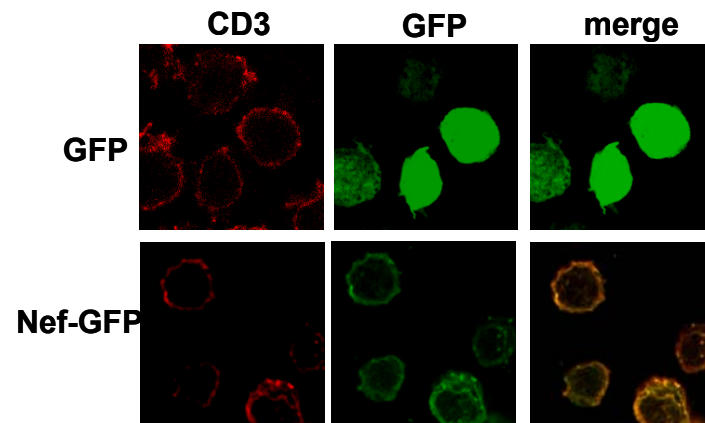


**Figure 32. Nef effect on the distribution of GM1 in Jurkat cells as shown by confocal microscopy.** **A.** Jurkat cells stably expressing GFP or Nef-GFP were seeded onto ploy-L-lysine coated coverslips at a density of  $1 \times 10^6$  cells/ml and allowed to attach for 1.5 hr, then fixed and stained for GM1. **B.** GFP- and Nef-GFP-expressing cells were seeded onto coverslips as above, treated with 8 mM M $\beta$ CD for 1 hr, and stained for GM1. Data were representative of 3 independent experiments.

**A**



**B**



**Figure 33. Nef effect on the distribution of CD81 in Jurkat cells as shown by**

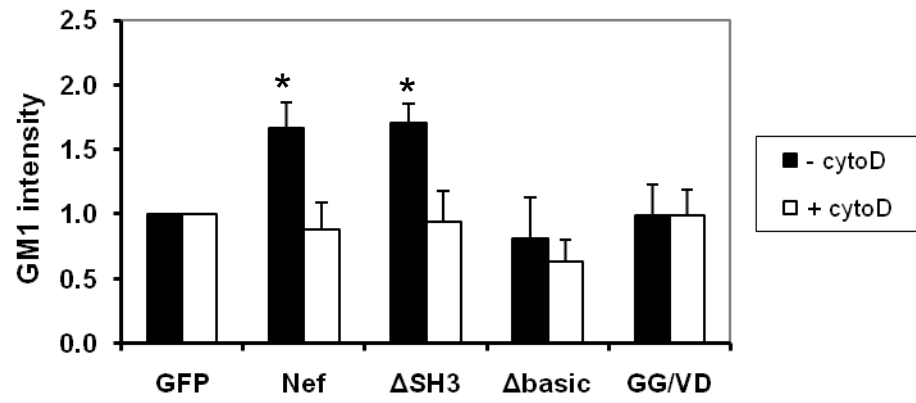
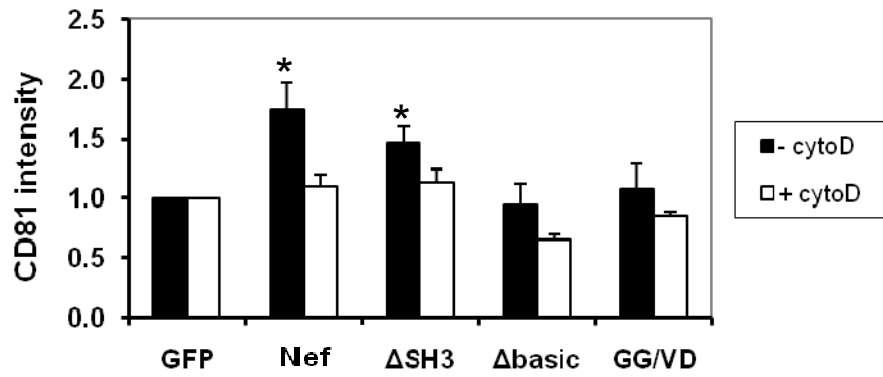
**confocal microscopy.** **A.** Jurkat cells stably expressing GFP or Nef-GFP were seeded onto poly-L-lysine coated coverslips at a density of  $1 \times 10^6$  cells/ml and allowed to attach for 1.5 hr, then fixed and stained for CD81. **B.** GFP- and Nef-GFP-expressing cells were seeded onto coverslips as above and stained for CD3. Data were representative of 3 independent experiments.

normal circumstances. The surface distribution of CD3 was not affected by Nef expression, suggesting that polarization is specific to VS components (Fig. 33B).

We next confirmed GM1 and CD81 polarization in Jurkat cells using our flow cytometry polarization strategy. We examined the GM1 and CD81 intensity of all five of our stable Jurkat cell lines. Both WT Nef and the SH3-binding domain mutant (Nef $\Delta$ SH3) showed significantly higher GM1 (Fig. 34A) and CD81 (Fig. 34B) intensity than the GFP control. Conversely, removal of either the N-terminal basic region (Nef $\Delta$ basic) or the myristoylation site (NefGG/VD) resulted in GM1/CD81 intensity similar to that of the GFP control, indicating that both the basic region of Nef and its myristoylation, but not the Nef SH3-binding domain, are necessary for Nef-induced lipid raft clustering.

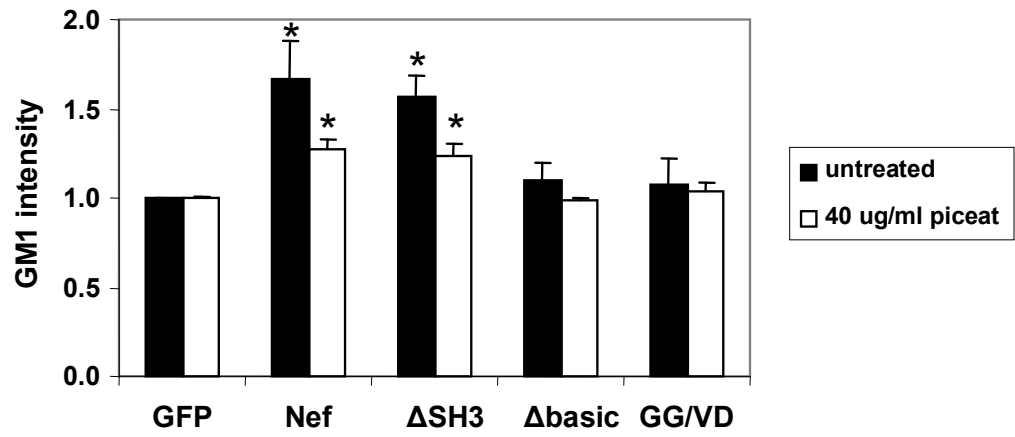
Since Jurkat cells are not as dependent upon the actin cytoskeleton to remain healthy as 293T cells, we were able to determine the contribution of actin reorganizations to polarization by cytochalasin D treatment for longer timepoints. We found that after 16 hr incubation with cytochalasin D all GM1 and CD81 clustering was abolished, indicating that this polarization is indeed driven by actin reorganizations. Because ZAP70 activation is required for VS-dependent transfer, we then determined whether the polarization of VS components would be affected by piceatannol treatment. We performed the flow cytometry polarization assay after treatment with 30  $\mu$ g/ml piceatannol and found that while GM1 showed decreased intensity changes upon piceatannol treatment, it was still significantly higher than that of GFP-expressing cells (Fig. 35A). However, upon close examination, we found that the overall GM1 intensity



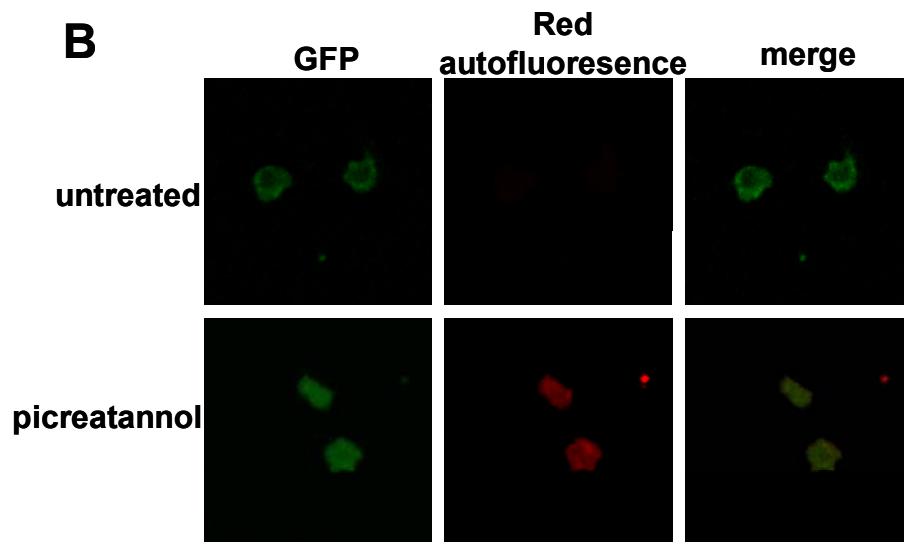
**A****B**

**Figure 34. Effect of Nef on the membrane distribution of GM1 and CD81 in Jurkat cells as measured by flow cytometry.** **A.** Jurkat cells stably expressing GFP, Nef-GFP, or Nef-GFP mutants were seeded into 24-well plates at a density of  $1 \times 10^6$  cells/ml and incubated overnight with or without 1  $\mu$ M cytochalasin D. Cells were fixed and stained for GM1, then gated for GFP<sup>+</sup> cells and analyzed by flow cytometry for GM1 intensity. The fold change in GM1 intensity was determined, with untreated GFP set to 1. **B.** Stable Jurkat cell lines were treated as above but stained with CD81, and the fold change in CD81 intensity was determined, with untreated GFP set to 1. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 4 independent experiments.

**A**



**B**

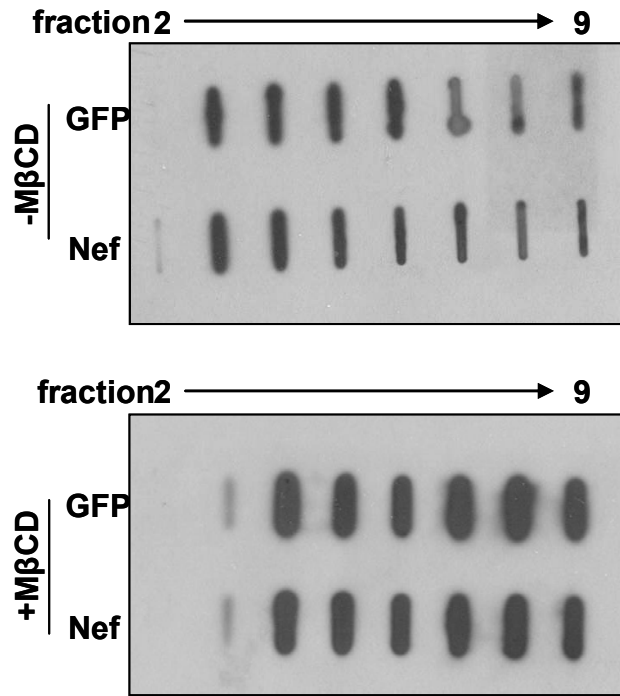
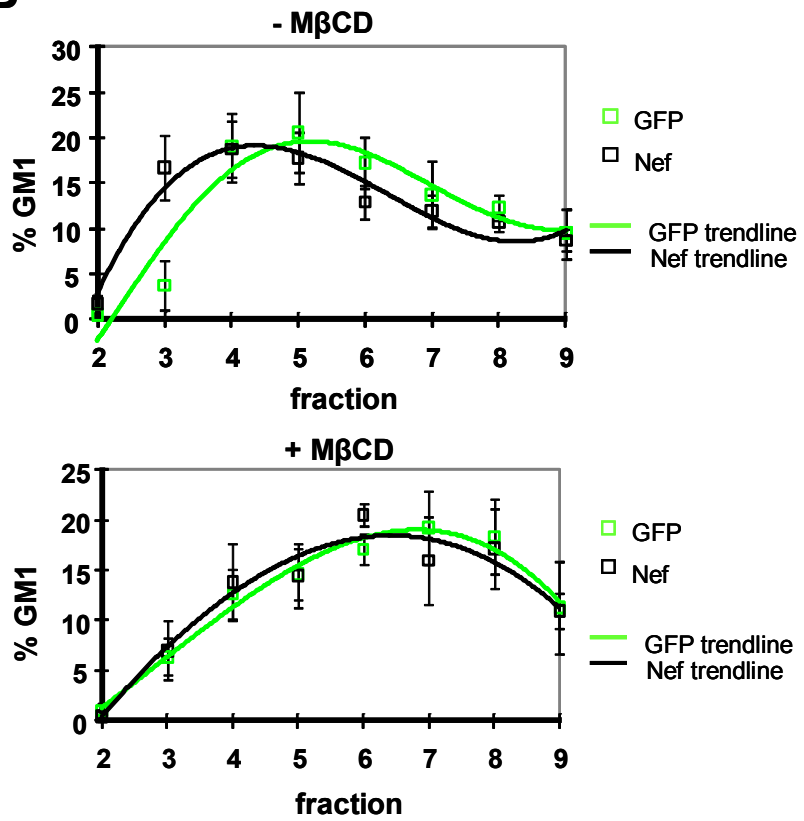


**Figure 35. Effect of piceatannol on Nef-induced alteration in GM1 membrane**

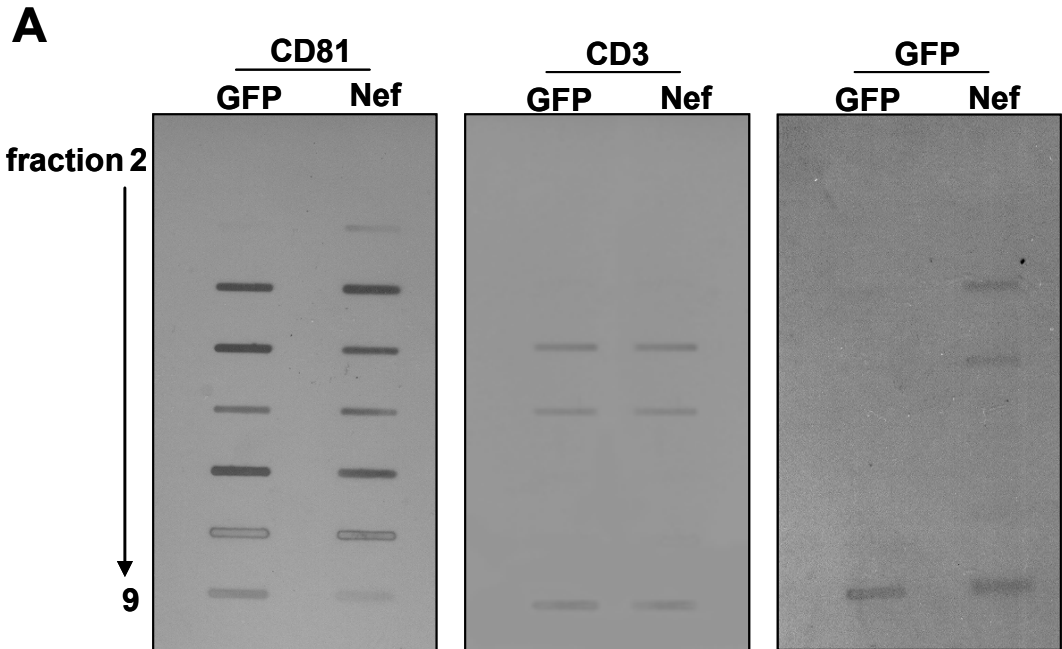
**distribution.** **A.** Jurkat cells stably expressing GFP, Nef-GFP, or Nef-GFP mutants were seeded into 24-well plates at a density of  $1 \times 10^6$  cells/ml and incubated overnight with or without 30  $\mu\text{g/ml}$  piceatannol. Cells were fixed and stained for GM1, then gated for GFP<sup>+</sup> cells and analyzed by flow cytometry for GM1 intensity. The fold change in GM1 intensity was determined, with untreated GFP set to 1. **B.** Stable Jurkat cell lines were treated with piceatannol as above, then seeded onto poly-L-lysine coated coverslips, allowed to attach for 1.5 hr, and fixed. Autofluorescence was detected using confocal microscopy. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 2 independent experiments.

of GFP-expressing cells was significantly higher after piceatannol treatment as compared to untreated cells, indicating that treatment increased the background intensity of GM1 staining. Since this assay measures small changes in the intensity of GM1 staining with and without Nef, any increase in the background level of staining would result in a smaller fold increase in intensity. This likely is what resulted in the lower change in GM1 intensity in piceatannol-treated Nef-expressing cells. To confirm that piceatannol causes autofluorescence, we examined piceatannol-treated GFP-expressing cells using confocal microscopy and found that while unstained, untreated GFP-expressing cells did not show any red color, the unstained treated cells did show easily detectable red fluorescence (Fig. 35B).

