

Examining the function of primary HIV-1 Nef isolates in the setting of viral control

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Abstract

HIV is a global pandemic. While combination anti-retroviral therapy can block viral replication, HIV establishes latent reservoirs that persist for life. A rare group of HIV-infected individuals called “elite controllers” spontaneously suppress plasma viremia to clinically undetectable levels. Understanding how elite controllers contain HIV could assist in developing more effective interventions, such as vaccines or eradication strategies.

The multi-functional HIV Nef protein is critical for maintenance of high viremia and progression to AIDS. By interacting with cellular protein trafficking machinery, Nef modulates cell surface protein expression to augment viral spread and immune evasion. Furthermore, by altering intracellular signaling events, Nef regulates the activation state of CD4⁺ T cells to promote viral pathogenesis.

Prior studies showed that HIV *gag* and *env* isolates from elite controllers were functionally attenuated compared to those from HIV progressors, in part due to viral polymorphisms selected in response to host immune pressure. However, a systematic investigation of primary Nef isolates had not been done. To explore the impact of natural sequence variation on five well-documented Nef functions, I examined Nef clones isolated from elite controllers and progressors. I observed that Nef clones from elite controllers displayed significantly lower activities for all functions. Furthermore, reduced Nef function was associated with the accumulation of polymorphisms selected in the context of the protective HLA-B*57 allele. To extend this analysis, I measured the ability of Nef isolates to modulate T cell receptor (TCR) signaling. I observed that wild type Nef inhibited NFAT transcription factor activity following T cell stimulation. Nef clones from progressors maintained this activity, but clones from elite controllers were impaired. Significant defects were mapped to polymorphisms in two regions: a key enzyme-binding domain and an N-terminal anchor domain.

In summary, I have shown that natural sequence variation in Nef results in substantial differences in protein function. Nef clones isolated from elite controllers displayed the poorest activity for multiple functions, indicating that attenuation of Nef may contribute to reduced pathogenesis in these cases. Therapeutic targeting of the Nef domains identified in my studies could contribute to HIV eradication strategies.

Keywords: HIV-1; Nef; Elite controllers; T cell receptor; Signal transduction

Dedication

This thesis is dedicated to Jeannette Markle and Brian Markle, who have always supported me following my heart, and who have always supported world peace.

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List of Acronyms

AICD	Activation-Induced Cell Death
AIDS	Acquired immunodeficiency disease syndrome
AP-1	Activator Protein 1 (transcription factor)
AP-1	Clathrin Adapter Protein-1
AP-2	Clathrin Adaptor Protein-2
cART	Combination antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation-4
CD8	Cluster of differentiation-8
CD28	Cluster of differentiation-28
CD74	Cluster of differentiation-74
CP	Chronic Progressor(s)
CTL	Cytotoxic T lymphocytes
EC	Elite controller(s)
<i>env</i>	HIV-1 envelope genes
<i>gag</i>	HIV-I structural genes
Hck	Hematopoietic Cell Kinase
HIV-1	Human Immunodeficiency Virus 1
HLA-I	Human Leukocyte Antigen class I
HXB2	HIV-1 reference strain
IL-2	Interleukin-2
LAT	Linker Activator of T cells
Lck	Lymphocyte-specific Protein Tyrosine Kinase (p56-Lck)
LTNP	Long-Term Non-Progressor
LTR	HIV-1 Long Terminal Repeat
MAPK	Mitogen-Activated Protein Kinase
MFI	Mean Fluorescence Intensity
NAK	Nef-associated kinase
Nef	HIV-1 Negative Factor protein
NFAT	Nuclear Factor of Activated T-Cells
NF-kB	Nuclear Factor-KappaB
NL43	HIV-1 lab-adapted strain

PACS-1	Phosphofurin acidic cluster sorting protein 1
PAK2	p21-Activated Kinase-2
PBMC	Peripheral Blood Mononucleated Cells
PKC- θ	Protein Kinase C-theta
PLA	Proximity Ligation Assay
<i>pol</i>	HIV-1 enzymatic genes
Rev	HIV-1 Regulator of Expression of Virion Proteins
RT	Reverse Transcriptase
SERINC5	Serine Incorporator-5
SH3	Src Homology 3 Domain
SF2	HIV-1 lab-adapted strain
SIV	Simian Immunodeficiency Virus
SLP-76	Leukocyte Protein-76
Tat	HIV-1 Trans-Activator of Transcription protein
TCR	T cell receptor
TGN	Trans-Golgi Network
Vif	HIV-1 Viral Infectivity Factor
Vpu	HIV-1 Viral Protein U
WT	Wild Type
ZAP-70	Zeta-chain-Associated Protein kinase 70

Chapter 1.

