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Infection of the Gut by HIV-1: The Pathogenic Role of the Nef Protein

Grant, Tannika

UNIVERSITY OF CALGARY

Infection of the Gut by HIV-1: The Pathogenic Role of the Nef Protein

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Tannika Grant

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Abstract

Human Immunodeficiency virus type 1 (HIV-1) targets several cells in the immune system and induces their progressive decline and the Acquired Immunodeficiency Syndrome (AIDS). HIV produces viral proteins including Nef; an important multifunctional protein and viral pathogenic determinant. The gastrointestinal tract and impairment of its epithelial barrier has been implicated in HIV pathogenesis, thus an investigation of the contribution of HIV-1 Nef on intestinal barrier dysfunction was performed. An *in vitro* model system was established and then used to analyse the pathogenic role of Nef. Trans-epithelial electrical resistance, Real time (RT)-PCR and fluorescence microscopy methods were used. It was found that Nef expression in colonic Caco-2 cells, Jurkat and U937 cells; reduced expression of the Caspases, increased viability of cells, while decreasing expression of the tight junction proteins. Thus HIV-1 Nef protein may contribute to HIV pathogenesis by disrupting barrier integrity and cell death of colonic epithelial cells and monolayers.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
Amp	ampicillin
BHK-21	Baby hamster kidney cells-21
BSA	Bovine serum albumin
Caspase	cysteine-aspartic-acid-protease
CD4	cluster of differentiation 4- glycoprotein expressed on cell surfaces
cDNA	complimentary DNA
CLDN	Claudin
CM	Conditioned Media
Ct	Threshold Cycle
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
ddH_20	Double distilled
DMEM	Dulbecco-Vogt modified Eagle medium
DNA	Deoxy-ribose nucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
FBS	Fetal Bovine Serum
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
HAART	Highly Active Antiretroviral Therapy
HAD	HIV-associated dementia
HIV	Human Immunodeficiency Virus
HRP	horseradish peroxidase
Hrs	Hours

IFN-γ	Interferon-gamma
Ig (G)	Immunoglobulin (subtype gamma)
IL	Interleukin
IMF	Immunofluorescence microscopy
JAM	Junction Adhesion Molecule
Kb	Kilobases
kDa	kiloDaltons
LB	Luria Bertani Broth
LPS	Lipopolysacchharide
LTNPs	Long Term Non Progressors
MDM	Monocyte derived Macrophage
MEM	Minimal Essential Medium
mRNA	messenger RNA
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt
ORF	open reading frame
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PBST	1X PBS-0.1%Tween-20
pcDNA3™	Mammalian transfection vector
PCR	Polymerase Chain Reaction
Pen	Penicillin
PFA	Paraformaldehyde
pSINrep5	Sindbis alphavirus expression vector
RNA	Ribonucleic acid
r/t	Room temperature
RT-PCR	Real-Time PCR
(S)	Stomach
SAC	Southern Alberta Clinic
SDS	sodium dodecyl sulfate

SDS-1α	Stromal-derived factor-1alpha
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIN	Sindbis
SIV	Simian Immunodeficiency Virus
Strep	Streptomycin
TAE	Tris-acetate-EDTA
TEER	Trans Epithelial Electrical Resistance
TNF-α	Tumor Necrosis Factor-alpha
TX	Triton X-100
V	Volts
WHO	World Health Organization
ZO	Zonula occludens

HIV genes and proteins

env	envelope gene
Env	Envelope protein
Gag	Group-associated antigens protein
gp 120	Glycoprotein 120 –surface subunit of Envelope
IN	Integrase
LTR	Long Terminal Repeat
Nef	Negative factor gene-accessory
Nef	Negative factor protein -accessory
NU	Nucleocapsid protein-structural
MA	Matrix protein- structural
ORF	Open reading frame
pol	Polymerase gene
PR	Protease
Rev	Regulator of viral expression gene- regulatory
RT	Reverse Transcriptase

sNef	Soluble Nef protein
Tat	Trans-Activator of Transcription gene- regulatory
Vif	Viral infectivity factor gene-accessory
Vpu	Viral Protein U gene -accessory
Vpr	Viral Protein R gene- accessory

HIV-1 Strains

JR-FL	Primary isolate cloned from the brain
NL4-3	Primary isolate derived from peripheral blood mononuclear cells
YU-2	Primary isolate derived from the brain

Chapter One: **Introduction**

Human Immunodeficiency virus type 1 (HIV-1) targets several cell types in the immune system and induces their progressive decline and functional defects, resulting in immunodeficiency and increased susceptibility to malignancy and opportunistic infections (i.e. Acquired Immunodeficiency Syndrome (AIDS)). Intensive research efforts are focused on vaccine and antiretroviral drug therapies, which have proven a complex challenge. The virus evolves rapidly to avoid the adaptive immune responses and develops antiretroviral drug resistance, ensuring its continuous replication. Differences in tropism, infection efficiency of various tissues and altered pathogenic properties of HIV-1 viral strains affect pathogenesis [1-4]. HIV encodes accessory proteins that have become of great interest as their role in enhancing viral replication became known. Of the 4 accessory proteins expressed, HIV-1 Nef has become established as a multifunctional viral factor that is involved in enhancing infectivity, virus replication and pathogenesis [5-10]

The gastrointestinal (GI) tract holds the body's largest lymphoid tissue infected by HIV-1; the gut associated lymphoid tissue (GALT) [4, 11-15]. Reports have established that the memory $CD4^+$ T cells, located in the GALT are preferentially infected and act as a major viral reservoir in HIV infected patients on HAART placing the GALT as pivotal in HIV-1 pathogenesis [12, 16-18]. In addition HIV-1 induces GI barrier defects and it has been reported that the damage to the barrier caused during the early acute phase of infection, has been implicated in the progressive systemic chronic immune activation found during the later stages of infection [4, 19-23]. In a preliminary investigation using GI tissues obtained from HIV^+ individuals interesting expression patterns for the HIV-1 *nef* gene were found in the gut (T. Grant

and G. van Marle, unpublished data). HIV-1 Nef protein has been implicated as an effector in many viral pathogenic pathways, yet questions remain about its possible role in GI pathogenesis. At the time of the investigation there was little known on the contribution of HIV-1 Nef on GI pathogenesis, thus an *in vitro* study was designed.

In this thesis the research hypothesis was that HIV-1 Nef is an enterotoxic molecule that plays a role in HIV-1 pathogenesis in the GI tract. The aims were to establish and evaluate an effective *in vitro* GI model system that was relevant to HIV-1 analysis and then using this system, investigate the direct and indirect effects of HIV-1 Nef protein on GI dysfunction.

1.1 HIV and AIDS

United Nations AIDS (UNAIDS) and World Health Organization (WHO) estimate that AIDS related causes have been responsible for the death of more than 30 million people since it was first recognized in 1981; making it among the more destructive epidemics that have occurred. Globally, around 34 million people are reported to currently live with HIV [24, 25]. In 2010 there were 2.7 million new HIV infections reported with an estimated 390 000 children included in this rate, and between 1.6 and 1.9 million people were reported to have had AIDS-related deaths [25]. This reflects a still large number of people with new infections and living with the infection, though the increased initiatives over the last decade have led to progress as more people are living with HIV, with HIV related deaths down by 21% [25]. New infection rates have stabilized in many parts of the world that had been heavily impacted by the virus, with decreased mortality and wider access to antiretroviral therapy [24]. Yet an increase of infection

rates (reported) has been noted in some higher income countries after declining steadily for years, thus awareness and education on the virus should be maintained [24]. There are two types of HIV that can be distinguished genetically; HIV-1, which is the cause of the current epidemic, and HIV-2; which is found in West Africa and is considered less pathogenic [2]. Both HIV-1 and HIV-2 are thought to have arisen from a zoonotic infection by simian immunodeficiency virus (SIV) [2, 26].

The development of AIDS following primary infection with HIV-1 varies widely but occurs after a relatively long period of clinical latency in most adults (median, 8 to 10 years) [2, 27, 28]. However, in some individuals, the progression of the disease course is very slow and AIDS develops after a much longer period. These HIV-1-infected individuals are termed slow progressors or long-term non progressors (LTNPs) whereas HIV-infected individuals that develop AIDS quickly are termed rapid progressors. HIV primarily infects various cells in the human immune system such as $CD4^+$ T cells, macrophages and dendritic cells [2, 29-31]. As illustrated in Figure 1.1, HIV infection leads to the progressive decline of $CD4^+$ T cells through multiple mechanisms that appear to involve direct viral killing of infected cells, increased rates of apoptosis in infected cells and uninfected cells, inflammation, sustained immune activation and host cell responses [4, 12, 13, 32, 33]. When $CD4^+$ T cell numbers decline below a critical level (<200 cells/μL) or upon the appearance of hallmark symptoms in areas with limited laboratories, the individual has become immune compromised and is defined as having AIDS (Acquired Immunodeficiency Syndrome) [2, 29, 31, 34]. The importance of direct viral killing of immune cells during infection was shown in the SIV model by Li et al. and Mattapallil et al. [35, 36].

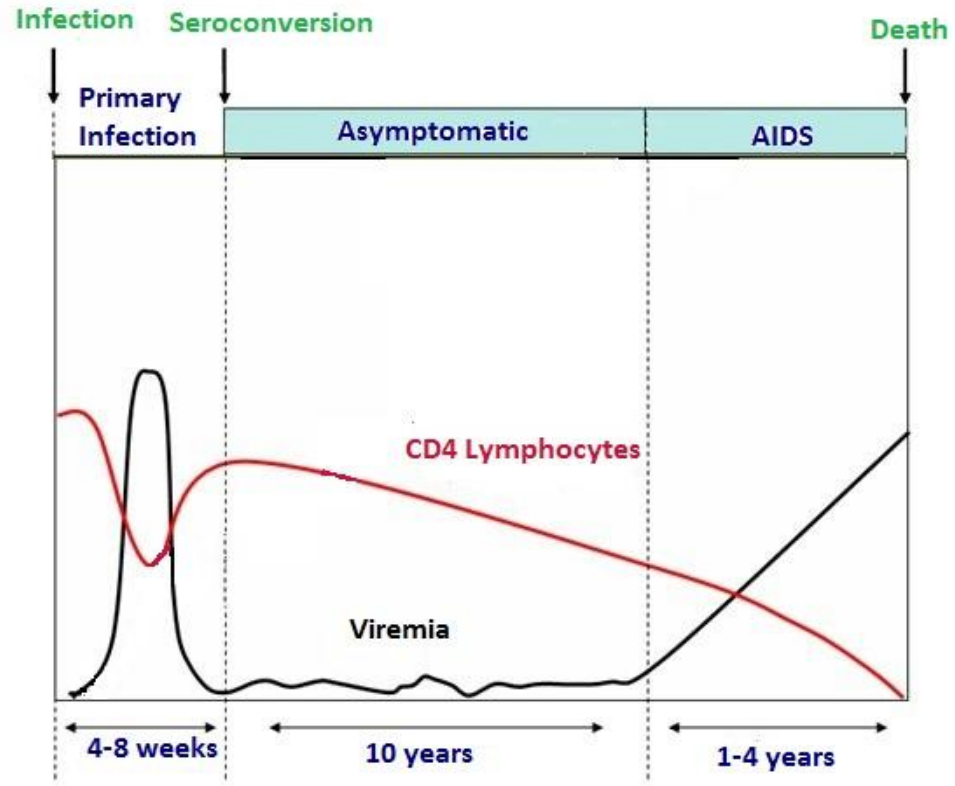


Figure 1.1 HIV-1 and AIDS Progression

The progression of HIV and AIDS from primary infection towards AIDS defined. After primary infection and the onset of the adaptive immune response, there is an asymptomatic period of clinical latency. Viremia levels remains stable as the virus is controlled by the immune system which may last for approximately 10 years in most adults. The $CD4^+$ T cell count rebounds at lower levels and then continues to decline at a slower pace over time. Once the CD4 T cell counts reach 200 cells/µl individuals are AIDS defined and susceptible to opportunistic infections and cancer with an average life expectancy of 1-4 years. Symptoms as defined by the WHO staging system may also be used to diagnose an individual in countries where laboratory support is limited. Figure adapted [37].

Brenchley and colleagues found that direct virally induced cell death was predominant during early infection, whereas indirect cell death induced through sustained immune activation contributed more in the later phase of infection [13]. Recent studies support both direct and indirect mechanisms contributing to the progression towards AIDS.

If untreated most HIV-infected individuals, develop and pass away, from AIDS related causes [3, 27, 34]. The main option available for the treatment of HIV/AIDS is combinations of antiretroviral therapy, consisting of pharmaceutical drugs that block various steps in the HIV life cycle termed highly active antiretroviral therapy (HAART). Antiretroviral treatment reduces both the morbidity and mortality of HIV infection; however routine access to medication is not available to everyone. Moreover, for the growing number of individuals living with the virus, the rapid development of antiretroviral drug resistance by HIV due to the high mutation rate of the virus is a problem [3, 24, 34].

1.1.1 HIV-1 Cell Infection

HIV-1 is an enveloped virus belonging to the Lentivirus family of retroviruses; it primarily infects cells with CD4 cell surface receptor molecules [2]. Infection of the cell is initially mediated by the surface envelope glycoprotein (gp120) binding to the CD4 receptor on a T-cell, macrophage, or dendritic cell (DC), and to a coreceptor on the membrane of the host cell. The primary receptors used by HIV are CC chemokine receptor 5 (CCR5) on macrophages and DCs or the CXC chemokine receptor 4 (CXCR4) on T cells. Other chemokine receptors such as CCR3 and GPR15/BOB have been found to be used to a smaller degree by the virus for entry [32, 38].

Illustrated simply in Figure 1.2a, upon attachment of the viral envelope (Env) to CD4 and the coreceptor CCR5 or CXCR4, the virus enters a target cell. Each virus particle-or virion contains two copies of an RNA genome that is surrounded by the capsid structural proteins. The viral genome is uncoated and has is reverse transcribed into DNA (proviral DNA) by a virally encoded reverse transcriptase (RT) present in the virus particle [34]. This proviral DNA is then integrated into the host-cell chromosomal DNA by a virally encoded integrase (IN) so that the genome can be transcribed by the host cell transcription machinery (Figure 1.2a and b) [2, 34]. The genome is 9749 nucleotides in length and codes for 9 primary gene products shown in (Figure 1.2b).

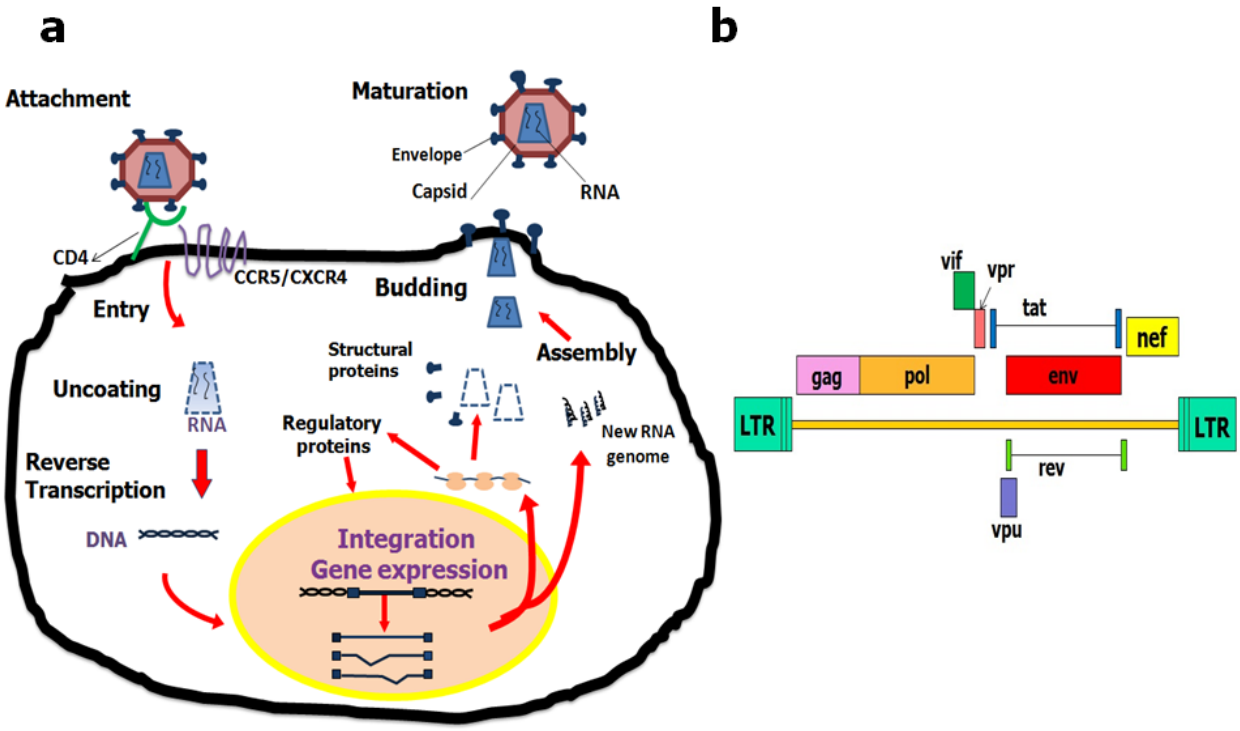


Figure 1.2 HIV-1 Viral life cycle and genome organization as integrated proviral DNA.

A. The viral life cycle upon infection of cells is illustrated in this simplified diagram. The virus binds to the cell and envelope fusion leads to release of the viral capsid into the cytoplasm. The RNA genome is released then reverse transcribed into double-stranded cDNA (proviral DNA). The cDNA is integrated into the host cell genome, referred to as provirus. Cell activation leads to transcription of the genome into mRNA encoding the viral proteins and genomic RNA copies. Viral genomes and proteins are transported to the cell surface membrane then packaged to form new virus particles that are released from the cells. B. The viral genome is 9749 nucleotides in length and has long terminal repeats (LTR) flanking the genome for integration, which acts as promoters and terminators for viral transcription. The virus encodes three essential prototypical retrovirus protein products Env (envelope), Gag and Pol (polymerase), two regulatory proteins Tat, Rev and four accessory proteins Vif, Vpr, Vpu, and Nef. Figure adapted [39, 40]

Once the virus has integrated the infection can go dormant. If the virus becomes active upon stimulation of the cell; viral RNAs including progeny viral genomes are produced, and the cell releases a large number of virus particles that can infect other cells. It has also been found that $CD4^-$ cells in the brain, GI tract and genital tract can also use the glycolipid galactosyl ceramide receptor with CXCR4 to enter host cells [21]. The interaction of the virus with these cells appears to involve a different region of Env gp120 subunit than those using the CD4 surface molecule[41]. The latent HIV-1 infection seems to occur in memory CD4 T cells and in macrophages, and these cells are thought to be an important viral reservoir [2, 42, 43].

HIV is highly variable, due to its high level of replication with generation of 10^9 to 10^{10} virions per cycle coupled to a high mutation rate of approximately 3×10^{-5} per nucleotide base per cycle of replication and high recombination events [34]. HIV's RT is error prone due to lack of proof reading resulting in low fidelity copies of the genome when copied into DNA. The resulting mutations are maintained in the viral genomic RNA, upon transcription of the proviral genomic DNA and lead to a large population of sequence variants [34, 44]. This complex scenario leads to many sequence variants of HIV in a single infected patient and contributes to the ability of the virus to evade the adaptive immune response and the rapid development of antiretroviral drug resistance [1, 3, 34].

Similar to many other viral diseases, it appears that the severity and time course of AIDS development is a reflection of individual host genetic susceptibility and differences in virus pathogenicity [28]. With the host genes controlling levels of virus replication and immune

response to HIV-1, while in turn the various HIV-1 proteins could elicit pathogenic host cell responses, altering pathogenic outcome [29, 31, 41].

1.2 The HIV-1 Nef Protein

Along with the three prototypical retroviral proteins (Gag, Pol and Env) and two regulatory proteins (Tat and Rev) HIV and SIV encode four accessory proteins Nef, Vif, Vpr, and Vpu (Figure 1.2b). Earlier HIV-1 research focused heavily on the essential proteins Gag, Pol, and particularly Env but as evidence on their functions increased the accessory proteins have become of interest. Studies *in vivo* or in primary cell types have shown that the accessory proteins can alter the severity of viral infection, replication and disease progression. Released soluble forms of these proteins are responsible for bystander effects on neighbouring immune cells in absence of productive infection, and more recently the Nef protein has been found to be released in exosomes as well [9, 45]. Of all the accessory proteins the *nef* gene is most abundantly expressed during HIV-1 infection. The Nef protein is 27 kDa in size and is produced early after virus infection and associates with the cell membrane through N-terminal myristoylation [9]. The Nef protein has been found to be a complex multifunctional protein that interacts with multiple host cellular pathways essential for cell survival and function, and has been shown to affect pathogenesis, viral replication and virion infectivity [5, 8-10, 46-48].

1.2.1 Functions of the Nef Protein

The Nef protein's multiple functions can be exerted at membrane-bound or cytoplasmic stages and depending on its intracellular localization it interferes with several cellular signal transduction pathways and modulates cell surface expression of different host proteins [48-53]. The Nef protein modulates the expression of a growing number of membrane associated proteins such as MHC I and CD4 through varying mechanisms. The Nef protein decreases the surface expression of MHC class 1 by sequestering it in the Golgi network of HIV-1 infected T cells, thereby interfering with the ability of Cytotoxic T Lymphocytes (CTLs) to recognize and destroy the infected cells [8, 54]. The Nef protein also down-regulates CD4 through endocytosis, and the subsequent lysosomal degradation likely prevents superinfection by other HIV virions, and also interferes with T cell receptor (TCR) signaling leading to immune evasion [9, 50, 52]. In addition, the up-regulation of Fas ligand (FasL) or Fas expression on the surface of infected cells inducing apoptotic death of $CD4^+$ and $CD8^+$ T cells during HIV-1 or SIV infection, was found by some researchers to be dependent on a functional *nef* gene [55]. The Nef protein has also been proposed to be involved in remodeling the actin cytoskeleton of the host cell to aid in cell-cell transmission of virus particles [53, 56, 57].

1.2.2 Effect on Pathogenesis

There is a significant amount of data supporting the importance of Nef as a pathogenic determinant in humans. In a study using the Sydney Blood Bank Cohort, eight HIV-1 infected individuals who were infected with a Nef-defective HIV-1 strain by a single blood donor were all long term non progressors (LTNPs) [58]. Further evidence came from subsequent studies with this cohort and of other individuals infected with HIV-1 strains with deletions or functional

defects in the Nef protein encoding region [6, 7, 46]. These results were confirmed in macaque animal models with *nef* deleted SIV strains displaying delayed disease progression. The Nef protein has been found to contribute to neurologic disease, where intracellular expression of Nef protein was cytotoxic to astrocytes *in vitro* and induced a neuroinflammatory response as well as neuronal injury *in vitro* and *in vivo* [59]. It was also found that various HIV-1 *nef* alleles induce a severe AIDS-like disease in transgenic (Tg) mice when expressed under the control of the CD4C regulatory elements in immature and mature $CD4^+$ T cells and in cells of the macrophage/dendritic lineage [5, 28, 60]. Pathological changes observed were numerous and varied between alleles, resulting in differing pathogenicities spanning most phenotypes associated with HIV-1 infection and AIDS in humans or in SIV-infected primates [28, 61].

Clarifying the main role of the HIV-1 Nef protein *in vivo* during the life cycle of the virus has been elusive despite the extensive amount of research. More recently its role in the enhancement of virion infectivity has become the widely accepted main biological function of the Nef protein [51]. While understanding the underlying mechanisms as well as the relevance to HIV-1 spread *in vitro* and *in vivo* has remained a challenge, evidence has established that HIV-1 Nef protein is a key viral factor that affects viral replication, virion infectivity and pathogenesis.

1.3 HIV-1 Pathogenesis and the Gastrointestinal Tract

It has been established that HIV-1 infects and replicates in different subsets of $CD4^+$ T cells preferentially. As the majority of $CD4^+$ T cells reside in the GALT, attention has shifted to this important viral reservoir and site for host-pathogen interactions during HIV-1 infection. They are distinct as a majority exhibit a memory phenotype, and due to their constant exposure to nutrients and microbial pathogens these lymphocytes are usually more activated [16]. They also abundantly express CCR5 (70%), while only 20% of PBLs express CCR5. This population of activated $CD4^+$ T cells provides HIV-1 with an ideal environment to establish infection [16]. In the last few years reports have further established these memory $CD4^+$ T cells, located in the GALT as a major viral reservoir in HIV infected patients on HAART, with findings that there are 10 times more of these $CD4^+$ T cells infected than found in blood placing the GALT as pivotal in HIV-1 pathogenesis.[12, 16, 18].

1.3.1 HIV-1 and GI Barrier Dysfunction

In addition to the implications of the events occurring at the GALT, there has been growing evidence that the damage to barrier integrity of the GI and genital tracts observed during HIV-1 are associated with a rise in microbial translocations across the GI tract [22]. The translocations of these products has been implicated as contributing greatly to the later phase of systemic inflammation and chronic immune activation of HIV-1 pathogenesis [22, 23, 62, 63].

HIV-1 induced GI dysfunction has been observed and at times falls under the term HIV-1 Enteropathy, although this was used to describe the symptoms of the associated gut dysfunction that appears in SIV and HIV-1 infected patients [19, 20, 64-66]. In 25-30% of cases HIV

infected patients displayed gastrointestinal symptoms such as chronic diarrhea, dehydration, and malabsorption that were unexplained by any identifiable enteric pathogen, and were often fatal features of AIDS in parts of the world [19, 21, 67]. Evidence that HIV-1 is enteropathogenic became clearer with increasing data of HIV- associated abnormal mucosal structure and function that included; villus atrophy and maturational defects in the epithelial cells resulting in carbohydrate and lipid malabsorption [11, 64, 68, 69]. Furthermore the observations that: HIV nucleic acids are found in the intestinal mucosa, there is an increase in severity of diarrhea as disease progresses, and that antiretroviral therapy leads to an improvement of epithelial function highlighted the role of HIV-1 in enteropathy [4, 11, 70, 71].

The contribution of direct infection of epithelial cells to enteropathogenesis is debatable as differentiated epithelial cells have a short life span of 3 to 5 days, seemingly insufficient to support efficient productive viral replication. Moreover, most infected cells are possibly of macrophage lineage and located in the lamina propria rather than epithelium [21]. This pointed to indirect mechanisms where epithelial functions are disrupted without direct infection of intestinal epithelial cells. There have been many studies using the virus and the HIV-1 Env protein gp 120, which can be released independent of infection that support the role of indirect mechanisms [20, 72]. The Env protein has been found to be enterotoxic, causing damage to the integrity of the mucosal barrier, and to epithelial cells of the GI tract, with increased permeability and proinflammatory markers [20, 21, 38]. As mentioned, there has been growing evidence that the HIV associated damage to the mucosal barrier integrity of the GI and genital tracts gives rise to microbial translocations during early infection that appears to contribute greatly to the later

phase of systemic HIV-1 pathogenesis [22, 23, 62, 63]. The underlying mechanisms and roles of the various viral proteins were of interest and remain to be clarified.