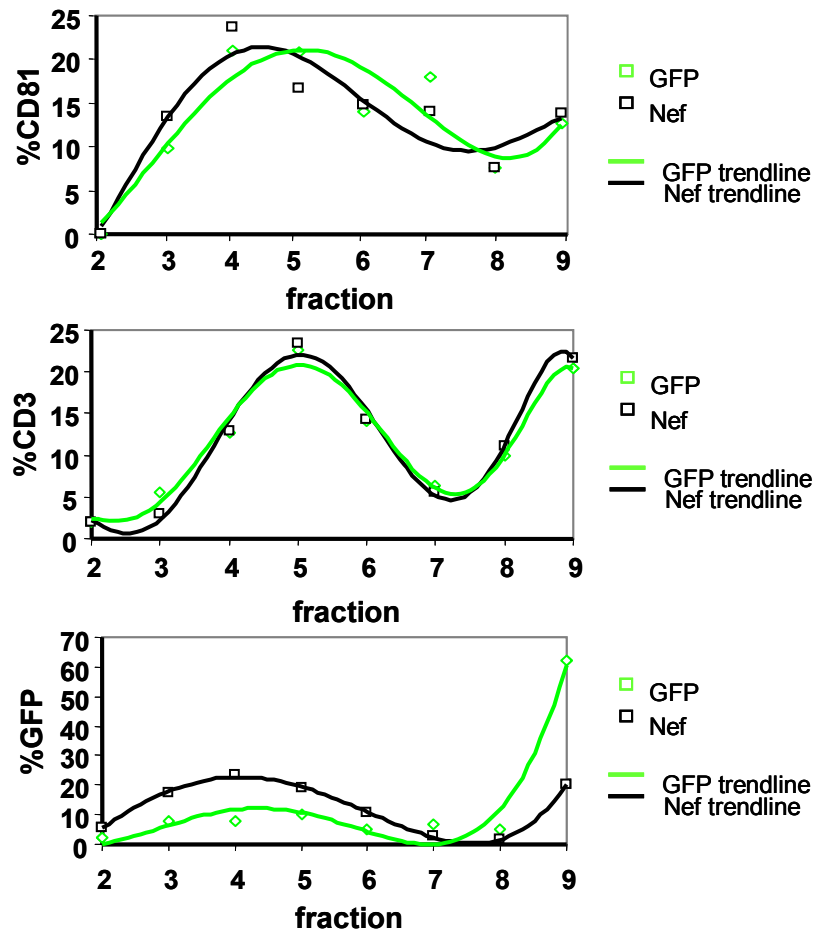
As an additional confirmation of the effect of Nef on lipid raft distribution, we next performed membrane fractionation or raft floatation assays. Slot blot analysis of membrane fractions showed that a larger percentage of GM1 localized to less dense fractions in Nef-expressing cells as compared to GFP-expressing cells (Fig. 36). Since lipid rafts localize to higher fractions due to their decreased density as compared to the surrounding non-raft membrane, the larger percentage of GM1 in the less dense fractions further supports the existence of larger lipid rafts, as larger rafts will exhibit an even greater decrease in density as compared to non-raft membranes. Since CD81 is known to localize largely to lipid rafts, we were also able to use this assay to confirm the effects of Nef on CD81 polarization. We found that CD81 also localized more to less dense fractions in Nef-expressing cells as compared to GFP-expressing cells (Fig 37). In contrast, CD3 distribution was not affected and remained within smaller rafts. Analysis

**A****B**

**Figure 36. Membrane fractionation in the presence and absence of Nef.** **A.** Jurkat cells stably expressing GFP or Nef-GFP were seeded into 6-well plates at a density of  $1 \times 10^6$  cells/ml and incubated overnight. One hour prior to harvesting, control cells were treated with  $8 \mu\text{M}$  M $\beta$ CD. The cells were cooled on ice for 20 min, then lysed in lipid raft lysis buffer and loaded onto a sucrose gradient for lipid raft fractionation, with 0.5 ml aliquots taken from the top after centrifugation. Aliquoted fractions were analyzed by GM1 slot blot. **B.** The percentage of total GM1 in each fraction was determined. Data were representative of 4 independent experiments.



**B**





**Figure 37. CD81 localization during membrane fractionation in the presence of Nef.**

**A.** Jurkat cells stably expressing GFP or Nef-GFP were seeded into 6-well plates at a density of  $1 \times 10^6$  cells/ml and incubated overnight. The cells were cooled on ice for 20 min, then lysed in lipid raft lysis buffer and loaded onto a sucrose gradient for lipid raft fractionation, with 0.5 ml aliquots taken from the top after centrifugation. Aliquoted fractions were analyzed by slot blot for CD81, CD3, and GFP. **B.** The percentage of total CD81, CD3, and GFP in each fraction was determined. Data were representative of 2 independent experiments.

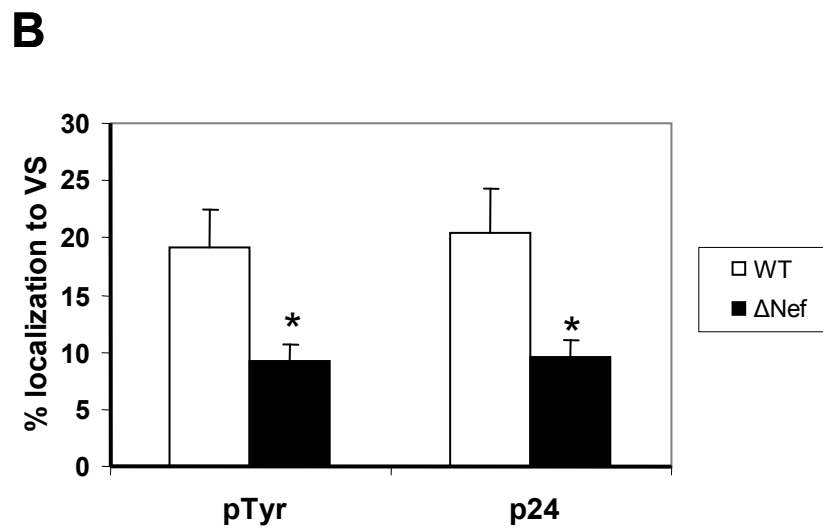
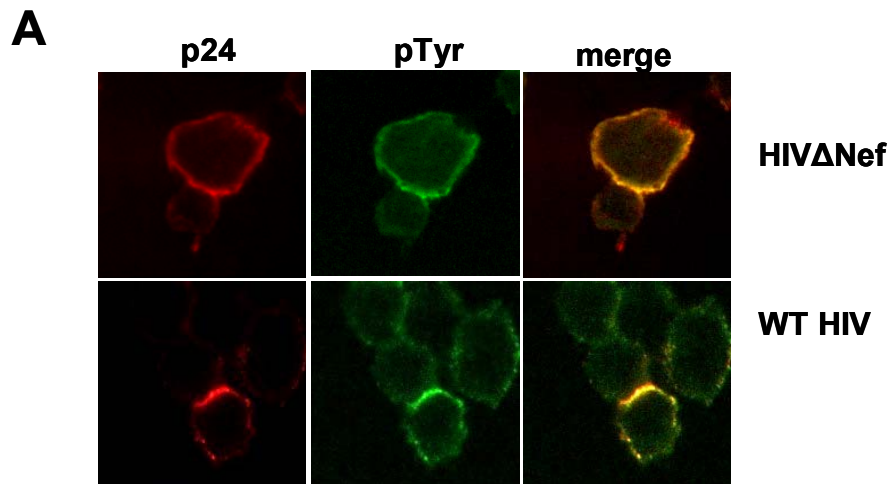
of these fractions for GFP shows that GFP is almost exclusively present in the cytoplasm, while Nef-GFP localized strongly to lipid rafts, although it is also present to a great extent in the cytoplasm (Fig. 37).

#### *2.5.5 Polarization of ZAP70*

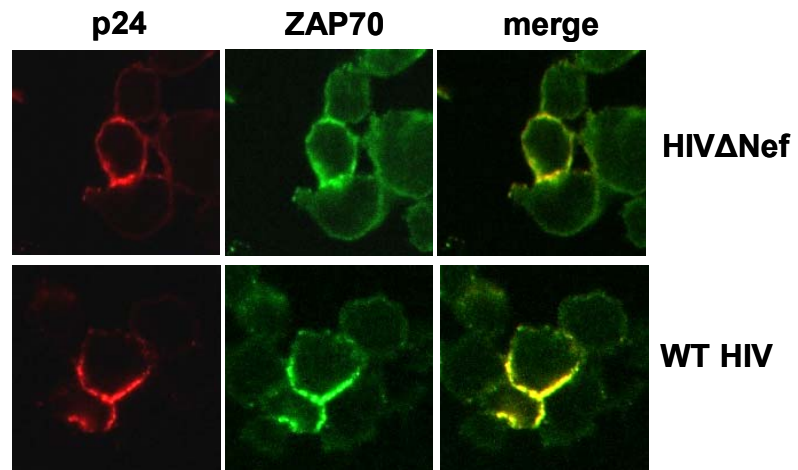
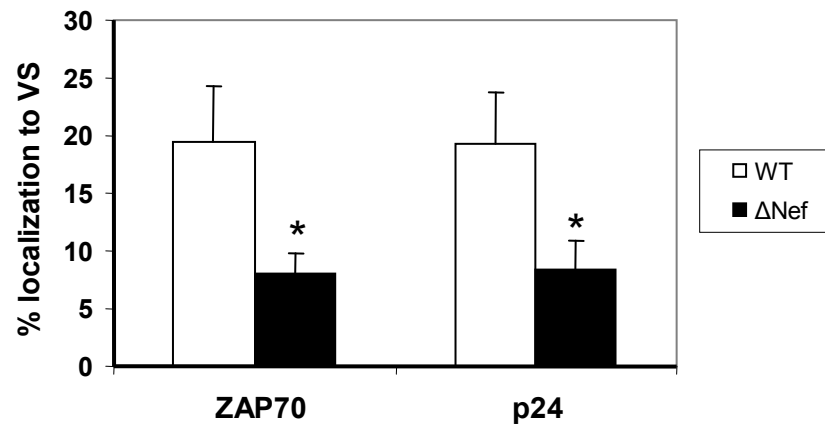
At this point, all the VS components examined were membrane molecules. Therefore, we then determined what effect Nef had on the localization of intracellular virological synapse components. ZAP70 tyrosine kinase is the only intracellular host protein other than actin that is known to be involved in the virological synapse; additionally, Nef is known to affect the distribution of ZAP70 during a signaling cascade resulting in MHC I downregulation. Therefore, we examined the cellular distribution of both ZAP70 and total phosphotyrosine in cells involved in virological synapses. We found that pTyr localized to the VS significantly more efficiently in WT HIV infected cells than in HIV $\Delta$ Nef infected cells (Fig. 38). Interestingly, p24 showed a similar pattern, suggesting that the two were recruited to the VS together. The pTyr localized to virological synapses is at least partially comprised of ZAP70, as ZAP70 staining shows an identical pattern of polarization (Fig. 39). This indicates that Nef expression alters the distribution of intracellular VS components as well as that of surface components.

### **2.6 Nef induces formation of actin protrusions**

Because of the importance of actin dynamics in both virological synapse formation and Nef-induced polarization of VS components we examined the distribution of F-actin, the form of actin responsible for the formation of membrane structures such as filopodia and



**Figure 38. Nef effect on the localization of pTyr to virological synapses.** **A.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% of cells were infected. Uninfected cells were incubated with infected cells in a 1:1 ratio, with  $1 \times 10^6$  cells in 200  $\mu$ l RPMI for 1.5 hr on poly-L-lysine treated coverslips to allow for VS formation. Cells were then fixed, permeabilized, and stained for p24 and total pTyr. **B.** The percentage of total pTyr that localized to the VS was quantified using Metamorph software. \*:  $p < 0.01$ . Data were mean  $\pm$  s.d. and representative of 4 independent experiments.

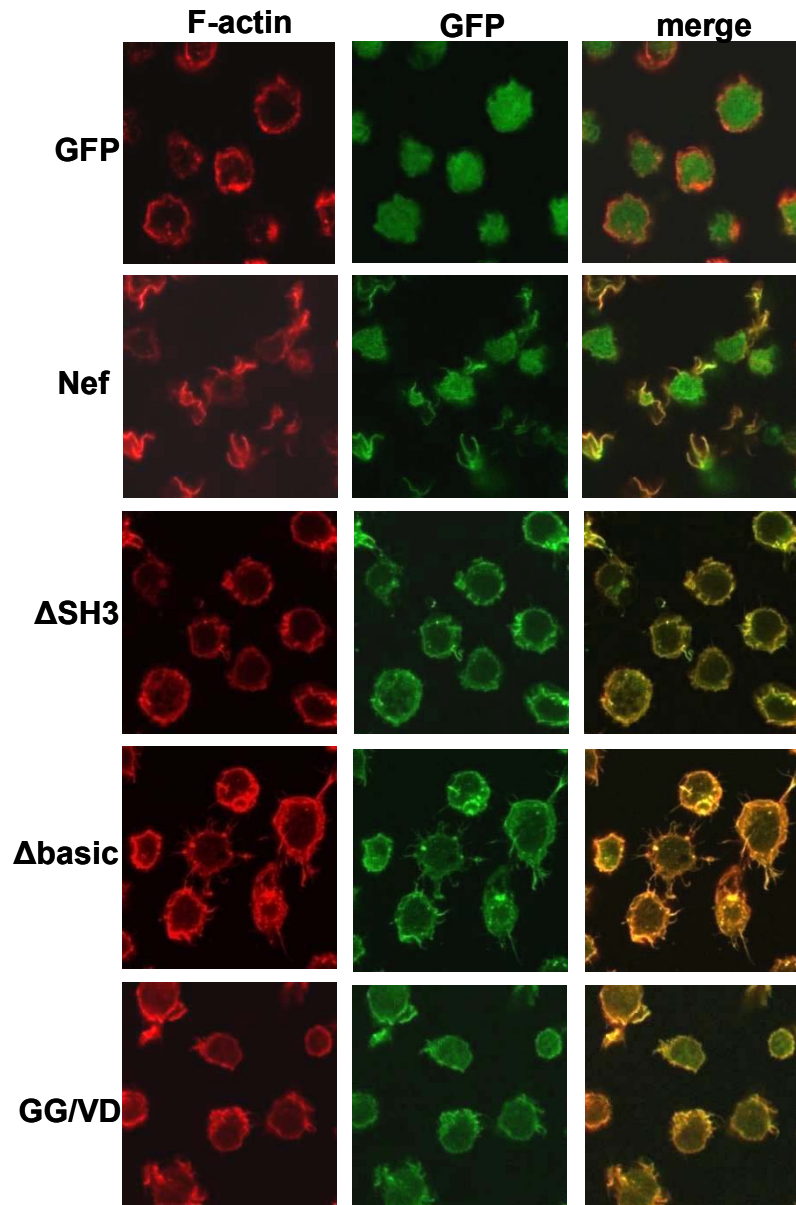
**A****B**

**Figure 39. Nef effect on the localization of ZAP70 to virological synapses.** **A.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% of cells were infected. Uninfected cells were incubated with infected cells in a 1:1 ratio, with  $1 \times 10^6$  cells in 200  $\mu$ l RPMI for 1.5 hr on poly-L-lysine treated coverslips to allow for VS formation. Cells were then fixed, permeabilized, and stained for p24 and ZAP70. **B.** The percentage of total ZAP70 that localized to the VS was quantified using Metamorph software. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 4 independent experiments.

ruffles, in each of the mutant Nef cell lines and found that Nef induced actin protrusions (Fig. 40A). These structures have since been reported in multiple studies, and have been shown to transfer Nef to target cells (Nobile, Rudnicka et al.; Xu, Santini et al. 2009). Our data further reveals that the SH3-binding domain of Nef and its myristoylation are required for formation of these protrusions. Interestingly, both the protrusions formed in Nef-expressing cells and the abortive protrusions formed in Nef $\Delta$ SH3-expressing cells appeared to be localized predominantly to one side of the cell. However, while the Nef $\Delta$ basic cells were capable of forming actin protrusions, they were much thinner than those in WT Nef-expressing cells and were evenly distributed around the cell. This supports our data that the basic region of Nef is involved in polarizing cells (Fig. 34) and also suggests that there is a separate mechanism responsible for the formation of actin protrusions as the requirements for these two processes are different.

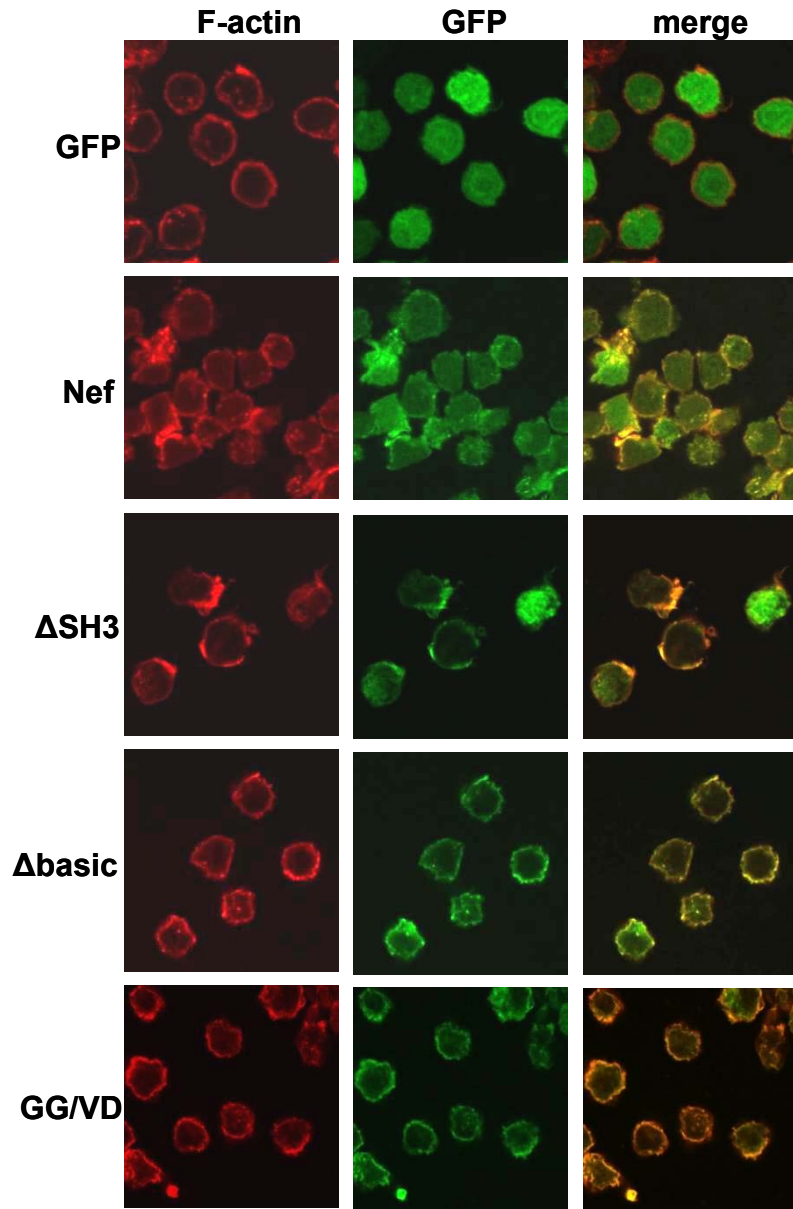
We theorized that these actin protrusions could be involved in either the establishment of virological synapses or the transfer of virions through virological synapses. Therefore, because of its involvement in viral transfer via VS, we wanted to determine the importance of ZAP70 on the formation of these protrusions. We found that treatment of piceatannol completely abolished all actin protrusions (Fig. 40B), indicating that ZAP70 was necessary for their formation and supporting the theory that they are indicative of VS transfer. Furthermore, close examination of the confocal data shows that although there are no protrusions formed after piceatannol treatment, F-actin distribution follows the same pattern as mentioned above; namely, that in WT Nef and Nef $\Delta$ SH3-expressing cells actin is polarized while Nef $\Delta$ basic cells show an even distribution of F-actin around the