Introduction

Section 1.8 of this chapter is adapted from the following published review article, of which I was first author and primary drafter.

Markle, T.J., Mwimanzi, P., Brockman, M.A., 2013. HIV-1 Nef and T cell activation: A history of contradictions. *Future Virology* 8(4). doi: 10.2217/fvl.13.20

Figures 1.8.1 and 1.8.2 appeared in this manuscript and are reprinted here in accordance with the publisher's statement: "Express permission is not required for this purpose [use of our materials in a thesis/dissertation], authors are able to re-use or adapt their article for use in their thesis/dissertation, provided a suitable acknowledgement to the original publication is included. Authors may also deposit their thesis/dissertation in an online, institutional repository if required by their institution."

1.1. HIV: A global pandemic

Acquired immunodeficiency disease syndrome (AIDS) is a medical condition that compromises the immune system, leading to opportunistic infections (Gottlieb et al., 1983; Friedman-Kien, 1983). In 1983, the aetiological cause of AIDS was identified as human immunodeficiency virus type I (HIV-1) (Barre-Sinoussi et al., 1983; Gallo et al., 1983), which infects and destroys CD4+ T cells (Giorgi et al., 1987). Clinical diagnosis of AIDS was defined as HIV-infection leading to reduction of the patient's CD4+ T cells count to below 200 cells per microlitre (Van Griensven et al., 1991).

Since the start of the epidemic it is estimated that over 70 million people have been infected with HIV, including over 35 million who have died of AIDS-related illnesses and 37 million currently living with HIV (UN AIDS, 2018). While initial hopes for vaccine or cure discovery were high (Fischinger et al., 1985), these have been elusive (Wang et al., 2018). The 1990s saw the introduction of combination antiretroviral therapy (cART) (Collier et al., 1996; D'Aquila et al., 1996; Staszewski et al., 1996), which is able to suppress HIV-1 replication and prevent progression to AIDS in most treated individuals (Vittinghoff et al., 1999). The number of yearly new infections peaked in 1996 at 3.4 million and AIDS-related deaths peaked in 2004 at 3.4 million; subsequently both numbers have been in decline, in part due to cART therapy and prevention initiatives (Montaner, 2011; Maartens et al., 2014; Yombi and Mertes, 2018). Nevertheless, HIV/AIDS remains an ongoing global health crisis, with 1.8 million new infections and 940,000 deaths in 2017 alone (UNAIDS, 2018), and overall HIV prevalence is increasing because people on cART are living longer (Maartens et al., 2014). Furthermore, a full two-thirds of people living with HIV are in sub-saharan Africa. While recent efforts to extend global cART access has resulted in over 20 million individuals on treatment (UNAIDS, 2018), availability of effective medications remains a challenge in lower-income countries (Minior et al., 2017).

In addition to geographic disparities, globally HIV is concentrated in vulnerable populations. The risk of acquiring HIV is dramatically higher among men who have sex with men (27 times), injection drug users (23x), female sex workers (13x), and transgendered women (13x) (UNAIDS, 2018). There are approximately 63,000 people living with HIV in Canada, and risk of infection is significantly higher in indigenous communities (Canada, 2018). There are also limitations inherent to cART. Although

cART controls viral replication in most patients, HIV nevertheless establishes cellular reservoirs that reactivate in the absence of therapy (Chun et al., 1997; Siliciano et al., 2003; Chun et al., 2011), meaning that cART must be maintained indefinitely. Life-long antiretroviral treatment is associated with an elevated risk of cardiovascular and renal diseases (Mocroft et al., 2010; Ross and McComsey, 2011; Tadesse et al., 2018) as well as drug resistance (Marconi et al., 2008). In summary, despite the progress of cART and declining annual incidences and deaths, the total number of HIV-infected individuals continues to grow, is concentrated in countries with fewer financial means to provide life-long therapy, and globally HIV is affecting particularly vulnerable populations. In this context, there is an urgent requirement for additional health interventions, such as vaccines to prevent infection or eradication strategies to cure patients.