1.3.2 Nef and the GI Tract

Previous investigations in our laboratory were conducted using samples from HIV-1 infected patients, demonstrated that the gut is compartmentalized within patients with respect to the Nef protein coding region [73]. I undertook a preliminary investigation to analyze the HIV-1 Nef protein for its pathogenic and inflammatory activity in the GI tract. The investigation was conducted using gut tissue samples from 8 HIV-1^{+} patients from the Southern Alberta Clinic (SAC) and control tissues obtained from 5 uninfected patients. Samples from this cohort consisted of esophagus (E), stomach (S), duodenum (D) and colon (C) tissue biopsies taken over 3 visits with 6 months in between each visit. An *in vivo* analysis of various gene expression profiles in the gastrointestinal tissues was performed using Real Time (RT)-PCR. Results of note from two time points, Visit 1 and Visit 5, are shown in Figure 1.3 (T. Grant and G. van Marle, unpublished data). I observed that the viral *nef* gene expression was differentially expressed in the gut compartments and was found to be elevated in colon tissues (Figure 1.3h); which was of note as the colon has been implicated in systemic disease pathogenesis [4, 13, 15, 33, 73, 74].

In addition I observed altered host and viral gene expression levels among various tissues of HIV-1 infected individuals compared to controls. Expression of proinflammatory molecules such as TNF-α, IL-1β and SDF-1α varied between tissues and over time (Figure 1.3 A, B, D unpublished data).

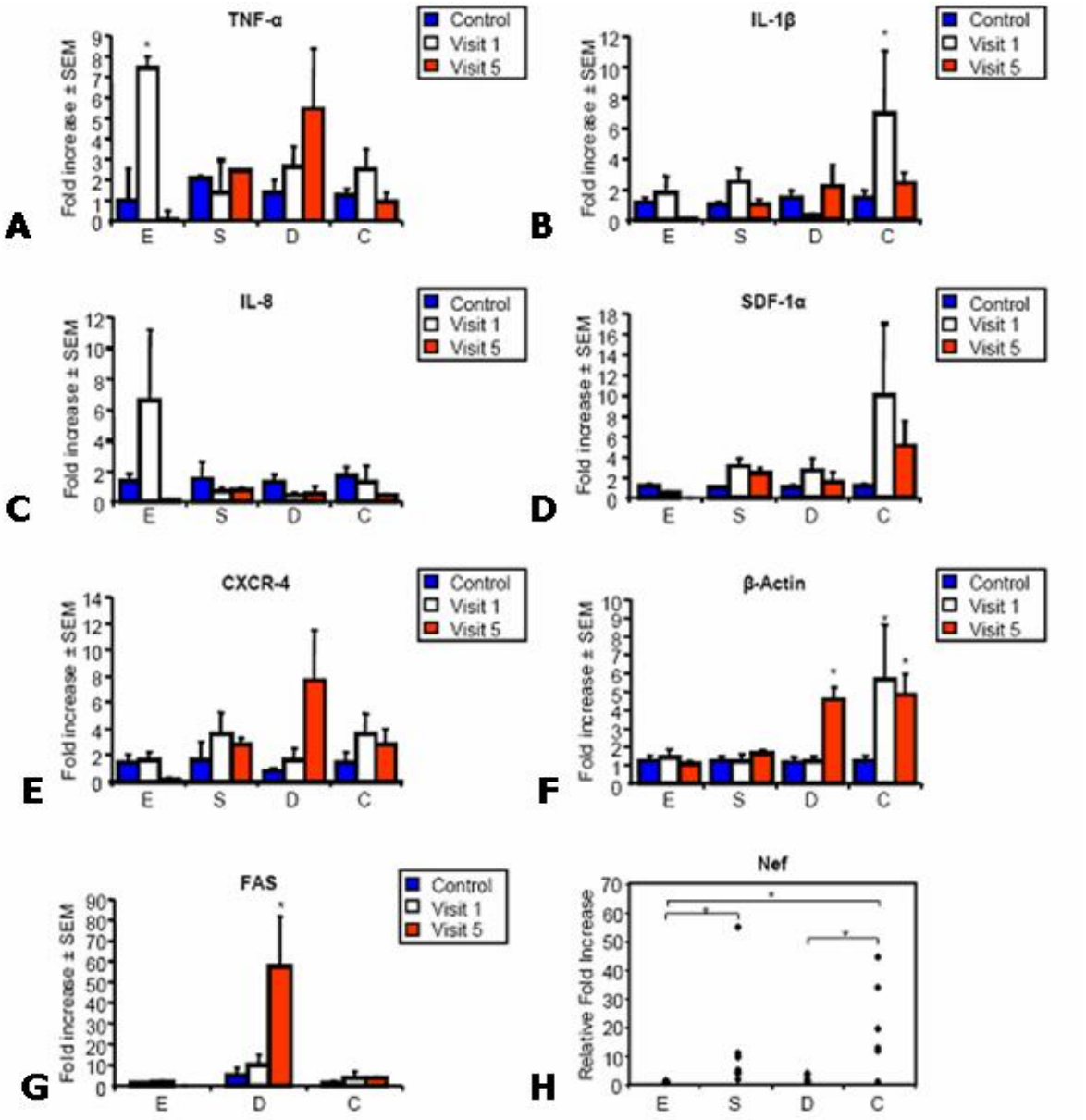


Figure 1.3 RT-PCR Analysis of viral and host genes expression in GI biopsies of HIV-1 infected individuals.

The investigation was conducted using gut tissue samples from 8 HIV-1^{+} patients from the Southern Alberta Clinic (SAC) and control tissues obtained from 5 uninfected patients. RNA was isolated from tissues and cDNA synthesized. Analysis shown is over two separate time periods (Visit 1 and Visit5) separated by 12 months in 4 different gut tissue compartments, esophagus

(E), stomach (S), duodenum (D) and colon (C), and then quantified relative to control (uninfected individuals) gene expression patterns. Differential gene expression of proinflammatory molecules in the different compartments over time was observed for TNF-α (A) IL-1β (B) and SDF-1α (D). F. β-Actin mRNA levels significantly increased over time in the duodenum and colon. G. Fas expression increased in the duodenum from V1 to V5. H. The viral *nef* gene expression is shown in the 4 gut compartments at Visit 5. Viral *nef* gene expression increased in the colon relative to levels in the esophagus. (T. Grant and G. van Marle, unpublished data). The experiments were repeated 2-3 times, per individual GI tissue sample. * = $p<0.05$, Dunn's multiple comparison test.

In Figure 1.3G Fas expression increased over time in the duodenum (T. Grant and G. van Marle, unpublished data), which is of note as this has been suggested to play a role in $CD4^+$ T cell depletion in the GI of the SIV models [17, 55, 75, 76]. It was also noted that alterations occurred to the initial housekeeping gene β-actin, whose expression consistently increased over time in the duodenum and colon tissues of HIV/AIDS patients (Figure 1.3F). This could be indicative of tissue repair as a response to HIV-1 induced injury, or inflammatory cell invasion, but may also be a direct effect of the Nef protein, which is known to interact with the cytoskeleton.

Analysis of the fold increases of gene expression from the colon in various patients using Spearman's Rho test revealed significant correlations between *nef* gene expression and SDF-1α, the HIV coreceptor CXCR4 and β-actin (Table 1.1) (T. Grant and G. van Marle, unpublished data). Correlation with β-actin mRNA expression is consistent with the aforementioned upregulation of expression in the colon. The correlation of Nef mRNA expression with SDF-1α mRNA expression is of significant interest. As other studies have shown its expression is induced by HIV-1 and affects HIV-1 pathogenesis in other organ systems [77]. This would also be consistent with the correlation between gag mRNA expression (i.e. HIV-1 genomic RNA expression) and SDF-1α. The correlation of Nef mRNA expression with CXCR4 is logical as SDF-1α is its ligand, and as such SDF-1α and CXCR-4 expression levels are correlated [78, 79]. A correlation was also observed between CCR5 and CXCR4 expression, which is of interest as these are the coreceptors needed for infection and have been shown to be affecting each other [32].

Table 1.1 Correlation Coefficients of mRNA Expression (Spearmans' Rho test).

Gene pair		rho*		Gene pair		rho*	
Nef	**β-Actin**	0.804	**p < 0.01**	**gag**	Nef	0.771	
	SDF-1α	0.855	**p < 0.01**		**SDF-1α**	0.943	**p < 0.01**
	IL-1β	0.624		**CXCR4**	**SDF-1α**	0.800	**p < 0.01**
	CCR-5	0.367			**CCR-5**	0.738	**p < 0.05**
	IL-8	0.571					
	CXCR4	0.667	**p < 0.05**	**IL-1β**	**MCP-1**	0.778	**p < 0.05**
	MCP-1	0.371			**IL-8**	0.833	**p < 0.05**

*** Spearmans' Rho, p value < 0.05 are indicated**

Correlations were observed from RT-PCR analysis between Nef mRNA expression and β-actin, SDF-1α, and CXCR4 mRNA expression levels. CXCR4 was correlated with its ligand SDF-1α and with the other primary HIV chemokine coreceptor CCR5. IL-1β was correlated with MCP-1 and IL-8 expression, similar to observations in other labs [80, 81], as IL-1β seems to be regulating MCP-1 and IL-8. (T. Grant and G. van Marle, unpublished data)

A recent study has been reported on the direct effects of soluble Nef (sNef) protein, which is released independent of viral infection and can exert its own functions during the viral life cycle. It was observed that direct exposure of sNef on genital and intestinal epithelial cells, increased coreceptor upregulation of CXCR4, as we observed in the patient GI tissues. They also found alterations in permeability, tight junctions and interestingly a modulatory effect for sNef, where sNef reduced the inflammatory effects of IFN-γ stimulated cells, suggesting that the virus has evolved defenses against the host IFN-γ responses [82, 83]. As discussed later, these findings have confirmed some of the patterns observed in this study for intracellular Nef protein.

The mechanisms and contributions behind the extensive HIV-1 induced impairment of the mucosal barrier that's been noted are still being clarified [23]. The observations, together with the evolutionary pattern and the elevated expression of the *nef* gene in the colon pointed towards a pathogenic role for Nef protein in the GI tract

1.4 The GI Tract

The GI tract has two essential roles; it is fundamental for digestion and it acts as a protective barrier; preventing damage and entry from harmful substances and pathogens. It represents the largest surface of the body in contact with the external environment, thus most human pathogens enter the body through the GI mucosal surface, especially in the intestines [84, 85]. In addition, along with the genital tract the GI tract represents one of the primary transmission routes for HIV-1. There are a variety of specific and nonspecific mechanisms used to establish the GI barrier, which include luminal mechanisms and digestive enzymes, the epithelial cells together with tight junctions in between them, and the GI mucosal immune system [86-88].

Upon entry into the lumen, barrier defense mechanisms include a thin mucous gel layer, secreted Immunoglobulin (Ig) A and antimicrobial agents such as the defensins which act as biological barrier, the rapid turnover of epithelial cells and the peristaltic movement of the GI tract to inhibit attachment and invasion by pathogens [85, 89]. The physical barrier is produced by the polarised single layer of epithelial cells, covered by a glycocalyx on their apical surface, and the cell junctions (Figure 1.4). The epithelial cells and their junctions play active roles in maintaining barrier integrity and the cells themselves induce the intestinal innate and adaptive immune systems [84, 90]. The lamina propria contains a large numbers of T cells, B cells, macrophage and DCs that interact with the epithelial cells, and monitor damage and microbial antigens [89]. They can also release factors to stimulate the growth and differentiation of the epithelial cells [89].

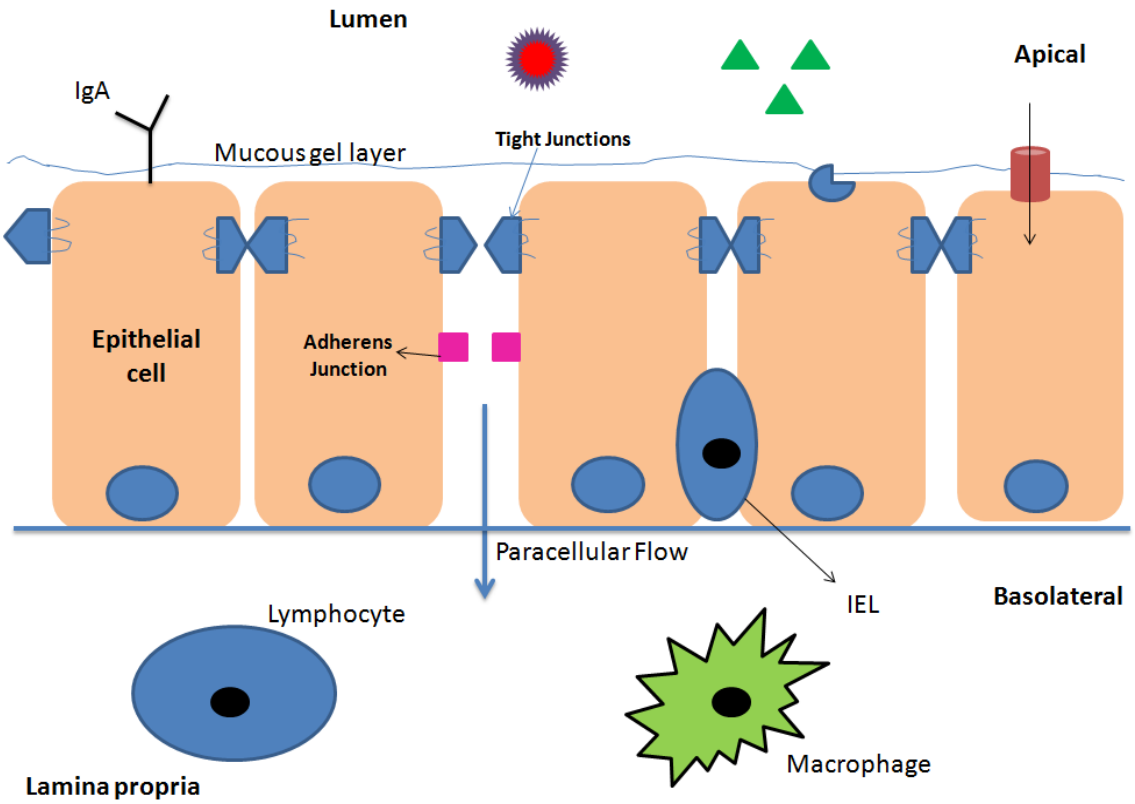


Figure 1.4 Schematic of the GI Mucosal Barrier

A simplified schematic diagram of the GI mucosal barrier. The polarized single layer of columnar epithelial cells is covered at the apical side with a thin mucus layer, secreted Ig A, and defensins that are part of the barrier defense mechanisms. The epithelial cells are sealed apically by the tight junction complex, which is followed below by the adherens junction (shown on one cell). The paracellular transport route is through the tight junctions, while the transcellular route is through various channels, transporters and pumps. The lamina propria contains the immune cells on guard, in cross talk with the epithelial cells. There are also intraepithelial lymphocyte (IEL) cells that can be found in the submucosal layer. Figure adapted from [85, 89].

The Th17 subset of T cells, play an important role in GI homeostasis as they regulate epithelial GI barrier and microbial invasion, providing protection against extracellular bacteria, fungi and mycobacteria [91, 92]. They secrete a number of proinflammatory cytokines and have gut-homing potential [91, 92]. While the innate and adaptive immune system plays a crucial role in defense, the epithelial mucosal layer in combination with the intercellular junctions and adhesion molecules are the first line of physical defense [90]. And thus a major function of the intestinal epithelia is to maintain an effective barrier that limits exposure of luminal antigens to the underlying tissue and circulation [85].

1.4.1 Mucosal Epithelial Layer

The epithelial layer in the intestines consists of a single layer of polarized columnar epithelial cells that are in constant flux [93]. The epithelial cells consist of primarily enterocytes- that are absorbent, with mucus secreting goblet cells and enteroendocrine cells in their midst. In the small intestine there are also Paneth and microfold cells, and the cells have microvilli, while the colon cells are flat [89]. As the epithelial cells are at the interface and face toxins and pathogens, a regular part of their maintenance involves high cell turn-over and shedding [93]. Thus their life span is short, 3-5 days once differentiated as they move up the crypt-villus axis (small intestine) or just the crypt in the colon, losing cell contact as they progress. The high cell turnover is programmed by apoptosis and this process is tightly controlled and balanced with cell proliferation to maintain the integrity of the barrier [94]. The epithelial cells interact with the immune cells and can also release proinflammatory markers and cytokines to induce their stimulation [89]. Intraepithelial lymphocytes in the submucosal layer are nearby to interact, thus this layer plays a very important role, not just a physical one. Epithelial cells have been shown *in*

vivo to express the coreceptor CXCR4, but it is still controversial whether they are just a reservoir for virus, or can support viral production in their short life cycle [95].

1.4.2 Tight Junctions

The tight junctions are of interest in the HIV induced pathogenesis as they are an important part of mucosal barrier function. The tight junction complex controls transport by the paracellular space by forming a seal between individual epithelial cells and in combination with the transcellular transport processes, generate distinct internal environments in the opposing compartments and defines the cells polarity (shown in Figure 1.4) [96, 97]. The tight junction complex involves an assembly of different peripheral membrane proteins, integral membrane proteins and the junctional adhesion molecules (JAMs) [97, 98]. Among the peripheral membrane proteins associated with tight junctions are the membrane-associated proteins zonula occludens (ZO)-1, ZO-2, and ZO-3.

The integral membrane proteins involved include, occludin and members of a large family of proteins called the claudins (CLDNs) [97, 98]. Both occludin and the CLDNs contain four transmembrane domains and are thought to be the points of cell-cell contact within the tight junction. ZO-1 has been shown to be a scaffolding protein that interacts with the cytoplasmic tails of occludin and the CLDNs [96]. In addition, ZO-1 interacts with ZO-2 and ZO-3, which then interact with various actin-binding proteins, linking the tight junction with the cytoskeleton [97, 98]. The variable expression patterns of the tight junction proteins in mucosal epithelial barriers form the varying permeability needed for tissue specific functions and are tightly regulated [97].

Barrier dysfunction leads to a change in homeostasis and the normal highly controlled intestinal permeability. The magnitude of permeation of molecules has been used in various models as means of assessing mucosal barrier integrity. Therefore, an integral component of intestinal homeostasis is maintenance and repair of the epithelial barrier itself, particularly in the setting of inflammation [99].

1.5 The Co Culture Barrier Model

The study of the human GI tract during HIV-1 infection is challenging as there are physical and psychological complications to obtaining biopsy samples from individuals. Procedures are invasive, and ethical considerations aside-the inability to alter the experimental conditions is a limitation, and reduces the types of investigations that can be performed. In addition investigations involving HIV are subject to rigorous procedures, specialized facilities and training, and resources not widely available. A standard animal model has not been established for studying HIV-1 GI pathogenesis. The currently used SIV non human primate models have been extremely helpful, yet have their own ethical concerns and limitations in extrapolating findings to humans on how the GI tract may respond [100]. There are species differences and additionally SIV strains must be used when working with primates as HIV infection does not follow the same pathogenesis in primates as it does in humans [100]. There have been *in vitro* models using intestinal cell lines and molecular biology techniques, that have been useful and have shed some light [101, 102]. There are advantages and disadvantages to all models, but for the following investigation the *in vitro* co-culture model system was used.

The co-culture system model involves epithelial cells seeded on tissue culture inserts with a permeable membrane of various pore sizes. The inserts have a substance that acts as an extra cellular matrix for the epithelial cells to adhere to, thus enabling a functional epithelial layer to be formed in the tissue culture plate [101, 103]. This allows the cells to establish polarity on the culture inserts and to then establish cell to cell contacts and form a differentiated monolayer. This permeable separation of the two compartments by the inserts enables a basal side and an apical side to be modeled, in addition to the ability to provide and receive nutrients or other factors of interest from one of the compartments [101, 103]. It is also possible to add other cells to mimic more complex tissue features as done with Caco-2 cells and human Raji B lymphocytes to mimic follicle- associated epithelium without the need for complex arrangements [104]. The co-culture model has been used extensively to study drug absorption and effects, immune cell interactions, and barrier defects in disease such as Inflammatory Bowel Disease [102, 105-107].

As a model epithelial barrier it is a reductionist model as it represents the intestinal epithelial cell monolayer only, but it has wide applicability and helps provide clarity to complex processes. In addition, the *in vitro* systems are used under standardized and more controlled conditions that can be experimentally manipulated more easily, and reduce the number of animals needed for experiments [101, 103].

1.6 Summary

HIV-1 Nef has been shown to have varied pathogenic effects in other tissues and cells, combined with the possible mechanisms of Nef on actin remodeling and cytoskeletal rearrangements, its effect at the intestinal epithelial barrier was of interest for this investigation. HIV-1 induced barrier dysfunction cannot be easily studied *in vivo*, and an effective animal model to study enteropathy is not available, but *in vitro* models using intestinal cell lines have shed some light. Thus after an initial *in vivo* gene expression analysis of HIV^+ infected GI tissues provided some correlations of note with respect to HIV-1 Nef in the GI tract an *in vitro* study was designed to further explore these observations.

At the onset of the study it was known that HIV-1 infects a broad range of cells in various tissues including the gut, though its role on colonic epithelial cells was less clear. In these analyses recombinant vectors were evaluated and used to infect or transfect the intestinal epithelial lines Caco-2, T84, HT-29, and the model T cells and monocytes; Jurkat and U937 cells, and used to analyze the direct and indirect contributions by the Nef protein to GI pathogenesis of HIV-1 infection.

1.7 Research Hypothesis and Aims

The hypothesis for this study was that the Nef protein is an enterotoxic molecule that plays a role in HIV-1 pathogenesis in the GI Tract.

Research Aims:

1. To evaluate various expression systems and model colonic epithelial cell lines to establish an effective reproducible *in vitro* strategy, that was relevant to assess the pathogenicity of HIV-1 Nef.

2. To use this *in vitro* system to investigate if the HIV-1 Nef protein contributes directly or indirectly to HIV-1 induced GI barrier dysfunction.

Chapter Two: **Experiment Methods and Materials**

2.1 Cell Culture Lines and Bacteria

2.1.1 Tissue Culture Cells

Three colon derived human epithelial cell lines were used over the course of these experiments and were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Caco-2 cells are an immortalized cell line isolated from a human colon adenocarcinoma displaying features of enterocytes in both small intestine and colon. The cells were maintained under tissue culture conditions in their growth medium of; Dulbecco's Modified Eagle Medium (DMEM), High Glucose, GlutaMAX supplemented with 10% Canadian Fetal Bovine Serum (FBS), 1% Minimal Essential Medium (MEM) Sodium Pyruvate 100 mM (100X), 1% Penicillin/Streptomycin (100X) (Pen/Strep) Liquid, and 1% L-Glutamine 200mM (100X).

T84 cells are a line isolated from human colon carcinoma which display colon epithelial crypt cell features. Cells were maintained in their growth media of a 1:1 volume of DMEM and Ham's F-12 media supplemented with 10% Canadian FBS, 1% MEM Sodium Pyruvate 100 mM (100X), 1% Pen/Strep (100X) and 1% L-Glutamine 200mM (100X). Lastly the human colorectal adenocarcinoma derived epithelial cell line HT-29 was maintained in growth media of McCoy's 5A (Modified) media supplemented with; 10% Canadian FBS, 1% MEM Sodium Pyruvate 100 mM (100X), 1% Pen/Strep (100X) and 1% L-Glutamine 200mM (100X).

In addition Baby Hamster Kidney (BHK)-21 adherent fibroblast cells were used in Sindbis infection experiments to produce viral stocks for infecting the colonic epithelial cells. The pSinRep5 viral vectors are optimally transfected in BHK-21 cell lines for production of virus stocks. These cells were kindly provided by Dr. Power at the University of Alberta. They were maintained in Eagle's Minimum Essential Medium (EMEM), 10% Canadian FBS, and 1% Pen/Strep (100X). The 4 adherent cell line described above were refreshed with growth media every 2-3 days until they reached confluence, at which point they were subcultured using Trypsin, 0.25% (1X) with EDTA 4Na, liquid for 5-10 mins at 37°C and 5% CO_2 then enzymatic digestion was inactivated by growth media. Fresh growth media was then mixed with cells based on desired dilution ratio.

Two model suspension cell lines were used for indirect *in vitro* experiments. The first was U937 a model monocyte cell line [108], and Jurkat E6 an immortalized line of T lymphocyte cells obtained from ATCC (Manassa, VA). Both cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 5-10% Canadian FBS and 1% Pen/Strep (100X) at 37°C and 5% CO_2. The media was replenished every 2 days and cells were subcultured by desired dilution ratio with fresh media. All cells listed above were maintained at 37°C in a humidified incubator with 5% CO_2 and routinely observed under phase contrast light microscopy (LM) to ensure proper growth. All cell culture reagents were obtained from Invitrogen (Burlington, ON).

2.1.2 Bacteria

The high efficiency chemically competent bacterial cell line; TOP10 (Invitrogen, Burlington, ON) were used for amplifying the pcDNA3 and Sindbis recombinant plasmids used in the study. Transformed bacterial cells were made into glycerol stocks and then grown on Luria Bertani medium (LB)-agar plates supplemented with 100 µg/ml ampicillin (Amp) for use in studies. Single colonies of transformed bacteria were grown overnight (12-15 hrs) in LB medium supplemented with 100µg/ml Amp (LB/Amp) on an orbital shaker at 250xg at 37°C. The bacterial cell lines, LB, and LB-agar powder were obtained from Invitrogen (Burlington, ON) and the Amp was purchased from Sigma (Oakville, ON).

2.2 Sindbis Virus Expression

A recombinant Sindbis (SIN) alphavirus expression vector was used to create viral stocks of the reporter protein Enhanced Green Fluorescent Protein (EGFP) in an evaluation of the infection efficiency of colon epithelial cells, for use as a viral infection vector for the Nef protein. The SINrep5 (pSINrep5) vector expressing EGFP (pSINrep5-EGFP), Env JR-FL(pSINrep5-JR-FL), Nef NL4-3 (pSinRep5-NL4-3) and Nef YU-2 (pSinRep5-YU-2) proteins were constructed and characterized previously by van Marle et al. in other cell types and were kindly provided by Dr. Power at the University of Alberta [59, 109].

2.2.1 Production of SINrep5-EGFP

The pSINrep5- protocol has been described previously [59, 110], briefly; 1µg of pSINrep5-EGFP and the helper virus construct (pDH-BB) were linearized with *Xho*I (New England Biolabs Pickering, ON) restriction enzyme per the manufacturer's protocol. Digested plasmids were gel purified by using the Qiaquick gel extraction kit (Qiagen Inc., Mississauga, ON) per instruction manual. The linearized plasmids were then treated with 100ng of Proteinase K (per kit protocol), gel purified and used as templates for the generation of capped RNA transcripts by *in vitro* run-off transcription using the SP6 mMESSAGE mMACHINE kit (Ambion Inc, Austin, TX). The obtained purified RNA transcripts were quantified and 10-15 µg of DH-BB RNA transcript along with 10-15 µg of SINrep5-EGFP RNA transcript were transfected to 3 x 10^6 viable BHK-21 cells/ml in PBS by electroporation using the Biorad Gene Pulser II (Bio-Rad Laboratories Canada Ltd., Mississauga, ON) at the following settings: two pulses at 800 Volts, 50 µF, and ∞ ohms [59, 110]. Cells were then immediately plated in their

culture media and incubated at 37 °C with 5% CO_2 for 24 hrs, at which point media containing SIN-EGFP was collected [59, 110].