**A**





**B**



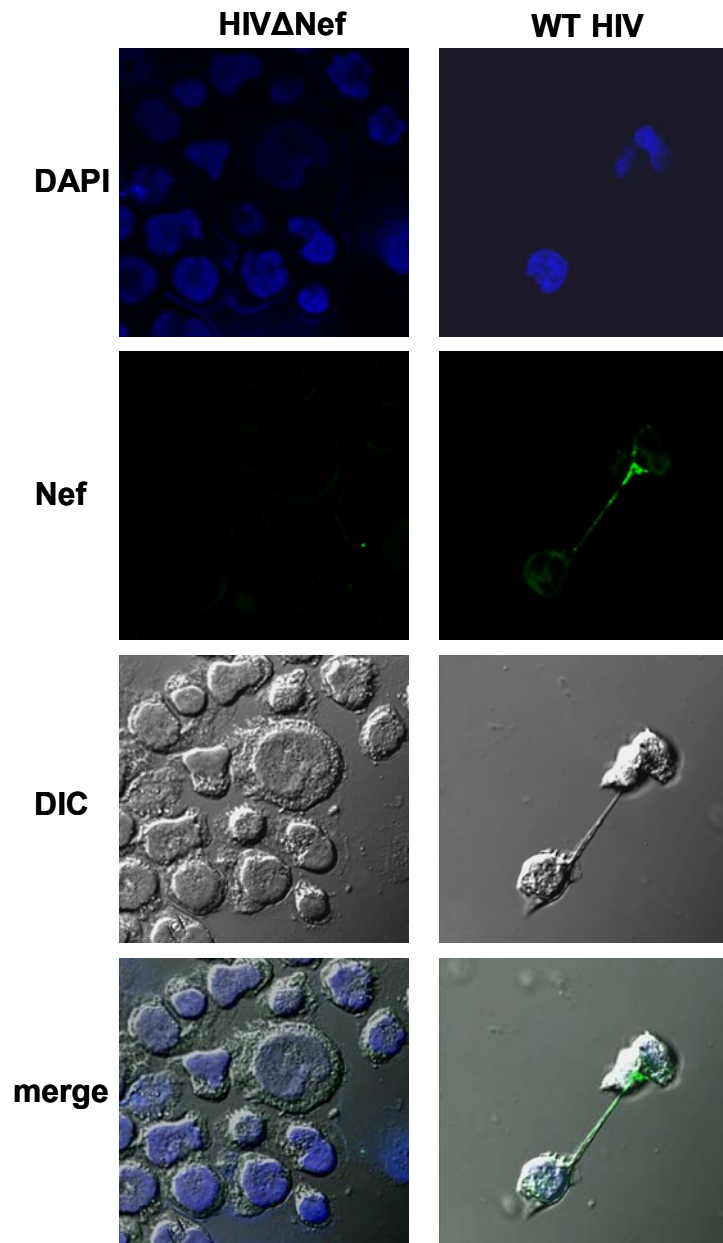
**Figure 40. Nef effect on actin organization.** **A.** Jurkat cells stably expressing GFP or Nef-GFP mutants were seeded onto poly-L-lysine-coated coverslips at a density of  $1 \times 10^6$  cells/ml and allowed to adhere for 1.5 hr, then fixed, permeabilized, and stained for F-actin. **B.** Stable Jurkat cell lines were treated with 30  $\mu\text{g/ml}$  piceatannol for 24 hr at a density of  $1 \times 10^6$  cells/ml, then seeded onto coverslips and stained as described above. Data were representative of 3 independent experiments.

periphery of the cell. This further confirms the requirements for Nef-mediated polarization of VS components and also confirms that there are separate requirements for polarization and transfer.

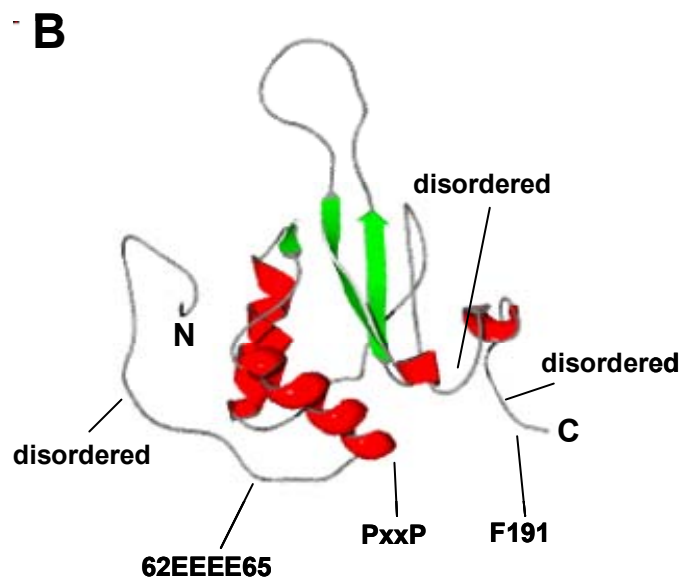
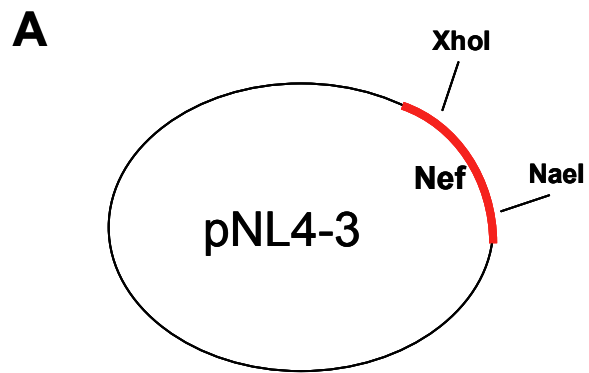
In an effort to confirm that these protrusions form in infected cells and that they are the same that are responsible for transferring Nef to target cells, we infected Jurkat cells with WT HIV and HIV $\Delta$ Nef, co-cultured them with target cells, and then stained for Nef (Fig. 41). When infected and uninfected cells were in close contact very few Nef-containing protrusions were observed, but when the cell density was lower the number of protrusions increased until approximately 5% of WT HIV infected cells exhibited protrusions connecting them to uninfected cells. We were able to detect Nef within the protrusions, and these were only found in WT HIV-infected cells as shown previously (Xu, Santini et al. 2009). Therefore, we concluded that the Nef-induced protrusions observed here are a means of transferring materials from the infected cell to an uninfected bystander cell.

## **2.7 VS formation using Nef mutant viruses**

Based on these findings we constructed a series of replication-competent Nef mutant viruses to further elucidate the mechanism of Nef enhancement of VS-dependent viral transfer (Fig. 42). Using these mutants we examined both the percentage of infected cells involved in synapses (Fig. 43) and the Gag transfer to target cells (Fig. 44). Our data shows that Nef-mediated recruitment of Vav (HIV.nef-*F191A*) and a functional SH3-binding domain (HIV.nef-*AxxA*) are necessary for proper VS formation and viral transfer. Interestingly, even though Nef results in greater localization of ZAP70 to the VS as



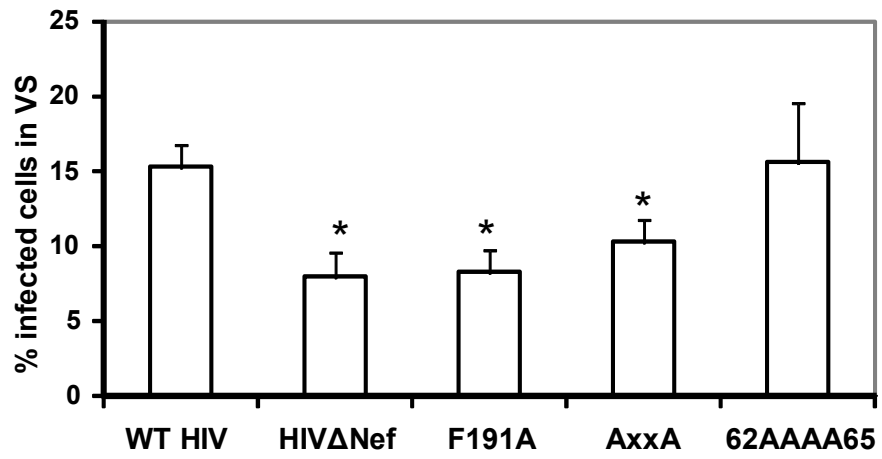
**Figure 41. Nef transfer to neighboring cells.** Jurkat cells were infected with WT HIV or HIV $\Delta$ Nef and cultured until close to 100% of cells were infected. Infected cells were washed and incubated with uninfected cells in a 1:1 ratio,  $1 \times 10^6$  total cells in 200  $\mu$ l RPMI, for 4 hr on poly-L-lysine treated coverslips to allow for Nef transfer. Cells were then fixed, permeabilized, and stained for Nef, then stained with DAPI. Data were representative of 2 independent experiments.



**C**

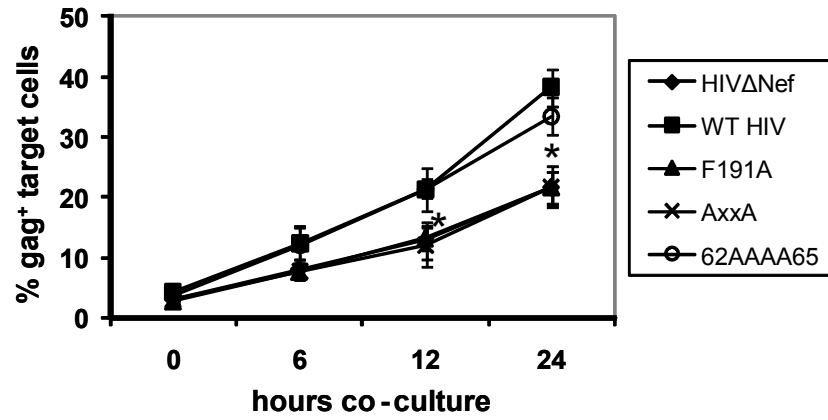
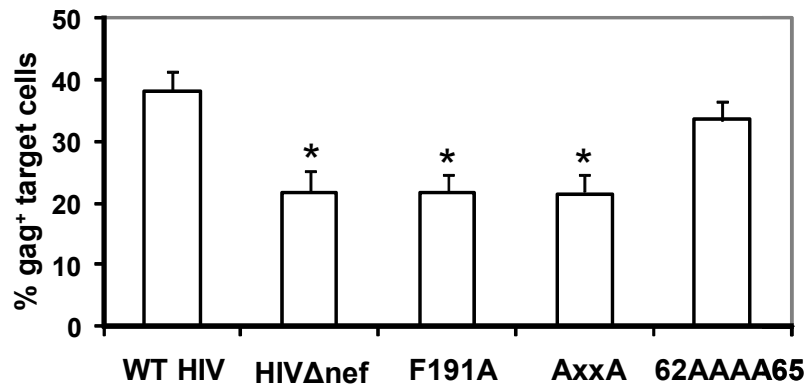
Target	Mutation	Function affected
62EEEE65	62AAAA65	PACS-2-dependent ZAP70 recruitment to MHC I downregulation cascade
PxxP	AxxA	SH3-binding
F191	F191A	Recruitment of Vav to microdomains for signaling

**Figure 42. Replication-competent Nef mutant viruses.** **A.** Plasmid map, **B.** mutation site, and **C.** function affected for replication-competent Nef mutant virus constructs. Nef structure adapted from Doms and Trono 2000 who compiled the proposed structure based on data from Grzesiek, Bax et al. 1996; Lee, Saksela et al. 1996; Barnham, Monks et al. 1997.





**Figure 43. Nef residues important for VS formation.** Jurkat cells were infected with WT HIV, HIV $\Delta$ Nef, or each of the Nef mutant viruses and cultured until close to 100% of cells were infected. Uninfected cells were labeled with CFSE and incubated with infected cells in a 1:1 ratio,  $1 \times 10^6$  total cells in 200  $\mu$ l RPMI, for 1.5 hr on poly-L-lysine treated coverslips to allow for VS formation. Cells were then fixed, permeabilized, and stained for p24, and the percentage of infected cells involved in a VS was determined for each virus. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

**A****B**

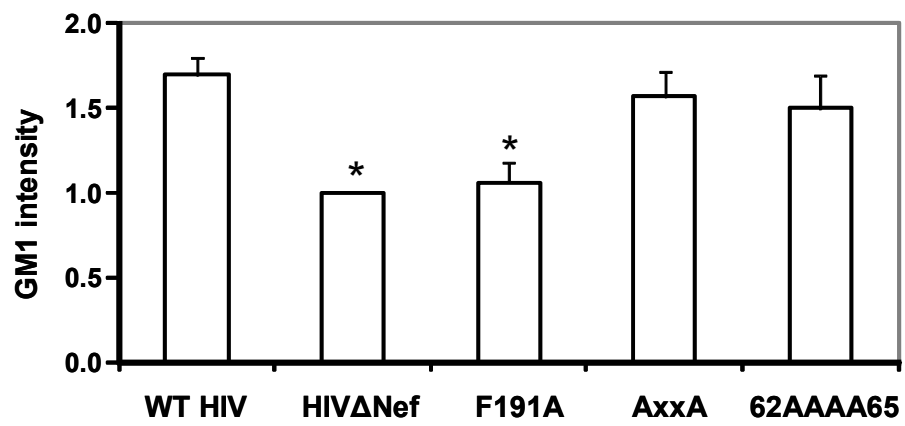
**Figure 44. Nef residues important for VS-dependent virus transfer.** **A.** Jurkat cells were infected with HIV $\Delta$ Nef, WT HIV, or Nef mutant viruses and cultured until close to 100% of cells were infected. Uninfected cells were labeled with CFSE and incubated with infected cells in a 1:1 ratio,  $1 \times 10^6$  total cells in 200  $\mu$ l RPMI in 96-well plates, for the indicated timepoints. Cells were then fixed, permeabilized, and stained for p24, then analyzed by flow cytometry to determine the percentage of Gag<sup>+</sup> target cells. **B.** A representation of the 24 hr timepoint is shown. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

shown above, direct Nef-mediated recruitment of ZAP70 (HIV.nef-62AAAA65) is not necessary for VS formation. This suggests that there is an indirect mechanism for ZAP70 recruitment to the virological synapse, possibly via its localization to lipid rafts.

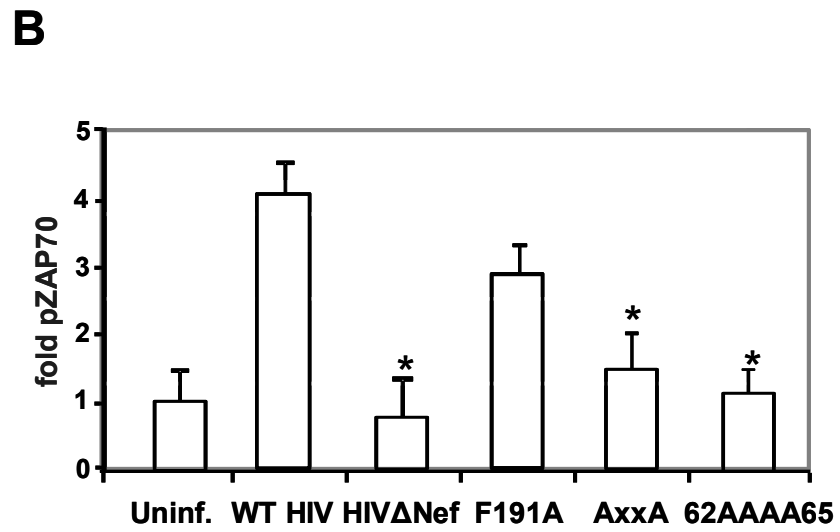
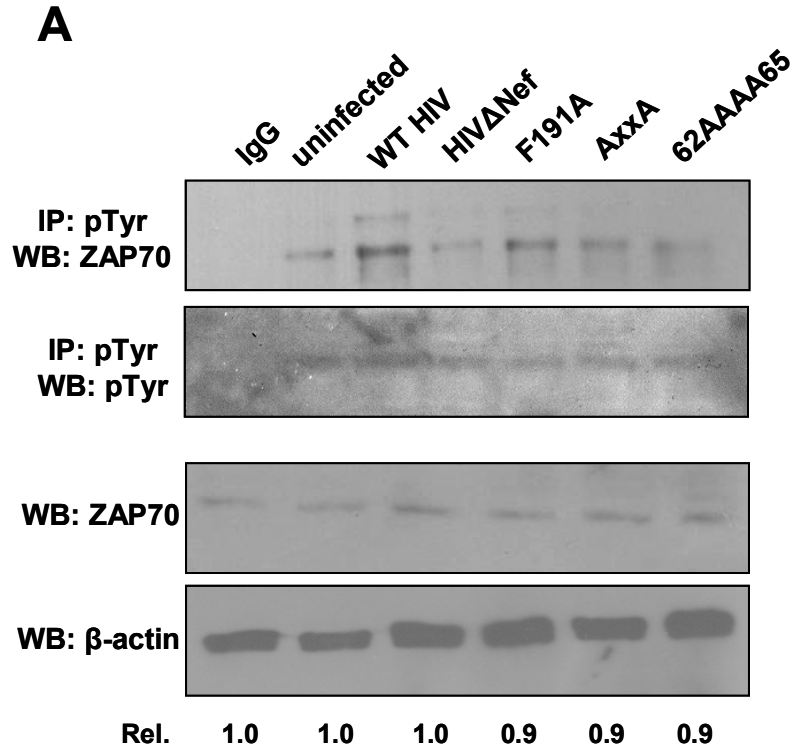
Because Vav is a well-known regulator of actin dynamics, we theorized that Nef-mediated recruitment of Vav to a signaling complex might be a key step in the polarization of synapse components that we had previously observed. We tested this theory by examining the GM1 intensity of infected cells and found that the HIV.nef-*F191A* mutation does indeed abolish Nef-mediated polarization (Fig. 45). To determine at which point the HIV.nef-*AxxA* is needed for the virological synapse, we looked at the levels of activated ZAP70 in cells infected with each of the HIV-1 mutants. We subjected infected Jurkat cell lysates to phosphotyrosine immunoprecipitation, followed by ZAP70 Western blot (Fig. 46). The results showed that, as expected, WT HIV increases the phosphorylation of ZAP70, while HIV $\Delta$ Nef has pZAP70 levels similar to uninfected cells. The HIV.nef-*F191A* mutant had no significant decrease in pZAP70 levels, consistent with our findings that polarization did not require ZAP70, while both HIV.nef-*AxxA* and HIV.nef-62AAAA65 exhibited decreased phosphorylation of ZAP70, though not to the extent of HIV $\Delta$ Nef.