1.2. HIV-1 genetics and life cycle

The HIV-1 genome consists of ~10 kb positive-sense single-stranded RNA that encodes for 9 genes (**Figure 1.2.1**). Three of these genes, *gag*, *pol*, and *env*, are found in all known retroviruses and are essential for viral replication, producing structural proteins, enzymes, and envelope glycoproteins, respectively. The regulatory genes *tat* and *rev* are required for making and trafficking viral gene transcripts. Accessory genes *nef*, *vif*, *vpr* and *vpu* are not essential for viral replication *in vitro*, but they promote HIV-1 pathogenesis *in vivo* (Frankel and Young, 1998).

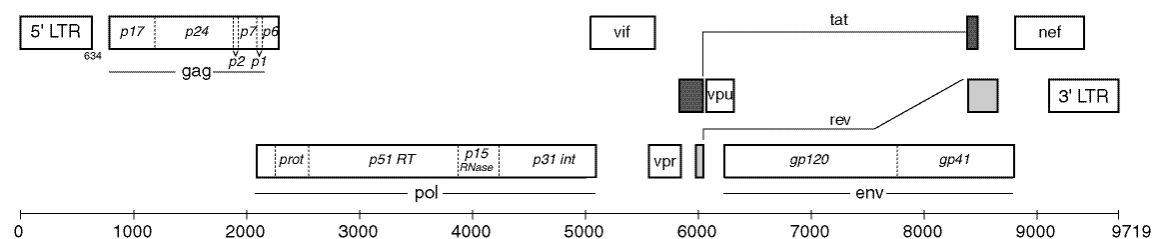


Figure 1.2.1. HIV-1 genome

The first step in the viral life cycle (**Figure 1.2.2**) occurs when **(1)** the viral envelope (Env) glycoprotein binds to the CD4 receptor and CCR5/CXC4 co-receptor on the surface of target CD4+ T cells (Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). **(2)** This allows the viral membrane to fuse with the target cell, releasing the viral core particle into the cytoplasm. **(3)** There the core particle can shed its protein coat and

release its contents, including the HIV single-stranded RNA genome (Camerini and Seed, 1990), which the viral reverse transcriptase enzyme uses as a template to produce double-stranded DNA (Jacobo-Molina and Arnold, 1991). **(4)** The HIV pre-integration complex enters the nucleus and **(5)** the viral Integrase enzyme facilitates insertion of the viral DNA into the host cell genome (Bushman et al., 1990). **(6)** The regulatory protein Tat enhances HIV transcription from the integrated HIV genome, also known as the “provirus” (Jones, 1989). HIV transcripts are **(7)** spliced and **(8)** trafficked out of the nucleus by Rev to facilitate **(9)** translation, leading to **(10)** assembly of new virions at the cell plasma membrane **(11)** from which they bud. **(12)** When virions have budded from the cell, the HIV protein Protease helps to cleave and process viral polypeptides, resulting in infectious virion particles (Peng et al., 1989).