2.2.2 Viral Titer and Epithelial Cell Infection

The viral titer for SINrep5-EGFP was determined by incubating 1x 10^6 non transfected BHK-21 cells grown on coverslips with serial dilutions of collected SINrep5-EGFP media. After 24 hrs cells were fixed with 4% Paraformaldehyde (PFA) (Sigma, Oakville, ON) and mounted on slides with ProLong Gold Antifade with 4′-6-diamidino-2-phenylindole (DAPI) reagent (Invitrogen, Burlington, ON) and the number of green EGFP-positive cells was counted under fluorescence microscopy. An average of 10^5-10^6 infectious virus particles/ml was obtained after counting the cells from 9 fields of view covering the slide and multiplying by the dilution factor. For epithelial cell infection, 1x 10^6 Caco-2 and T84 cells were grown on coverslips in multiwell plates (MP) (Corning, Tewksbery, MA) and incubated with serial dilutions of SIN-EGFP media to determine infectivity. Cells were fixed after 48 hrs and visualized by fluorescence microscopy, infection efficiency of epithelial cells was determined relative to the infectivity of SIN-EGFP on BHK-21 cells for the same viral titer.

2.3 pcDNA3 Expression Vector:

The pcDNA3 (Invitrogen, Burlington, ON) mammalian transfection vector was used to make constructs for the expression of two prototypic HIV-1 Nef proteins, and two control proteins EGFP and HIV-1 Env in the cell lines described previously. The Nef NL4-3 encoding sequence is derived from HIV-1 strain NL4-3 which was isolated from human blood cells and consists of 206 amino acids [111, 112]. In comparison Nef YU-2 encoding sequence was molecularly cloned and genotypically characterized from uncultured brain tissue of a patient with AIDS dementia complex and consists of 215 amino acids [59]. The Env-JR-FL encoding sequence is derived from the monocyte/macrophage-tropic (JR-FL) HIV-1 strain. The JR-FL strain was isolated from brain tissue of an HIV^+ patient with HIV-associated dementia (HAD), and has cytotoxic effects in brain tissue and neural cell lines and was used in these experiments as a positive control [59, 113, 114]. And lastly EGFP was used in the study as both a reporter protein for transfection and then as the negative vector control. This plasmid has been used for expression in a wide range of mammalian cell lines under control of the Cytomegalovirus (CMV) promoter and was kindly provided by Dr. S.R.W. Chen (University of Calgary).

2.3.1 pcDNA3 Constructs

2.3.1.1 PCR Amplification of Nef

The general Nef primers designed previously in laboratory were modified by the addition of a 5'-*EcoR* 1 and 3'-*Xho I* restriction enzyme recognition sequence at their ends to provide overhangs for directional cloning into pcDNA3 at the *EcoR I* and *Xho I* sites in the multiple cloning polylinker region (Figure 2.1).

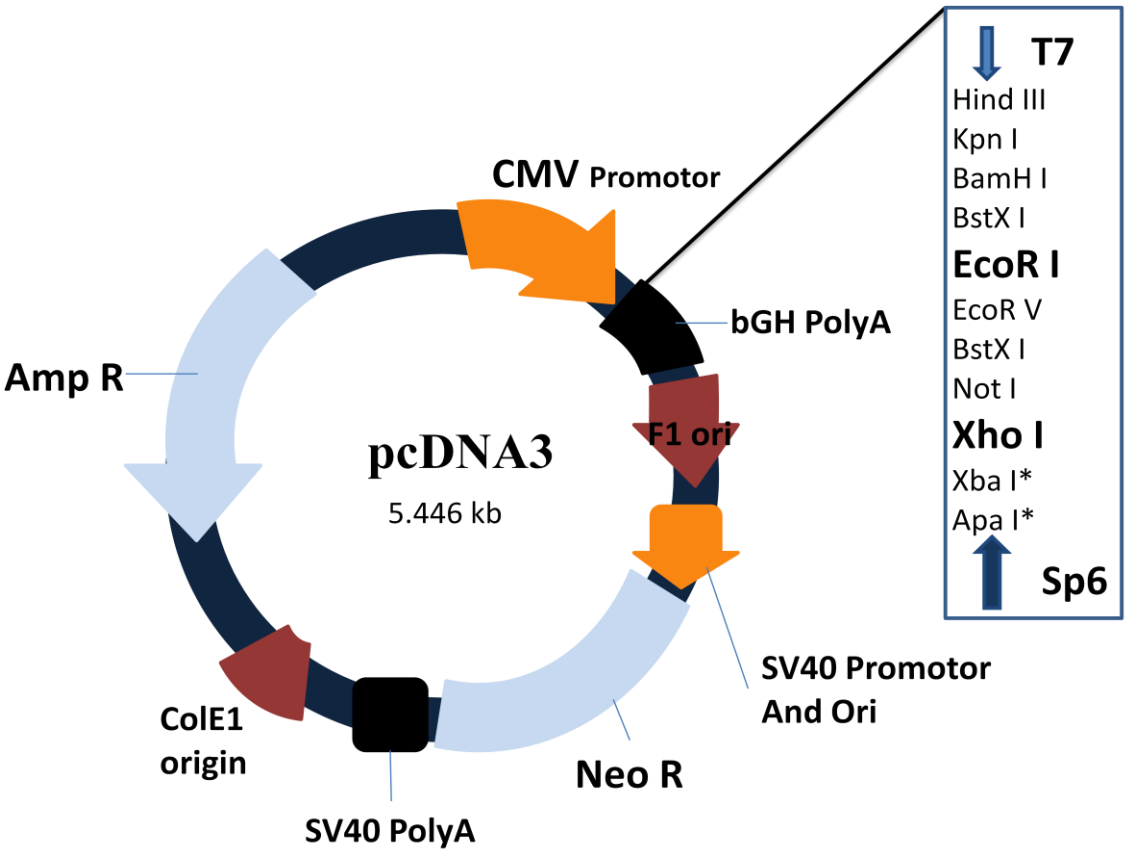


Figure 2.1 The pcDNA3 Transfection Vector

The Nef YU-2 and Nef NL4-3 open reading frames (ORF)s were subcloned into the pcDNA3 mammalian transfection vector (Invitrogen, Burlington, ON) at the multi cloning site using the *EcoRI* and *XhoI* restriction site for directional cloning. The vector was used to express the Nef proteins, YU-2 and NL4-3 as well as the Env JR-FL protein and Enhanced Green Fluorescent Protein (EGFP) as controls.

Primers were synthesized by the University of Calgary Core DNA Services (Calgary, Canada). The primers were then used to PCR amplify the Nef NL4-3 and Nef YU-2 encoding regions from the recombinant pSINrep5 vectors described above. The reaction mixture consisted of; 5µl 10X Pfx amplification buffer, 1.5 µl of 10 mM DNTP mixture, 1 µl of 50mM $MgSO^4$, 1.5 µl of primer mix (10 mM each), DNA template: 150ng for pSinRep5-Nef YU-2 and 250ng for pSinRep5-Nef NL4-3, ddH_20 Rnase free then 0.4 µl of Platinum *Pfx* DNA Polymerase in a total reaction volume of 50µl. PCR reagents were from Invitrogen (Burlington, ON).

The amplification protocol consisted of: an initial denaturation step of 5 min at 95°C, then 35 cycles of 1 min at 95°C, 1 min at 50°C for annealing (determined by gradient PCR), 1 min at 68°C for extension, followed by a final extension step of 10 min at 68°C. The forward primer used was: EcoR1-Nef8748F 5'-GCTGGAATTCGAAGAATAAGAC -3' and the reverse primer was Xho I-Nef9425R 5'-GAATTCTCGAGTCCCACCCCATC-3'. The PCR products were analyzed on a 1% TAE agarose (Invitrogen, Burlington, ON) gel by electrophoresis at 90 V for visual confirmation of fragment, at 697 bp and 725 bp for Nef NL4-3 and Nef YU-2 respectively. All gels were visualized by Ethidium Bromide (Sigma, Oakville, ON) and SafeView Nucleic Acid Stain (Applied Biological Materials Inc., Richmond, BC).

2.3.1.2 Subcloning

The Nef PCR fragments were prepared for ligation; briefly- the fragments were digested with *Xho I* and *EcoR I* restriction enzymes for 1 hr, and fragments were run on a 1% TAE agarose gel at 90V for isolation with the Qiaquick gel extraction kit (Qiagen Inc., Mississauga, ON). For the vector; 1 µg of pcDNA3 was digested with *Xho I* and *EcoR I* for 1.5hrs and

analyzed on a 1% TAE agarose gel by electrophoresis at 90 V to ensure proper digestion. Digested plasmid was then isolated from gel using the Qiaquick gel extraction kit as per insert fragments.

Ligation reactions were performed using a 3:1 insert to plasmid DNA ratio, thus 300ng of insert DNA was used for 100ng of plasmid DNA, and then 1.5µl of T4 DNA Ligase (Invitrogen, Burlington, ON), 1.5µl 10X T4 Ligase Buffer and ddH_20 was combined in a reaction volume of 15µl at 16°C over night. Following ligation, 10µl of ligation mixture was used to transform 100µl of TOP 10 competent bacteria cells by heat shock method for plasmid DNA amplification. Briefly, the DNA and cells were combined and stored on ice for 10 mins, then incubated in a 42°C water bath for 45 seconds and stored back on ice for 2mins. Then cells were incubated with 1ml of an LB/50% glycerol (Sigma, Oakville, ON) mixture for 1 hr at 37°C with shaking at 2500 rpm. A 100µl aliquot of the cell culture was then spread on LB-Agar plates supplemented with 100µg/ml of Amp and bacteria were grown for 13-15 hrs at 37°C.

Single colonies were selected from the plates and grown in LB for 12-15 hours at 37°C in an orbital shaker at 2500 rpm, and plasmid DNA isolated with the Qiagen Plasmid Miniprep kit for colony screening. Clones were analyzed by restriction digestion analysis for presence of insert and positive clones sent for sequencing to ensure correct insertion and sequence. Sequencing was performed by the University of Calgary DNA core services laboratory. Kris Cannon provided assistance with the PCR protocol design and technical assistance screening the final Nef clones. In addition she performed the pcDNA3-EGFP and pcDNA3-Env JR-FL constructions.

2.4 Transfection Reactions

Recombinant pcDNA3 DNA was transfected into the cell lines by lipofection and electroporation to express the proteins of interest. The plasmid DNA was prepared for transfection. Transformed TOP 10 *E. coli* were amplified in 100ml-250ml of LB/Amp media for plasmid DNA isolation. Bacterial cultures were grown as described above, and plasmid DNA was isolated using the GenElute Plasmid Miniprep Kit (Sigma, Oakville, ON) using the manufacturer's protocol. The DNA was eluted in RNase free water (Invitrogen, Burlington, ON), quantified by spectrophotometer and optical density checked to ensure purity of DNA. For the lipofection reactions the DNA was also subjected to phenol chloroform extraction and isopropanol precipitation-as needed for higher concentrations and quality. For the electroporation reactions, DNA was further concentrated by vacuum centrifugation and resuspended in RNase free water and then quantified again with a purity check.

2.4.1 Lipofection

Cells were transiently transfected by lipofection using Lipofectamine 2000 and Lipofectamine LTX with Plus Reagent (Invitrogen, Burlington, ON), the given protocols were followed and then further optimizations were performed. Briefly; cells were washed twice in sterile PBS, and then viable cells counted by hemocytometer using 4% Trypan blue (Invitrogen) staining exclusion test of cell viability [115]. Cells were then resuspended in their medium at $5x10^5$-$1x10^6$ cells per well the day before transfection. The three epithelial cell lines obtained from ATCC (Manassas, VA) were used between passages 5-15, while Jurkat and U937 cells were used between passages 15-24. The following conditions were then varied and evaluated for improved transfection efficiencies. Plasmid DNA concentration was varied with 2, 3, 4, 5 and

7μg tested. The ratio of DNA (in μg): Lipofectamine 2000 (in μl) was varied from 1:2-1:5. Complexes were diluted in Opti-MEM Reduced Serum Medium (Invitrogen), and the incubation period for the DNA: liposome complex formation was varied from the recommended time of 20mins and then increased up to 6 hrs. The mixtures were then added to the seeded cells and left for 24 hrs, and then fresh media was added to each well. A ratio of DNA (in μg): Lipofectamine 2000 (in μl) of 1:4, with an increased incubation period of plasmid DNA with lipid reagent of 4-6 hrs gave the best results regardless of DNA concentration. EGFP transfection efficiencies were determined by fluorescent microscopy and lastly by flow cytometry.

2.4.2 Neon Electroporation System

The Neon electroporation system was used in addition to transiently transfect cells with the pcDNA3 constructs following the provided instructions for DNA, and cell preparation. The system differs from other electroporation systems as it uses a pipette tip chamber as opposed to a cuvette. The Neon transfections were performed with Caco-2, T84, and HT-29 cells obtained from ATCC (Manassas, VA) kept between passages 4-10, and Jurkat and U937 cells used between passages 8-20. Cells are prepared 1-2 days before such that they are between 70-90% confluence for electroporations. On the day of transfection viable cells were counted by hemocytometer using 4% Trypan Blue (Invitrogen, Burlington, ON) staining exclusion test of cell viability [115], washed with PBS, and were collected by centrifugation at the concentration needed for resuspension in Resuspension BufferR (Invitrogen) at the cell density given by protocol (listed in Table 2.1 for cells used).

Table 2.1 Electroporation Parameters

Cell Line	Pulse Voltage V	Pulse Width (ms)	Pulse number	Cell Density (cells/ml)	Tip Type
Caco-2	1300	20	2	$5x\ 10^6$	100 µl
HT-29	1300	20	2	$5x\ 10^6$	100 µl
T84	1300	20	2	$5x\ 10^6$	100 µl
Jurkat	1350	10	3	$2\ x10^7$	100 µl
U937	1400	10	3	$5x\ 10^6$	10 µl

Final electroporation parameters used for the pcDNA3 transfection of cells using the Neon electroporation system. The 24-well optimization protocol that is preprogrammed into the system to determine optimal electroporation parameters was performed for epithelial cells and final parameters used are shown. The parameters used for the Jurkat and U937 cells were from the protocol provided from the Neon cell database online.

Cells are gently pipetted to obtain a single cell suspension then mixed with plasmid DNA and electroporated according to determined parameters. Cells are immediately pipetted into their growth medium (without antibiotics) and returned to CO_2 incubator for expression. The 24-well optimization protocol that is preprogrammed into the system to determine optimal electroporation parameters was performed for epithelial cells and final parameters used are shown in Table 2.1. The parameters used for the Jurkat and U937 cells were from the protocol provided from the Neon cell database online. All cells were transfected with 3µg of plasmid DNA for final experiments. The electroporation tips and buffers were specific to system and obtained by Invitrogen in the Neon transfection kit for electroporation tip type 100µl or 10µl. Transfection efficiency for EGFP was determined after 24 and 48 hrs under fluorescence microscopy by counting fields of EGFP positive cells, and for final counts and Neon system evaluation by flow cytometry.

2.5 Co-Culture System

The epithelial cells were cultured under normal conditions described above. For co-culture studies, cells were seeded on 12 or 24 well ThinCert tissue culture inserts in CellStar plates (Greiner Bio-One, Frickenhausen, Germany). Both transparent and translucent inserts were used with 0.4µm pore size polyethylene terephthalate (PET) semi-permeable membranes. For the direct transfection studies the cells were cultured alone after transfection in their growth medium (Figure 2.2). Staurosporine (Sigma, Oakville, ON) was used to induce apoptosis as positive control and applied apically to mock cells at 1µM and 0.5µM and plated along with transfected cells. The top compartment had 500µl of medium, and bottom compartment had 1.0 ml medium, that was replenished every 2-3 days. For the 24 well set up media was replenished every other day-2 days, depending on media colour.

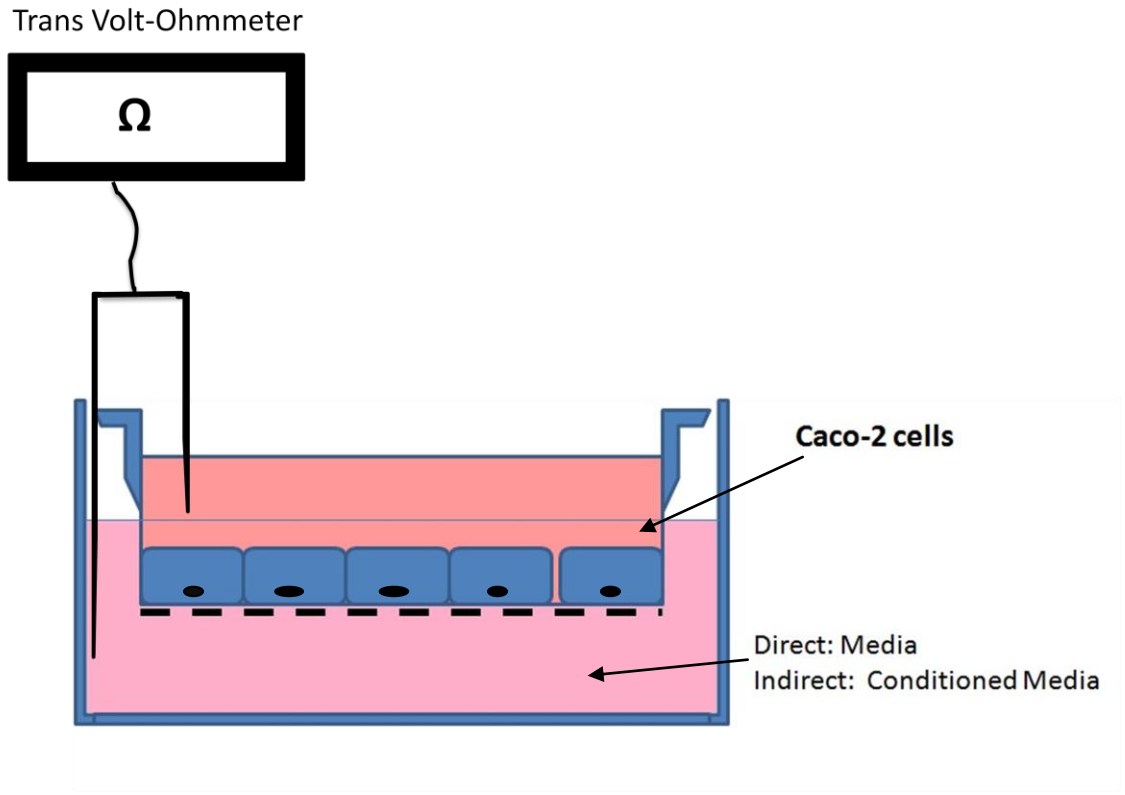


Figure 2.2 Co-Culture System and Trans-epithelial Electrical Resistance.

Colonic epithelial Caco-2 cells are shown in the co-culture system. For the direct studies Caco-2 cells were co-cultured on cell culture insert filter membranes in multiwell plates after transfection in growth medium. For the indirect effects of the HIV-Nef protein, the Caco-2 cells were cultured with conditioned media harvested from either transfected Jurkat cells or transfected U937 cells. Trans-epithelial electrical resistance (TEER) was measured using an Epithelial Volt-Ohmmeter, across the cell monolayer to assess the integrity of tight junctions at 24 hr time points, multiple readings were taken and averaged per well and time point.

2.5.1 Indirect Toxicity

To study the indirect toxicity, conditioned media (CM) harvested from transiently transfected Jurkat and U937 cells was prepared at varying dilutions. Briefly; $1x10^6$ viable Jurkat cells and $1x10^5$ viable U937 cells were counted by hemocytometer using 4% Trypan blue (Invitrogen, Burlington, ON) staining exclusion test of cell viability [115], and then transfected with recombinant pcDNA3 vectors or electroporation buffer for mock control. Cells were cultured for 2.5 days in 1mL of growth medium, and the resulting CM was harvested. CM was then diluted in media at 10, 25 and 50% concentrations and evaluated initially by overlaying on epithelial cells for varying days; 2, 3, and 5 days. Cells were lysed and analyzed by RT-PCR.

The Caco-2 cells were added to the inserts grown in media, allowed to form a monolayer and then CM, diluted to various concentrations of 10%, 25%, and 50% in media was applied. The CM was added to both the top (apical) and bottom (basolateral) compartment for comparative analysis after upon which cells were added to just the basolateral compartment. For both the direct and indirect co-culture studies using the 12 well plates, 0.5 ml of media was added to the top compartment and 1.5 ml were added to the bottom compartment with the media changed every 2-3 days. For experiments performed using the 24 well plates, 0.25 ml was added to the top of insert and 1 ml was added to the bottom compartment with the media changed every 1-2 days.

2.6 Transepithelial Electrical Resistance Studies

2.6.1 Direct Toxicity

The Trans-epithelial electrical resistance (TEER) method is a widely used standard tool to assess the barrier function of *in vitro* epithelium; and was used in this study to assess the effects of Nef on epithelial barrier function. The epithelial cells were transfected at $5x10^5$ as described above and then a single cell suspension was carefully applied to 12 and 24 well transparent Thincert inserts (Greiner Bio-One). TEER was measured using the EVOM2 Epithelial Volt-Ohmmeter with STX2 electrodes set (World Precision Instruments, Sarasota, FL) across the cells. Measurements were recorded every 24 hrs for 4 days after transfection. The media was changed every 2 days.

2.6.2 Indirect Toxicity

For the indirect studies, $5x10^5$ of a single epithelial cell suspension was carefully applied to the ThinCert inserts and grown under normal conditions until monolayers were differentiated. Upon plateau of TEER around 6-8 days after seeding, the baseline TEER was recorded and CM was applied to the basolateral compartment. Measurements were taken every 24 hrs for 4 days, and CM was replenished every 2 days. Measurements were all taken after letting the media settle to room temperature (r/t) for 5 minutes. The electrodes were inserted in different positions in each well and the readings were averaged to control for solution and monolayer edge effects [116]. The TEER value for the gap between the membrane filter insert is subtracted (the blank) from raw total values and then the tissue resistance is multiplied by the area of filter membrane. The TEER values were expressed as % of initial (baseline) TEER, before addition of CM. The

direct toxicity TEER studies were repeated in 5 experiments with duplicate and triplicate (for Nef) inserts. The indirect TEER studies were repeated 4 times with 2-3 inserts per condition.

2.7 Real-time Quantitative PCR (RT-PCR)

2.7.1 Nucleic Acid Isolation

After the cells were transfected or co-cultured with CM for 48 hrs, they were rinsed with 1 ml of PBS and then lysed in 0.5 ml of TRIzol Reagent (Invitrogen, Burlington, ON), using the manufacturer's protocol to isolate total RNA, DNA, and proteins. Total RNA was dissolved in RNase free water (Invitrogen Burlington, ON); then RNA was quantified using a BD spectrophotometer (BD, Mississauga, ON) and Nanodrop (Thermo Scientific, Wilmington, DE). The RNA was used to create complimentary DNA (cDNA) copies by an established three step procedure previously used in the lab for Real Time PCR (RT-PCR) experiments [117]. In the first step 2 µg of RNA was treated with 1 unit of DNase I, 1x DNase buffer and 0.5 units of RNaseout Rnase inhibitor for 1 hour at 37°C. It is then incubated with 2µl of Random Oligonucleotide Hexamer (0.1U/µl) primers and 5µl of 10 mM deoxyribonucleotide triphosphates (dNTPs), for 5 min at 70°C. Lastly it was incubated with; 10µl of 5X RT buffer, 4µl of 0.1M DTT, 0.5µl of Rnaseout RNase inhibitor and 1µl of Superscript II reverse transcriptase (200 U/µl) in a total reaction volume of 50µl for 1.5 hr at 37°C followed by 10 min at 70°C. The cDNA was diluted 1:1 with RNase free water and 5µl was used for each RT-PCR reaction. All cDNA synthesis reagents were from Invitrogen (Burlington, ON, Canada).

2.7.2 Oligonucleotide Primers

The Nef and Env specific primers used were designed using the HIV Sequence database and NCBI Primer-BLAST. Correct sequence alignment over intron/exon junctions were checked by BLAST analysis. All primers were synthesized by the University of Calgary Core DNA Services (Calgary, Canada) and resuspended in RNase free water. The other primers sequences were found through literature reviews and primer databases. Primers were optimized and analyzed by gradient PCR and gel electrophoresis, varying temperatures and concentrations and sequences. Final primers used are listed in Table 2.2, with the references for the sequence source. All primer concentrations were used at 5µM.

2.7.3 RT-PCR Protocol

The RT-PCR protocols used over the course of the experiments were established and described previously [59] with some adjustments. Semi quantitative RT-PCR was performed over the course of the study using Biorad's; iCycler IQ, iQ5 and CFX systems (Bio-Rad, Mississauga, ON, Canada), with 25 µl reactions used. Each PCR reaction contained 5 µl of template cDNA, 13.5 µl of SH Supermix [1X PCR buffer (Invitrogen, Burlington, ON), 5mM Magnesium Chloride (Invitrogen), 40mM of each dNTP (Invitrogen), Tween-20 (Sigma, Oakville, ON), and glycerol (Sigma)], 7.5 µl of SYBR-Green (1/50 000 dilution; Molecular Probes, Burlington, ON), 1 µl of fluorescein (1/10 000; Bio-Rad), 1 µl of forward and reverse primer mix and 0.2 µl of Taq Polymerase (Invitrogen). Non template controls were also included in each reaction plate.

Table 2.2 Oligonucleotide Primers used for RT-PCR Analysis

Gene	Primer sequence 5'-3'	Tm	Reference
β-Actin Forward	CTGGAACGGTGAAGGTGACA	60	A
β-Actin Reverse	AAGGGACTTCCTGTAACAATGCA	60	
GAPDH Forward	GAAGGTGAAGGTCGGAGTC	60	A
GAPDH Reverse	GAAGATGGTGATGGGATTTC	60	
egfp forward	CTGCTGCCCGACAACCAC	60	[118]
egfp reverse	TCACGAACTCCAGCAGGAC	60	
GFPRT Forward	TCTGTCTCCGGTGAAGGTGAAG	58	[119]
GFPRT Reverse	GGCATGGCAGACTTGAAAAAG	58	
NL4-3-Nef 8928 Forward	ACAATGCTGCTTGTGCCTGGCT	59.86	B
NL4-3-Nef 9174 Reverse	ACCCCTGGCCCTGGTGTGTA	59.08	
Nef YU-2 v1 Forward	GAGCCGAGCCAGCAGCAGAA	59.42	B
Nef YU-2 v1 Reverse	GGTCAGTGGCCATCTAGTCCCCC	60.05	
ENV #4Forward	ACCCGACAGGCCCGAAGGAA	59.2	B
ENV #4 Reverse	TGAGGGCTTCCCACCCCCTG	59.8	
Caspase 3 Forward	AGAACTGGACTGTGGCATTGAG	60	[120]
Caspase 3 Reverse	GCTTGTCGGCATACTGTTTCAG	60	
Caspase 8 Forward	CCTGGGTGCGTCCACTTT	60	C
Caspase 8 Reverse	CAAGGTTCAAGTGACCAACTCAAG	60	
Caspase 9 Forward	GCGACCTGACTGCCAAGAAA	60	D
Caspase 9 Reverse	TCACAATCTTCTCGACCGACA	60	

Gene	Primer sequence 5'-3'	Tm	Reference
CLDN1 Forward	TTCGTACCTGGCATTGACTGG	60	[121]
CLDN1 Reverse	TTCGTACCTGGCATTGACTGG	60	
CLDN3 Forward	CTGCTCTGCTGCTCGTGTCC	60	[121]
CLDN3 Reverse	TTAGACGTAGTCCTTGCGGTCGTAG	60	
CLDN4 Forward	GGCTGCTTTGCTGCAACTGTC	60	[121]
CLDN 4 Reverse	GAGCCGTGGCACCTTACACG	60	
JAM 1 Forward	ACCAAGGAGACACCACCAGAC	60	[122]
JAM1 Reverse	GAGGCACAAGCACGATGAGC	60	
Occludin Forward	TCAAACCGAATCATTATGCACCA	60	D
Occludin Reverse	AGATGGCAATGCACATCACAA	60	
ZO-1 Forward	CAAGATAGTTTGGCAGCAAGAGATG	60	[123]
ZO-1Reverse	ATCAGGGACATTCAATAGCGTAGC	60	

*All primers were used at a concentration of 5µM.A: Dr van Marle, B, Tannika Grant, C: RTPrimerDB ID: 86, D: Primer BankID: 4505487a1

The amplification protocol consisted of an initial denaturation step of 3 min at 95 °C, followed by 40-45 cycles of denaturation at 95 °C for 1 min, 45 seconds (s) at the annealing temperature (T_m) of the primers, and extension at 72 °C for 45 s. A final extension step of 72 °C for 2 min was applied and then the temperature was raised to 68°C for 1 min and the amplicons were subjected to a melt curve analysis to ensure proper amplification. In this step the temperature was raised from 68 to 99 °C in 1 °C increments, and the data was acquired for 8 s at each temperature increment.