## **2.8 VS formation in PBMC**

Finally, we confirmed these findings using primary cells. Therefore, we isolated PBMC and, following activation infected them with WT HIV and each of our Nef mutant viruses. We analyzed each infection for number of virological synapses formed



**Figure 45. Nef residues necessary for GM1 polarization.** Jurkat cells were infected with HIV $\Delta$ Nef, WT HIV, or Nef mutant viruses and cultured until 100% of cells were infected, and  $1 \times 10^6$  cells were fixed and stained for GM1, then analyzed by flow cytometry for GM1 intensity. The fold change in GM1 intensity was determined, with HIV $\Delta$ Nef set to 1. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.



**Figure 46. Relationship between Nef-induced activation of ZAP70 and VS-**

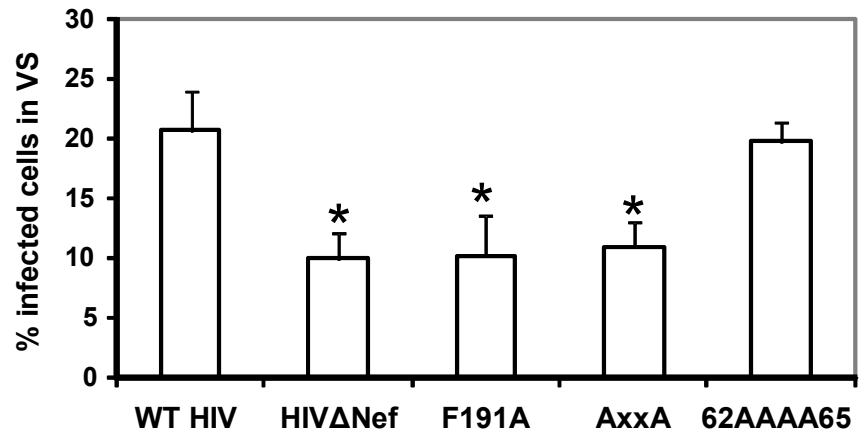
**dependent transfer.** **A.** Jurkat cells were infected with HIV $\Delta$ Nef, WT HIV, or Nef mutant viruses and cultured until 100% of cells were infected. The cells were lysed and subjected to phosphotyrosine immunoprecipitation, followed by Western blot for ZAP70. **B.** Densitometry analysis of pZAP70 was normalized using a phosphotyrosine band of ~55 kDa that was not affected by Nef expression. Western blot analysis was performed on ZAP70 normalized to actin. \*:  $p < 0.05$ . Data were mean  $\pm$  s.d. and representative of 3 independent experiments.



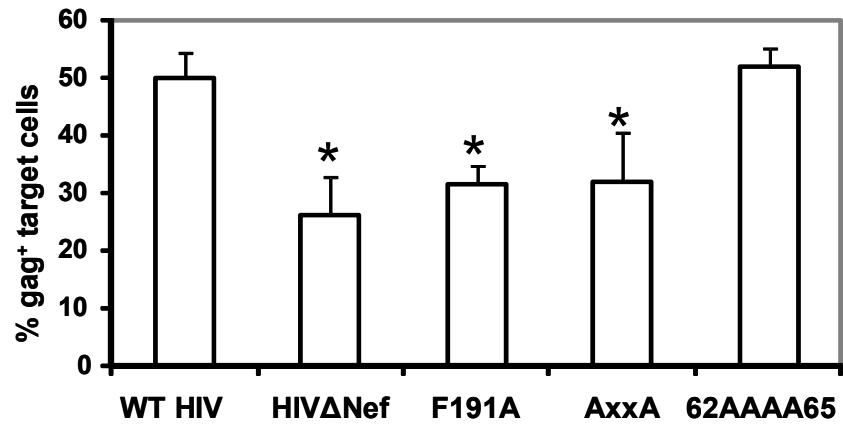
(Fig. 47A) and the percent Gag transfer (Fig. 47B) as described above for Jurkat cell infections. We found that Nef increased the number of infected cells engaged in virological synapses in primary cells to an extent similar to that of Jurkat cells, while the difference in VS-dependent Gag transfer was even higher than that of Jurkat cells.

Taken together, the data obtained from our experiments reveals that Nef is acting by two distinct mechanisms. Firstly, Nef induces polarization of VS components through an actin-dependent process that requires its N-terminal basic region and myristoylation. This process is driven by Nef recruitment of Vav. Secondly, Nef induces protrusions that require activation of ZAP70 to form, and likely are an indication of VS-dependent viral transfer. Further analysis using replication-competent Nef mutant viruses showed that Nef-specific recruitment of ZAP70 was not involved in promoting VS formation or transfer; however, the Nef SH3-binding domain activated ZAP70, allowing the formation of actin protrusions. When both pathways are active, the result is optimal formation of virological synapses and efficient transfer of virions to target cells at the synapses.

**A**



**B**



**Figure 47. Nef effect on VS formation in PBMC.** **A.** Primary cells were infected with WT HIV, HIV $\Delta$ Nef, or Nef mutant viruses and cultured until close to 100% of cells were infected. Uninfected cells were labeled with CFSE and incubated with infected cells in a 1:1 ratio, 1 x 10<sup>6</sup> total cells in 200  $\mu$ l RPMI, on poly-L-lysine-coated coverslips for 1.5 hr to allow for VS formation. Cells were then fixed, permeabilized, and stained for p24, and the percentage of infected cells involved in a VS was determined for each virus type. **B.** Additional CFSE-labeled target cells were incubated with infected cells in a 1:1 ratio, 1 x 10<sup>6</sup> total cells in 200  $\mu$ l RPMI in 96-well plates, for 24 hr and stained for p24 to determine the percentage of Gag<sup>+</sup> target cells. \*: p<0.05. Data were mean  $\pm$  s.d. and representative of 3 independent experiments.

## DISCUSSION

### **Summary of the results**

The challenges of current anti-HIV treatments mandate development of alternative and novel anti-HIV therapies. Nef is a very important HIV-1 protein, and its biggest pathogenic factor *in vivo*. Therefore, in this study we focused on two different aspects of Nef: the virion incorporation of a Nef mutant called Nef7 and Nef's role in virological synapse formation. In the first part of the study, we confirmed that Nef7 incorporated into progeny virions at a higher level than WT Nef (Fig. 10), and demonstrated that Nef7.A3G also incorporated into HIV-1 virions to a similar extent (Fig. 13). In contrast, A3G alone was present in virions in a very small amount when expressed in the presence of Vif. The higher incorporation of Nef7.A3G over A3G alone was partially accounted for by a decreased ability of Vif to degrade the fusion protein, likely due to steric hindrance of Vif binding to A3G. Fusion of A3G to Nef7 may have physically blocked Vif making its binding to A3G more difficult. However, this slight increase in the cellular expression of Nef7.A3G over A3G is not enough to account for the large difference in virion incorporation. Therefore, Nef7.A3G virion incorporation is likely largely due to the Nef7 phenotype.

We further showed that the Nef7.A3G fusion protein retains the less pathogenic phenotype of Nef7, as Nef7.A3G fails to activate PAK2 or to downregulate CD4 or MHC I (Fig. 14, 15, 16). Importantly, we show that the Nef7.A3G fusion protein retains the anti-HIV activity of A3G even in the context of HIV-1 Vif expression and is independent

of both the cell types used and the method of virion entry (Fig. 18, 19, 20). Furthermore, we show that Nef7.A3G-containing VLP potently inhibit HIV-1 replication in Jurkat cells (Fig. 22).

The next aspect of Nef function during HIV-1 infection that we investigated was what, if any, affect it had on HIV-1 virological synapses. We first determined the ability of WT HIV and HIV $\Delta$ Nef to form virological synapses and found that there was a significantly decreased ability of HIV-1 to form synapses in the absence of Nef (Fig. 23). This decreased ability to form virological synapses translated to a lower amount of Gag protein transferred to target cells (Fig. 25) as well as delayed replication kinetics in culture conditions that favor cell-cell replication over cell-free replication (Fig. 26).

The ability of Nef to enhance the formation of virological synapses and the subsequent transfer of HIV-1 virions to target cell was confirmed in primary CD4<sup>+</sup> T lymphocytes (Fig. 47). In fact, the requirement for Nef appeared to be greater in primary cells than in cell lines, as WT HIV showed an approximately 63% increase in Gag transfer to target cells over HIV $\Delta$ Nef in primary cells, whereas in Jurkat cells the difference was approximately 50%.

Further experiments revealed that Nef possessed two distinct abilities that could promote HIV-1 virological synapse formation and viral transfer. The first involved polarization of many virological synapse components, including GM1, CD81, ZAP70, and p24 (Fig. 27-29, 32-34, 37-39). The polarization of most of these is likely dependent upon lipid raft

clustering, as all of the mentioned components are localized to lipid rafts. Furthermore, we found that Nef did not specifically recruit ZAP70 to the virological synapse, nor was there any indication that Nef directly affected the cellular localization of either CD81 or p24 (Fig. 43-45). Polarization of lipid rafts was found to require the membrane localization of Nef and was promoted by Nef recruitment of Vav followed by Vav-induced actin reorganization (Fig. 45). ZAP70, however, was not involved in the process (Fig. 35).

The second aspect of Nef that could promote virological synapse formation was its ability to form actin protrusions. Formation of these protrusions required ZAP70 and is likely an indication of virological synapse-dependent viral transfer to target cells. The Nef SH3-binding domain and Nef myristoylation are both required for this process, the first to ensure ZAP70 activation and the second to ensure the proper cellular localization of Nef (Fig. 40).

### **Virion incorporation of Nef7 and Nef7 fusion proteins**

Nef7 has been previously shown to incorporate into viruses at an estimated 1100 molecules per virion, as compared to the estimated 10 molecules of WT Nef per virion. They estimated the number of WT Nef molecules present in HIV virions by comparing the relative densities of the WT Nef and p24 bands, and then used this constant to estimate the number of Nef7 molecules present in HIV virions. Because protein detection can become saturated this difference may not translate linearly to detection on Western blots, and the study did not determine fold incorporation by densitometry

analysis of the Western blots (Peretti, Schiavoni et al. 2005). Using densitometry analysis our results showed higher virus incorporation of Nef7 than WT Nef by about 9 fold, with both <sup>V153<sup>L</sup></sup> and <sup>E177<sup>G</sup></sup> point mutations being required for the increased incorporation (Fig. 10). The higher virion incorporation of Nef7 is most likely due to its increased localization to lipid rafts; however, the mechanism behind this increased localization has yet to be determined. Our results also show that Nef7.A3G maintains the higher virion incorporation property of Nef7 (Fig. 13). A3G by itself was used as a control, and as expected, failed to encapsidate efficiently. A recent study has shown that fusion of A3A, a Vif-resistant member of the APOBEC3 family that does not restrict HIV-1 infectivity to the HIV-1 protein Vpr resulted in the alteration of its virion localization from the matrix to the viral core, which granted Vpr.A3A anti-HIV activity (Aguiar, Lovsin et al. 2008). A second recent study showed that fusion of A3G to a virion-targeting peptide derived from Vpr (R88-A3G) resulted in restriction of HIV-1 infectivity (Ao, Yu et al. 2008). This fusion protein is susceptible to Vif-mediated degradation, and its higher virion incorporation is due to increased R88-A3G targeting to the virion by the remaining undegraded R88-A3G. Compared to Vpr, Nef7 is incorporated into virion at a higher level (Singh, Tungaturthi et al. 2001; Peretti, Schiavoni et al. 2005). In addition, the higher level of virion incorporation of Nef7 as compared to WT Nef offers Nef7 an advantage over the native HIV-1 Nef protein for virion incorporation. In contrast, Vpr.A3A and R88-A3G are likely to be equal in efficiency to the native HIV-1 Vpr protein in virion incorporation. This is very important when the anti-viral activity of these chimeras is evaluated in the context of HIV-1 infected cells. Furthermore, our results showed that Nef7.A3G had anti-HIV activity in

both replication-defective single round HIV infection and the HIV-1 replication assay. However, whether Vpr-A3A would be an effective anti-HIV agent in the context of HIV-1 replication is not established.

### **Pathogenicity of Nef7 fusion proteins**

Aside from its virion incorporation, a key property of Nef7 that allows it to be used as a carrier for therapeutic proteins is its relative lack of toxicity. While Nef is the most important pathogenic factor of HIV-1, Nef7 has been shown to be defective for a number of key Nef properties. One of the best known characteristics of Nef is its ability to activate the cellular kinase PAK2, and disease progression in macaques has been associated with reversion of Nef mutants to a PAK2-activating phenotype (Khan, Sawai et al. 1998). Previous work has shown that Nef7 does not activate PAK2 (D'Aloja, Santarcangelo et al. 2001), and our data supports that conclusion. In an in vitro kinase assay, both Nef7 and the Nef7.A3G fusion protein failed to activate PAK2, confirming that Nef7 is less pathogenic than WT Nef and that fusion of A3G to Nef7 does not alter this phenotype (Fig. 14). This loss of function does not appear to correlate with a loss of Nef7 binding to PAK2, as PAK2 is detected in immunoprecipitates of both Nef7 and Nef7.A3G. Likewise, the expression of Nef7 does not affect the intracellular levels of PAK2. Taken together, these data suggest that the loss of PAK2 activation in Nef7 is due to an inability of bound Nef7 to activate PAK2. Our conclusion is in agreement with the study in which the Nef residues 85, 89, 187, 188, and 191 are found to be critical for Nef binding to PAK2 (Agopian, Wei et al. 2006), as none of these residues are affected in Nef7 as compared to WT Nef.



A second well known property of Nef, the downregulation of surface molecules such as CD4 and MHC I, has also been shown to be defective in Nef7 (D'Aloja, Santarcangelo et al. 2001). Our results show that in HeLa cells WT Nef downregulates both CD4 and MHC I, with MHC I downregulation being much more efficient (Fig. 15, 16). Both Nef7 and Nef7.A3G show no significant decrease in the surface expression of either CD4 or MHC I. Nef7 had previously been shown to downregulate CD4 slightly (D'Aloja, Santarcangelo et al. 2001), although much less efficiently than WT Nef. In the system that we used, Nef7 shows a statistically insignificant decrease in CD4 expression. This difference is likely due to the different cell types used, as HeLa cells have been shown to have a high recycling rate for CD4. Therefore, any internalized CD4 is quickly replaced at the cell surface, resulting in a smaller overall decrease in surface expression of CD4.

### **Inhibition of HIV-1 infectivity using Nef7.A3G**

Because Nef7.A3G is incorporated into progeny viruses, we expected that it would retain the A3G ability to restrict the infectivity of HIV-1, and this was indeed the case. In a single-cycle HIV infectivity assay Nef7.A3G-containing HIV virions showed a dramatic reduction in infectivity (Fig. 18-20). This reduction was consistent across all cell types used and with both VSV-G pseudotyped viruses and viruses expressing X4- and R5-tropic HIV receptors, confirming that fusion to Nef7 restores the anti-HIV phenotype of A3G in the presence of Vif. In order to determine whether the Nef7.A3G fusion protein was Vif-resistant, we expressed Nef7.A3G, Nef7.A3G(D128K), Nef7.A3G(E259Q), and A3G in the presence or absence of Vif and analyzed both cellular expression levels and virion incorporation (Fig. 17). Nef7.A3G(D128K) had the highest level of intracellular

expression, followed by Nef7.A3G and Nef7.A3G(E259Q). The A3G protein by itself had lower expression than any of the Nef7 fusion proteins, indicating that Nef7.A3G fusion proteins are partially Vif-resistant, possibly due to structural constraints on Vif binding. However, the difference between virion incorporation of Nef7.A3G and Nef7.A3G(D128K) is not as great as the differences in their cellular expression. This suggests that the slight Vif-resistance of Nef7.A3G is not solely responsible for its increased virion incorporation.

We theorized that specific targeting of Nef7.A3G fusion proteins to HIV infected cells could inhibit the spread of HIV infection by allowing it to be incorporated into the progeny viruses produced from those cells and thus restrict the further spread of HIV from all targeted cells. To test this, we created VLP that were pseudotyped with CD4 and CXCR4, allowing their targeting to HIV-infected cells through an “inverse fusion” process where the CD4 and CXCR4 on the VLP bind to the gp120 expressed on the surface of HIV infected cells. We infected Jurkat T cells with HIV-1, then treated them with pseudotyped VLP containing Nef7.A3G, and monitored virus production over multiple rounds of infection (Fig. 22). In this study we used a ratio of input virus to VLP of 1:10. This ratio is very important, as a higher ratio would result in a higher magnitude of antiviral effect. The results confirm that Nef7.A3G-containing VLP inhibit the spread of HIV in infected Jurkat cells. Furthermore, the specific targeting of infected cells by inverse fusion should minimize bystander toxicity, increasing the usefulness of this strategy as a therapeutic platform to inactivate HIV-1 and suppress HIV-1 replication.

### **Effect of Nef on HIV-1 virological synapse formation**

We report here that Nef enhances the formation of and transfer of virus through HIV-1 virological synapses. In cells infected with WT HIV virus, there was a significant increase in the number of synapses formed as compared to cells lacking Nef (Fig. 23). To ensure that this increase in the number of VS formed translated to a similar increase in the amount of virus transferred to target cells, we took advantage of a previously described assay for VS-dependent transfer. In this system, the contribution of cell-free virus to target cell infection has been shown to be negligible, at less than 1% (Sol-Foulon, Sourisseau et al. 2007). In our experiments, WT HIV showed significantly increased transfer of virions to target cells as compared to HIV $\Delta$ Nef virus (Fig. 25). Importantly, we included as negative controls known inhibitors of VS transfer: cytochalasin D, which inhibits the actin restructuring required for VS formation; and piceatannol, a Syk tyrosine kinase inhibitor that has particularly high activity against ZAP70. Both of these inhibitors resulted in VS transfer of virus that was similar to that of HIV $\Delta$ Nef.