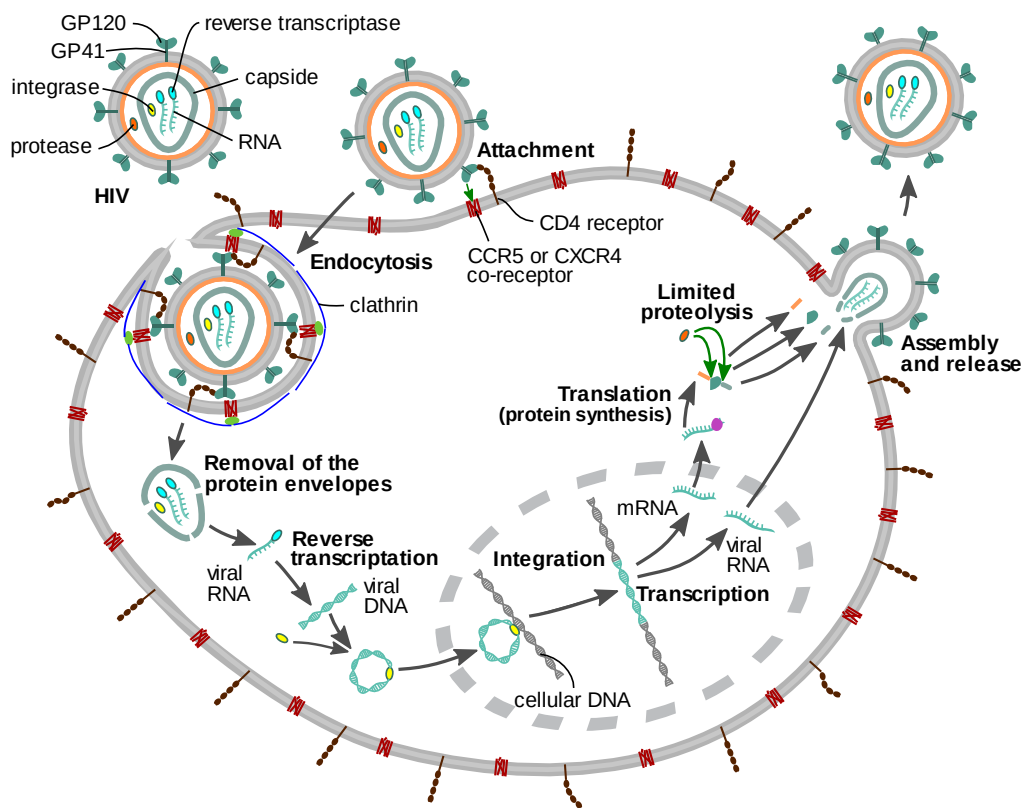


Figure 1.2.2. HIV-1 replication cycle

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1.3. HIV-1 and immune evasion

HIV uses several mechanisms to evade the host immune response, which together contribute to the inability of natural responses to clear infection, as well as to the challenge of developing a sterilizing vaccine or eradication therapy.

1.3.1. High error rate and mutations

HIV-1 is a retrovirus and therefore utilizes reverse transcriptase, which is highly error prone (Preston et al., 1988; Roberts et al., 1988). Furthermore, the virus has a rapid replication rate of up to 10^{10} virions/day (Ho, 1997). These factors result in the generation of highly polymorphic viruses, a small subset of which may escape detection by both humoral and T cell arms of the immune system (Leslie et al., 2004), or antagonism by antiretroviral drugs (Clavel and Hance, 2004). In the case of T cell evasion, viral polymorphisms can impair peptide processing, epitope presentation on host human leukocyte antigen (HLA) molecules, or recognition by cytotoxic T lymphocytes (CTL) (Carlson et al., 2015).

1.3.2. Combating host viral restriction factors

Several HIV regulatory/accessory protein antagonize host viral restriction factors. Nef down-modulates HLA class I molecules, thereby reducing the recognition by CTL (Cohen et al., 1999; Le Gall et al., 1998; Schwartz et al., 1996). Nef also down-regulates SERINC5 to increase infectivity of virions (Rosa et al., 2015; Usami et al., 2015). The HIV-1 Vif protein antagonizes APOBEC3, a host restriction factor that hypermutates retroviral genes (Sheehy et al., 2002). Finally, HIV-1 Vpu protein downregulates the retroviral antagonist Tetherin from the plasma membrane, allowing HIV virions to bud more efficiently (Neil et al., 2008).

1.3.3. Integration and latency

As mentioned, the HIV proviral genome integrates into the host cell genome. During productive HIV replication, the viral 5' LTR promoter is activated by host transcription factors such as NFAP, AP-1, and NFkB (Nabel and Baltimore, 1987; Poli et al., 1990) to transcribe RNA encoding HIV proteins Tat, Nef, and Rev (Kao et al., 1987;

Kim et al., 1989; Klotman et al., 1991). Subsequently, the transactivator protein Tat enhances transcription of unspliced and spliced HIV RNA transcripts, which Rev exports out of the nucleus for translation (Felber et al., 1989; Malim et al., 1989). However, in a subset of infected cells the HIV provirus can remain transcriptionally silent (Bushman et al., 1990). The propensity for “latency” seems to be affected by integration site (Wagner et al., 2014), epigenetics (Kauder et al., 2009), differential Tat activity (Chen et al., 2017), and cell activation state (Chavez et al., 2015), the latter possibly regulated by the viral Nef protein (Markle et al., 2013) -- among other factors. The natural immune system does not seem to be able to clear this latent reservoir (Kuang et al., 2018).