2.7.4 Data and Statistical Analysis

Curve analysis was performed using the Biorad software included for each system and then threshold values (Ct) were further quantified using the ΔΔCt method, which gives relative fold changes (RFC) of gene expression levels compared to the control group gene expression [124]. All Ct values were first normalized against the Ct of GAPDH, a routinely used housekeeping gene in RT-PCR experiments which displays a constant expression pattern under various conditions [59]. For this study the control group used was mock non-transfected cells, and control RFCs were averaged around 1.00 [59, 124]. To confirm for the correct product, amplicons were analyzed by gel electrophoresis in addition to the melt curve analysis. Real time PCR experiments were repeated a minimum of 4 times. Statistical significance was determined by comparisons among groups using one way ANOVA with Tukey post test, or the non parametric Kruskal-Wallis test and Dunn's multiple comparison post test.

2.8 Fluorescence Microscopy

2.8.1 EGFP Transfection

Cells infected and transfected with SINRep5-EGFP and pcDNA3-EGFP were visualized under fluorescence for the determination of EGFP transfection efficiency. The cells were grown on sterilized cover slips in 6, 12 or 24 multi well plates for analysis of expression. After desired time points described above, the cells were washed with 1X PBS and then cover slips transferred to fresh plates. Cells were fixed with 4% PFA (Sigma, Oakville, ONT) for 15 mins at room temperature (r/t), washed again with 1 ml of PBS and applied to glass slides (VWR, Edmonton, AB) with glycerol or ProLong Gold Antifade with DAPI (Invitrogen, Burlington, ON). Cells were visualized using an Olympus BX51 upright fluorescence microscope with the DP-71 camera and software for imaging, and with an inverted Olympus IX5 microscope with DP-72 camera and software (Olympus, Markham, ON). Images were processed further using Image J software (Image J, Bethesda, Maryland, USA).

2.8.2 Indirect Immunofluorescence

To visualize HIV-1 Nef, Env and Caspase 3, cells were subject to indirect immunofluorescence microscopy. Cells were grown on circular cover slips or on the inserts, and were then processed for analysis 48 hrs after transfection or after incubation with 25% CM. Cells were stained as follows; cover slips or inserts were transferred to a new plate and cells were washed with PBS then fixed with 4 % PFA for 15 mins at r/t. Cells were transferred to a new plate, washed with PBS and then subject to permeabilization with PBS supplemented with 0.5% Triton X-100 (TX) (Sigma, Oakville, ONT) for 10 mins at r/t. Blocking was performed

with PBS supplemented with 5% goat serum (Invitrogen, Burlington, ON) and 5% bovine serum albumin (BSA) (Sigma) for 30 mins r/t.

Cells were incubated with the following primary antibodies (Abs) consisting of a mouse monoclonal or rabbit polyclonal antibody directed against the HIV-1 Nef protein (NIH # 1539 and abcam # ab4669) at 1:200 dilution, a monoclonal Ab against HIV-1 envgp120 (NIH #2640) at 1:250 and a rabbit polyclonal antibody to Caspase 3 at 1:1000, was diluted in PBS supplemented with 1% BSA and Goat Serum for 1.5 hrs at r/t. The cells were washed three times for 5-10 mins with PBS supplemented with 0.1% TX, and then incubated with secondary Ab for 1 hr in dark at r/t. The secondary Abs were Goat anti-rabbit Ig G conjugated to Alexa 546 and Goat anti-mouse Ig G conjugated to Alexa488 used at 1:1000 dilution. Cells were washed 3 times for 10 mins, and then cover slips were mounted on slides with the ProLong Antifade with DAPI reagent. For the membrane inserts; the membranes were removed with a scalpel, mounted on a slide and sealed with a cover slip for visualization. Mock transfected control cells transfected with electroporation buffer only were stained with primary Abs and secondary Abs alone as a control for non specific detection. Cells were visualized as described previously above.

The coverslips and slides used were obtained from VWR (Edmonton, AB) and the PBS was made from tablets obtained from Sigma (Oakvile, ONT). The secondary Alexa fluors used were obtained from Molecular Probes (INV, Burlington, ON). Rabbit polyclonal to Caspase 3 (ab49822) and rabbit polyclonal to HIV-1 Nef (# ab4669) was obtained from Abcam (Cambridge, MA). The following reagent was obtained through the AIDS Research and

Reference Reagent Program, AIDS Program, NIAID, NIH: HIV-1JR-CSF Nef Monoclonal Antibodies from Dr. Kai Krohn and Dr. Vladimir Ovod [125]. The following reagent was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: HIV-1 gp120 Monoclonal Antibody from Dr. Dennis Burton and Carlos Barbas [126].

2.9 Western Blotting

Cells were processed for western blotting 48 hrs after transfection or co-culture. Cells were washed with 1X PBS, detached and resuspended over ice in 1X Cell Lysis Buffer for 30 mins. Cell lysis buffer was prepared ((20mM Tris-HCl (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM Na_3VO_4, 10mM NaF, 1mM PMSF), with 1X protease inhibitor cocktail added fresh at the time of lysis, reagents used were all from Sigma (Oakville, ONT). Cell debris was collected by centrifugation and then the concentration of cell lysates was quantified using the Bradford (Biorad, Mississauga, ON) assay. Cell lysates were resuspended in 2X sodium dodecyl sulfate (SDS) sample loading buffer and boiled for 5 mins. Equal amounts of protein were loaded (20, 30 and 35µg) and resolved on a 12% SDS-polyacrylamide gel. Proteins were transferred to Hybond nitrocellulose membrane (Amersham Biosciences, Baie d'Urfé, PQ) using a Semi Dry Transfer apparatus (Biorad, Mississauga, ON) at constant voltage 15V for 45 mins, and then the membranes were subject to immunodetection.

Membranes were washed in PBS supplemented with 0.1% Tween-20 (PBST) (Sigma) 3 times for 5 minutes, and then incubated for blocking in a solution consisting of PBS

supplemented with 0.1% Tween 20 (PBST) and 5% non-fat dried milk (Carnation) for 1 hr at r/t. Membranes were then incubated with primary Abs in the blocking solution overnight at 4°C with gentle rocking. The primary Abs used were mouse monoclonal anti-HIV-1JR-CSF Nef (NIH #1539) at 1:250 and mouse monoclonal anti-Caspase 3 (Abcam Cambridge, MA) at 1:1000 with mouse anti-β-actin (Sigma) as control (1:5000). After 5 washes in the PBST, membranes were incubated with the secondary Ab. Secondary Ab used was Goat anti-mouse whole Ig G conjugated to horse radish peroxidase (Sigma) at 1: 2 000 for 30 mins at r/t. After 5 washes of 5-10 mins with PBST, proteins were detected using ECL enhanced chemiluminescence reagent (Amersham Biosciences), and proteins were exposed to X-Ray film (Kodak Biomax) for varying exposure times and developed.

All PBS was prepared using tablets from Sigma (Oakville, ONT). The following reagent was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: HIV-1JR-CSF Nef Monoclonal Antibodies from Dr. Kai Krohn and Dr. Vladimir Ovod [125].

2.10 MTT Assay

To quantify the cell proliferation after transfection, the MTT assay was used. MTT is a yellow tetrazolium salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) that is reduced by a mitochondrial enzyme into an insoluble colored product formazan [127], after solubilisation with detergent solutions the absorbance of the purple color change is read by spectrophotometry to quantitate the living cells. The TACS MTT Cell Proliferation Assay kit (Trevigen, Gaithersburg, MD) was used following the protocol provided. Transfected cells were titered to optimal concentration of $1x10^5$ cells in 100µl per well, then seeded in a 96 CellBIND flat bottom (Corning Inc, Tewksbury, MA) multi-well plate.

Cells were incubated at 37 °C at 5% CO_2, for 2 days, then 10µl of MTT reagent was added and the plate was set back in the incubator for 3 hrs. Upon solid colour reaction, solubilization reagent was added and the plate was left in the dark for solubilization. The absorbance of the product was read on a Biotek multi-well plate reader (Winooski, VT, USA) at 570nm and 670nm as the reference absorbance. The cells were added to four wells each, along with four control wells added with culture media alone as the blank, which was subtracted from all of the readings. The average absorbance was determined and the assay was repeated four times.

2.11 Flow Cytometry

Flow cytometry was used to determine the transfection efficiency for EGFP expression for final transfection vector evaluations. Cells were transfected as described above with pcDNA3-EGFP and allowed to express, then after 1, 2, and 3 days were processed for determination of transfection efficiency. Cells were collected by centrifugation at 200x g, washed in PBS, then resuspended in a single cell suspension in 0.6 ml of PBS supplemented with 0.1% Sodium Azide (Sigma, Oakville, ONT). Samples were sent for analysis, performed by the University of Calgary Flow Cytometry Core Services.

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California USA). For TEER analysis, the comparison among groups over days was performed using One-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post testing. For real time-PCR analysis, comparisons among groups were done using one way ANOVA with Tukey post test, or the non parametric Kruskal-Wallis test and Dunn's multiple comparison post test. Comparisons for the MTT assay were analyzed using the non parametric test Kruskal-Wallis, with Dunn's multiple comparisons post test. All data was expressed as mean ± standard error of the mean (SEM), and probability (p) value of less than 0.05 was considered significant.

Chapter Three: **Results**

3.1 The Evaluation of Expression Systems for an *In Vitro* Analysis of GI Epithelial Barrier Function

An *in vitro* based study was designed to assess the contribution of the HIV-1 Nef protein to HIV-1 induced GI pathogenesis. A system that was effective for intracellular HIV-1 protein expression in human intestinal epithelial cells, and had wide applicability, for subsequent and future investigations in the laboratory was elucidated by an evaluation of two expression vector systems, three intestinal cell lines and culture conditions for studying HIV-1 induced GI dysfunction. The expression vector systems were first evaluated for the efficient expression of the two HIV-1 prototypic proteins Nef NL4-3 and Nef YU-2, along with the enterotoxic HIV-1 envelope protein (Env JR-FL), in various model cells to be used in subsequent experiments. As the model colonic epithelial cells Caco-2 and T84, and model T cells and monocytes lines-Jurkat and U937 are known to be more resistant to transfection by standard protocols, studies were conducted using EGFP as a reporter gene to assess the transfection efficiencies of the various systems. The recombinant Sindbis alphavirus expression vector pSINrep5-EGFP was investigated along with the mammalian transfection vector pcDNA3-EGFP using various transfection methods.

3.1.1 Sindbis Virus System: EGFP expression and low infectivity in GI epithelial cells

Recombinant Sindbis alphavirus expression vectors, shown previously to effectively infect various neural cell lines for the study of HIV-1 neuropathogenesis were used to infect BHK-21, Caco-2 and T84 cells [59, 109]. For this system BHK-21 cells were transfected with pSINrep5-EGFP viral transcripts to create virus stocks collected from media after 24hrs as shown in Figure 3.1a and b. The SIN-EGFP viral stocks were then used to infect the colonic epithelial cells at various dilutions, with undiluted stock used on cells shown in Figure 3.1c and d. As BHK-21 cells are highly infected by SIN infection, the infection efficiencies were determined relative to BHK infection for the same viral titer and cells [109, 110]. SIN-EGFP was found to weakly infect the cells under the conditions used, with low to barely detectable EGFP infection efficiencies of 0.6% and 0.001% for Caco-2 and T84 cells respectively, shown in Table 3.1. A system with higher protein expression was desired, as HIV-1 proteins are more cytotoxic than EGFP and will lead to even lower expression. Optimization attempts did not lead to vast improvements in expression thus this system was discontinued and focus moved solely to the pcDNA3 vector constructs.

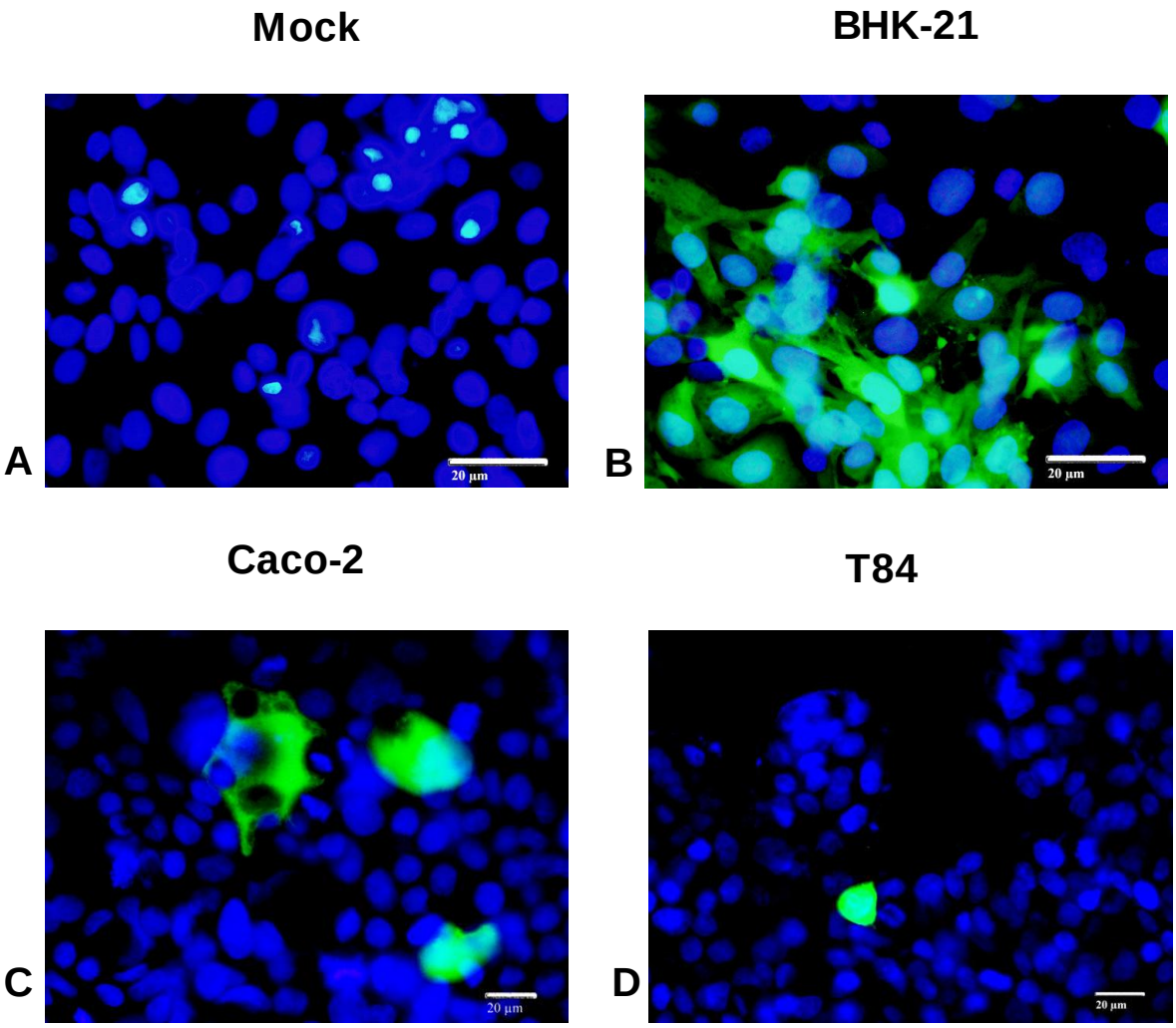


Figure 3.1 SIN-EGFP Infection.

Representative images of Sindbis-EGFP transfections and infection results are shown above as visualized by fluorescence microscopy with DAPI staining of nuclei in blue. A and B. BHK-21 cells were transfected with cells alone (mock A) and 15 µg of pSinRep5-EGFP and helper viral RNA transcripts (B) to create viral stocks of SIN-EGFP in media which was collected after 24hrs. C and D. Viral stock of SIN-EGFP was incubated with the colonic epithelial cells Caco-2 and T84 in serial dilutions for 48 hrs for infection. SIN-EGFP was found to weakly infect the cells under the conditions used, with low to barely detectable EGFP infection efficiencies of

0.6% and 0.001% for Caco-2 and T84 cells respectively. Images are representative of a minimum of 5 independent experiments. Scale bar = 20 µm

Table 3.1 Transfection Efficiencies in various cell lines

Cell Line	Transfection Method	Time hrs	Efficiency
	pSinRep5-EGFP[A]		
BHK-21	Electroporation and infection	48	100%
Caco-2	Infection	48	0.06%
T84	Infection	48	0.001%
	pcDNA3-EGFP[B]		
Caco-2	Lipofection; LTX™	48	5-7%
	Lipofection; Lipofectamine2000™	48	16-28%
	Electroporation; Neon™	**48**	**45-60%**
T84	Lipofection; LTX™	48	<1%
	Lipofection; Lipofectamine2000™	48	<5%
	Electroporation; Neon™	**48**	**46-50%**
HT-29	**Electroporation; Neon™**	**48**	40-60%
Jurkat	Lipofection; LTX™	48	5-6%
	Lipofection; Lipofectamine2000™	48	1-8%
	Electroporation; Neon™	24	28%
	Electroporation; Neon™	**48-72**	**53-66%**
U937	Lipofection; LTX™	48	<5%
	Lipofection; Lipofectamine2000™	48	8%
	Electroporation; Neon™	24	23%
	Electroporation; Neon™	**48-72**	**38-60%**

[A] Infection efficiencies were determined by percent of infected cells relative to number of infected BHK-21cells for same pSinRep5-EGFP titer. [B] Transfection efficiency of pcDNA3-EGFP was determined by fluorescence microscopy and flow cytometry.

3.1.2 The pcDNA3 Transfection Vector System: Construction and EGFP Expression

Protein expression using the pcDNA3 vector became the primary vector, and was evaluated and optimized next. The encoding sequences for EGFP, Env JR-FL, Nef NL4-3 and YU-2 were first subcloned into the pcDNA3 vector. Upon confirmation of correct insert and orientation in vectors by sequencing analysis, pcDNA3-EGFP was used to transfect the Caco-2, T84 and HT-29 cell lines. The lipofection method followed by a new electroporation system was used for plasmid DNA transfection, and EGFP expression evaluated.

3.1.2.1 Lipofection

Lipid-mediated delivery of DNA into the epithelial cells was evaluated for efficient EGFP expression using two different lipofection reagents. As described in the methods, various optimization strategies were used with Lipofectamine 2000™ and Lipofectamine LTX™ to transfect the cells of interest. Caco-2 cells transfected with pcDNA3-EGFP using Lipofectamine 2000™ reagent is shown in Figure 3.2b and using Lipofectamine LTX™ reagent in Figure 3.2c, visualized under fluorescence microscopy. The efficiency using various concentrations of DNA for lipid transfection were tested, 3ug of DNA had been used most frequently, as lower and higher concentrations of DNA did not appear to greatly contribute to improved expression. Transfection efficiencies were low at first but increased with Lipofectamine 2000 as DNA was incubated with liposomes for 2-4 hours before adding to seeded cells, and then allowed to complex overnight. Medium was replaced after 24 hours and cells were then analyzed for EGFP expression after 48 hours by fluorescence microscopy. Transfection efficiencies, as summarized in Table 3.1, were improving in Caco-2 cells, starting around 11% and approaching 30% with Lipofectamine 2000™, as opposed to the 5-6% obtained with Lipofectamine LTX™.

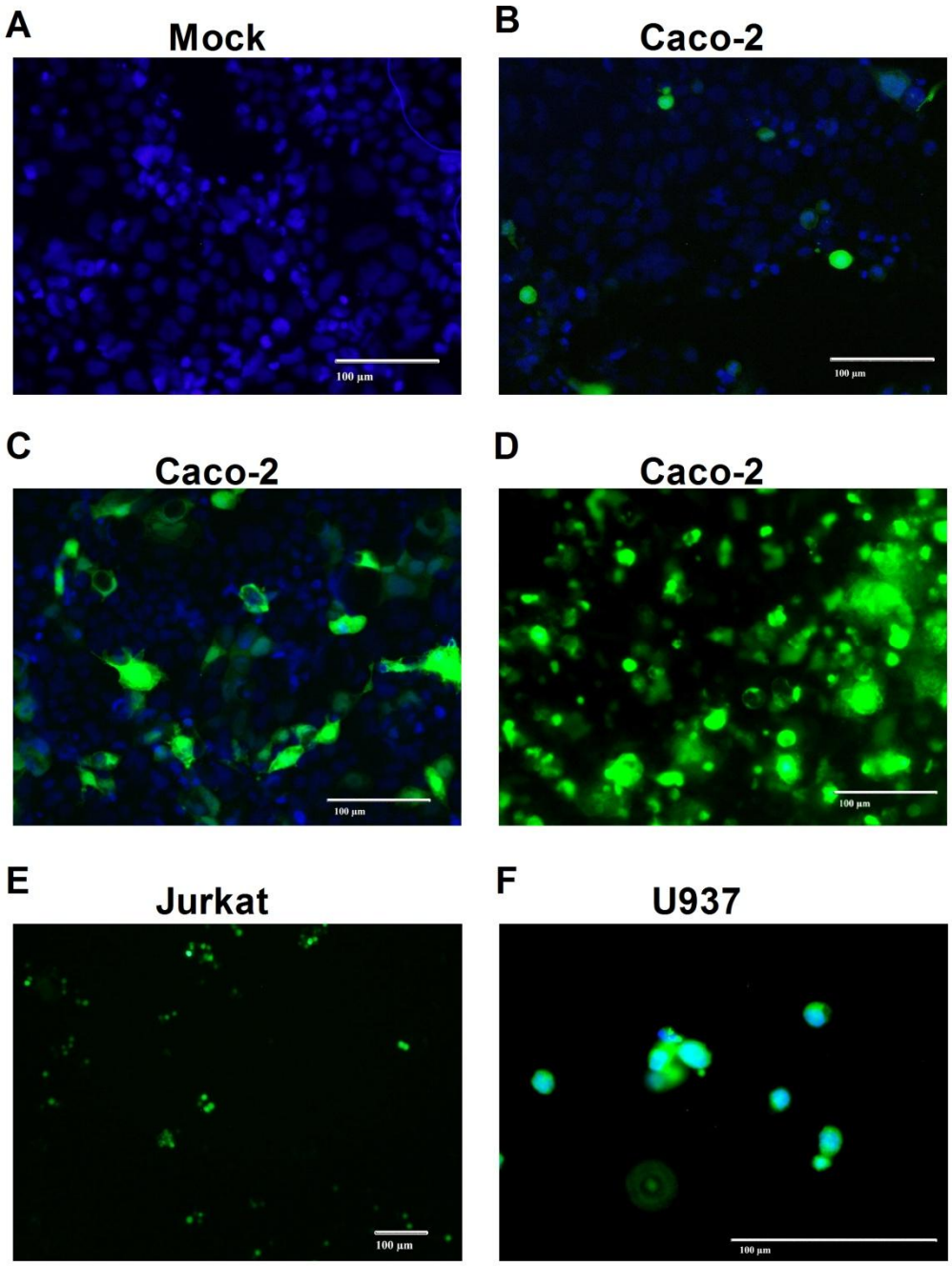


Figure 3.2 Transfection system evaluations using pcDNA3-EGFP.

A-C. Transfection of Caco-2 epithelial cells with 3µg of pcDNA3-EGFP DNA using lipofection reagents. Transfected cells plated on coverslips were fixed after 48 hrs and visualized by fluorescence microscopy with DAPI blue staining of nuclei for EGFP expression. Transfection

efficiencies were low at 5-6% using Lipofectamine LTX™ (B). Optimizations using Lipofectamine 2000™ (C) did lead to improvements, starting around 11% and approaching 30%. D-F. Transfections of the cells with 3µg of DNA using the Neon™ electroporation system was evaluated to obtain improved EGFP expression. D. The transfection efficiency of EGFP in Caco-2 cells was from 45-60%, cell image was taken in 6 well plates without DAPI stain. E and F. After 48 hrs, transfection efficiencies of 66% for Jurkat cells (E) and 38% for U937 cells (F) were obtained. The Jurkat cell image was taken in suspension in tissue culture flask without DAPI, while U937 cells were visualized on slide with DAPI.

Scale bar=100 µm

The EGFP expression was still too low, particularly in the Jurkat and U937 cells as seen by the transfection efficiency of 5-8% in Table 3.1. The expression of the HIV proteins were expected to be lower than EGFP; in addition higher expression levels were desired for the indirect studies using media from Nef transfected Jurkat and U937 cells, thus the Neon™ electroporation system was evaluated next.

3.1.2.2 Electroporation System: Neon

The Neon™ electroporation system was evaluated next as an alternate method of delivering the recombinant pcDNA3 vectors into our cells of interest. Transfection of Jurkat and U937 cells with pcDNA3-EGFP was performed first. The protocol and electroporation parameters provided online by the manufacturer for Jurkat and U937 cells specifically were used, and a transfection efficiency of 66% for Jurkat and 38% for U937 cells was obtained, as determined by flow cytometry (Table 3.1). The epithelial cells were then evaluated as well, and as shown in Table 3.1, the efficiencies were notably higher for Caco-2, T84 and HT-29 respectively. As a protocol was not provided for Caco-2 or T84 cells from manufacturer the HT-29 protocol was used initially and then optimized, with the final parameters used described previously in methods.

High quality DNA that is concentrated at the µg/µl level without ethanol precipitation was essential for higher efficiencies obtained using this system. The Sigma GenElute Plasmid Miniprep kit was found suitable, followed by vacuum centrifugation to concentrate the DNA as it left less salts compared to midi prep kits tried. Various concentrations of DNA were evaluated

for improved expression in the last section for the lipofection transfections, with 3ug determined to be an effective concentration and was used as the starting concentration for the electroporation procedure. As the results from the first transfections in Jurkat and U937 cells were high enough, 3µg of DNA was carried through and kept consistent for the rest of the experiments in this study. The relative EGFP mRNA expression using Neon system in the various cells discussed is shown in Figure 3.3.