HIV-1 spread through virological synapses is faster than viral spread through cell-free virus *in vivo*, but in most tissue culture conditions the infected cell density is too low to favor VS-dependent over cell-free transfer. Additionally, by the time the cell density and number of infected cells becomes high enough to result in high VS formation rates, the amount of cell-free virus overwhelms any change in replication kinetics that might be seen due to inhibition of VS formation. Therefore, in order to see whether or not Nef's effect on VS formation resulted in differences in viral spread, we monitored replication kinetics in a situation that favored VS-dependent virus spread over cell-free virus spread:

high cell density; 33% p24<sup>+</sup> (infected) cells at the start of the experiment, with the fusion inhibitor T-20 also added to one set in order to eliminate any virus spread resulting from cell-free virus. We found that in these VS-favorable conditions there is a small but significant lag in the replication kinetics of HIV $\Delta$ Nef infections as compared to WT infections (Fig. 26B). This lag becomes more pronounced and longer-lived when T-20 is included to eliminate any cell-free infections (Fig. 26B).

There is some controversy regarding the means of virion entry into target cells during HIV-1 VS, with some studies reporting uptake via endocytosis (Chen, Hubner et al. 2007; Bosch, Grigorov et al. 2008; Hubner, McNerney et al. 2009) and some reporting a need for gp120-CCR5/CXCR4 dependent fusion (Martin, Welsch et al.; Massanella, Puigdomenech et al. 2009). Because we saw significant virus spread in our cultures even in the presence of T-20, we conclude that in our system virion entry into target cells during VS is at least partially fusion-independent. This does not, however, eliminate the possibility that virion entry is a combination of endocytosis and fusion. Taken together, our data show that Nef enhances the spread of HIV-1 through virological synapses, and that this enhancement is relevant to HIV-1 spread in both the short and long term.

### **Nef-induced polarization of virological synapse components**

Previous studies have indicated that Nef may affect lipid raft dynamics (Simons and Ehehalt 2002; Riethmuller, Riehle et al. 2006). Specifically, Nef increases the size of lipid rafts during IS formation and the localization of tyrosine kinases to rafts (Djordjevic et al. 2004). Because of the importance of lipid raft clustering in VS formation we

believed that this might be an important mechanism of Nef enhancement of VS formation. To test this, we examined the distribution of GM1 in Nef-expressing cells using three different methods: confocal microscopy (Fig. 27A, 32), FACS analysis of GM1 intensity (Fig. 29, 34A), and raft floatation assays (Fig. 36). All three techniques show that Nef increases the size of lipid rafts, likely by inducing clustering of small rafts to form larger ones.

CD81 is another important component of virological synapses and is responsible for preventing the formation of syncytia during cell-cell contacts, thus allowing the infected cell to detach and form a VS with another target cell (Weng, Kremontsov et al. 2009). We analyzed the surface distribution of CD81 in Nef-expressing cells in the same way as GM1 and found that Nef expression also polarized surface CD81 (Fig. 28, 33, 34B, 37).

We then wished to determine whether Nef affected ZAP70 distribution during virological synapse formation. We therefore examined ZAP70 staining during VS formation in WT HIV and HIV $\Delta$ Nef infections and found that there was a large increase in both ZAP70 and total pTyr localization to the VS in the presence of Nef (Fig. 38, 39). Interestingly, the localization of p24 to the VS was also increased to a similar degree, suggesting that the two were recruited together. Given the localization of ZAP70 to lipid rafts, the preponderance of CD81 in lipid rafts (Jolly and Sattentau 2007), and the assembly of HIV-1 virions there, it is likely that the polarization of all these VS components is accomplished by the clustering of lipid rafts, which then indirectly bring the other components to the synapse.

### **Nef residues involved in clustering of virological synapse components**

To determine the Nef domains responsible for the polarization of virological synapse components, we took advantage of a previously constructed panel of Jurkat cells stably expressing a series of Nef mutants fused to GFP. We performed our flow cytometry polarization assay on each mutant, using both GM1 and CD81 and found that the alteration of both GM1 and CD81 distribution requires myristoylation of Nef as well as a functional N-terminal basic region (Fig. 34). Interestingly, both these determinants are involved in the localization of Nef to the plasma membrane (Geyer, Fackler et al. 2001; Giese, Woerz et al. 2006), suggesting that membrane localization of Nef is extremely important in this process (see Fig. 5). We also demonstrated that Nef-induced clustering of lipid rafts and CD81 is driven by actin reorganization, as treatment with cytochalasin D completely abolished all polarization.

The strict requirement for membrane localization, as well as the dependence upon actin reorganization, led us to believe that Nef-induced activation of Vav may be responsible for the polarization of VS components. Furthermore, it has been previously shown that clustering of lipid rafts in immunological synapse formation was largely dependent upon Vav activation (Villalba, Bi et al. 2001). We therefore created a replication-competent HIV-1 construct with a mutation at F191, the residue responsible for the recruitment of Vav to its signaling cascade in lipid rafts (Krautkramer, Giese et al. 2004). As expected, HIV.nef-*F191A* exhibited decreased virological synapse formation (Fig. 43), with a corresponding decrease in the amount of VS-dependent Gag transfer (Fig. 44). To determine what, if any, effect mutating F191 had on the polarization of virological

synapse components, we performed our flow cytometry-based GM1 polarization assay and showed that the Nef-induced polarization of VS components was indeed mediated by Vav, as HIV.nef-*F191A* showed reduced clustering of GM1, on level with that of HIV $\Delta$ Nef (Fig. 45).

### **Nef-induced protrusions as an indication of viral transfer**

In addition to virological synapses, there exists a second means of direct cell-cell spread of HIV-1. These are more long-range and are known as nanotubes or filopodial bridges (Sherer, Lehmann et al. 2007; Davis and Sowinski 2008; Sowinski, Jolly et al. 2008). It has been hypothesized that these protrusions are remnants of virological synapses, left after cell separation, or that they are the initial step in VS formation, establishing cell-cell connections that allow the infected and uninfected pair to come together more efficiently (Haller and Fackler 2008). Intriguingly, it has recently been reported by multiple groups that Nef induces protrusions in expressing cells (Nobile, Rudnicka et al. 2010; Xu, Santini et al. 2009); these have been shown to transfer Nef to bystander cells (Xu, Santini et al. 2009). It is likely that these filopodia are analogous to the nanotubules that form between infected and uninfected cells. We examined F-actin in Nef-expressing cell lines and confirmed this phenomenon (Fig. 40A). Additionally, we showed that the formation of Nef protrusions requires a functional SH3-binding domain in Nef. Because of the possibility of these protrusions being analogous to VS transfer, we treated cells with piceatannol to examine the involvement of ZAP70 in this process. We found that treatment with piceatannol completely abolished the protrusions, which provided

additional support for the theory that Nef protrusions are associated with VS viral transfer (Fig. 40B).

### **ZAP70 involvement in Nef enhancement of HIV-1 virological synapses**

In an effort to further elucidate the mechanism of Nef enhancement of virological synapse formation, we generated a series of replication-competent Nef-mutant viruses (Fig. 42). Our first mutant, mentioned previously, was HIV.nef-*F191A*, which is defective in Nef-mediated recruitment of Vav to lipid raft microdomains. Our second mutant generated was HIV.nef-*AxxA*, which we used to confirm the importance of Nef activation of ZAP70 by its SH3 domain. Thirdly, we wished to confirm the idea that ZAP70 was not specifically recruited to the VS, but instead was recruited indirectly. To this end, we generated the HIV.nef-*62AAAA65* mutation, which abolishes Nef-mediated recruitment of ZAP70 to its signaling complex (Atkins, Thomas et al. 2008). We examined these HIV-1 mutants for VS formation as well as VS-dependent viral transfer (Fig. 43, 44). We found that, as expected, HIV.nef-*AxxA* was similar to HIV $\Delta$ Nef in both cases. We also confirmed the indirect mechanism of ZAP70 localization to the virological synapse, as HIV.nef-*62AAAA65* behaves like WT HIV during VS assays.

Analysis of pZAP70 levels in infected Jurkat cells showed that while HIV.nef-*F191A* had little effect on ZAP70 phosphorylation as compared to WT HIV, infection with both the HIV.nef-*AxxA* and HIV.nef-*62AAAA65* viruses resulted in decreased pZAP70 (Fig. 46). Since HIV.nef-*62AAAA65* is known to have decreased ZAP70 activation in at least one pathway, that of MHC I downregulation, it is not unexpected that the whole cell pZAP70

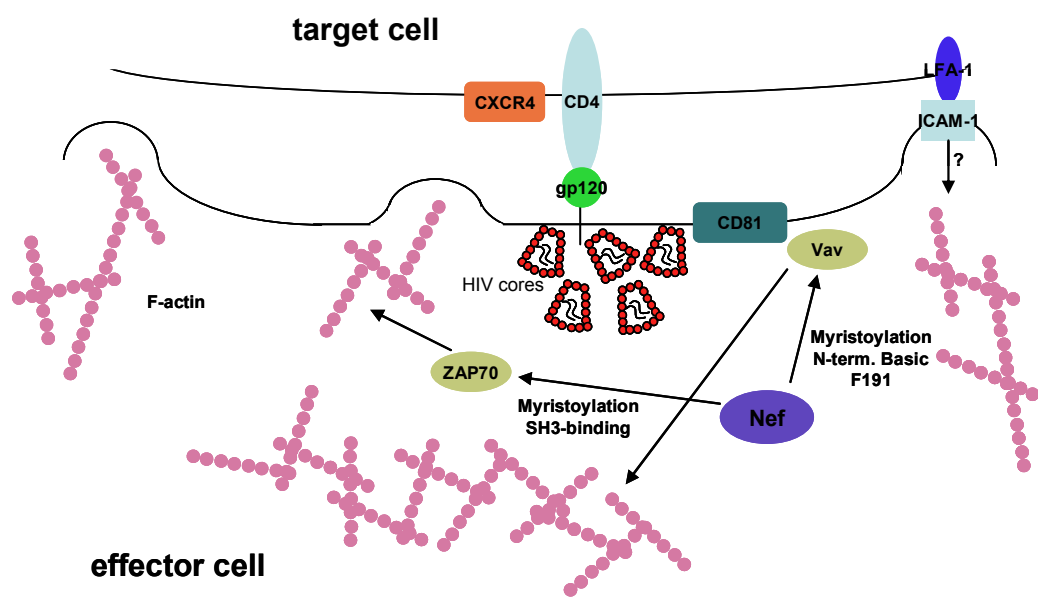


levels would be affected. However, based on the data from our VS assays, we conclude that this decrease may be localized to a different subcellular location than the ZAP70 needed for VS transfer, as HIV.nef-62AAA65 had no effect on virological synapses. This is reasonable since the acidic domain of Nef responsible for the recruitment of ZAP70 in the MHC I downregulation cascade acts through its binding to PACS-2, which largely locates to the ER and mitochondria (Simmen, Aslan et al. 2005; Atkins, Thomas et al. 2008). Conversely, since HIV.nef-*AxxA* showed both decreased pZAP70 and lower VS transfer, we propose that the pool of ZAP70 that is localized in lipid rafts and is necessary for virological synapses requires the PxxP domain of Nef to function fully.

### **Virological synapse formation in primary CD4<sup>+</sup> T lymphocytes**

Finally, we confirmed the effect of Nef on VS formation in primary cells. Interestingly, we found that Nef has a greater effect on VS formation in primary cells as compared to Jurkat cells, as during Gag transfer analysis in Jurkat cells HIV $\Delta$ Nef results in approximately 63% of WT transfer, whereas in primary cells this decreased to approximately 50% of WT (Fig. 47). Additionally, we found that the total Gag transfer to target cells is increased in primary cells as compared to Jurkat cells. This supports an idea mentioned before that primary cells may favor VS-dependent viral spread over cell-free spread, while the opposite is true for cell lines (Puigdomenech, Massanella et al. 2009). This theory may also explain why VS-dependent viral spread is more relevant in many *in vivo* situations than cell-free viral spread. Our data reveals an interesting mechanism of Nef-mediated enhancement of HIV-1 virological synapses, wherein Nef exhibits a two-pronged approach to amplify both formation of, and transfer of virus

through, HIV-1 virological synapses (Fig. 48). This effect may partially explain the greater infectivity of WT HIV-1 over Nef-deleted HIV-1 in primary cells.



**Figure 48. Proposed working model for Nef-mediated enhancement of HIV**

**virological synapses.** Nef induces polarization of VS components through an actin-dependent process that requires its N-terminal basic region. This process is driven by Nef recruitment of Vav. Additionally, Nef induces protrusions that require activation of ZAP70 to form, and likely are an indication of VS-dependent viral transfer. When both Nef functions are working properly, there is optimal formation of virological synapses and spread of HIV virus through them.

## PERSPECTIVES

HAART has reduced the AIDS-associated mortality in developed countries by up to 80-90%, although the effect is much lower in developing countries where anti-retroviral drugs are cost-prohibitive and rare (Hammer, Eron et al. 2008; Ho and Bieniasz 2008). Proper application of HAART therapy can reduce plasma viral loads to undetectable levels, but it cannot eradicate the virus. Furthermore, replication can sometimes continue undetected in lymphoid tissues, and if treatment is stopped for any reason viral loads become very high. Unfortunately, the high cost of HAART and non-compliance issues due to toxicity often contribute to the rise of HAART-resistant HIV-1 strains. This has made the development of alternative anti-HIV therapies a priority. Because of the shortcomings of current anti-HIV therapies, there has recently been increased interest in exploiting cellular proteins involved in the HIV-1 lifecycle in order to develop new therapies. Importantly, due to the recent revelation of the importance of virological synapses in the spread of HIV-1 any future therapies would need to take into account the differing requirements for cell-dependent and cell-free virus spread in order to be completely effective *in vivo*. At the current time, however, little is known about the cellular determinants of HIV-1 virological synapse regulation, and even less is known about the viral determinants of VS regulation. Therefore, the regulatory mechanisms of HIV-1 virological synapses need to be elucidated in order to develop the most effective anti-HIV therapies.

### **Nef7 as a platform for therapeutic protein delivery**

What we have shown here is a proof-of-concept that Nef7 works well as a carrier platform for delivery of therapeutic molecules. This strategy has many potential uses aside from the Nef7.A3G fusion protein. Because of its high virion incorporation, Nef7 fusion proteins can be used to target several components of the HIV-1 virion, such as Nef7.EDN targeting of the HIV-1 tRNA primer. Moreover, Nef fusion proteins can be adapted to deliver anti-HIV therapeutic proteins or components directly into HIV-1 or HIV-infected cells. This is a great advantage over traditional means of antiviral therapy, as this direct targeting of infected cells greatly diminishes bystander cell toxicity, and thus could result in fewer potential side effects during treatment. In a clinical setting VLP, such as those used in gene therapy, could be used to deliver Nef7.A3G to target cells. This strategy would require continual treatment, but since the treatment involved delivery of protein there would be no concerns about Nef7 reverting to WT Nef.

An alternative strategy would be to deliver Nef7.A3G DNA that is driven by the HIV-1 LTR and allowed to integrate into the host cell chromosome. If that cell is later infected by HIV-1 transcription of the Nef7.A3G gene would commence, and result in inhibition of viral spread. This strategy is more like a preventative treatment. It would be more proactive and stop the infection immediately; however, since the treatment involves delivery of Nef7 DNA there is the chance of revertants to WT Nef given the selective pressures to produce WT Nef *in vivo*. Since Nef7 is derived from F12-HIV<sub>nef</sub>, a mutated Nef gene that did manage to persist *in vivo*, it is possible that Nef7 would also manage to persist without reverting back to a WT Nef phenotype but this would have to be tested.

Another potential use of Nef7, aside from delivery of therapeutic proteins to viral particles, is as a protein carrier for lipid raft localization. Since Nef7 owes its enhanced virion incorporation largely to its increased localization to lipid rafts as compared to WT Nef, Nef7 could work equally well for delivering proteins to lipid rafts as for delivering proteins to HIV-1 virions. This could potentially be a very useful tool, as lipid rafts tend to have very large concentrations of signaling molecules. Indeed, many important signaling processes require the integrity of lipid rafts or the aggregation of lipid rafts into large signaling platforms. As such, any disorder that involves aberrant signaling from signal cascades dependent upon lipid rafts could potentially be affected by Nef7 fusion proteins.

Further characterization of the molecular mechanism responsible for Nef7's higher virion incorporation and the structure-function relationship of Nef7 shall surely help fine tune this strategy to minimize any residual pathogenic activity of Nef7 and to maximize the benefits of this novel strategic anti-HIV platform for its potential clinical translation.

#### **Potential of Nef7 as a laboratory research tool**

Nef7 fusion proteins could also be used for novel experimental procedures in the laboratory. For example, a Nef7.GFP fusion protein could be used to track viral cores at early stages of infection, and a Nef7.luciferase fusion protein could be used as a sensitive assay for measuring virus production. The high virion incorporation of Nef7 would allow it to be utilized for any procedure that requires quantification or identification of viral particles. This would not even necessarily need to be HIV-1 virions, as MLV virions also

exhibit high virion incorporation of Nef7. It would be interesting to determine whether Nef7 could be used as a general tool for viral tracking, or whether it only works with enveloped viruses, or only with retroviruses.

### **Nef involvement in immunological and virological synapses**

Given that Nef has been shown to inhibit the formation of immunological synapses, at first glance it seems contradictory that Nef also promotes the formation of virological synapses. However, further examination of the mechanisms of Nef down-modulation of the immunological synapse reveals that Nef mainly accomplishes this by altering the sub-cellular localization of CD3 and Lck. In the context of the virological synapse this would not be relevant, as CD3 does not localize to the HIV-1 virological synapse. It has not been determined what, if any, involvement Lck has in virological synapses, but as the majority of Lck is associated with CD4, it is unlikely that Lck would have any great role in the effector cell, as CD4 is mostly downregulated from the surface in these cells and so does not localize to the virological synapse on that side. Lck may very well be required in the target cell as CD4 is a key component of the target cell virological synapse.