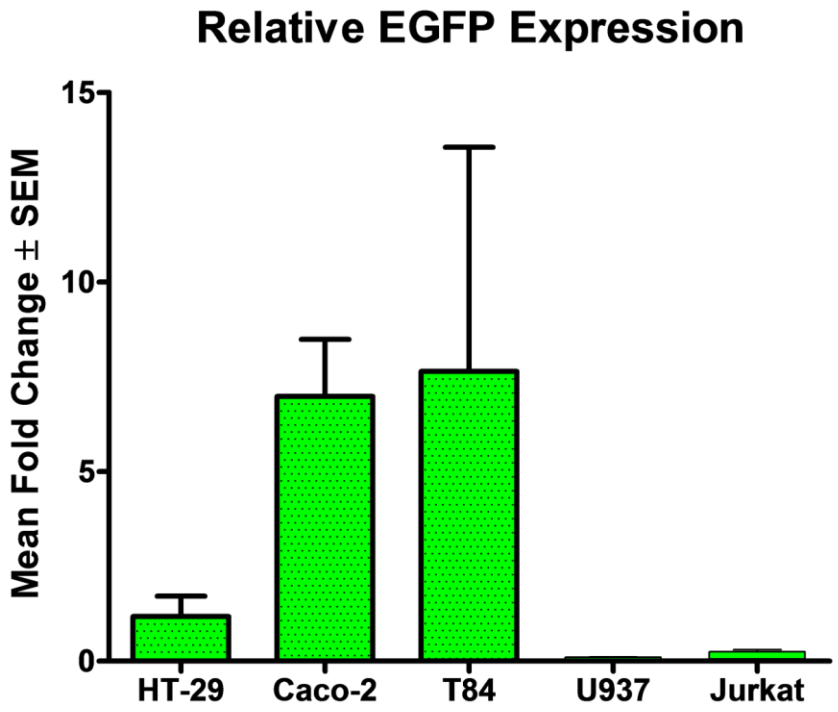


Figure 3.3 Relative EGFP mRNA expression in cells using Neon™ Electroporation System

RT-PCR analysis EGFP expression in colonic epithelial cells, as expressed relative to HT-29 EGFP mRNA expression levels, including Jurkat and U937 EGFP expression. The mean fold change of EGFP mRNA expression was 1.33 in HT-29 cells, 6.9 in Caco-2 cells and 7.64 in T84 cells. In addition U937 cells and Jurkat cells had a mean fold change of 0.11 and 0.25 relative to HT-29 EGFP expression. EGFP was normalized against GAPDH expression. Results shown are from the analysis of EGFP mRNA expression in 4 independent experiments.

For this system, the epithelial cells are electroporated in suspension as opposed to when seeded and polarized in a monolayer, as preferred for direct toxicity studies. Overall the high efficiencies obtained in a wide range of cells, in addition to the ease of use and consistency observed with this method was desirable. In addition, all data for subsequent studies were being compared to data obtained from both mock and vector (pcDNA3-EGFP) control transfected cells. Control cell transfections were performed along with the Nef transfections under the same conditions, and would reflect distortions due to the electroporation step that have been described to occur in epithelial cells previously [128]. The Neon™ electroporation system using the pcDNA3 constructs obtained the highest transfection efficiencies, while being easy to use and reproducible; as such this system was used for all subsequent analyses.

3.1.3 Caco-2 Cells: the model intestinal epithelial cells

Over the course of the evaluations Caco-2 cells were found to be the best cell line for our experimental model as the cells were responsive to the conditions used and displayed applicability for different experimental conditions for future investigations. Caco-2 cells were more responsive to infection by SINrep5-EGFP, and transfection of pcDNA3-EGFP by both lipofection and Neon electroporation. While T84 and HT-29 cells demonstrated comparable EGFP transfection efficiencies to Caco-2 cells, TEER analysis of both cell lines resulted in below average values with decreased ability to form an effective polarized and differentiated monolayer. Differences between all transfected groups including controls were insignificant. In addition mRNA expression values were extremely low across all groups and genes analyzed for the HT-29 cells, while the Caco-2 and T84 response was more robust and differences were able to be analyzed. The T84 cells were used for the following studies solely in confirmation

experiments after results obtained with Caco-2 to ensure that results seen were not cell line specific.

3.2 Expression of HIV-1 Nef Protein

After determining the expression system, methods and cell lines to be used for the *in vitro* study, HIV-1 protein expression was evaluated. The recombinant pcDNA3 vector constructs with two prototypic Nef protein isolates (pcDNA3-Nef NL4-3 and pcDNA3-Nef YU-2) in addition to the positive enterotoxic Env (pcDNA3-Env JR-FL), including pcDNA3-EGFP as the vector control, were transfected in 5.5 $x10^6$ Caco-2 cells. The transfected cells were seeded on cell culture plates or microscope coverslips in wells and after 48 hrs, cells were either lysed or fixed for analysis by western blotting or indirect immunofluorescence microscopy. Nef protein expression was barely visible to undetectable as shown in Figure 3.4a for Caco-2 cells by indirect immunofluorescence microscopy. There were many viable cells remaining on the coverslips 48 hrs after transfection, after fixing and then after the indirect immunostaining procedures. Yet when observing under the microscope the fluorescence staining was at very low intensities suggesting that the cells were only expressing low levels of the Nef proteins. In order to evaluate the conditions and antibodies used a comparative side study was performed using BHK-21 cells. As shown in Figure 3.4b, transfection efficiency was high around 60% and easily visualized by indirect immunofluorescence microscopy.

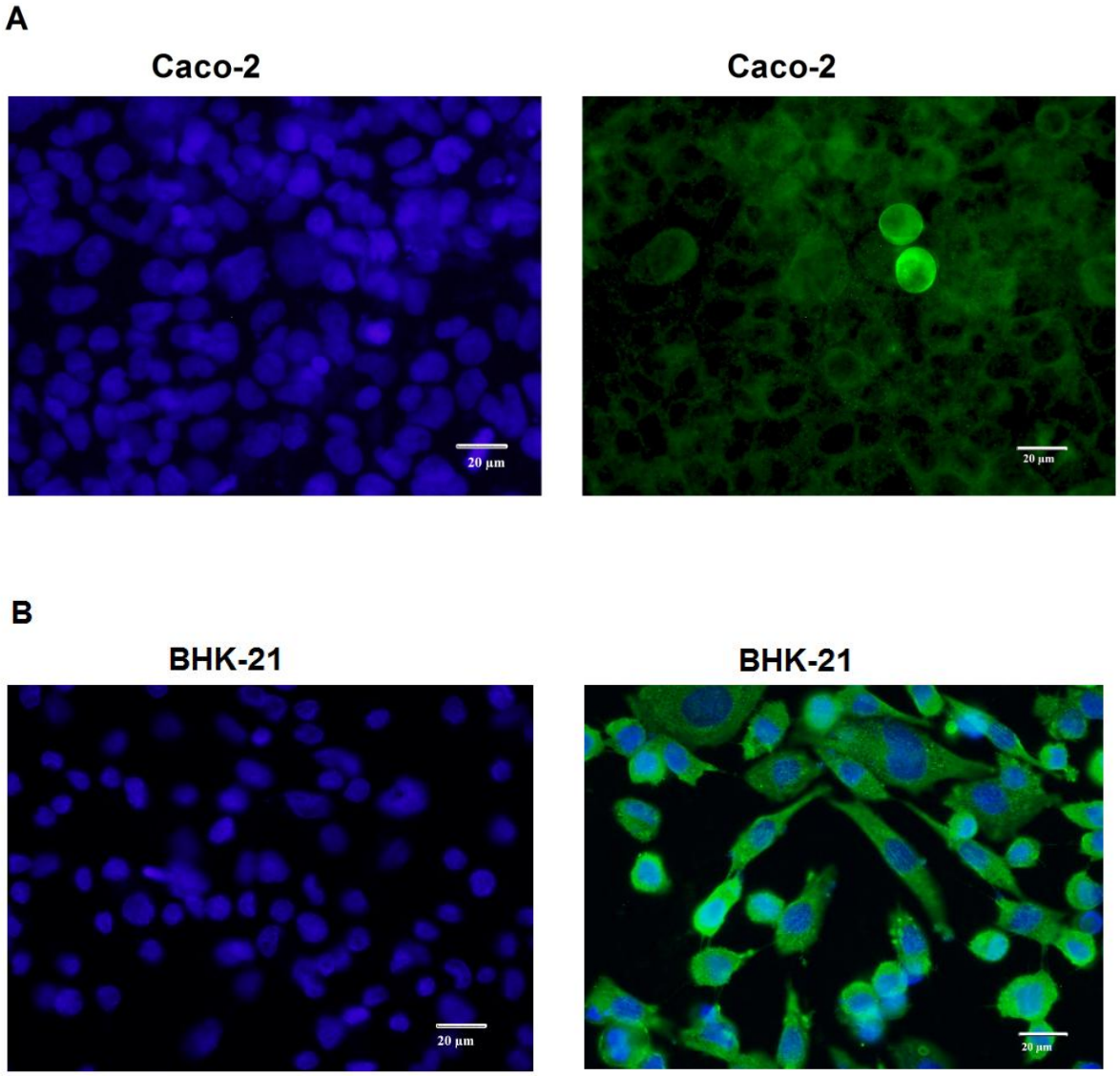


Figure 3.4 HIV-1 Nef protein expression.

A. Transfected Caco-2 cells were seeded on microscope coverslips and cells were fixed for analysis by indirect immunofluorescence microscopy after 48 hrs. As shown, with DAPI staining of nuclei, low to barely detectable Nef protein expression was observed as cells displayed low intensity staining. B. A comparative study was performed using the easily transfected cell line BHK-21 under the same conditions as the Caco-2 cells. Nef protein expression in BHK-21 cells

was highly efficient at 60%, thus while the protocols were working, transfection efficiency was low in the colonic Caco-2 epithelial cell line. Scale bar= 20 µm

RT-PCR was performed next to determine if *nef* and *env* mRNA can be amplified out of cells using designed *nef* NL4-3, *nef* YU-2 and *env* JR-FL sequence specific primers. *nef* NL4-3 and *nef* YU-2 mRNA were found in Caco-2 cells at 5% relative to total EGFP mRNA expression (Figure 3.5a). In addition to melt curve analysis to ensure specificity and the absence of primer dimers, the PCR products were analyzed for correct amplicon band size by 1% TAE agarose gel electrophoresis shown in Figure 3.5b. The presence of *nef* mRNA was confirmed and these results were consistently obtained. RT-PCR analysis was then used to confirm mRNA expression for the transfection reactions. The presence of HIV-1 Nef NL4-3, YU-2 and Env JR-FL in the Jurkat and U937 cells were also verified by RT-PCR analysis. After confirming the presence of *env* and *nef* mRNA in the cells, the culture supernatants were collected as conditioned media for co-culture experiments to study the indirect toxicity of the Nef protein.

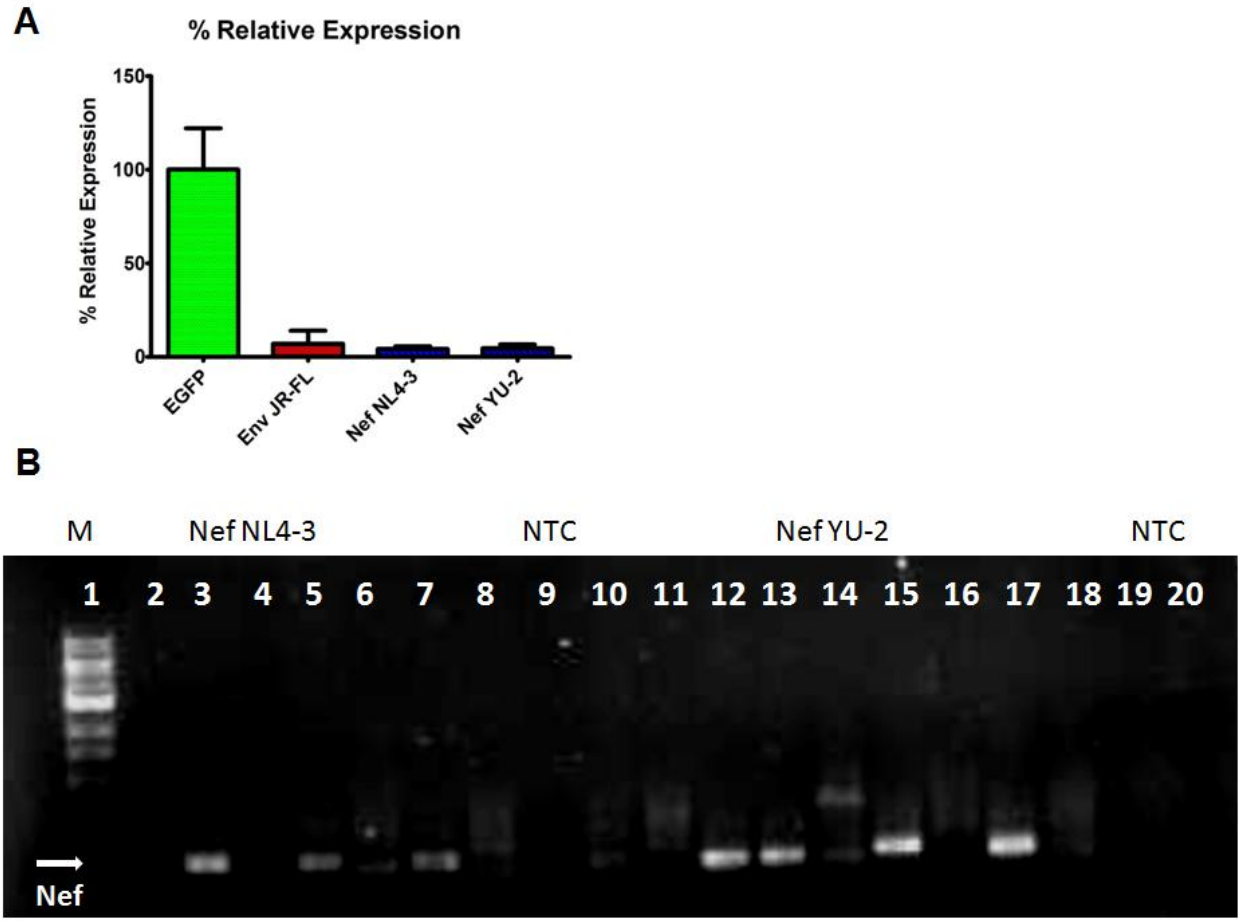


Figure 3.5 Nef mRNA Expression

A. Nef mRNA expression was analyzed by real time PCR using sequence specific primers and then quantified relative to EGFP expression in Caco-2 cells, the two *nefs* and *env* JR-FL were found in cells at 5% (Nef NL4-3 and Nef YU-2) and 6% for e*nv* JR-FL, n=4 independent experiments. The presence of *nef* mRNA was confirmed and these results were consistently obtained. RT-PCR analysis was then used to confirm mRNA expression for the transfection reactions. B. The *nef* mRNA real time PCR products were analyzed for correct amplicon band size by 1% TAE agarose gel electrophoresis. The Nef NL4-3 amplicons are shown in lanes 3, 5 and 7 which contain the amplicons from the reactions using the Nef specific primers and *nef* NL4-3 template, and lanes 12, 13, 15 and 17 contain the Nef amplicons from the reactions with

the Nef YU-2 template. In lane 1 the DNA marker (M) was loaded. In lanes 2 and 4 the reactions containing cDNA template from mock transfected (lane 2) and EGFP transfected (lane 4) Caco-2 cells combined with Nef specific primers were loaded on the gel to test for non specific amplicons. The reactions did not result in non specific amplicons as expected. Non template control (NTC) PCR reactions using Rnase free water were loaded in lanes 9, 19 and 20. Amplicons from reactions containing GAPDH (lanes 8, 10, 11, 14) and β-actin (lanes 16, and 18) primers were loaded and were a faint smear.

3.3 The Direct *In Vitro* Effects of HIV-1 Nef Protein on GI Epithelial Barrier Integrity

A set of *in vitro* experiments were performed to investigate the direct toxicity effects of the two HIV-1 Nef proteins from the NL4-3 and YU-2 viral isolates on the Caco-2 epithelial monolayer and cells. Monolayer integrity and cell death was assessed using; TEER analysis, RT-PCR and IMF for the expression and activation of the Caspase proteins the mediators of cell death, and the MTT assay and lastly tight junction gene expression analysis was investigated.

3.3.1 Nef protein expression does not alter the TEER of epithelial monolayers

As described earlier, it has been noted that impaired epithelial maturation, actin rearrangements, and microtubule depolymerizations are important manifestations in HIV enteropathy and barrier dysfunction [19-21, 66]. As the actin cytoskeleton has an essential role in tight junction function, a co-culture model system was used to measure the Trans-Epithelial Electrical Resistance (TEER) across the Caco-2 epithelial monolayers. TEER is a measure of the paracellular flux of molecules through the tight junction complex of an epithelial monolayer, and is a quantification of the ability for molecules to flow through the barrier [129]. Alterations of TEER are interpreted as an alteration in tight junction permeability and thus of monolayer integrity [130]. The recombinant pcDNA3 vectors expressing EGFP, Env JR-FL and the two Nef proteins were transfected into Caco-2 cells, which were then plated on semi-permeable cell culture membrane inserts. TEER values were recorded at 24 hr time points after transfection as the Caco-2 monolayers formed. As illustrated in Figure 3.6, Nef transfected monolayers had a minor reduction in TEER that was not at a significant level compared to mock or vector (EGFP) transfected monolayers. While Env expression resulted in a significant reduction as expected $p < 0.05$.

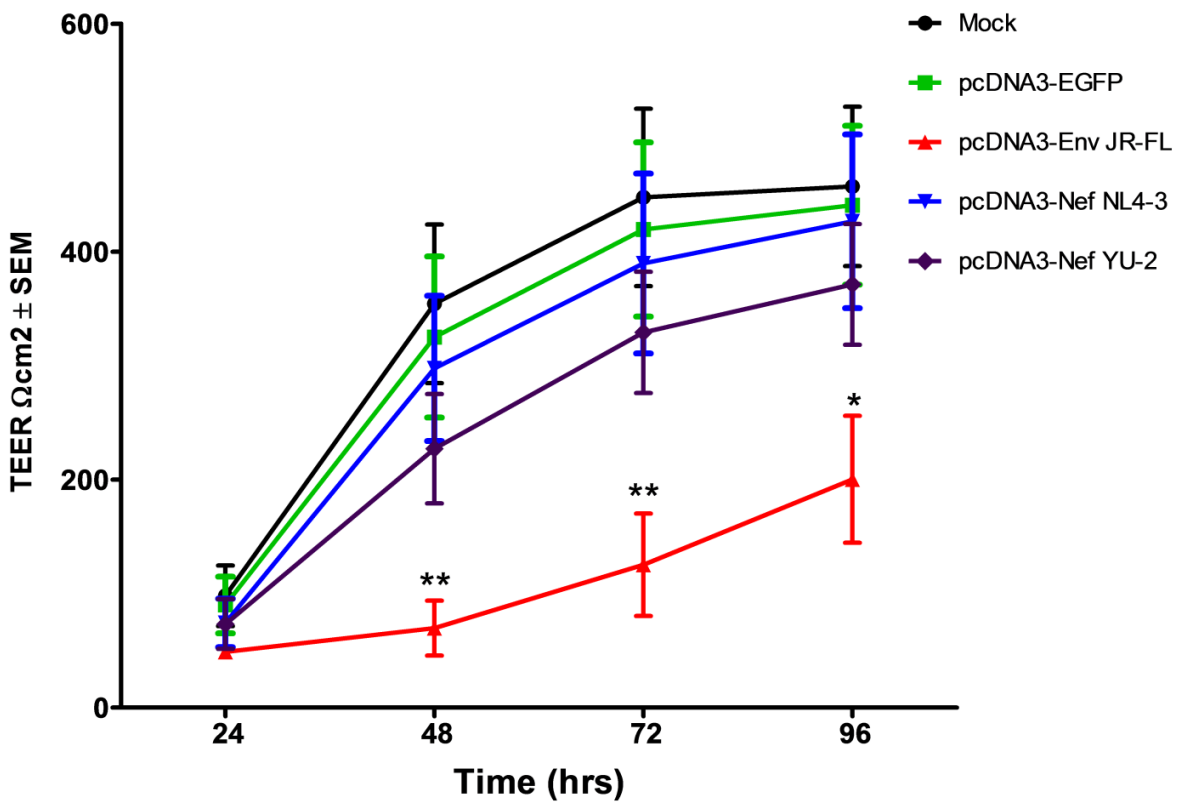


Figure 3.6 TEER of transfected Caco-2 cell monolayers.

Trans-Epithelial Electrical Resistance was measured after transfection of Caco-2 cells with proteins of interest as cells settled and the monolayer formed. While TEER was reduced Nef did not alter the TEER of Caco-2 monolayers significantly compared to mock and pcDNA3-(EGFP) control monolayers. Expression of enterotoxic Env JR-FL did significantly decrease TEER from 48-96 hrs *$p<0.05$ and **$p<0.01$ relative to controls. Data points are expressed as mean TEER values for each day ± SEM, n=5 independent experiments with 6-10 monolayers per protein group. Two-way repeated measures ANOVA was performed with Bonferroni post test.

3.3.2 Nef protein expression decreases the expression of the Caspases in epithelial cells

In vivo GI epithelial cells experience high turnover with a relatively short life span of 3-5 days, but cell death and increased cell turnover due to elevated rates of cell death, could still form a part of the GI barrier dysfunction that has been observed. As the Caspase proteins are the mediators of cell death, semi quantitative RT-PCR was performed to analyze the gene expression of Caspase 3, 8 and 9. RNA was isolated to synthesize cDNA 48 hrs after transfection. In Figure 3.7a, results for Caspase 3 mRNA expression levels were interestingly reduced compared to control cells. Fold changes (FC) in expression levels is expressed relative to mock transfected control cells (RFC), which was set around a mean of 1.00, and the vector pcDNA3-EGFP transfected control cells had a mean RFC of 1.21. Nef NL4-3 transfected cells had reduced mRNA expression levels with an RFC of 0.79, while Nef YU-2 transfected cells had a mean RFC of 0.60 which was significant versus both control cells $p<0.05$. Env JR-FL transfected cells had an increased mRNA RFC of 1.51, expected from the known enterotoxic protein, $p <0.05$. Caspase 8 and 9 mRNA expression levels followed the same patterns, with Nef transfected cells expressing less mRNA, while Env JR-FL had increased levels relative to vector control cells but the differences were smaller and not significant.

An MTT assay was performed in addition to investigate cell proliferation 48hrs after transfection, shown in Figure 3.7b. Nef transfected cells were shown to be viable at same levels as control cells, while Env JR-FL transfected cells demonstrated a 32% reduction in proliferation $p<0.05$ in the Caco-2 cells. Jurkat and U937 cells were assayed 3 days after transfection and in Figure 3.7c can see that in all groups cells were viable and proliferating.

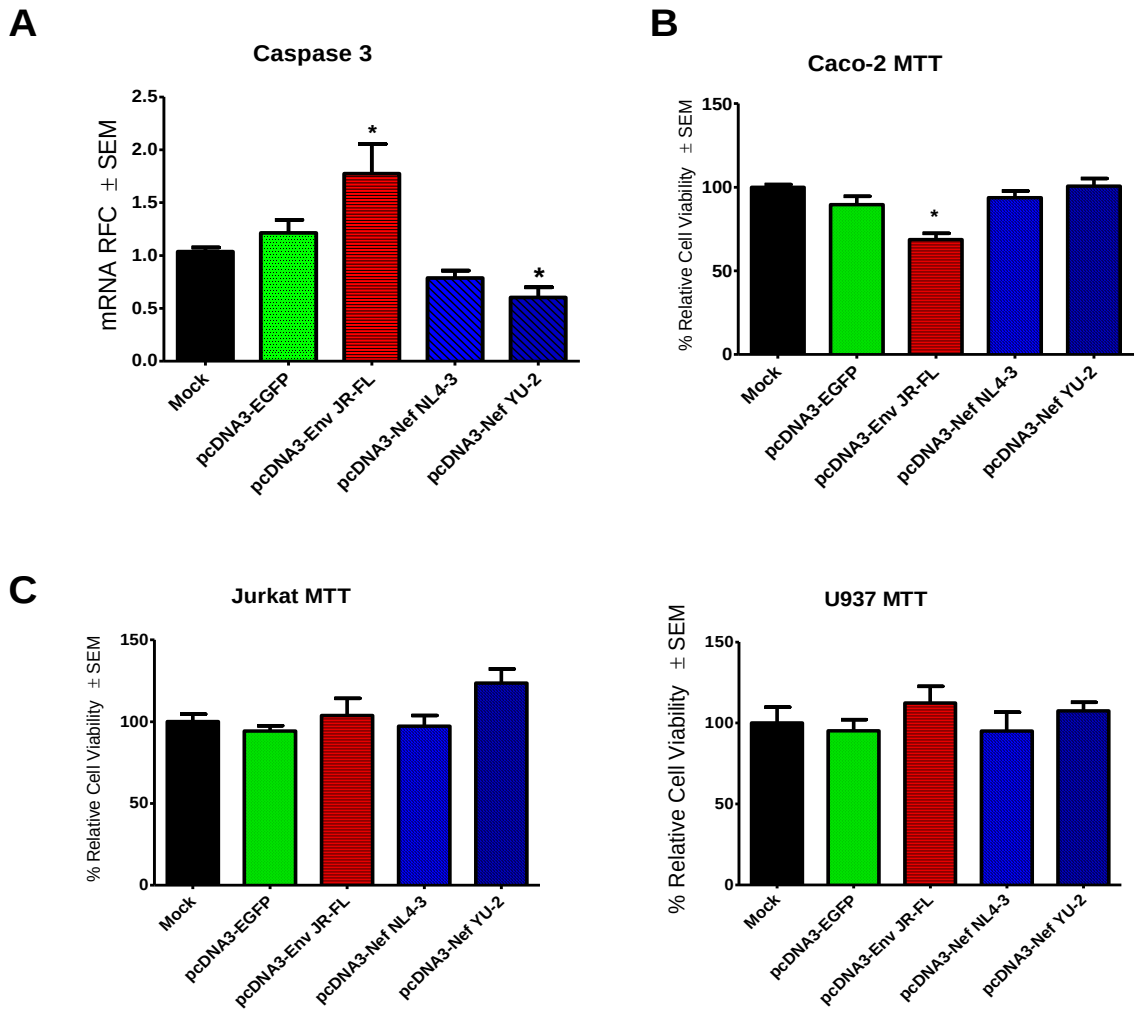


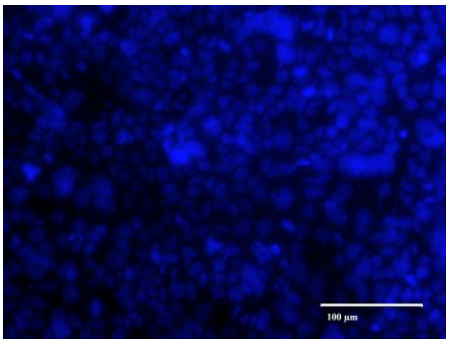
Figure 3.7 The effects of Nef protein on cell death

A. RT-PCR analysis of Caspase 3 gene expression in transfected Caco-2 cells demonstrated that mRNA expression was reduced in Nef NL4-3 and significantly reduced in Nef YU-2 transfected cells relative to mock and pcDNA3 (EGFP) control cells. The Env JR-FL transfected cells demonstrated an increase in expression. Caspase expression was normalized against GAPDH and expressed relative to mock transfected control cell. *$p < 0.05$, one way ANOVA using Tukey post test, n=6 independent experiments. B. Cell viability was also assessed by MTT assay and demonstrated that Nef transfected cells were viable and proliferating at same levels of control

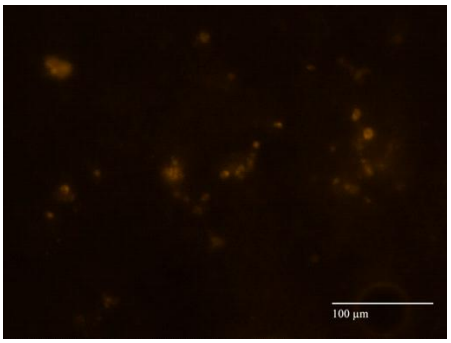
cells, while Env JR-FL caused a significant reduction in cell proliferation (*$p<0.05$ n= 4 MTT reactions) by Kruskal Wallis test and Dunn's multiple comparisons post test. C. Nef transfected Jurkat and U937 cells were also viable and proliferating.