From our data and previous work published by others, it appears as though Nef is responsible for a delicate balancing act in infected cells, wherein immunological synapses are destabilized at the same time that IS downstream signaling is enhanced, and virological synapse formation is promoted. The two processes may even amplify each other. Since virological synapses share at least some components with immunological synapses, the destabilization of immunological synapses may free up components for



virological synapses that may otherwise be scarce. This may be even more important in the context of an infected individual, because lymphoid organs are such important sites of viral replication *in vivo*. These sites have extremely high cell density, which would be a great asset for virological synapse formation, but they are also the main site of antigen presentation to CD4<sup>+</sup> T lymphocytes by antigen presenting cells. This would result in a preference for immunological synapse formation over that of virological synapse formation, unless there was something to discourage their formation. It would be very interesting to determine the rates of VS formation with and without concurrent IS stimulation. It may be that Nef has an even greater impact on virological synapse formation *in vivo* than has been shown here because of its ability to destabilize the immunological synapse. Therefore, this new function of Nef may partially explain its impact on HIV-1 replication and pathogenesis in infected individuals.

Virological synapses may also play an important role in horizontal and vertical transmission of HIV-1. Infected cells can exist in the bodily fluids that transmit HIV-1, particularly contaminated blood but others as well. These infected cells could then transfer HIV-1 to susceptible cells in the newly exposed individual much more efficiently than cell-free virus in those same fluids. Additionally, infected cells are much less fragile than cell-free virus, which does not persist outside of the body in an infectious form for more than a few minutes. Therefore, infected cells may provide not only a more efficient means of transmitting HIV-1, but also a more stable one.

### **Nef transfer to target cells**

During HIV-1 infection, only a relatively small percentage of cells are infected at any given time. However, there are widespread effects on the immune system, even in uninfected cells. Our lab and others have shown that Nef can be transferred to uninfected target cells through nanotube protrusions, and this may be at least a partial cause of the immune dysregulation seen. This is supported by the findings that Nef induces apoptosis in bystander cells and can inhibit antibody class switching to IgG/IgA in bystander B cells.

It is possible that Nef may also play a role in establishing a virological synapse on the part of the target cell. The nanotubes that seem to play a role in virological synapse-dependent viral transfer also have been shown to transfer Nef to bystander cells. As mentioned previously, there is a theory that these protrusions are remnants of virological synapses, left after cell separation, or that they are the initial step in VS formation, establishing cell-cell connections that allow the infected and uninfected pair to come together more efficiently. Given that conduits can form without stimulation from a virological synapse, or even without infection, it seems likely that Nef-induced protrusions are involved at the earlier stage of virological synapses. If this is indeed the case, it is possible that Nef could be transferred to the target cells of a virological synapse prior to its establishment per se, and “prime” it for engagement with the effector cell. An interesting next step could be to determine whether Nef is transferred to the target cells of a virological synapse, and if so, what affect this may have on the target cell’s ability to engage in the synapse.

### **Signaling pathways at the virological synapse**

Even with these new insights into the required components of the virological synapse, there is still very little known about the signaling pathways involved in the effector cell and even less known about those involved in the target cells. Adhesion molecules have also been shown to play a role in downstream signaling at the virological synapse, as antibodies directed against ICAM-1 signaling, which still allow conjugate formation, result in impaired virological synapse formation. Therefore, there is an unknown pathway signaling through the adhesion molecules of a VS.

The other signaling pathway known to be involved in virological synapses includes ZAP70. Prior to this work, ZAP70 was known to increase the transfer of virions to target cells, but did not affect conjugate formation. The authors of this study proposed that ZAP70 increased the localization of Gag to the synapse, as they found more intense points of Gag staining in the presence of ZAP70 than without (Sol-Foulon, Sourisseau et al. 2007). However, close analysis of the VS confocal images shows Gag at the tips of protruding portions of the infected cell. Therefore, this more intense staining of Gag in the presence of ZAP70 may not be an overall increase of Gag to the VS, but instead an indication of ZAP70 increasing the transfer of Gag through protrusions into target cells. It is still unknown how ZAP70 activation causes the formation of actin protrusions, so it would be beneficial to examine this pathway in more detail in order to further elucidate the mechanisms of virological synapse formation.

### **Nef enhancement of infectivity in primary cells**

The most elusive Nef property investigated to date is its ability to increase the infectivity of HIV-1 in primary cells. This difference in infectivity was most evident in infections of unstimulated PBMC at low MOI. The finding that Nef-containing virions exhibited greater infectivity in single round infectivity assays partially explains this phenomenon. Unfortunately, it also led to a focus on the effect of Nef in cell-free virus, and since that time almost all efforts to elucidate the mechanism behind Nef-mediated enhancement of replication in primary cells has involved the mechanism behind the increased infectivity of cell-free virus. However, even taking into account the equivalent infectivity between the Nef<sup>+</sup> and Nef<sup>-</sup> viruses, comparison of PBMC infections in early studies still reveals delayed viral replication in the absence of Nef, suggesting that there is a secondary mechanism involved in the Nef-mediated enhancement of infectivity in primary cells that involves secondary viral spread (Miller, Warmerdam et al. 1994).

Our findings that Nef enhances the spread of HIV-1 through virological synapses may offer an explanation for this observed difference in replication, even with adjusting for the increased infectivity of cell-free virions. If, as we and others have proposed, primary cells preferentially utilize virological synapses over cell-free virus, this may be a more relevant explanation for the increased infectivity of Nef<sup>+</sup> viruses in primary cells than an increase in free-virus infectivity. It would be interesting to attempt to separate the relative contributions of cell-free and cell-cell viral infection when determining differential replication kinetics in primary cells in order to resolve this question. This could potentially be addressed by using a transwell culture, wherein infected cells are

separated from target cells by a membrane that allows cell-free virus to pass through but not infected cells. The replication kinetics of the cell-free HIV-1 could then be determined, and then differences between the replication kinetics of mixed infected and uninfected cultures and the cell-free virus culture would demonstrate the contribution of VS-mediated virus spread.

## REFERENCES

- . "AIDS Epidemic Update 2009." from  
<http://www.unaids.org/en/KnowledgeCentre/HIVData/EpiUpdate/EpiUpdArchive/2009/default.asp>.
- . "HIV/AIDS Data and Statistics." from <http://www.who.int/hiv/data/en/>.
- . "Resource tracking and projections." from  
<http://www.unaids.org/en/KnowledgeCentre/HIVData/Tracking/>.
- Agopian, K., B. L. Wei, et al. (2006). "A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2." J Virol **80**(6): 3050-61.
- Aguiar, R. S., N. Lovsin, et al. (2008). "Vpr.A3A chimera inhibits HIV replication." J Biol Chem **283**(5): 2518-25.
- Aguiar, R. S. and B. M. Peterlin (2008). "APOBEC3 proteins and reverse transcription." Virus Res **134**(1-2): 74-85.
- Aiken, C. and D. Trono (1995). "Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis." J Virol **69**(8): 5048-56.
- Alfsen, A., P. Iniguez, et al. (2001). "Secretory IgA specific for a conserved epitope on gp41 envelope glycoprotein inhibits epithelial transcytosis of HIV-1." J Immunol **166**(10): 6257-65.
- Alkhatib, G., C. C. Broder, et al. (1996). "Cell type-specific fusion cofactors determine human immunodeficiency virus type 1 tropism for T-cell lines versus primary macrophages." J Virol **70**(8): 5487-94.

- Alkhatib, G., C. Combadiere, et al. (1996). "CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1." Science **272**(5270): 1955-8.
- Anderson, E. C. and A. M. Lever (2006). "Human immunodeficiency virus type 1 Gag polyprotein modulates its own translation." J Virol **80**(21): 10478-86.
- Anderson, J. L., A. T. Johnson, et al. (2007). "Both linear and discontinuous ribosome scanning are used for translation initiation from bicistronic human immunodeficiency virus type 1 env mRNAs." J Virol **81**(9): 4664-76.
- Anderson, S. J., M. Lenburg, et al. (1994). "The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef." J Virol **68**(5): 3092-101.
- Ao, Z., Z. Yu, et al. (2008). "Vpr14-88-Apobec3G fusion protein is efficiently incorporated into Vif-positive HIV-1 particles and inhibits viral infection." PLoS ONE **3**(4): e1995.
- Arold, S. T. and A. S. Baur (2001). "Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein." Trends Biochem Sci **26**(6): 356-63.
- Askjaer, P., T. H. Jensen, et al. (1998). "The specificity of the CRM1-Rev nuclear export signal interaction is mediated by RanGTP." J Biol Chem **273**(50): 33414-22.
- Atkins, K. M., L. Thomas, et al. (2008). "HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: analysis using short interfering RNA and knock-out mice." J Biol Chem **283**(17): 11772-84.

- Barda-Saad, M., A. Braiman, et al. (2005). "Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton." Nat Immunol **6**(1): 80-9.
- Barnham, K. J., S. A. Monks, et al. (1997). "Solution structure of a polypeptide from the N terminus of the HIV protein Nef." Biochemistry **36**(20): 5970-80.
- Basu, V. P., M. Song, et al. (2008). "Strand transfer events during HIV-1 reverse transcription." Virus Res **134**(1-2): 19-38.
- Benaroch, P., E. Billard, et al. "HIV-1 assembly in macrophages." Retrovirology **7**: 29.
- Berg, L. J., L. D. Finkelstein, et al. (2005). "Tec family kinases in T lymphocyte development and function." Annu Rev Immunol **23**: 549-600.
- Berkhout, B. (1996). "Structure and function of the human immunodeficiency virus leader RNA." Prog Nucleic Acid Res Mol Biol **54**: 1-34.
- Berkhout, B., R. H. Silverman, et al. (1989). "Tat trans-activates the human immunodeficiency virus through a nascent RNA target." Cell **59**(2): 273-82.
- Bieniasz, P. D. (2009). "The cell biology of HIV-1 virion genesis." Cell Host Microbe **5**(6): 550-8.
- Bishop, K. N., R. K. Holmes, et al. (2004). "Cytidine deamination of retroviral DNA by diverse APOBEC proteins." Curr Biol **14**(15): 1392-6.
- Blaak, H., F. de Wolf, et al. (1997). "Temporal relationship between human immunodeficiency virus type 1 RNA levels in serum and cellular infectious load in peripheral blood." J Infect Dis **176**(5): 1383-7.



- Blaak, H., A. B. van't Wout, et al. (2000). "In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline." Proc Natl Acad Sci U S A **97**(3): 1269-74.
- Blanpain, C., F. Libert, et al. (2002). "CCR5 and HIV infection." Receptors Channels **8**(1): 19-31.
- Bleul, C. C., L. Wu, et al. (1997). "The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes." Proc Natl Acad Sci U S A **94**(5): 1925-30.
- Blot, V., L. Delamarre, et al. (2004). "Human Dlg protein binds to the envelope glycoproteins of human T-cell leukemia virus type 1 and regulates envelope mediated cell-cell fusion in T lymphocytes." J Cell Sci **117**(Pt 17): 3983-93.
- Bolinger, C. and K. Boris-Lawrie (2009). "Mechanisms employed by retroviruses to exploit host factors for translational control of a complicated proteome." Retrovirology **6**: 8.
- Bosch, B., B. Grigorov, et al. (2008). "A clathrin-dynamin-dependent endocytic pathway for the uptake of HIV-1 by direct T cell-T cell transmission." Antiviral Res **80**(2): 185-93.
- Braaten, D., E. K. Franke, et al. (1996). "Cyclophilin A is required for an early step in the life cycle of human immunodeficiency virus type 1 before the initiation of reverse transcription." J Virol **70**(6): 3551-60.

- Brasey, A., M. Lopez-Lastra, et al. (2003). "The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle." J Virol **77**(7): 3939-49.
- Brenchley, J. M., D. A. Price, et al. (2006). "Microbial translocation is a cause of systemic immune activation in chronic HIV infection." Nat Med **12**(12): 1365-71.
- Brenchley, J. M., T. W. Schacker, et al. (2004). "CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract." J Exp Med **200**(6): 749-59.
- Briant, L., V. Robert-Hebmann, et al. (1998). "Involvement of extracellular signal-regulated kinase module in HIV-mediated CD4 signals controlling activation of nuclear factor-kappa B and AP-1 transcription factors." J Immunol **160**(4): 1875-85.
- Brown, D. A. and E. London (1998). "Functions of lipid rafts in biological membranes." Annu Rev Cell Dev Biol **14**: 111-36.
- Brown, D. A. and E. London (2000). "Structure and function of sphingolipid- and cholesterol-rich membrane rafts." J Biol Chem **275**(23): 17221-4.
- Buck, C. B., X. Shen, et al. (2001). "The human immunodeficiency virus type 1 gag gene encodes an internal ribosome entry site." J Virol **75**(1): 181-91.
- Bunnell, S. C., D. I. Hong, et al. (2002). "T cell receptor ligation induces the formation of dynamically regulated signaling assemblies." J Cell Biol **158**(7): 1263-75.
- Campbell, E. M., R. Nunez, et al. (2004). "Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity." J Virol **78**(11): 5745-55.

- Campi, G., R. Varma, et al. (2005). "Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling." J Exp Med **202**(8): 1031-6.
- Cantin, R., J. F. Fortin, et al. (1997). "The acquisition of host-derived major histocompatibility complex class II glycoproteins by human immunodeficiency virus type 1 accelerates the process of virus entry and infection in human T-lymphoid cells." Blood **90**(3): 1091-100.
- Cao, Y., L. Qin, et al. (1995). "Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection." N Engl J Med **332**(4): 201-8.
- Caputi, M., M. Freund, et al. (2004). "A bidirectional SF2/ASF- and SRp40-dependent splicing enhancer regulates human immunodeficiency virus type 1 rev, env, vpu, and nef gene expression." J Virol **78**(12): 6517-26.
- Carlini, F., A. Nicolini, et al. (1996). "The non-producer phenotype of the human immunodeficiency virus type 1 provirus F12/HIV-1 is the result of multiple genetic variations." J Gen Virol **77** ( Pt 9): 2009-13.
- Carr, J. M., H. Hocking, et al. (1999). "Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood lymphocytes." Virology **265**(2): 319-29.
- Cavrois, M., J. Neidleman, et al. (2004). "HIV-1 virion fusion assay: uncoating not required and no effect of Nef on fusion." Virology **328**(1): 36-44.

- Chazal, N., G. Singer, et al. (2001). "Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity." J Virol **75**(8): 4014-8.
- Chen, P., W. Hubner, et al. (2007). "Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses." J Virol **81**(22): 12582-95.
- Cherepanov, P., G. Maertens, et al. (2003). "HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells." J Biol Chem **278**(1): 372-81.
- Chester, A., V. Weinreb, et al. (2004). "Optimization of apolipoprotein B mRNA editing by APOBEC1 apoenzyme and the role of its auxiliary factor, ACF." RNA **10**(9): 1399-411.
- Chowers, M. Y., C. A. Spina, et al. (1994). "Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene." J Virol **68**(5): 2906-14.
- Clapham, P. R. and A. McKnight (2001). "HIV-1 receptors and cell tropism." Br Med Bull **58**: 43-59.
- Cullen, B. R. (2006). "Role and mechanism of action of the APOBEC3 family of antiretroviral resistance factors." J Virol **80**(3): 1067-76.
- D'Aloja, P., E. Olivetta, et al. (1998). "gag, vif, and nef genes contribute to the homologous viral interference induced by a nonproducer human immunodeficiency virus type 1 (HIV-1) variant: identification of novel HIV-1-inhibiting viral protein mutants." J Virol **72**(5): 4308-19.

- D'Aloja, P., A. C. Santarcangelo, et al. (2001). "Genetic and functional analysis of the human immunodeficiency virus (HIV) type 1-inhibiting F12-HIVnef allele." J Gen Virol **82**(Pt 11): 2735-45.
- Dahmus, M. E. (1996). "Phosphorylation of mammalian RNA polymerase II." Methods Enzymol **273**: 185-93.
- Dahmus, M. E. (1996). "Reversible phosphorylation of the C-terminal domain of RNA polymerase II." J Biol Chem **271**(32): 19009-12.
- Davis, D. M. and S. Sowinski (2008). "Membrane nanotubes: dynamic long-distance connections between animal cells." Nat Rev Mol Cell Biol **9**(6): 431-6.
- Day, J. R., C. Munk, et al. (2004). "The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity." J Virol **78**(3): 1069-79.
- de Ronde, A., B. Klaver, et al. (1992). "Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes." Virology **188**(1): 391-5.
- Deeks, S. G. (2006). "Challenges of developing R5 inhibitors in antiretroviral naive HIV-infected patients." Lancet **367**(9512): 711-3.
- Demirov, D. G., J. M. Orenstein, et al. (2002). "The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner." J Virol **76**(1): 105-17.
- Derdeyn, C. A., J. M. Decker, et al. (2004). "Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission." Science **303**(5666): 2019-22.

- Djordjevic, J. T., S. D. Schibeci, et al. (2004). "HIV type 1 Nef increases the association of T cell receptor (TCR)-signaling molecules with T cell rafts and promotes activation-induced raft fusion." *AIDS Res Hum Retroviruses* **20**(5): 547-55.
- Doms, R. W. and D. Trono (2000). "The plasma membrane as a combat zone in the HIV battlefield." *Genes Dev* **14**(21): 2677-88.
- Douek, D. C., J. M. Brenchley, et al. (2002). "HIV preferentially infects HIV-specific CD4+ T cells." *Nature* **417**(6884): 95-8.
- Douek, D. C., L. J. Picker, et al. (2003). "T cell dynamics in HIV-1 infection." *Annu Rev Immunol* **21**: 265-304.
- Dull, T., R. Zufferey, et al. (1998). "A third-generation lentivirus vector with a conditional packaging system." *J Virol* **72**(11): 8463-71.
- Dykstra, M., A. Cherukuri, et al. (2003). "Location is everything: lipid rafts and immune cell signaling." *Annu Rev Immunol* **21**: 457-81.
- Ehrlich, L. I., P. J. Ebert, et al. (2002). "Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation." *Immunity* **17**(6): 809-22.
- Endres, M. J., S. Jaffer, et al. (1997). "Targeting of HIV- and SIV-infected cells by CD4-chemokine receptor pseudotypes." *Science* **278**(5342): 1462-4.
- Esposito, D. and R. Craigie (1998). "Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein-DNA interaction." *EMBO J* **17**(19): 5832-43.
- Fackler, O. T., A. Alcover, et al. (2007). "Modulation of the immunological synapse: a key to HIV-1 pathogenesis?" *Nat Rev Immunol* **7**(4): 310-7.