Transfected Caco-2 cells were also analyzed for Caspase 3 protein expression after 48 hrs by indirect immunofluorescence staining and fluorescence microscopy. As shown in representative images in Figure 3.8, Caspase 3 expression levels in Nef transfected Caco-2 cells was found to be lower or comparable to control cell staining. Thus Nef expression appeared to actually reduce the effects of Caspase 3 expression in Caco-2 epithelial cells.

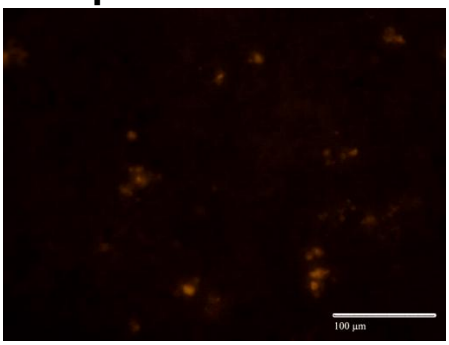
Mock



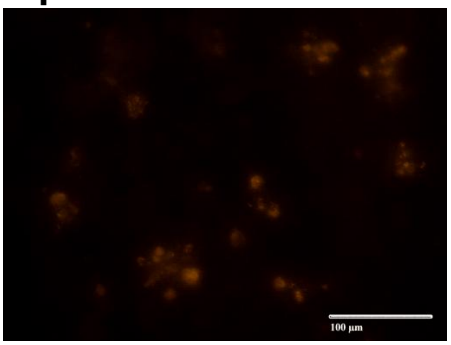
Mock



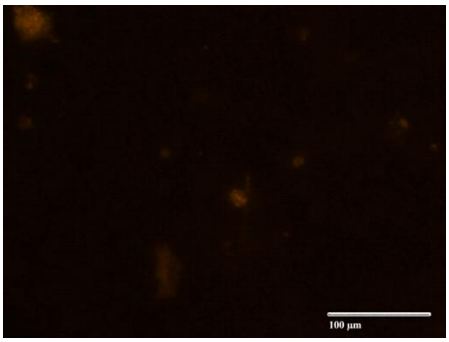
pcDNA3-EGFP



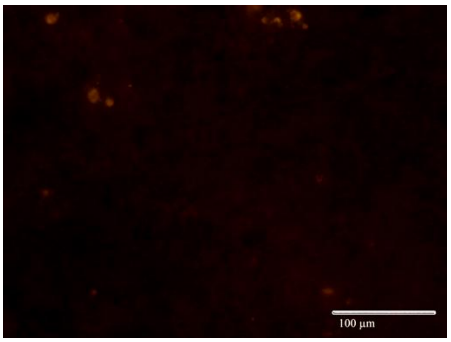
pcDNA3-Env JRFL



pcDNA3 Nef NL4-3



pcDNA3-Nef YU-2



0.5µM Staurosporine

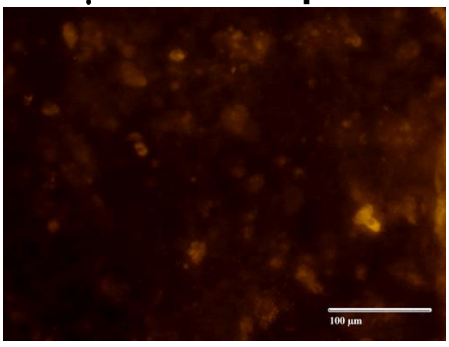


Figure 3.8 The effects on Caspase 3 protein expression.

Transfected Caco-2 cells were also analyzed for Caspase 3 protein expression after 48 hrs by indirect immunofluorescence microscopy. Mock cells are stained with DAPI to display background levels of cells in upper left panel. Caspase 3 expression levels in Nef NL4-3 and Nef YU-2 transfected Caco-2 cells were found to be lower or comparable to the mock and vector transfected control cells. Thus Nef expression appeared to actually reduce the effects of Caspase 3 expression in Caco-2 epithelial cells. The positive control cells cultured with 0.5μM staurosporine or transfected with pcDNA3-Env JRFL had increased Caspase 3 expression.

3.3.3 Nef alters tight junction gene expression levels in epithelial cells

In GI tissues from HIV^+ infected patients, we previously found increased β-actin expression observed significantly in the duodenum and colon samples which had a correlation with increased *nef* gene expression (T Grant and G. Van Marle, unpublished data). An investigation of the direct effects of Nef on the mRNA expression levels of various tight junction proteins was conducted. Semi quantitative RT-PCR analysis of the mRNA expression patterns of the tight junction proteins; ZO-1, Occludin, JAM-1, CLDN-1, CLDN-3 and CLDN-4, was conducted 48hrs after transfection. As demonstrated in Figure 3.9a and b, Nef NL4-3 and Nef YU-2 transfected cells had significant reduced ZO-1 expression, with mean RFC of 0.43 and 0.34 compared to control cells $p<0.05$. This pattern was observed for JAM-1 mRNA expression in Nef NL4-3 transfected cells illustrated in Figure 3.9b. Nef NL4-3 had a 0.39 RFC, $p<0.05$. Nef YU-2 did have a reduced RFC of 0.67 though not significant compared to controls. Representative results for the CLDN's are represented by CLDN-3, as it is constitutively expressed in high numbers in the intestine. CLDN-3 expression was also reduced in Nef NL4-3, Nef YU-2 and Env JR-FL transfected cells and the effect was significant $p<0.05$ with an RFC of 0.39, 0.29 and 0.15 respectively (Figure 3.9c). Occludin mRNA expression was also analyzed, as shown in Figure 3.8d; mRNA expression was comparable to control levels. Although the TEER levels were comparable to controls, there was an altered response in gene expression of the tight junction components in the Caco-2 cells after Nef expression

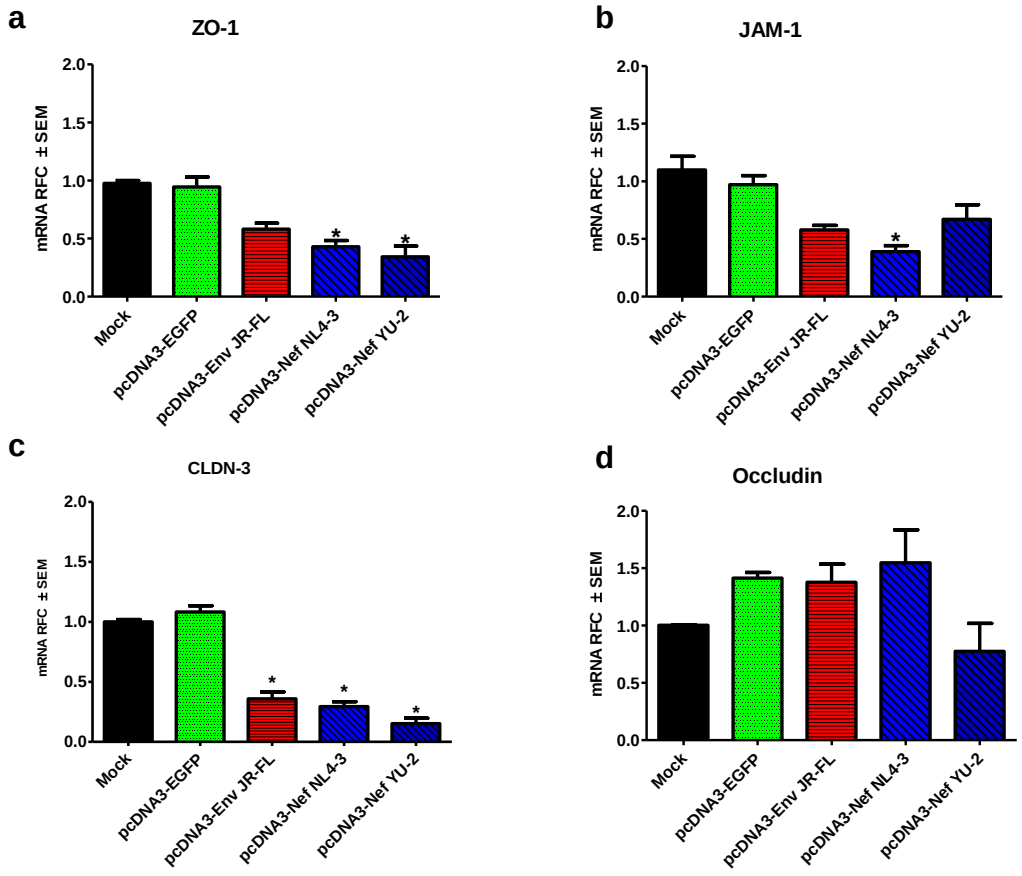


Figure 3.9 The effects of transfected proteins on tight junction mRNA expression.

RT-PCR analysis of Caco-2 cells 2 days after transfection demonstrated alterations in expression of the tight junction proteins. A. ZO-1 was significantly down regulated in cells expressing both Nef viral isolates compared to mock and pcDNA3-EGFP transfected cells. B) Nef NL4-3 transfected cells had reduced expression of JAM-1 RFC of 0.39, relative to controls while Nef YU-2 was reduced but the effect at this size was insignificant. C. CLDN-3 mRNA expression was reduced significantly in Nef NL4-3, YU-2 and Env JR-FL transfected Caco-2 cells. D) Occludin expression levels were comparable across the protein groups. n=4 independent experiments *P<0.05 by Kruskal-Wallis test and Dunn's multiple comparison post test.

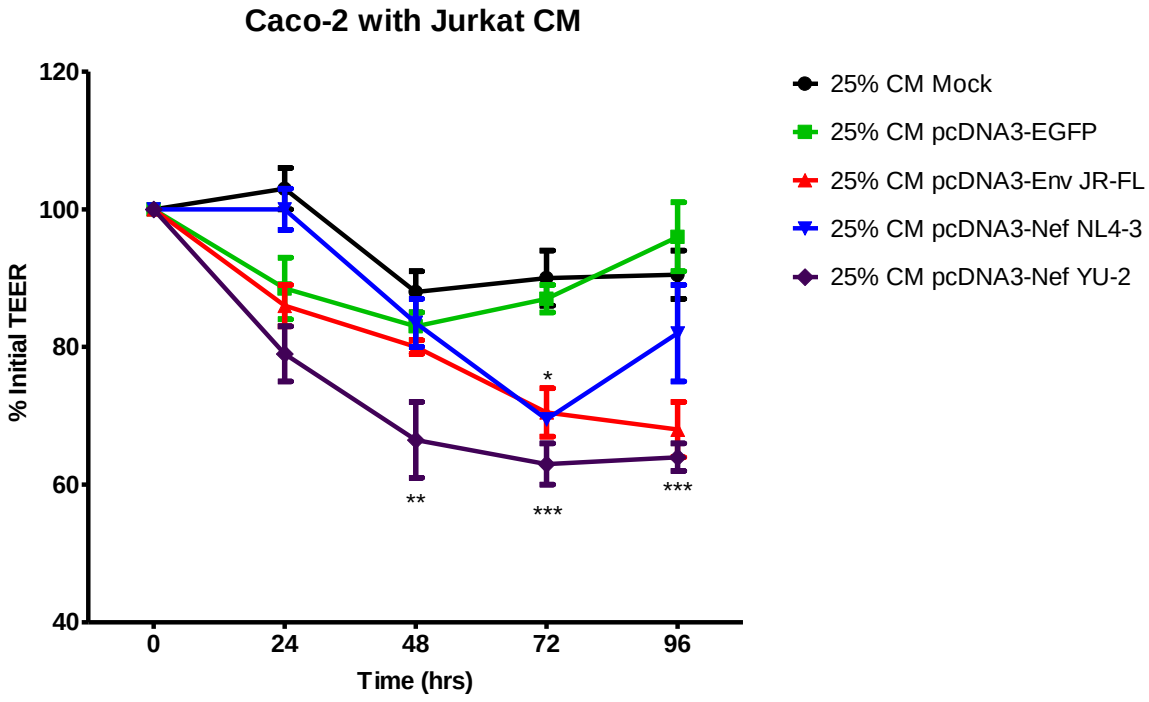
3.4 The Indirect Role of HIV-1 Nef Protein on *In Vitro* GI Epithelial Barrier Integrity

HIV-1 infects T cells and cells of monocyte/macrophage lineage in the various tissues, and particularly in the GI tract in the lamina propria of the mucosa and preferentially in the GALT. To investigate the indirect toxicity of the HIV-1 Nef protein on Caco-2 epithelial cells and monolayers another set of experiments were conducted with modifications to the above conditions for a comparative analysis. In these experiments the Caco-2 cells were co-cultured with conditioned media (CM) harvested from Nef transfected Jurkat (T cell model line) and U937 (monocyte model line) cells. The CM was diluted at 10, 25, and 50% with cell media and applied to the basolateral compartment of a co-culture system, with differentiated Caco-2 epithelial monolayers cultured on permeable membrane inserts.

3.4.1 Nef CM altered the TEER of Caco-2 epithelial monolayers

As previous evidence places a larger role for indirect mechanisms contributing to the barrier defects observed during HIV-1 infection TEER analysis experiments were performed with both Jurkat and U937 CM at the varying dilutions. Caco-2 monolayers were co-cultured on inserts for 5-7 days upon differentiation and baseline TEER values were recorded, the results are shown in Figure 3.10. Results are represented as % of initial baseline TEER and were then compared to the control monolayer responses over time. Significant reductions were observed in the Caco-2 monolayers co-cultured with 25% CM from Nef transfected Jurkat and U937 cells compared to controls.

A


Caco-2 with Jurkat CM
% Initial TEER
Time (hrs)
120
100
80
60
40
0
24
48
72
96
*
**
***
***
25% CM Mock
25% CM pcDNA3-EGFP
25% CM pcDNA3-Env JR-FL
25% CM pcDNA3-Nef NL4-3
25% CM pcDNA3-Nef YU-2

B

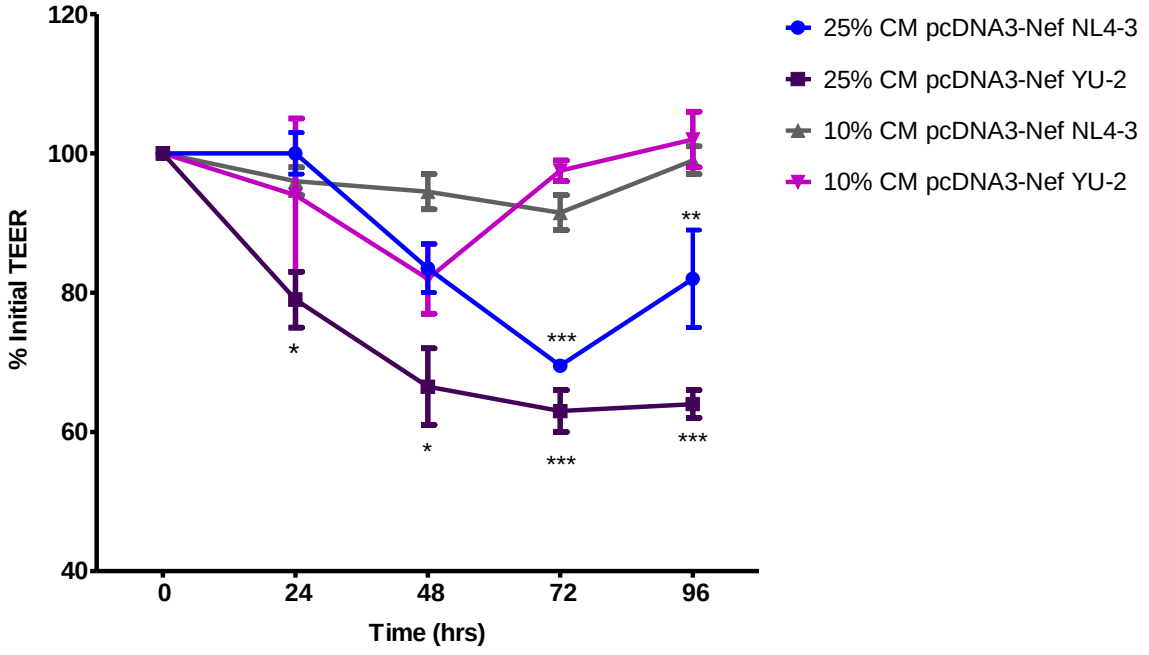

Caco-2 with Jurkat CM
% Initial TEER
Time (hrs)
120
100
80
60
40
0
24
48
72
96
*
*
***
***
**
***
25% CM pcDNA3-Nef NL4-3
25% CM pcDNA3-Nef YU-2
10% CM pcDNA3-Nef NL4-3
10% CM pcDNA3-Nef YU-2

Figure 3.10 TEER of Caco-2 monolayers co-cultured with conditioned media from Jurkat transfected cells.

Results are represented as % of initial baseline TEER and were then compared to the control monolayers co-cultured with 25% mock CM and 25% pcDNA3-EGFP CM responses over time. A. The change in % baseline TEER over time across monolayers co-cultured with 25% Nef YU-2 conditioned media (CM) from Jurkat cells were significantly reduced from 48-96 hrs to 60% of initial TEER. The TEER across monolayers cultured with 25 % Nef NL4-3 CM were altered significantly at 72 hrs. The TEER response between the two Nef isolates was significantly different at 24, 48 and 96 hrs, with Nef YU-2 causing a larger reduction compared to the response from the Nef NL4-3 isolate. B. There was a concentration effect with the % initial TEER of monolayers cultured with 25% Nef NL4-3 CM significantly lower than the TEER of monolayers cultured with 10% Nef NL4-3 CM at 72 and 96 hrs. The response from monolayers cultured with 25% Nef YU-2 % initial TEER was statistically lower than with 10% Nef YU-2 CM at all time points.

*p<0.05, **p<0.01, ***p<0.001, Two Way repeated measures ANOVA with Bonferroni post test. n= 10-14 monolayers per transfection group.

Monolayers co- cultured with 25% Nef YU-2 CM from Jurkat transfected cells demonstrated a significant alteration in TEER with a decrease to 60% of initial mean TEER compared to the monolayers cultured with 25% control CM from mock and pcDNA3-EGFP at 48 to 96hr time points, whereas 25% Nef NL4-3 CM resulted in a significant decrease of initial TEER at the 72 hr time point in the Caco-2 monolayers. The % initial TEER values from monolayers cultured with the two 25% Nef CMs were significantly different from each other at 24, 48 and 96 hrs. There was a concentration effect observed (Figure 3.10b), as TEER values differed significantly between the monolayers co-cultured with 10% and 25% Nef YU-2 CM, at all time points, $p<0.05$ and for the monolayers co-cultured with 25% Nef NL4-3 CM the % initial TEER was significantly reduced from its value at 10% CM, at 72 and 96 hrs respectively. Lastly, time was a statistically significant factor $p<0.05$. Thus Nef transfection of the Jurkat cells appeared to cause alterations over time to the integrity of the monolayers that was not observed when Nef was expressed endogenously in the Caco-2 cells.

This was also observed in Caco-2 monolayers co-cultured with CM from U937 transfected cells, as shown in Figure 3.11a. Similar patterns were observed with the Nef transfected monolayers showing significant decreases in TEER response. And the 25% Nef YU-2 CM again resulted in a more pronounced effect from 24-96 hrs, with a low of 39% at 48 hrs before reaching 56% of initial TEER. Monolayers co-cultured with 25% Nef NL4-3 CM again demonstrated less of a reduction in TEER, with a significant difference from controls appearing at 96 hrs after CM was applied reaching 63% initial TEER $p<0.05$. Env JR-FL CM resulted in significant reduced TEER response from 24 hours-96 hours $p<0.05$-0.01.

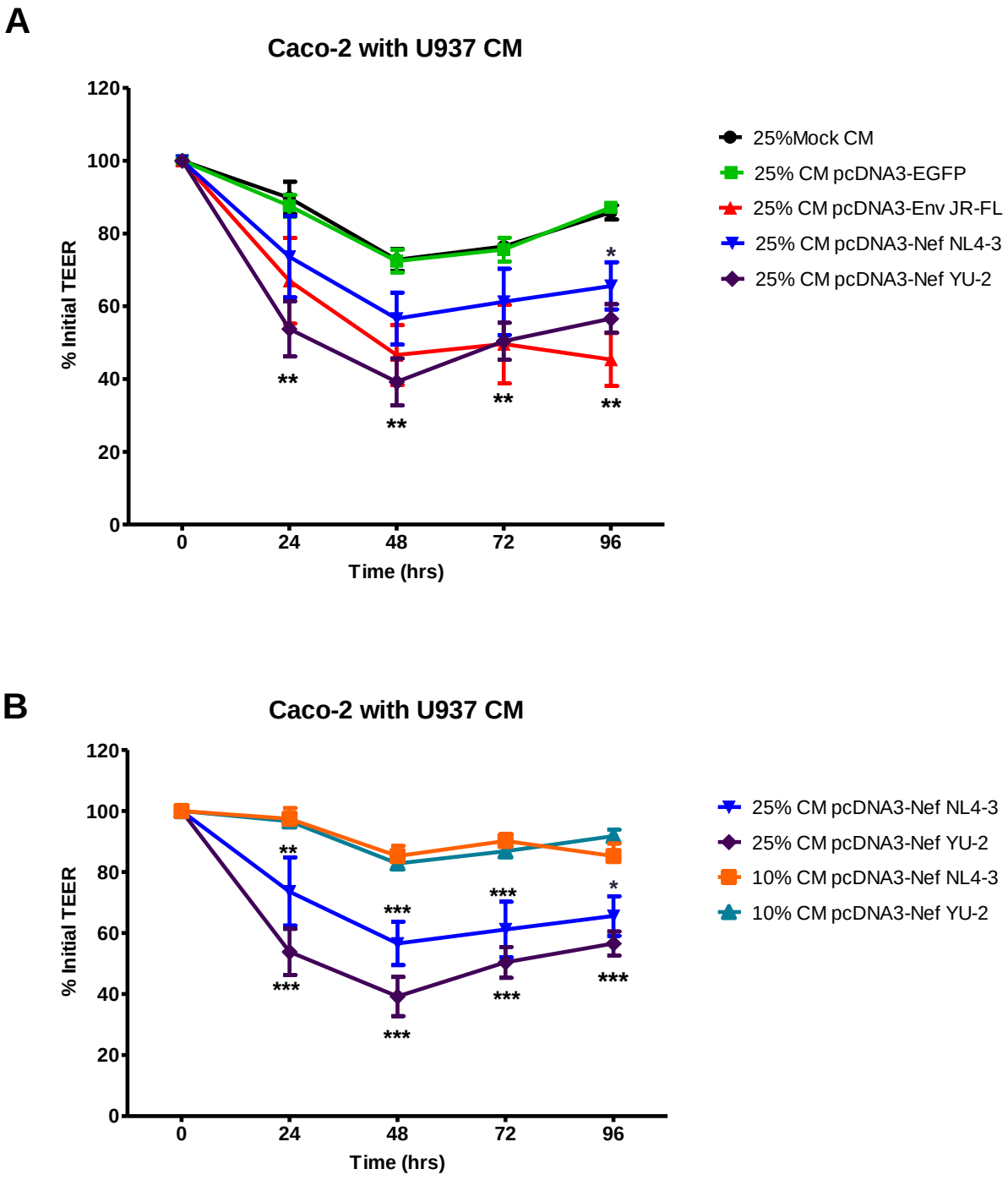


Figure 3.11 The TEER of Monolayers co-cultured with U937 conditioned media

Monolayers co-cultured with 25% CM from Nef transfected U937 cells also displayed significant alterations in TEER relative to control cultured monolayers. A significant reduction in

% baseline TEER values for Caco-2 monolayers was observed, with a decrease to 39% at 24hrs to 56% at 96 hrs, for Nef YU-2 CM, while monolayers with Nef NL4-3 CM had a significant reduction to 63% of initial TEER at 96 hrs. The difference in TEER response between the Nef isolates at 25% was significant at 24 hrs, (74% vs 54%), but were not statistically different at other time points. Monolayers co-cultured with Env JR-FL CM were significantly reduced at 72 and 96 hrs with Jurkat CM, and at 24-96 hrs with U937 CM $p<0.05$at 24 hrs to $p<0.01$at 48-96 hrs. Values were compared to control monolayers co-cultured with 25% mock CM and 25% pcDNA3-EGFP CM. B. Concentration was a factor as the TEER results from Caco-2 monolayers cultured with 25% CM were significantly lower than their values observed at 10% CM dilution, for both Nef strains. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, Two Way repeated measures ANOVA with Bonferroni post test. n= 10-14 monolayers

Concentration was also a factor as values at 25% Nef CM were significantly reduced from their values at 10% CM for all time points and this effect was observed for both Nef isolates (Figure 3.11b), while the TEER response in monolayers cultured with 25% CM TEER differed significantly from each other at 24 hrs $p<0.05$.

3.4.2 Nef conditioned media leads to reduction of Caspase expression in Caco-2 cells

The CM was added to the Caco-2 cells and assessed for cell death, as any observed changes may be the result of molecules released from the Nef protein expressing cells. RT-PCR analysis of Caspase mRNA expression levels relative to levels in cells co-cultured with mock CM was performed. Representative results for Caspase 3 are shown, with Caco-2 cells co-cultured with varying dilutions of Jurkat CM in Figure 3.12a. Of note, cells cultured with 10 and 50% Nef YU-2 CM had reduced RFCs of 0.62 and 0.32 $p<0.05$. All expression levels were raised at 25% CM, but were relative due to fluctuations in expression levels. In Figure 3.12b, cells cultured with both 10% CM from Nef transfected U937 cells had reduced mRNA expression with an RFC of 0.57 and 0.75 respectively $p<0.05$. At 25% CM concentration, while the cells co-cultured with Env JR-FL CM and Nef NL4-3 CM had reduced expression levels with RFC's of 0.79 and 0.62, the cells cultured with Nef YU-2 CM displayed significantly reduced mRNA levels at 0.32 $p<0.05$. Cells cultured with both 50% Nef CM had increased expression with RFC of 3.16 and 3.54. The same expression patterns were observed for Caspase 8 and 9 expression levels.

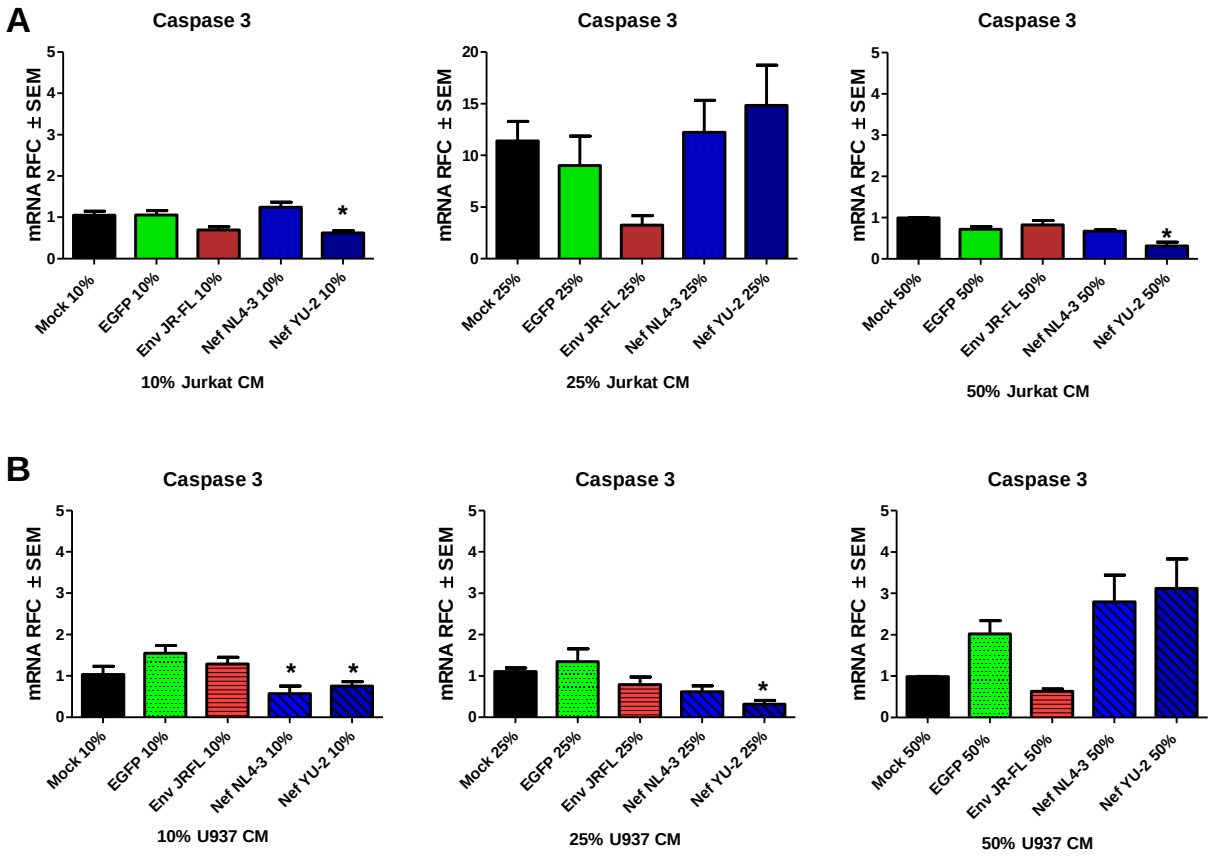


Figure 3.12 Real time PCR of Caspase 3 mRNA expression in Caco-2 cells cultured with conditioned media from Jurkat and U937 transfected cells.

A. Cells cultured with 10 and 50% Nef YU-2 CM from Jurkat cells had RFC of 0.62 and 0.32 $p<0.05$. While at 25% CM all expression levels were raised. B. Caco-2 cells cultured with 10% Nef NL4-3 and Nef YU-2 CM from U937 cells, had RFC of 0.57 and 0.75 respectively $p<0.05$. In 25% CM while Env JR-FL and Nef NL4-3 had RFC's of 0.79 and 0.62, Nef YU-2 was significant at 0.32 $p<0.05$. Cells cultured with both 50% Nef CM had an increased expression with RFC of 3.16 and 3.54. *$p<0.05$ n=5-6 independent experiments by ANOVA using Tukey post test.

The Caco-2 monolayers co-cultured with Nef CM were still intact after the 96 hrs end point, compared to monolayers cultured with 25% staurosporine CM which were degraded. Representative images are shown in Figure 3.13 of Caspase 3 expression in the Caco-2 monolayers 48 hrs after co-culture, as visualized by indirect immunofluorescence microscopy. The monolayers with Nef CM had relative to low Caspase 3 expression detected at 48 hrs, along with the control monolayers. The staurosporine co-cultured monolayers had intense staining at 48 hrs (Figure 3.13) and lots of cell shedding in the wells upon media change. Env JR-FL CM co-cultured monolayers had comparable low levels of Caspase expression after 48 hrs, although as time progressed the monolayers were degraded more with relatively more dead cells in the media. The monolayers of all groups, except the Staurosporine co-cultured cells, were still intact when observed under microscope, up to 7 days after application of CM. Thus cell death did not appear to be the cause of reduced TEER values observed.

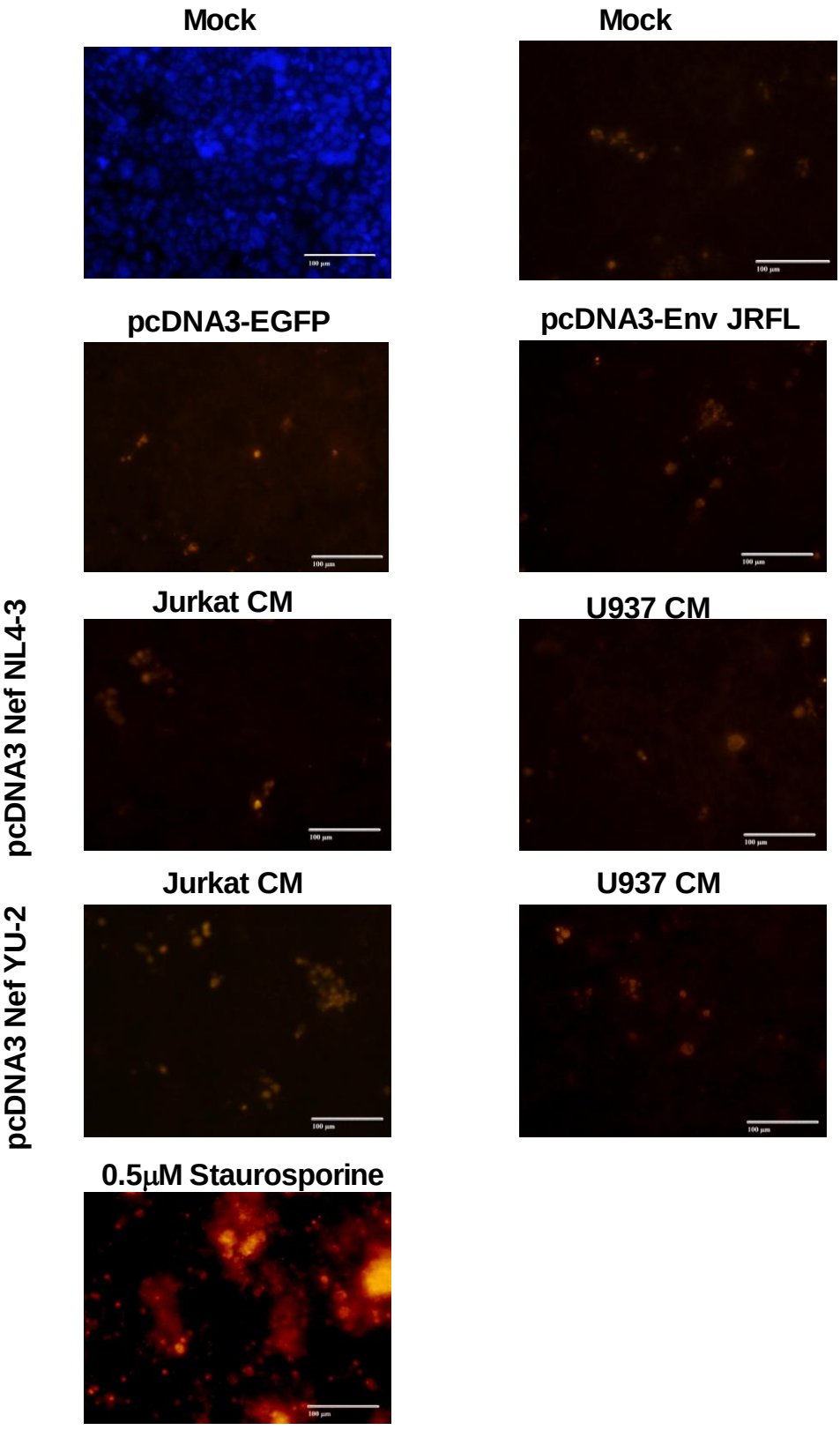

Mock
Mock
pcDNA3-EGFP
pcDNA3-Env JRFL
pcDNA3 Nef NL4-3
Jurkat CM
U937 CM
pcDNA3 Nef YU-2
Jurkat CM
U937 CM
0.5μM Staurosporine

Figure 3.13 Caspase 3 protein expression in Caco-2 monolayers co-cultured with conditioned media.

Representative images of Caspase 3 protein expression are shown as visualized by indirect immunofluorescence microscopy for comparative visualization. Caco-2 monolayers grown on cell culture insert filter membranes were co-cultured with 25% CM for 48 hrs. The Caco-2 monolayers were fixed on the cell culture inserts and subject to indirect immunofluorescence staining of Caspase3. Protein expression on inserts with 25% CM from Nef NL4-3, YU-2 and Env JR-FL proteins were comparable or lower than mock and vector control monolayers. Thus Nef CM did not appear to cause cell death after 48 hrs incubation. Monolayers cultured with 0.5μM staurosporine displayed intense staining. Mock panel with DAPI staining of nuclei represents background cells on inserts. Scale bar = 100μm

3.4.3 Nef conditioned media alters expression of tight junction proteins in epithelial cells

RT-PCR was performed on Caco-2 cells cultured with CM to analyze the effect on the expression levels of various tight junction proteins. Alterations of note were found for ZO-1 and JAM-1. In Figure 3.14a, ZO-1 mRNA expression was significantly altered in cells cultured with 10% Nef YU-2 CM from Jurkat cells with an RFC of 0.68, and Env JR-FL with RFC of 0.74 $p<0.05$. In Figure 3.14b, cells cultured with 10% Nef NL4-3 CM from U937 cells had an RFC of 0.60, while Nef YU-2 and Env JR-FL were comparable to controls $p<0.05$.

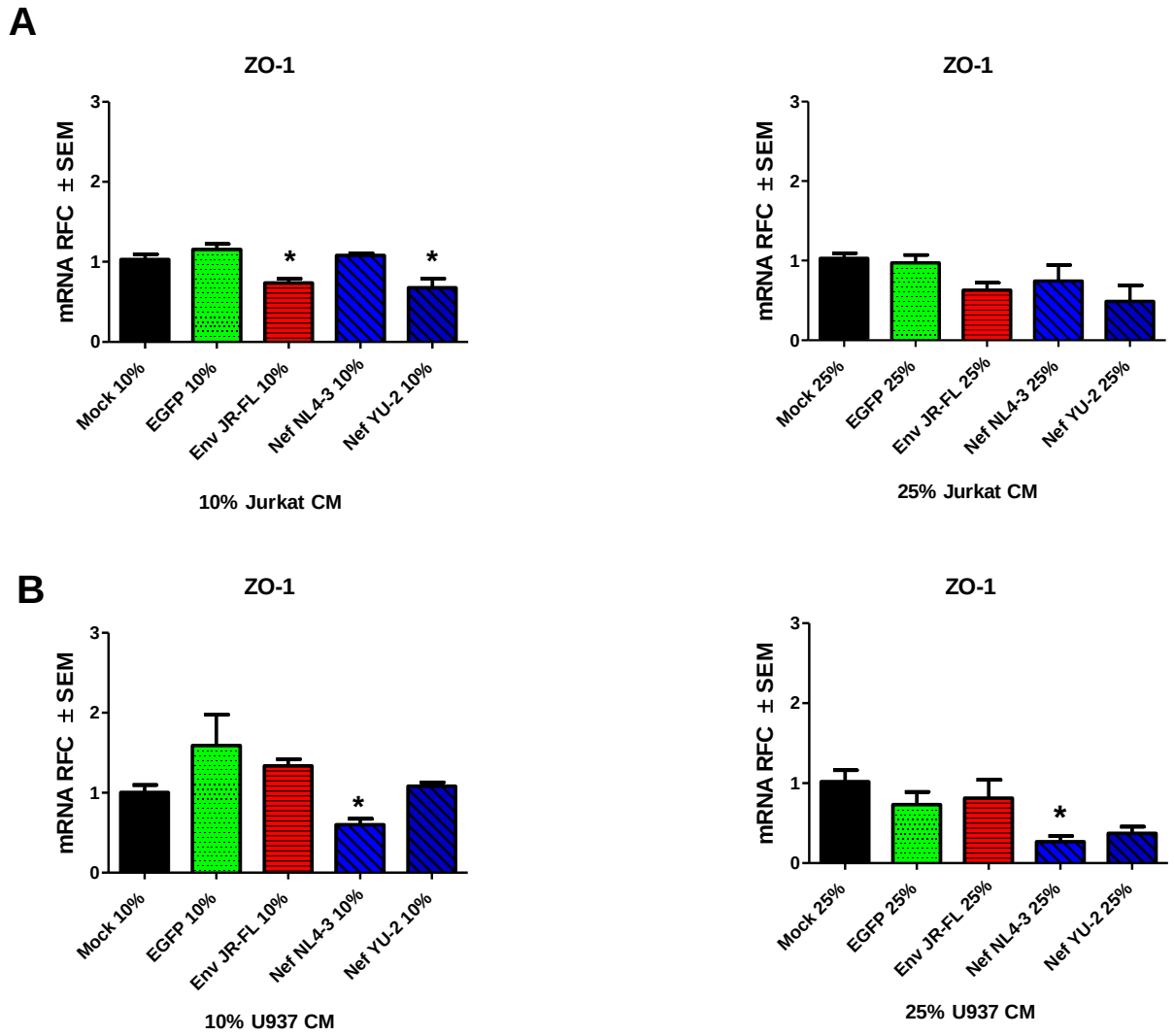


Figure 3.14 ZO-1 mRNA Expression in Caco-2 cells co-cultured with conditioned media.

A. ZO-1 mRNA expression was reduced in Caco-2 cells co-cultured with 10% CM from Jurkat cells, in Nef YU-2 CM with an RFC of 0.68, and Env JR-FL CM with RFC of 0.73, while Nef NL4-3 had an RFC of 1.08, relative to the control RFC. ZO-1 mRNA expression levels in Caco-2 cells co-cultured in 25% CM from Jurkat cells in Env JR-FL and Nef NL4-3 and Nef YU-2 CM had an RFC of 0.63, 0.72 and 0.47 respectively relative to controls. B. ZO-1 expression levels in Caco-2 cells co-cultured in CM from U937 cells. Cells cultured in 10% Nef NL4-3 CM

had reduced gene expression, with an RFC of 0.60 which was significant, while cells with Nef YU-2 CM had comparable expression levels to mock at an RFC of 1.08, and Env JR-FLCM resulted in an increase with an RFC of 1.34. In 25% CM the ZO-1 gene expression levels were further reduced with an RFC of 0.27 for Caco-2 cells co-cultured in 25% Nef NL4-3 CM, while cells co-cultured with Nef YU-2CM had an RFC of 0.37.

*$p<0.05$ n=5-6 independent experiments by ANOVA using Tukey post test. And n=4 independent experiments by Kruskal-Wallis test using Dunn's multiple comparisons post test.

For JAM-1 mRNA expression levels illustrated in Figure 3.15, changes were observed from 25% Nef YU-2 CM from Jurkat cells with an RFC of 0.28 $p<0.05$. In cells cultured with 25% Nef YU-2 CM from U937, the RFC was decreased while 25% Nef NL4-3 CM co-cultured cells had increased expression. CLDN-3 expression levels were significantly reduced as well; with the Caco-2 monolayers cultured with 10% Nef YU-2 CM had reduced expression at 0.48 RFC, while expression levels of mRNA in Caco-2 cells transfected with Nef NL4-3 CM were variable and within control margins. CLDN-3 expression in cells cultured with 25% CM was 0.49 for Nef YU-2 CM, and Nef NL4-3 was 1.91 RFC, and was significantly different from each other. As with the transfected cells, changes detected in Occludin expression levels were comparable to controls in the Jurkat and U937 CM groups. Thus Nef CM appeared to be causing significant alterations in tight junction mRNA expression in the Caco-2 cells in response.

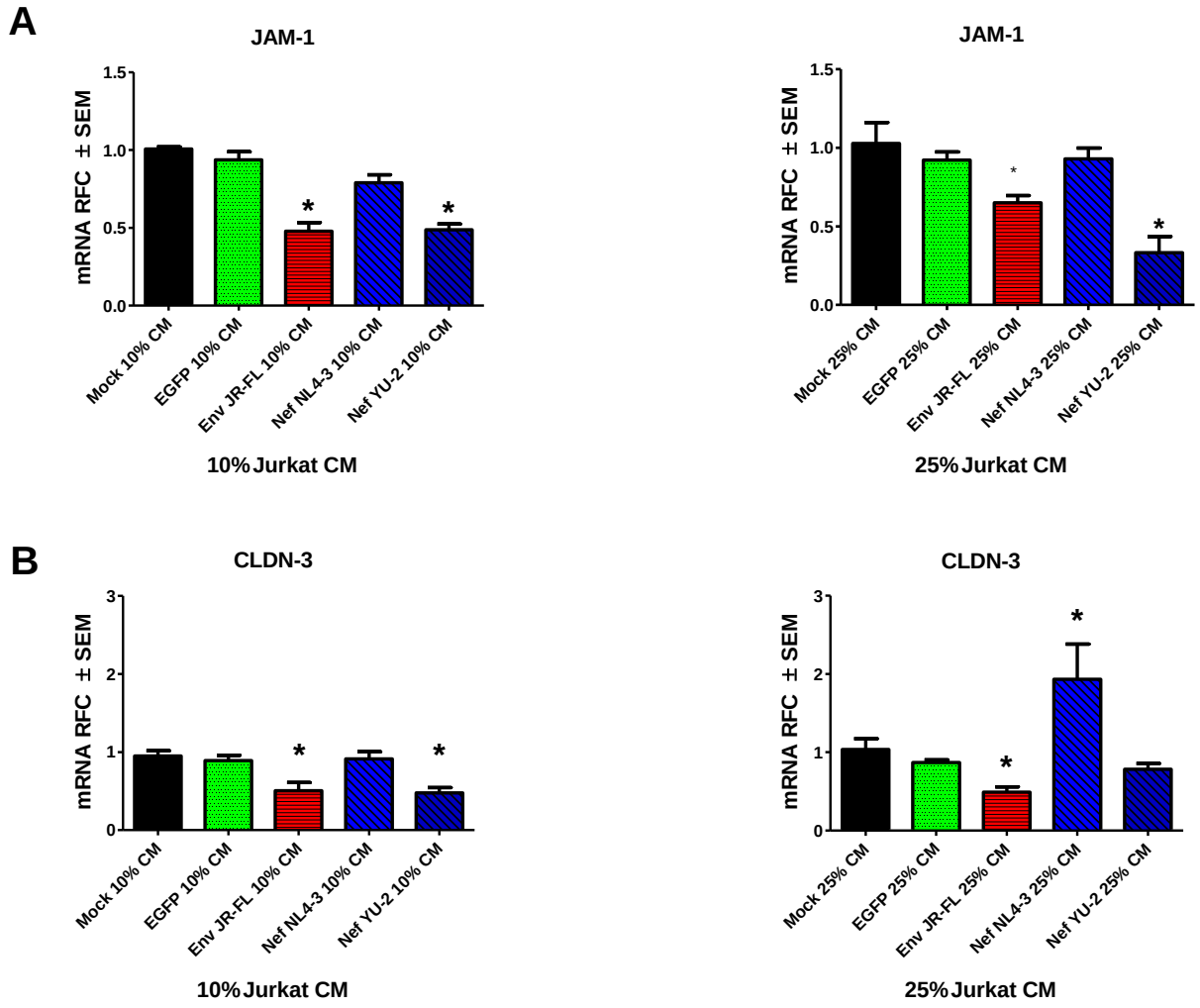


Figure 3.15 JAM-1 and CLDN-3 mRNA expression in Caco-2 cells co-cultured with conditioned media

JAM-1 mRNA expression was significantly reduced. In Caco-2 cells co-cultured with 10% CM from Jurkat cells, the JAM-1 gene expression levels were of 0.79 RFC for Nef NL4-3 CM, Nef YU-2 had RFC of 0.49, and Env JR-FL had an RFC of 0.48. The RFC was 0.28 in Caco-2 cells co-cultured with 25% Nef YU-2 CM from transfected Jurkat cells, relative to control monolayers. Results for Nef NL4-3CM were comparable to controls, but significantly differed

from Nef YU-2. For CLDN-3 expression, the Caco-2 monolayers cultured with 10% Nef YU-2 CM had reduced expression at 0.48 RFC, and cells in 10% Env JR-FLCM were reduced with RFC of 0.51. Expression levels of mRNA in Caco-2 cells transfected with Nef NL4-3 CM were variable and within control margins. CLDN-3 expression in cells cultured with 25%CM was 0.49 for Nef YU-2 CM, and Nef NL4-3 was 1.91 RFC.

*P<0.05 For CLDN-3 n=4 independent experiments using Kruskal-Wallis test with Dunn's multiple comparison's post test. For JAM n=6 independent experiments, using ANOVA with Tukey post test.

Chapter Four: **Discussion**

The study of the human GI tract during HIV-1 infection is challenging. As HIV induced GI dysfunction cannot be easily studied *in vivo*, *in vitro* models using intestinal cell lines have been used in the past and have shed some light on HIV-1 Enteropathy. Thus after an initial *in vivo* gene expression analysis of HIV^+ infected GI tissues provided some correlations of note with respect to HIV-1 Nef in the GI Tract an *in vitro* study was designed to further explore these observations.

4.1 The Establishment of an Optimized *In Vitro* system for Analyzing HIV-1 induced GI Pathogenesis

4.1.1 In Vitro Expression System Evaluation

In the first part of the study an optimized *in vitro* system was established in order to conduct the investigation of the contributions of HIV-1 Nef on GI dysfunction. The T84 cell and Caco-2 cell lines were both used to evaluate protein expression using two expression vector systems. In this study Sindbis (SIN) alphavirus SINrep5 vector constructs were evaluated and used to infect the GI epithelial cell lines [109]. We found that recombinant SIN exhibited a low ability to infect and efficiently express EGFP in the GI epithelial cells used. The Sindbis virus expression vector was proposed as the primary system, chosen as it would be a closer mimic of an HIV infection, and was previously characterized as exhibiting wide cellular tropism that had successfully been used to study HIV neuropathogenesis [59, 109]. While it was hoped that this system could be used in these studies for proof of principle as the two Nef protein sequences and

Nef recombinant vectors have been previously well characterized, the alternate expression system had to be used [59, 109]. The low infection efficiency of SIN-EGFP viral stocks on the colonic epithelial cells suggests that the Sindbis virus envelope glycoproteins were unable to efficiently bind or fuse to receptors on the Caco-2 and T84 cells to deliver the viral genome into the cell. It has been reported that genetically modifying the glycoproteins to target specific cells by the addition of monoclonal IgG Abs that will bind to the specific cell surface has been accomplished [131], which may be a possible solution to increase permissiveness of the cells. It is also possible that the SIN virus is able to enter the cells but the colonic epithelial cell environment and interferon response may inhibit viral replication and protein expression [132].

There are possibilities for improved infectivity by using more concentrated viral stock using chemical reagents such as DEAE-Dextran, or to evaluate another viral based system for future experiments. The time and cost for the viral transcripts and preparations, in addition to the inconsistency observed at times must be balanced with the types of results and system desired. Results using viral vector systems also have to be assessed to ensure the effects are not due to host-cell interactions with the vector itself. Thus using a secondary system such as the pcDNA3 vector, to confirm that findings are not due to remaining viral structural proteins [133]. Thus while viral vectors are very desirable to model HIV as opposed to transfection, they have their own set of issues that have to be considered. In addition to some issues mentioned above around tissue and cell tropism and time needed for virus production, one must ensure that the viral vectors are causing low cytotoxicity and immune responses in the cells used.

The pcDNA3 mammalian transfection vector was evaluated as a secondary system. After the construction of the recombinant pcDNA3 vectors was performed, by cloning of the encoding sequences from the recombinant pSinRep5-Nef vectors, transfection of epithelial cells, Jurkat and U937 cells by lipofection was performed. EGFP expression was low and variable in Caco-2 cells, while minimal expression was observed in T84, Jurkat and U937 cells. Lipid mediated delivery of DNA has been widely used, but it is known this method rarely works optimally under new and untested conditions [134]. Optimizations are usually necessary for every new combination of cell, nucleic acid and conditions used, in addition due to the range of proprietary lipid transfection reagents available one must purchase and test the combinations as some work better with certain cells and conditions [134]. As the recombinant plasmids were constructed and untested, the need for optimization trials was expected for improved transfection efficiency. Yet the results were lower than desired and inconsistent at times. In addition expression in the Jurkat and U937 cells was extremely low for EGFP and not conducive to later applications looking at the indirect toxicity of Nef. The LTX reagent provided a protocol specific for Caco-2 cells and other hard to transfect cell lines which it was designed for, yet the combination clearly did not work for my conditions. Results were better with the older Lipofectamine 2000 formulation but the transfection efficiency in U937 and Jurkat cells were still too low for the indirect studies. Another proprietary lipid mixture than the two tried in this study, such as FuGENE® 6, may have provided higher transfection efficiencies in the colonic epithelial cells, U937 and Jurkat cells.

Transfection of pcDNA3-EGFP in 5 cell lines, as HT-29 cells were included at this point, was evaluated next with a novel electroporation system to try to obtain improved expression

results. High EGFP expression efficiency was obtained for all cell lines. The results were very consistent, with high expression. The optimizations needed were minimal to get started in comparison to the other systems. Electroporation uses short electric pulses to create transient pores in the cell membrane, allowing the uptake of the nucleic acids containing the ORF of our proteins [128]. The difference apparently for the high expression observed immediately, is due to the small size and materials of electroporation tips, thus allowing higher voltages to be used to create more transient pores than other electroporation systems. The small size allows less damage to the cells which increases the amount of cell survival and cells expressing the proteins of interest for these more sensitive cell lines [135]. The limitation of using this method for our conditions was that the epithelial cells are electroporated in suspension as opposed to when seeded and differentiated in a monolayer, as preferred for direct studies, though with the expression challenges faced, it was still a beneficial system.

4.1.1.1 Plasmid DNA Concentration

Cytotoxicity of the sequences was an apparent issue, and observed in the SIN plasmids as well by van Marle et al. [59]. The plasmids containing the Nef and Env ORFs were cytotoxic to the bacteria cells resulting in low cell yields upon amplification in LB. Thus the high concentration of DNA needed for transfections was more challenging to isolate than usual. Large cultures of 150-200ml at a time were needed to obtain the high quantities of plasmid DNA needed for the transfections. The DNA was then concentrated for electroporation reactions as only 10% of the volume in the tip (10 or 100µl tips) can be DNA. Colleagues in the lab evaluated the use of different nutrient rich culture media, while improved slightly yields were

still low. Thus a compromise had to be made between obtaining higher expression levels, cell toxicity and the concentration of DNA used per reaction tip.