- Fackler, O. T., W. Luo, et al. (1999). "Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions." Mol Cell **3**(6): 729-39.
- Fais, S., M. R. Capobianchi, et al. (1995). "Unidirectional budding of HIV-1 at the site of cell-to-cell contact is associated with co-polarization of intercellular adhesion molecules and HIV-1 viral matrix protein." AIDS **9**(4): 329-35.
- Fantini, J., D. G. Cook, et al. (1993). "Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor." Proc Natl Acad Sci U S A **90**(7): 2700-4.
- Fantini, J., D. Hammache, et al. (2000). "Role of glycosphingolipid microdomains in CD4-dependent HIV-1 fusion." Glycoconj J **17**(3 -4): 199-204.
- Fantini, J., M. Maresca, et al. (2000). "Glycosphingolipid (GSL) microdomains as attachment platforms for host pathogens and their toxins on intestinal epithelial cells: activation of signal transduction pathways and perturbations of intestinal absorption and secretion." Glycoconj J **17**(3 -4): 173-9.
- Fauci, A. S., G. Pantaleo, et al. (1996). "Immunopathogenic mechanisms of HIV infection." Ann Intern Med **124**(7): 654-63.
- Fenard, D., W. Yonemoto, et al. (2005). "Nef is physically recruited into the immunological synapse and potentiates T cell activation early after TCR engagement." J Immunol **175**(9): 6050-7.
- Feng, Y., C. C. Broder, et al. (1996). "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor." Science **272**(5263): 872-7.

- Fischer, M., B. Joos, et al. (2004). "Cellular viral rebound after cessation of potent antiretroviral therapy predicted by levels of multiply spliced HIV-1 RNA encoding nef." J Infect Dis **190**(11): 1979-88.
- Flores, O., G. Lee, et al. (1999). "Host-cell positive transcription elongation factor b kinase activity is essential and limiting for HIV type 1 replication." Proc Natl Acad Sci U S A **96**(13): 7208-13.
- Flynn, N. M., D. N. Forthal, et al. (2005). "Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection." J Infect Dis **191**(5): 654-65.
- Foster, J. L., S. J. Anderson, et al. (1994). "Specific suppression of human CD4 surface expression by Nef from the pathogenic simian immunodeficiency virus SIVmac239open." Virology **201**(2): 373-9.
- Foster, J. L. and J. V. Garcia (2007). "Role of Nef in HIV-1 replication and pathogenesis." Adv Pharmacol **55**: 389-409.
- Frank, I., H. Stoiber, et al. (1996). "Acquisition of host cell-surface-derived molecules by HIV-1." AIDS **10**(14): 1611-20.
- Frankel, A. D. and J. A. Young (1998). "HIV-1: fifteen proteins and an RNA." Annu Rev Biochem **67**: 1-25.
- Friedl, P., A. T. den Boer, et al. (2005). "Tuning immune responses: diversity and adaptation of the immunological synapse." Nat Rev Immunol **5**(7): 532-45.
- Gallo, S. A., C. M. Finnegan, et al. (2003). "The HIV Env-mediated fusion reaction." Biochim Biophys Acta **1614**(1): 36-50.



- Ganser-Pornillos, B. K., M. Yeager, et al. (2008). "The structural biology of HIV assembly." Curr Opin Struct Biol **18**(2): 203-17.
- Garcia, J. V. and A. D. Miller (1991). "Serine phosphorylation-independent downregulation of cell-surface CD4 by nef." Nature **350**(6318): 508-11.
- Garriga, J., J. Peng, et al. (1998). "Upregulation of cyclin T1/CDK9 complexes during T cell activation." Oncogene **17**(24): 3093-102.
- Gaudin, C., M. H. Mazaauric, et al. (2005). "Structure of the RNA signal essential for translational frameshifting in HIV-1." J Mol Biol **349**(5): 1024-35.
- Geballe, A. P. and M. K. Gray (1992). "Variable inhibition of cell-free translation by HIV-1 transcript leader sequences." Nucleic Acids Res **20**(16): 4291-7.
- Geijtenbeek, T. B., D. S. Kwon, et al. (2000). "DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells." Cell **100**(5): 587-97.
- Geyer, M., O. T. Fackler, et al. (2001). "Structure--function relationships in HIV-1 Nef." EMBO Rep **2**(7): 580-5.
- Giese, S. I., I. Woerz, et al. (2006). "Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1(SF2) Nef." Virology **355**(2): 175-91.
- Grakoui, A., S. K. Bromley, et al. (1999). "The immunological synapse: a molecular machine controlling T cell activation." Science **285**(5425): 221-7.
- Greenway, A. L., G. Holloway, et al. (2003). "HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication." J Biosci **28**(3): 323-35.

- Groom, H. C., E. C. Anderson, et al. (2009). "Rev regulates translation of human immunodeficiency virus type 1 RNAs." J Gen Virol **90**(Pt 5): 1141-7.
- Groot, F., S. Welsch, et al. (2008). "Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses." Blood **111**(9): 4660-3.
- Grossman, Z., M. Meier-Schellersheim, et al. (2006). "Pathogenesis of HIV infection: what the virus spares is as important as what it destroys." Nat Med **12**(3): 289-95.
- Grzesiek, S., A. Bax, et al. (1996). "The solution structure of HIV-1 Nef reveals an unexpected fold and permits delineation of the binding surface for the SH3 domain of Hck tyrosine protein kinase." Nat Struct Biol **3**(4): 340-5.
- Guadalupe, M., E. Reay, et al. (2003). "Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy." J Virol **77**(21): 11708-17.
- Gupta, N. and A. L. DeFranco (2003). "Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation." Mol Biol Cell **14**(2): 432-44.
- Haase, A. T. (1999). "Population biology of HIV-1 infection: viral and CD4+ T cell demographics and dynamics in lymphatic tissues." Annu Rev Immunol **17**: 625-56.
- Haase, A. T. (2005). "Perils at mucosal front lines for HIV and SIV and their hosts." Nat Rev Immunol **5**(10): 783-92.

- Hallenberger, S., M. Moulard, et al. (1997). "The role of eukaryotic subtilisin-like endoproteases for the activation of human immunodeficiency virus glycoproteins in natural host cells." J Virol **71**(2): 1036-45.
- Haller, C. and O. T. Fackler (2008). "HIV-1 at the immunological and T-lymphocytic virological synapse." Biol Chem **389**(10): 1253-60.
- Haller, C., S. Rauch, et al. (2007). "HIV-1 Nef employs two distinct mechanisms to modulate Lck subcellular localization and TCR induced actin remodeling." PLoS One **2**(11): e1212.
- Haller, C., S. Rauch, et al. (2006). "The HIV-1 pathogenicity factor Nef interferes with maturation of stimulatory T-lymphocyte contacts by modulation of N-Wasp activity." J Biol Chem **281**(28): 19618-30.
- Hammache, D., G. Pieroni, et al. (2000). "Reconstitution of sphingolipid-cholesterol plasma membrane microdomains for studies of virus-glycolipid interactions." Methods Enzymol **312**: 495-506.
- Hammarstedt, M. and H. Garoff (2004). "Passive and active inclusion of host proteins in human immunodeficiency virus type 1 gag particles during budding at the plasma membrane." J Virol **78**(11): 5686-97.
- Hammer, S. M., J. J. Eron, Jr., et al. (2008). "Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel." JAMA **300**(5): 555-70.
- Harder, T., P. Scheiffele, et al. (1998). "Lipid domain structure of the plasma membrane revealed by patching of membrane components." J Cell Biol **141**(4): 929-42.

- Harrich, D., J. Garcia, et al. (1989). "Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat." J Virol **63**(6): 2585-91.
- Harris, R. S., K. N. Bishop, et al. (2003). "DNA deamination mediates innate immunity to retroviral infection." Cell **113**(6): 803-9.
- Harris, R. S., S. K. Petersen-Mahrt, et al. (2002). "RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators." Mol Cell **10**(5): 1247-53.
- He, J., Y. Chen, et al. (1997). "CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia." Nature **385**(6617): 645-9.
- He, J., S. Choe, et al. (1995). "Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity." J Virol **69**(11): 6705-11.
- Hicks, C. B., C. Gay, et al. (2007). "Acute HIV infection: the impact of anti-retroviral treatment on cellular immune responses." Clin Exp Immunol **149**(2): 211-6.
- Ho, D. D. and P. D. Bieniasz (2008). "HIV-1 at 25." Cell **133**(4): 561-5.
- Hoffman, T. L., C. C. LaBranche, et al. (1999). "Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein." Proc Natl Acad Sci U S A **96**(11): 6359-64.
- Horejsi, V. (2003). "The roles of membrane microdomains (rafts) in T cell activation." Immunol Rev **191**: 148-64.
- Hubner, W., G. P. McNerney, et al. (2009). "Quantitative 3D video microscopy of HIV transfer across T cell virological synapses." Science **323**(5922): 1743-7.

- Huppa, J. B. and M. M. Davis (2003). "T-cell-antigen recognition and the immunological synapse." Nat Rev Immunol **3**(12): 973-83.
- Huthoff, H. and M. H. Malim (2007). "Identification of amino acid residues in APOBEC3G required for regulation by human immunodeficiency virus type 1 Vif and Virion encapsidation." J Virol **81**(8): 3807-15.
- Igakura, T., J. C. Stinchcombe, et al. (2003). "Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton." Science **299**(5613): 1713-6.
- Iyengar, S., J. E. Hildreth, et al. (1998). "Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells." J Virol **72**(6): 5251-5.
- Jacks, T., M. D. Power, et al. (1988). "Characterization of ribosomal frameshifting in HIV-1 gag-pol expression." Nature **331**(6153): 280-3.
- Janes, P. W., S. C. Ley, et al. (1999). "Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor." J Cell Biol **147**(2): 447-61.
- Jarmuz, A., A. Chester, et al. (2002). "An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22." Genomics **79**(3): 285-96.
- Johnson, D. C. and M. T. Huber (2002). "Directed egress of animal viruses promotes cell-to-cell spread." J Virol **76**(1): 1-8.
- Jolly, C., K. Kashefi, et al. (2004). "HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse." J Exp Med **199**(2): 283-93.
- Jolly, C., I. Mitar, et al. (2007). "Adhesion molecule interactions facilitate human immunodeficiency virus type 1-induced virological synapse formation between T cells." J Virol **81**(24): 13916-21.

- Jolly, C., I. Mitar, et al. (2007). "Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1." J Virol **81**(11): 5547-60.
- Jolly, C. and Q. J. Sattentau (2004). "Retroviral spread by induction of virological synapses." Traffic **5**(9): 643-50.
- Jolly, C. and Q. J. Sattentau (2005). "Human immunodeficiency virus type 1 virological synapse formation in T cells requires lipid raft integrity." J Virol **79**(18): 12088-94.
- Jolly, C. and Q. J. Sattentau (2007). "Human immunodeficiency virus type 1 assembly, budding, and cell-cell spread in T cells take place in tetraspanin-enriched plasma membrane domains." J Virol **81**(15): 7873-84.
- Keppler, O. T., N. Tibroni, et al. (2006). "Modulation of specific surface receptors and activation sensitization in primary resting CD4+ T lymphocytes by the Nef protein of HIV-1." J Leukoc Biol **79**(3): 616-27.
- Kestler, H. W., 3rd, D. J. Ringler, et al. (1991). "Importance of the nef gene for maintenance of high virus loads and for development of AIDS." Cell **65**(4): 651-62.
- Khan, I. H., E. T. Sawai, et al. (1998). "Role of the SH3-ligand domain of simian immunodeficiency virus Nef in interaction with Nef-associated kinase and simian AIDS in rhesus macaques." J Virol **72**(7): 5820-30.
- Kirchhoff, F., T. C. Greenough, et al. (1995). "Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection." N Engl J Med **332**(4): 228-32.

- Krammer, P. H., R. Arnold, et al. (2007). "Life and death in peripheral T cells." Nat Rev Immunol **7**(7): 532-42.
- Krautkramer, E., S. I. Giese, et al. (2004). "Human immunodeficiency virus type 1 Nef activates p21-activated kinase via recruitment into lipid rafts." J Virol **78**(8): 4085-97.
- LaBranche, C. C., T. L. Hoffman, et al. (1999). "Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity." J Virol **73**(12): 10310-9.
- Larkin, M., R. A. Childs, et al. (1989). "Oligosaccharide-mediated interactions of the envelope glycoprotein gp120 of HIV-1 that are independent of CD4 recognition." AIDS **3**(12): 793-8.
- Laspia, M. F., A. P. Rice, et al. (1989). "HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation." Cell **59**(2): 283-92.
- Lee, C. H., K. Saksela, et al. (1996). "Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain." Cell **85**(6): 931-42.
- Leng, Q., G. Borkow, et al. (2001). "Immune activation correlates better than HIV plasma viral load with CD4 T-cell decline during HIV infection." J Acquir Immune Defic Syndr **27**(4): 389-97.
- Lewinski, M. K., M. Yamashita, et al. (2006). "Retroviral DNA integration: viral and cellular determinants of target-site selection." PLoS Pathog **2**(6): e60.
- Lewis, P., M. Hensel, et al. (1992). "Human immunodeficiency virus infection of cells arrested in the cell cycle." EMBO J **11**(8): 3053-8.

- Li, J., Y. Liu, et al. (2002). "Direct participation of Sam68, the 68-kilodalton Src-associated protein in mitosis, in the CRM1-mediated Rev nuclear export pathway." J Virol **76**(16): 8374-82.
- Li, J., Y. Liu, et al. (2002). "Expression of exogenous Sam68, the 68-kilodalton SRC-associated protein in mitosis, is able to alleviate impaired Rev function in astrocytes." J Virol **76**(9): 4526-35.
- Liu, Y., H. Liu, et al. (2004). "CD4-independent infection of astrocytes by human immunodeficiency virus type 1: requirement for the human mannose receptor." J Virol **78**(8): 4120-33.
- Llano, M., M. Vanegas, et al. (2004). "LEDGF/p75 determines cellular trafficking of diverse lentiviral but not murine oncoretroviral integrase proteins and is a component of functional lentiviral preintegration complexes." J Virol **78**(17): 9524-37.
- Llano, M., M. Vanegas, et al. (2006). "Identification and characterization of the chromatin-binding domains of the HIV-1 integrase interactor LEDGF/p75." J Mol Biol **360**(4): 760-73.
- Lozach, P. Y., A. Amara, et al. (2004). "C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles." J Biol Chem **279**(31): 32035-45.
- Lu, X., X. Wu, et al. (1996). "CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1." Curr Biol **6**(12): 1677-84.



- Lubben, N. B., D. A. Sahlender, et al. (2007). "HIV-1 Nef-induced down-regulation of MHC class I requires AP-1 and clathrin but not PACS-1 and is impeded by AP-2." Mol Biol Cell **18**(9): 3351-65.
- Lyles, R. H., A. Munoz, et al. (2000). "Natural history of human immunodeficiency virus type 1 viremia after seroconversion and proximal to AIDS in a large cohort of homosexual men. Multicenter AIDS Cohort Study." J Infect Dis **181**(3): 872-80.
- Majorovits, E., M. Nejmeddine, et al. (2008). "Human T-lymphotropic virus-1 visualized at the virological synapse by electron tomography." PLoS One **3**(5): e2251.
- Malim, M. H., S. Bohnlein, et al. (1989). "Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function." Cell **58**(1): 205-14.
- Malim, M. H., J. Hauber, et al. (1989). "The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA." Nature **338**(6212): 254-7.
- Mallardo, M., E. Dragonetti, et al. (1996). "An NF-kappaB site in the 5'-untranslated leader region of the human immunodeficiency virus type 1 enhances the viral expression in response to NF-kappaB-activating stimuli." J Biol Chem **271**(34): 20820-7.
- Mancebo, H. S., G. Lee, et al. (1997). "P-TEFb kinase is required for HIV Tat transcriptional activation in vivo and in vitro." Genes Dev **11**(20): 2633-44.
- Manes, S., G. del Real, et al. (2000). "Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection." EMBO Rep **1**(2): 190-6.

- Mangino, G., Z. A. Percario, et al. (2007). "In vitro treatment of human monocytes/macrophages with myristoylated recombinant Nef of human immunodeficiency virus type 1 leads to the activation of mitogen-activated protein kinases, IkappaB kinases, and interferon regulatory factor 3 and to the release of beta interferon." J Virol **81**(6): 2777-91.
- Manninen, A., G. H. Renkema, et al. (2000). "Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway." J Biol Chem **275**(22): 16513-7.
- Mariani, R., D. Chen, et al. (2003). "Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif." Cell **114**(1): 21-31.
- Marin, M., K. M. Rose, et al. (2003). "HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation." Nat Med **9**(11): 1398-403.
- Martin, N., S. Welsch, et al. "Virological synapse-mediated spread of human immunodeficiency virus type 1 between T cells is sensitive to entry inhibition." J Virol **84**(7): 3516-27.
- Massanella, M., I. Puigdomenech, et al. (2009). "Antigp41 antibodies fail to block early events of virological synapses but inhibit HIV spread between T cells." AIDS **23**(2): 183-8.
- McDonald, D., L. Wu, et al. (2003). "Recruitment of HIV and its receptors to dendritic cell-T cell junctions." Science **300**(5623): 1295-7.
- Mehandru, S., M. A. Poles, et al. (2004). "Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract." J Exp Med **200**(6): 761-70.

- Mehle, A., J. Goncalves, et al. (2004). "Phosphorylation of a novel SOCS-box regulates assembly of the HIV-1 Vif-Cul5 complex that promotes APOBEC3G degradation." Genes Dev **18**(23): 2861-6.
- Miele, G., A. Mouland, et al. (1996). "The human immunodeficiency virus type 1 5' packaging signal structure affects translation but does not function as an internal ribosome entry site structure." J Virol **70**(2): 944-51.
- Miller, M. D., M. T. Warmerdam, et al. (1994). "The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages." J Exp Med **179**(1): 101-13.
- Miura-Shimura, Y., L. Duan, et al. (2003). "Cbl-mediated ubiquitinylation and negative regulation of Vav." J Biol Chem **278**(40): 38495-504.
- Modem, S., K. R. Badri, et al. (2005). "Sam68 is absolutely required for Rev function and HIV-1 production." Nucleic Acids Res **33**(3): 873-9.
- Monks, C. R., B. A. Freiberg, et al. (1998). "Three-dimensional segregation of supramolecular activation clusters in T cells." Nature **395**(6697): 82-6.
- Muramatsu, M., K. Kinoshita, et al. (2000). "Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme." Cell **102**(5): 553-63.
- Neil, S. J., T. Zang, et al. (2008). "Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu." Nature **451**(7177): 425-30.
- Nejmeddine, M., A. L. Barnard, et al. (2005). "Human T-lymphotropic virus, type 1, tax protein triggers microtubule reorientation in the virological synapse." J Biol Chem **280**(33): 29653-60.

- Nejmeddine, M., V. S. Negi, et al. (2009). "HTLV-1-Tax and ICAM-1 act on T-cell signal pathways to polarize the microtubule-organizing center at the virological synapse." Blood **114**(5): 1016-25.
- Neumann, M., J. Harrison, et al. (1994). "Splicing variability in HIV type 1 revealed by quantitative RNA polymerase chain reaction." AIDS Res Hum Retroviruses **10**(11): 1531-42.
- Nguyen, D. H. and J. E. Hildreth (2000). "Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts." J Virol **74**(7): 3264-72.
- Nobile, C., D. Rudnicka, et al. "HIV-1 Nef inhibits ruffles, induces filopodia, and modulates migration of infected lymphocytes." J Virol **84**(5): 2282-93.
- Okochi, K., H. Sato, et al. (1984). "A retrospective study on transmission of adult T cell leukemia virus by blood transfusion: seroconversion in recipients." Vox Sang **46**(5): 245-53.
- Ono, A. (2009). "HIV-1 Assembly at the Plasma Membrane: Gag Trafficking and Localization." Future Virol **4**(3): 241-257.
- Pantaleo, G., C. Graziosi, et al. (1993). "New concepts in the immunopathogenesis of human immunodeficiency virus infection." N Engl J Med **328**(5): 327-35.
- Pantaleo, G., S. Menzo, et al. (1995). "Studies in subjects with long-term nonprogressive human immunodeficiency virus infection." N Engl J Med **332**(4): 209-16.
- Park, I. W. and J. J. He (2009). "HIV-1 Nef-mediated inhibition of T cell migration and its molecular determinants." J Leukoc Biol **86**(5): 1171-8.

- Parkin, N. T., E. A. Cohen, et al. (1988). "Mutational analysis of the 5' non-coding region of human immunodeficiency virus type 1: effects of secondary structure on translation." EMBO J **7**(9): 2831-7.
- Peretti, S., I. Schiavoni, et al. (2005). "Cell death induced by the herpes simplex virus-1 thymidine kinase delivered by human immunodeficiency virus-1-based virus-like particles." Mol Ther **12**(6): 1185-96.
- Peretti, S., I. Schiavoni, et al. (2006). "Selective elimination of HIV-1-infected cells by Env-directed, HIV-1-based virus-like particles." Virology **345**(1): 115-26.
- Perkins, N. D., A. B. Agranoff, et al. (1994). "Transcription factor AP-2 regulates human immunodeficiency virus type 1 gene expression." J Virol **68**(10): 6820-3.
- Peterlin, B. M. and D. Trono (2003). "Hide, shield and strike back: how HIV-infected cells avoid immune eradication." Nat Rev Immunol **3**(2): 97-107.
- Phillips, D. M. (1994). "The role of cell-to-cell transmission in HIV infection." AIDS **8**(6): 719-31.
- Pitisuttithum, P., P. Gilbert, et al. (2006). "Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand." J Infect Dis **194**(12): 1661-71.
- Popovic, M., G. Lange-Wantzin, et al. (1983). "Transformation of human umbilical cord blood T cells by human T-cell leukemia/lymphoma virus." Proc Natl Acad Sci U S A **80**(17): 5402-6.
- Price, D. H. (2000). "P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II." Mol Cell Biol **20**(8): 2629-34.

- Puigdomenech, I., M. Massanella, et al. (2009). "On the steps of cell-to-cell HIV transmission between CD4 T cells." Retrovirology **6**: 89.
- Qiao, X., B. He, et al. (2006). "Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells." Nat Immunol **7**(3): 302-10.
- Quaranta, M. G., B. Mattioli, et al. (2006). "The immunoregulatory effects of HIV-1 Nef on dendritic cells and the pathogenesis of AIDS." FASEB J **20**(13): 2198-208.
- Raney, A., A. Y. Shaw, et al. (2007). "Structural constraints on human immunodeficiency virus type 1 Nef function." Virology **368**(1): 7-16.
- Rauch, S., K. Pulkkinen, et al. (2008). "Human immunodeficiency virus type 1 Nef recruits the guanine exchange factor Vav1 via an unexpected interface into plasma membrane microdomains for association with p21-activated kinase 2 activity." J Virol **82**(6): 2918-29.
- Readinger, J. A., G. M. Schiralli, et al. (2008). "Selective targeting of ITK blocks multiple steps of HIV replication." Proc Natl Acad Sci U S A **105**(18): 6684-9.
- Reddy, T. R., W. D. Xu, et al. (2000). "General effect of Sam68 on Rev/Rex regulated expression of complex retroviruses." Oncogene **19**(35): 4071-4.
- Renkema, G. H. and K. Saksela (2000). "Interactions of HIV-1 NEF with cellular signal transducing proteins." Front Biosci **5**: D268-83.
- Revy, P., T. Muto, et al. (2000). "Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2)." Cell **102**(5): 565-75.

Riethmuller, J., A. Riehle, et al. (2006). "Membrane rafts in host-pathogen interactions."

Biochim Biophys Acta **1758**(12): 2139-47.

Rockstroh, J. K. and S. Mauss (2004). "Clinical perspective of fusion inhibitors for

treatment of HIV." J Antimicrob Chemother **53**(5): 700-2.

Roeth, J. F. and K. L. Collins (2006). "Human immunodeficiency virus type 1 Nef:

adapting to intracellular trafficking pathways." Microbiol Mol Biol Rev **70**(2):

548-63.

Rossi, D. and A. Zlotnik (2000). "The biology of chemokines and their receptors." Annu

Rev Immunol **18**: 217-42.

Rudnicka, D., J. Feldmann, et al. (2009). "Simultaneous cell-to-cell transmission of

human immunodeficiency virus to multiple targets through polysynapses." J Virol

**83**(12): 6234-46.

Saito, T. and T. Yokosuka (2006). "Immunological synapse and microclusters: the site

for recognition and activation of T cells." Curr Opin Immunol **18**(3): 305-13.

Salvi, R., A. R. Garbuglia, et al. (1998). "Grossly defective nef gene sequences in a

human immunodeficiency virus type 1-seropositive long-term nonprogressor." J

Virol **72**(5): 3646-57.

Sanchez-Velazquez, N., E. B. Udofia, et al. (2004). "hRIP, a cellular cofactor for Rev

function, promotes release of HIV RNAs from the perinuclear region." Genes Dev

**18**(1): 23-34.

Sato, H., J. Orenstein, et al. (1992). "Cell-to-cell spread of HIV-1 occurs within minutes

and may not involve the participation of virus particles." Virology **186**(2): 712-24.

- Sattentau, Q. J. and R. A. Weiss (1988). "The CD4 antigen: physiological ligand and HIV receptor." Cell **52**(5): 631-3.
- Sawai, E. T., I. H. Khan, et al. (1996). "Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques." Curr Biol **6**(11): 1519-27.
- Scarlatti, G., E. Tresoldi, et al. (1997). "In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression." Nat Med **3**(11): 1259-65.
- Schrager, J. A., V. Der Minassian, et al. (2002). "HIV Nef increases T cell ERK MAP kinase activity." J Biol Chem **277**(8): 6137-42.
- Schumacher, A. J., G. Hache, et al. (2008). "The DNA deaminase activity of human APOBEC3G is required for Ty1, MusD, and human immunodeficiency virus type 1 restriction." J Virol **82**(6): 2652-60.
- Schwartz, O., V. Marechal, et al. (1995). "Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell." J Virol **69**(7): 4053-9.
- Schwartz, O., V. Marechal, et al. (1996). "Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein." Nat Med **2**(3): 338-42.
- Schwartz, S., B. K. Felber, et al. (1992). "Mechanism of translation of monocistronic and multicistronic human immunodeficiency virus type 1 mRNAs." Mol Cell Biol **12**(1): 207-19.
- Sheehy, A. M., N. C. Gaddis, et al. (2002). "Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein." Nature **418**(6898): 646-50.



- Sherer, N. M., M. J. Lehmann, et al. (2007). "Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission." Nat Cell Biol **9**(3): 310-5.
- Simmen, T., J. E. Aslan, et al. (2005). "PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis." EMBO J **24**(4): 717-29.
- Simmons, A., B. Gangadharan, et al. (2005). "Nef-mediated lipid raft exclusion of UbcH7 inhibits Cbl activity in T cells to positively regulate signaling." Immunity **23**(6): 621-34.
- Simon, J. H., N. C. Gaddis, et al. (1998). "Evidence for a newly discovered cellular anti-HIV-1 phenotype." Nat Med **4**(12): 1397-400.
- Simon, V., D. D. Ho, et al. (2006). "HIV/AIDS epidemiology, pathogenesis, prevention, and treatment." Lancet **368**(9534): 489-504.
- Simons, K. and R. Ehehalt (2002). "Cholesterol, lipid rafts, and disease." J Clin Invest **110**(5): 597-603.
- Simons, K. and E. Ikonen (1997). "Functional rafts in cell membranes." Nature **387**(6633): 569-72.
- Singh, S. P., P. Tungaturthi, et al. (2001). "Virion-associated HIV-1 Vpr: variable amount in virus particles derived from cells upon virus infection or proviral DNA transfection." Virology **283**(1): 78-83.
- Sol-Foulon, N., M. Sourisseau, et al. (2007). "ZAP-70 kinase regulates HIV cell-to-cell spread and virological synapse formation." EMBO J **26**(2): 516-26.
- Sourisseau, M., N. Sol-Foulon, et al. (2007). "Inefficient human immunodeficiency virus replication in mobile lymphocytes." J Virol **81**(2): 1000-12.

- Sowinski, S., C. Jolly, et al. (2008). "Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission." Nat Cell Biol **10**(2): 211-9.
- Spina, C. A., T. J. Kwah, et al. (1994). "The importance of nef in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes." J Exp Med **179**(1): 115-23.
- Spina, C. A., H. E. Prince, et al. (1997). "Preferential replication of HIV-1 in the CD45RO memory cell subset of primary CD4 lymphocytes in vitro." J Clin Invest **99**(7): 1774-85.
- Steinbrook, R. (2007). "One step forward, two steps back--will there ever be an AIDS vaccine?" N Engl J Med **357**(26): 2653-5.
- Stevenson, M. (2003). "HIV-1 pathogenesis." Nat Med **9**(7): 853-60.
- Subramaniam, D., H. Zhou, et al. "Cholesterol rich lipid raft microdomains are gateway for acute phase protein, SERPINA1." Int J Biochem Cell Biol **42**(9): 1562-70.
- Swigut, T., N. Shohdy, et al. (2001). "Mechanism for down-regulation of CD28 by Nef." EMBO J **20**(7): 1593-604.
- Tamma, S. M., N. Chirmule, et al. (1997). "CD4 cross-linking (CD4XL) induces RAS activation and tumor necrosis factor-alpha secretion in CD4+ T cells." Blood **90**(4): 1588-93.
- Teng, B. B., S. Ochsner, et al. (1999). "Mutational analysis of apolipoprotein B mRNA editing enzyme (APOBEC1). structure-function relationships of RNA editing and dimerization." J Lipid Res **40**(4): 623-35.

- Thoulouze, M. I., N. Sol-Foulon, et al. (2006). "Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse." Immunity **24**(5): 547-61.
- Tobiame, M., J. E. Lineberger, et al. (2003). "Nef does not affect the efficiency of human immunodeficiency virus type 1 fusion with target cells." J Virol **77**(19): 10645-50.
- Towers, G., M. Bock, et al. (2000). "A conserved mechanism of retrovirus restriction in mammals." Proc Natl Acad Sci U S A **97**(22): 12295-9.
- Towers, G. J., T. Hatzioannou, et al. (2003). "Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors." Nat Med **9**(9): 1138-43.
- Vanegas, M., M. Llano, et al. (2005). "Identification of the LEDGF/p75 HIV-1 integrase-interaction domain and NLS reveals NLS-independent chromatin tethering." J Cell Sci **118**(Pt 8): 1733-43.
- Varma, R., G. Campi, et al. (2006). "T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster." Immunity **25**(1): 117-27.
- Vasiliver-Shamis, G., M. W. Cho, et al. (2009). "Human immunodeficiency virus type 1 envelope gp120-induced partial T-cell receptor signaling creates an F-actin-depleted zone in the virological synapse." J Virol **83**(21): 11341-55.
- Venzke, S., N. Michel, et al. (2006). "Expression of Nef downregulates CXCR4, the major coreceptor of human immunodeficiency virus, from the surfaces of target cells and thereby enhances resistance to superinfection." J Virol **80**(22): 11141-52.

- Villalba, M., K. Bi, et al. (2001). "Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T cells." J Cell Biol **155**(3): 331-8.
- von Essen, M., C. M. Bonefeld, et al. (2004). "Constitutive and ligand-induced TCR degradation." J Immunol **173**(1): 384-93.
- Wei, B. L., V. K. Arora, et al. (2005). "Activation of p21-activated kinase 2 by human immunodeficiency virus type 1 Nef induces merlin phosphorylation." J Virol **79**(23): 14976-80.
- Welker, R., M. Harris, et al. (1998). "Virion incorporation of human immunodeficiency virus type 1 Nef is mediated by a bipartite membrane-targeting signal: analysis of its role in enhancement of viral infectivity." J Virol **72**(11): 8833-40.
- Weng, J., D. N. Krementsov, et al. (2009). "Formation of syncytia is repressed by tetraspanins in human immunodeficiency virus type 1-producing cells." J Virol **83**(15): 7467-74.
- Wilfingseder, D., M. Spruth, et al. (2003). "Complement-mediated enhancement of HIV-1 neutralisation by anti-HLA antibodies derived from polytransfused patients." Int Arch Allergy Immunol **131**(1): 62-72.
- Wu, X., Y. Li, et al. (2005). "Weak palindromic consensus sequences are a common feature found at the integration target sites of many retroviruses." J Virol **79**(8): 5211-4.
- Xu, W., P. A. Santini, et al. (2009). "HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits." Nat Immunol **10**(9): 1008-17.

- Yamamoto, N., M. Okada, et al. (1982). "Transformation of human leukocytes by cocultivation with an adult T cell leukemia virus producer cell line." Science **217**(4561): 737-9.
- Yedavalli, V. S., C. Neuveut, et al. (2004). "Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function." Cell **119**(3): 381-92.
- Yilmaz, A., C. Bolinger, et al. (2006). "Retrovirus translation initiation: Issues and hypotheses derived from study of HIV-1." Curr HIV Res **4**(2): 131-9.
- Yu, Z., N. Sanchez-Velaz, et al. (2005). "The cellular HIV-1 Rev cofactor hRIP is required for viral replication." Proc Natl Acad Sci U S A **102**(11): 4027-32.
- Zenklusen, D. and F. Stutz (2001). "Nuclear export of mRNA." FEBS Lett **498**(2-3): 150-6.
- Zhang, L., J. Saadatmand, et al. (2008). "Function analysis of sequences in human APOBEC3G involved in Vif-mediated degradation." Virology **370**(1): 113-21.
- Zhang, Z., T. Schuler, et al. (1999). "Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells." Science **286**(5443): 1353-7.
- Zhou, Y., H. Zhang, et al. (2005). "Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells." J Virol **79**(4): 2199-210.
- Zhu, Y., T. Pe'ery, et al. (1997). "Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro." Genes Dev **11**(20): 2622-32.

## CURRICULUM VITAE

Linden Ann Green

### **Educational Background**

- 2006-2010 *Indiana University*, Indianapolis, IN  
Ph.D., Dept. of Microbiology and Immunology  
Advisor Johnny J. He, Ph.D.
- 2000-2004 *Indiana University* Bloomington, IN  
B.S. in Microbiology, with honors

### **Research Experience**

- 2006-2010 *Indiana University*, Indianapolis, IN  
Graduate Research Assistant under Johnny J. He, Ph.D.
- 2004-2005 *Indiana University Purdue University Indianapolis*, Indianapolis IN  
Laboratory Technician under Larry Jones, M.D., Ph.D.
- 2002-2004 *Indiana University*, Bloomington, IN  
Undergraduate researcher under David Daleke, Ph.D

### **Teaching Experience**

- 2008 *Indiana University*, Indianapolis, IN  
Teaching Assistant in Microbiology J210, "Nursing Microbiology"

### **Grants and Fellowships**

- 2008-2010 NIH T32 Immunology and Infectious Diseases Training Grant

### **Publications**

Fan Y, Zou W, Green LA, Kim BO, He JJ. "Activation of Egr-1 Expression in Astrocytes by HIV-1 Tat: New Insights into Astrocyte-Mediated Tat Neurotoxicity." *J Neuroimmune Pharmacol.*, 2010 Apr 23. [Epub ahead of print]

Park IW, Han C, Song X, Green LA, Wang T, Liu Y, Cen C, Song X, Yang B, Chen G, He JJ. "Inhibition of HIV-1 entry by extracts derived from traditional Chinese medicinal herbal plants." *BMC Complement Altern Med.*, 2009; **9**:29.

Green LA, Liu Y, He JJ. "Inhibition of HIV-1 infection and replication by enhancing viral incorporation of innate anti-HIV-1 protein A3G: a non-pathogenic Nef mutant-based anti-HIV strategy." *J Biol Chem*, 2009; **284**(20):13363-72.

Fan Y, Green LA, Liu Z, He JJ. "ER stress caused by HIV-Tat-induced upregulation of GFAP triggers neuron toxicity." (to be submitted)

Park IW, Timani KA, Green LA, Fan Y, He JJ. "HIV-1 Nef is transferred from Nef-expressing T cells to hepatocytes and upregulates HCV replication in these cells." (to be submitted)

Green, L. A., Park, I-W., Fan, Y., Henao-Mejia, J., and He, J. J. 2010. "HIV-1 Nef facilitates virological synapse formation and enhances cell-mediated HIV-1 infection" (to be submitted).