4.1.1.2 Nef Protein Expression

Upon evaluation of HIV-1 Nef and Env expression, extremely low to undetectable visual signal levels was observed using immunofluorescence microscopy and immunoblotting techniques, throughout the investigation regardless of optimization attempts to the various protocols. In the end RT-PCR analysis using sequence specific primers was used for confirmation of the presence of *nef* and *env* mRNA in the transfected cells, with mRNA expression levels consistently obtained around 5% relative to EGFP expression in the Caco-2 cells. To test if the issue with Nef expression was due to the reagents and methods used, a dual study was performed using the same transfection protocols in the easily transfected cell line BHK-21 and with the epithelial cells. Visualization of both Nef and Env proteins were observed by immunofluorescence microscopy in the BHK-21 cells, with transfection efficiencies of approximately 60% and 20% relative to EGFP for Nef and Env respectively. In the GI cells they were still barely detectable using the same conditions.

It would appear that the combination of the toxicity of the proteins expressed was not able to be overcome in these cell lines for high enough expression and thus signal detection. The lower viable intracellular expression of these proteins in these cell lines was expected relative to EGFP as the HIV ORFs were toxic in the bacterial cells in the SIN vector as well. Plasmid DNA amplification of the recombinant HIV SIN and pcDNA3 vector constructs in the bacteria cells was consistently lower. This was also observed previously by van Marle et al. [59] with the

recombinant SIN vectors as well. The difference for their studies was that SIN virus particles were infectious enough in the neural cells that 10^6 virus particles were able to be used to overwhelm the cells leaving enough viable that high expression of HIV-1 Nef and Env JR-FL was possible and visually detected. As the *in vivo* infectivity of the HIV is low as described previously, anywhere from 0.0001-0.1% there was enough expression to observe an effect but it was not ideal, as it is preferable to start with higher expression and dilute the effect. This also limited the ability to visualize co-localization patterns as desired.

There have been previous reports on the difficulties with obtaining and maintaining high levels of Nef expression in certain cell types, and a variety of methods used to overcome this including constructing Nef-fusion proteins to control and amplify signal, and using DNA and RNA viral vectors [133, 136-138]. In a recent study, using electroporation to transfect peripheral blood mononuclear cells (PBMCs), they observed higher yields of GFP compared to HIV-1 Gag and Nef protein, with HIV-1 Gag being expressed more efficiently by the cells than Nef, though the toxicity of the mRNA was equal among the HIV proteins [139]. To address this issue and improve immunodetection of Nef and Env in future studies the ORF's can be cloned again, and evaluated in vectors under different promoters and with fusion tags, for improved expression and signal amplification, with the ability to do immunoprecipitation if needed.

It is well established that the choice of plasmid is important for expression, and that expression success is dependent on the type of cell and gene one is expressing [140]. Transfection of the pcDNA3 vectors with the Neon electroporation system was relatively easier than some methods, and the results were consistent. Multiple plasmids were able to be

electroporated sequentially in a short period of time including the control cells under regular cell culture conditions. Improvements to the expression levels obtained are possible, but the results were enough to observe differences for an investigation of intracellular HIV-1 Nef expression.

4.1.2 The Caco-2 Model Intestinal Epithelial Cell Line

After evaluating the Caco-2, HT-29 and T84 cell lines for studying effects of HIV-1 Nef protein, the Caco-2 cells were found to be most widely applicable and responsive to experimental conditions and analyses performed in this investigation. The T84 and HT-29 cells were sensitive to electroporation parameters and thus the TEER values were abnormally low. This led to the decreased ability to differentiate between differences in TEER for direct toxicity studies. The T84 cells most resemble colonic crypt cells and usually form tight monolayers with much higher baseline TEERs than Caco-2 cells. Increased permeability of Caco-2 intestinal monolayers and a drop in TEER of up to 30% was observed following varying electroporation pulses by Ghartey-Tagoe et al, with recovery after a return to normal conditions observed from 1 hr to 24 hrs after electroporation [128]. This transient increase in permeability was assessed as a possible way to exploit the use of electroporation to increase drug transport across monolayers [128]. This suggests that the T84 cells were more sensitive, with a decreased ability to recover from the disruptions caused by the electric pulses to form a differentiated monolayer compared to the Caco-2 cells although they are both colonic epithelial lines.

More recently it's been reported that after analysis of Caco-2, HT-29 and T84 cells for use as *in vitro* intestinal cell models for future experiments for GI disorders, and for studying HIV, Caco-2 cells were also confirmed to be the most responsive in their investigations under unstimulated and inflamed conditions [82, 83, 105]. The Caco-2 cells are an immortalized line derived from a human epithelial colorectal adenocarcinoma [141]. These cells were chosen initially for evaluation as they were derived from human intestinal epithelial cells and have been shown previously to express coreceptors that are used by HIV for entry, the CXCR4 and Gal Cer coreceptors [142]. They also exhibit features that are similar to colonocytes in earlier passage stages and go on to display features of the small intestinal enterocytes; producing brush borders and enzymes when fully differentiated up to 21 days in culture [106, 141, 143]. As it has been reported that the cancerous state of these epithelial cells can lead to alterations of their tissue characteristics at high passage numbers, care is needed when working with them [102, 144]. It has also been reported that their ability to differentiate and their permeability can be altered at higher passage numbers, leading to altered results and difficulty in comparing data [102, 144]. In these investigations the passage numbers were kept low and within the same range, and methods of cell culture and isolations were kept as consistent as possible.

4.1.3 Alternative (Co-culture) Models

As described in the introduction *in vivo* GI epithelial barrier function is more complex, and involves interactions with the intestinal immune cells. In a desire to address limitations to the simple co-culture model for studying GI disorders, Leonard et al recently constructed a three-dimensional co-culture model of intestinal mucosa in an inflamed state to represent the more *in*

vivo state of an immune response mediated chronic inflammation [105]. They developed a more complex system by stimulating the epithelial lines HT-29, Caco-2 and T84 with proinflammatory compounds, and included primary macrophages and dendritic cells [105]. This type of model would be useful and appropriate for modeling the later stages of HIV infection with the chronic immune activation phase. Recently, microtechnology approaches to studying HIV *in vitro* have been described and assessed [75, 145]. The benefits to these methods are described as enabling greater control over the pattern and placement of cells in a co-culture system. Stybayeva et al recently described constructing a micropatterned co-culture using HT-29 epithelial cells and captured Jurkat T cells on printed arrays to establish a novel in vitro model for HIV pathogenesis [145]. Presence of HIV in Jurkat cells was confirmed by RT-PCR analysis as well in this study [145].

The system is a simple model as described, and findings need to be confirmed *in vivo* for applicability but they provide valuable guidelines, and the method has been noted as the best test involving human cells that does not directly involve human subjects [102, 107]. While in this investigation we chose to use Jurkat and U937 CM, a three dimensional culture would be interesting to use at a later point with human primary peripheral blood mononuclear cells (PBMCs), Peripheral blood lymphocytes (PBLs) and Monocyte Derived Macrophages (MDMs).

4.2 The Role of HIV-1 Nef on the Integrity of an *In Vitro* GI Epithelial Barrier

In the second part of the study, the contribution of the HIV Nef protein to GI epithelial barrier dysfunction was investigated after I observed increased viral *nef* expression in HIV^+ GI colon tissues that correlated with actin and CXCR4 expression in a preliminary gene expression study (T. Grant and G. van Marle, unpublished results). The *in vitro* GI epithelial barrier system, determined in the first part was used to provide further knowledge on HIV-1 Nef effects and function.

4.2.1 The Effects of HIV-1 Nef on TEER of Epithelial Monolayer

An assessment of the direct and indirect effects of endogenous Nef on epithelial monolayer integrity was performed by TEER analysis, which primarily measures the ion permeability of the tight junctions [98]. The TEER was first measured by forming Caco-2 epithelial monolayers upon expression of intracellular HIV-1 Nef proteins, and secondly in an indirect assay upon co-culture of differentiated Caco-2 monolayers with varying dilutions of CM, harvested from Nef transfected Jurkat and U937 cells. We found that intracellular expression of the two Nef proteins did not result in a significant alteration of TEER of the Caco-2 monolayers, whereas varying dilutions of CM led to significant reductions in TEER with the Nef YU-2 allele after 24 hrs and as time progressed. These results would suggest that endogenous Nef does not exert a direct effect on paracellular permeability of the Caco-2 epithelial monolayers. To test the whole intestinal permeability that takes other factors of the epithelial barrier into account, a permeability assay using a probe would be needed in conjunction to the TEER recordings as the two may not be correlated. An example of some of

the varying molecular weight probes used to assess permeability across the epithelial monolayer include mannitol, sodium fluorescein, dextran and inulin [116, 144].

In contrast significant reductions in TEER were observed from the Caco-2 monolayers co-cultured with Nef CM, particularly the Nef YU-2 isolate. Thus it would appear that the Caco-2 monolayers reacted to inflammatory cytokines and other stimulatory factors released in the CM from the host Jurkat and U937 cells expressing intracellular Nef. It has been established by previous reports that intracellular Nef expression in these cell types; T cells and cells of the monocytes/macrophage lineage, causes the cells to release various proinflammatory cytokines and chemokines including; TNF-α, IL-1β and IFN-γ [146-149]. The effects of these cytokines on the intestinal cells have been reported in studies of GI mucosal inflammation and HIV enteropathy over the years [150-152].

Barrier defects and reductions in TEER across the intestinal barrier after exposure to active virus and to Env gp120 protein have been reported [69, 72, 153-155]. In a recent study it has been reported that the reduction in TEER, and disruption to the barrier function observed after direct intestinal cell exposure to the virus and the Env protein was caused from TNF-α secreted by the epithelial cells, as this effect was blocked by anti-TNF-α, antibodies [155]. One can propose from these results that the TEER response observed from the Nef CM may also be due to the presence of TNF-α produced by the Jurkat and U937 cells in response to Nef protein expression, though further investigations are required.

In the recent study using soluble Nef (sNef) it was noted that sNef significantly up-regulated permeability, but did not alter TEER, as was found in this investigation [83]. It was also reported that sNef partially reduced the effects of IFN-γ exposure on the permeability of the epithelial monolayers to the levels found with sNef added alone. It is proposed that the virus has adapted to counter the effects of the interferon response [82]. It would be of interest to see if the same modulation of TEER would be observed if the indirect assay was repeated with Caco-2 cells expressing intracellular Nef, and co-cultured with CM. While the two forms of Nef have been reported to exert varying functions, depending on their location and the cell type [146-149], it would be likely that this effect could be seen with endogenous Nef in the colonic epithelial cells.

4.2.2 The Effect of HIV-1 Nef on Epithelial Cell Death

Epithelial cell death, upon direct transfection of HIV-1 Nef and indirect exposure to varying concentrations of Nef CM was assessed. In an analysis of the gene expression levels of the Caspase effectors of cell death, Caspase 3, 8 and 9, we found significantly reduced or comparable gene expression levels relative to mock transfected control cell levels. In addition analysis of Caspase 3 protein expression by immunofluorescence microscopy (IMF) and western blotting, confirmed that Caspase 3 protein expression levels were reduced or comparable to controls. Nef transfected cells were also viable and proliferating at the same levels as control cells. Gene expression analysis and IMF revealed the same patterns of reduced Caspase expression levels in Caco-2 cells co-cultured with Nef CM from Jurkat cells and U937 cells.

The results suggest that Nef expression in epithelial cells does not cause cell death. That alternatively, as observed from gene expression analysis, Caspase expression was being down-regulated in Nef transfected epithelial cells, and in the epithelial cells cultured with CM. In the GI tract the high epithelial cell proliferation levels are balanced by equal rates of high cell death by apoptosis, in a tightly regulated effort to maintain barrier integrity [94]. Thus it appears that Nef is attempting to protect the cell from the programmed cell death signals by reducing the expression of the 3 Caspase genes evaluated to improve cell survival. The down regulation of the Caspases observed in the epithelial cells from the co-cultures with CM, suggests that Nef is also stimulating anti apoptosis factors in the Jurkat and U937 cells that was released into the media.

Nef has been shown in reports to inhibit apoptosis in $CD4^+$ T cells and in MDMs to protect the infected cells [8, 54, 156-158]. Using *in vitro* models, it has been shown that HIV-1 Nef and Tat protein expression offers resistance to apoptosis to monocytes/macrophages, and that these cells are protected from both virus and host cell factors by anti apoptosis genes [158, 159]. Nef interferes with various signaling pathways to inhibit apoptosis in T cells, as it's been shown to inhibit p53, TNF-α and FasL mediated apoptosis to protect the infected cell while activating apoptosis of cytotoxic lymphocyte cells [8, 54, 156, 157]. In a report on the persistently apoptosis resistant T cells found during HIV infection, the resistance was found to be independent of viral replication and involved a modulation of the mitochondrial pathway with reduced Caspase 3 and mitochondrial membrane potential activation [160]. Thus it would appear that the indirect down regulation of Caspase expression seen in the Caco-2 cells with CM may also be from the factors released from intracellular Nef expression in the Jurkat and U937 cells. While in their study Quaranta and colleagues did not find alterations in the percent of apoptotic

cells upon direct exposure to sNef, they did observe a Nef- mediated inhibition of IFN-γ induced apoptosis [83].

It has been found, using the SIV animal model, that during early infection there is massive cell death and increased proliferation of gut epithelium with apoptosis being the main mechanism [161]. The peak gut epithelial cell apoptosis coincided with interactions of the virus and GPR15/BOB, the alternative HIV co-receptor, with virion binding to the basal surface of the epithelium in the small and large intestine [161]. Thus it appears that Nef is acting to balance the high rates of apoptosis activated by the binding of Env gp120 to the cells, and tries to modulate or reduce the cell death by reducing Caspase expression and releasing factors that stimulate anti apoptotic genes. Maintaining or increasing the life cycle of infected and nearby cells would be a part of its function as an enhancer of virion infectivity, aiding viral spread to nearby cells and allowing virus time to pass through the cell or induce factors that would recruit other target cells [8].

4.2.3 The Effect of Nef on Tight Junctions

The tight junctions are important in maintaining epithelial barrier integrity and functions. Changes in the shape of tight junction strands is often the result of changes in tight junction protein expression and the Claudin proteins in particular have been implicated [162]. Therefore an assessment of the gene expression levels of various components of the epithelial tight junction complex was performed. We found that intracellular expression of the two Nef proteins resulted in significant reductions of the tight junction genes; ZO-1, JAM-1, and CLDN-3, in the Caco-2 cells. This effect was also observed in epithelial cells co-cultured with varying dilutions of CM

from Nef transfected Jurkat and U937 cells. Confirmation by immunofluorescence microscopy of ZO-1 by a colleague demonstrated irregular increased spacing between the cell-cell contacts (M. Al Najeer and G van Marle, unpublished data). Occludin expression levels were widely variable in our investigation, which represents the limitations from the experiment numbers.

JAM deficient mice have been shown to have reduced barrier function, with increased rates of epithelial cell proliferation and apoptosis, in addition to intestinal immune cell activation [85, 163]. While the Claudin proteins are well known to be crucial to the tight junction complex, as they regulate the actual pores of the tight junction and change their expression patterns in response to various events such as inflammatory cytokines [164]. Lastly ZO-1 forms the direct link between the Claudins, occludin and the actin cytoskeleton, thus the combined down-regulation of these genes should result in loss in barrier function. Yet in the direct study, the down-regulation of the tight junction proteins was not enough for a noticeable alteration in the ion movement though the paracellular route.

It has been noted in GI inflammation studies, that tight junction alterations are not enough to cause the severe inflammatory states of disease [85]. Endogenous Nef may have been suppressing or modulating the expression of inflammatory molecules that the Caco-2 cells would be producing. Whereas the barrier alterations observed from CM were likely mediated from the alterations of the tight junction expression combined with the rise in the inflammatory cytokines present in the media from Jurkat and U937 cells. Immune cells produce a large quantity of cytokines in response to viral binding and the inflammatory molecules released are known to induce reductions to tight junction protein expression [165].

It's been reported that tight junction gene expression levels were reduced to the same levels upon exposure to Env gp120, active HIV-1 virus or inactive virus [155]. Thus they report that the tight junction alterations were associated with attachment of the virus, as opposed to virus production [155]. Nef induced alterations to the tight junction components, directly through intracellular expression in the Caco-2 epithelial cells and by indirect means through stimulation of immune cells, would aid in this cytoskeletal rearrangements and virus attachment leading to more permissive epithelial cells for cell-cell contacts [166].

Brenchley and colleagues report extensive damage to the mucosal barrier found in fixed tissues after infection using the SIV animal model, but only low levels of replicating virus were found in the lamina propria cells [23]. Thus making it likely that Nef and the HIV proteins enhancing the ability of viral transfer passage through the cells to get to the actual target cells of the GALT [23]. That Nef appears to directly cause a reduction in epithelial cell death and unaltered TEER suggests that its direct role appears to be more protective balancing the survival of the epithelial cells where virus may have already attached or infected. This method of impairing the integrity of the barrier is less damaging compared to the virotoxic effects of the Env protein reported by other laboratories [21, 35, 38, 155] and observed in this study. These findings confirm its known function as an enhancer of virion survival and infectivity.

4.2.4 Nef Isolate Variability

The two prototypic HIV-1 Nef protein strains used over the course of the investigation demonstrated variable effects to the Caco-2 epithelial cells and monolayers over much of the study. These differences were often significant from each other, in addition to the controls. Cells and monolayers transfected with the Nef YU-2 allele exhibited more pronounced responses than those transfected with the Nef NL4-3 allele, with significantly reduced TEER and expression levels of Caspase and tight junction genes. The tissue specific variability observed between the two Nef alleles was observed previously by van Marle et al [59]. As it was found that intracellular expression of Nef protein from the YU-2 brain-derived HIV-1 strain was cytotoxic to astrocytes *in vitro* and induced a neuroinflammatory response as well as neuronal injury *in vitro* and *in vivo* [59]. In addition analysis of the inflammatory response induced from patient isolated Nef protein coding sequences was found to be variable and dependent on the sequence of the Nef protein, consistent with viral evolution towards a more conserved and possibly more pathogenic Nef protein in the brain [109].

As illustrated in a schematic of the two Nef sequence alignments in Figure 4.1, Nef NL4-3 and Nef YU-2 have some sequence variability. The Nef protein is highly conserved in lentiviruses and even slight polymorphisms in the protein have been associated with different functions, cytotoxicities and cellular distributions, which may affect cell fate and pathogenesis [59]. In a transgenic mice model, Jolicoeur et el found numerous varied pathological changes observed between *nef* alleles, which resulted in differing pathogenicity spanning most phenotypes associated with HIV-1 infection and AIDS in humans or in SIV-infected primates [28].

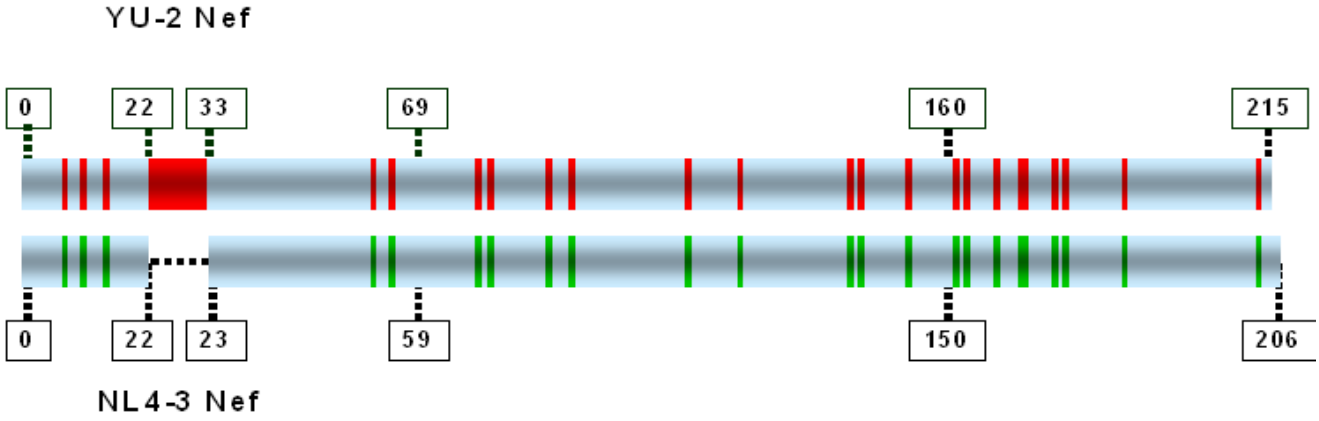


Figure 4.1 Schematic Representation of the Prototypic Nef Proteins YU-2 and NL4-3

The brain derived Nef YU-2 consist of 215 amino acids and it contains an additional 9 amino acids from position 22-33, compared to the blood derived Nef NL4-3 protein. In addition they differ by another 24 amino acid residues indicated by the red and green bars. The two Nef proteins display varying pathogenicities which may be related to these differences in protein sequence.

These findings in this study corroborate previous reports where different Nef alleles have varied pathogencities, with implications of where a GI derived Nef allele would line up. In the Southern Alberta clinic (SAC) gut cohort, significant viral loads in the gut mucosa were still observed for patients with low plasma viral loads [167]. Survival data from the SAC gut cohort also demonstrates that high viral loads and particular viral species in the GI tract are linked to reduced survival prognosis [167] supporting the view of the importance of these variations in the GI tract to overall disease pathogenesis (ie. development of AIDS).

4.3 Conclusion

The aims of the study were to; evaluate and establish an effective reproducible *in vitro* system for studying HIV-1 GI pathogenesis, and to then perform an analysis on the effects of HIV-1 Nef proteins at the GI tract. While the first aim was wider in scope than proposed leading to a smaller second investigation the findings have been able to be reported and has been the foundation for expanded investigations in the laboratory presently underway.

Over the course of the evaluations, challenges with the viral based SIN system and low infectivity of GI cells led to the expanded evaluations of pcDNA3 vector constructs, which were subsequently used as the primary expression vector for future experiments. In addition after evaluating the cell lines, the human colonic Caco-2 epithelial cell line, used in a co-culture system was found to be an effective *in vitro* model of the GI epithelial barrier. The cells were most responsive when combined with the expression vectors, to our conditions and the types of analyses desired, while intracellularly expressing the HIV-1 proteins. The system enabled reproducible manipulations and was widely applicable to a number of investigations.

An investigation into the contributions of the Nef protein was then performed. Our results suggest that the Nef protein contributes directly and more notably, indirectly to impairments of the epithelial barrier. Intracellular expression and factors from Nef expressing cells; provide resistance against epithelial cell death, and alters their tight junction expression, likely for enhanced viral attachment, transfer and survival of virus in host epithelial cells.

The Nef protein appears to perform its functions more moderately and selectively, than the Env protein, which consistently exerts more cytotoxic responses. This variability shown contributes to its reputation as a challenging multifaceted protein, and it's early mislabelling as a negative viral factor. Extensive studies have established its importance in HIV pathogenesis, and it is an ideal target for therapeutics.

4.4 Future directions

An exploration of additional vectors with fusion tags, including an alternate viral vector system would be beneficial. As HIV-1 infection has an impact on lymphatic and monocyte derived cells the effects of the different Nef proteins on human uninfected primary cells should be analyzed. For increased *in vivo* applicability repeating the experiments with isolated PBMCs, MDMs, and PBLs would be of interest. Fractionation studies, and 2D gel analysis to analyze the factors in the CM and to detect if soluble Nef has been released from the Jurkat and U937 cells and/or using the primary cells. Repeating the TEER analysis of the co-culture experiments using CM and cytokines would be of interest to determine if we would observe the same modulation of the IFN-γ response recently reported. Permeability assays using a tracer molecule in addition to recording TEER would help provide a more complete result. The effects would also need to be able to be blocked and then restored to ensure it's a Nef specific effect. In addition an investigation into the cytoskeletal rearrangements apparently induced by Nef in the epithelial cells would be a relevant extension to provide direct evidence of Nef involvement for virion transfer.

Future experiments will involve the use of GI derived Nef protein coding sequences previously cloned in the laboratory from the HIV-1 infected patients in the gut cohort [73]. The *in vitro* findings from this experiment and the ongoing investigations can be explored and confirmed by; RT-PCR and western blots using the RNA and proteins isolated from the patient samples, immunohistochemistry using the paraffin embedded gut tissues stored, and immunofluorescence microscopy. A comparison between the two prototypic sequences and the alignment of those obtained from the gut derived Nef sequences isolated from patient samples and their pathogenic effects will help delineate regions in the Nef protein that are important for pathogenesis.

4.5 Significance:

The HIV-1 Nef protein has been implicated in many studies as a major pathogenic factor as it accelerates clinical progression towards AIDS in hosts infected with immunodeficiency viruses. It has been shown to have multiple other effects thus a thorough understanding of Nef function within both the infected host and the infected host cell is needed to further characterize these effects and their role in acceleration of viral pathogenesis *in vivo*. Nef protein's particular effects on the GI tract, the major site for CD4 T cell depletion and replication, are not well characterized.

HIV is predominantly transmitted through sexual contact at various mucosal surfaces with sexual transmission at the oral, GI and genital tract as the primary routes of contact. The GALT acts as a viral reservoir with a notable contribution to systemic pathogenesis. In addition

to the events at the GALT, the damage caused by HIV at the GI barrier has been implicated as enabling microbial translocations, and the accumulation of these products in the circulation from the GI lymphatics, creating systemic inflammation that is proposed to have an association with the chronic immune activation phase and pathogenesis of HIV-1 [22, 23]. While much has to be clarified, the damage that was observed was unexplained by viral infection alone, with small amounts of virus found replicating in the lamina propria cells using the SIV animal model [23].

Apoptosis of the enterocytes has been one of the proposed mechanisms for impairment, and the Env protein has been shown to cause apoptosis in (SIV/HIV) models across intestinal epithelial cells and mucosal surfaces during the acute early phase of infection. In this investigation we found that HIV-1 Nef down regulates the cell death signals and contributes to the epithelial barrier dysfunction by altering tight junction gene expression. Reduction of tight junction proteins alters the effectiveness of the epithelial barrier. Although further studies are needed, the epithelial cells seem to be damaged as the virus and its viral proteins, prepare the host cells for viral transfer and induce factors for stimulating the immune cells as it establishes in the GALT.

The widely accepted primary biological function of Nef is as an enhancer of virion infectivity. In our study we found *in vivo* correlations of viral *nef* up regulation in the colon and duodenum with increased CXCR4 up-regulation. *In vitro* studies have confirmed that Nef induced up-regulation of the CXCR4 coreceptor as opposed to the CCR5 receptor, on DC's and on colonic epithelial cells [82, 168]. The up-regulation of the CXCR4 receptor favours the X4

tropic viruses that bind to T cells and induce the viral synctium for fusion, thus favouring viral transmission by cell-cell contact [8, 166].

The number of new HIV infections continues to rise in subpopulations of North America, Eastern Europe, Central Asia, Oceania, Middle-East and North Africa, while it has remained stable in other regions of the world [25] underscoring the importance of global initiatives and response rates. On its own HIV has a low infectivity rate, thus viral factors like Nef, are needed and highly conserved to increase virulence, thus it remains a notable target for therapeutics. Improved GI health and mucosal integrity improves the host responses, which helps increase the control of the virus and improves survival length. HIV is a complex virus, which is constantly evolving and developing immune evasion strategies, thus combined therapeutic approaches will be necessary to challenge the virus. For the millions of people surviving with the virus for long periods of time, drug resistance is a concern, and necessitates the development of new strategies and maintained awareness to maintain the progress that has been made.

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