1.3.4. CD4+ T cell decline

Another dimension of immune evasion is that HIV-1 selectively infects helper CD4+ T cells, which are key players in the adaptive immune response against pathogens (Klatzmann et al., 1984). In a tragic feedback loop, destruction of the host's CD4-T cell population by HIV impairs the host's ability to combat HIV.

1.4. Cytotoxic T cell (CTL) responses to HIV-1 infection

Nevertheless, the host does mount a vigorous adaptive immune response against the virus. In HIV+ individuals, circulating CD8+ T lymphocytes (CTL) were found that targeted Gag and Env epitopes on infected cells (Walker et al., 1987). Subsequently, CTL that target multiple HIV proteins were found as early as three weeks post infection, but neutralizing antibodies were not found; this temporal association between CTL response and control of acute infection suggested that CTL are responsible for control of acute viremia (Koup et al., 1994; Borrow et al., 1994). Indeed, many of the viral mutations selected in early infection are associated with CTL responses (Phillips et al., 1991; Borrow et al., 1997; Goulder et al., 1997). In the context of SIV infection, CTL responses also governed viral control (O'Connor et al., 2002). Gag and Nef are highly targeted by CTL during HIV-1 infection (Frahm et al., 2004; Kiepiela et al., 2007). In particular, during acute infection, CTL responses against Nef are often dominant (Lichterfeld et al., 2004). However, in most people, this immune response is not sufficient to control infection because the virus mutates to escape HLA-restricted CTL responses (McMichael and Philips, 1997).

Given that HIV+ individuals cannot clear established infection, it is unlikely that strategies to boost the natural immune response could realize sterilizing protection or cure. However, some T cells responses are better than others, and a therapeutic vaccine could prompt CTL responses more effective at crippling the virus, leading to viral control analogous to cART (Johnston and Fauci, 2007). Understanding the mechanisms by which some infected individuals progress more slowly and/or control viral spread may offer clues to treatments.

1.5. Long-term nonprogressors (LTNP)

Initially, researchers investigated patients who took longer to progress to AIDS (CD4+ count below 200/ul), called long-term survivors or non-progressors (Pantaleo et al., 1995; Munoz et al., 1995). About 5-15% of infected individuals were found to have stable CD4+ T cell counts and no AIDS symptoms (Cao et al., 1995). There were reports that viral genetics contributed to slow disease progression: some LTNP had been infected with defective virus, for example *nef*-deleted virus (Kirchhoff et al., 1995; Learmont et al., 1999) or other “unusual” polymorphisms (Alexander et al., 2000).

However, evidence emerged that host genetic factors contributed to differential disease progression. Consistently, genetic association studies showed that the host genes most correlated with varying disease progression were HLA alleles (Carrington and O'Brien, 2003). Notably, HLA class I molecules function to present viral epitopes to CTL -- further evidence on the importance of CTL responses in natural viral control. Unfortunately, some LTNP did over time progress to AIDS (Lafrere et al., 1997).

1.6. HIV-1 elite controllers

Once HIV RNA assays were developed in the 1990s, it was possible to quantify plasma viral load which proved to be an excellent predictor of disease progression (Mellors et al., 1996). Interestingly, LTNP displayed a wide range of viral loads (Choudhary et al., 2007), including a subset of LTNP who had no detectable plasma viral load at all (<50 copies/ml, the limit of detection for commercial assays) even in the absence of therapy. These rare patients (1 in 300) were named HIV-1 elite controllers (EC) (Deeks and Walker, 2007). Many EC showed no evidence of CD4+ T cell decline

for over two decades. Therefore, it became a priority to investigate the role of viral and host genetics in EC.

1.6.1. Viral genetics in EC

Given that the virus replication is almost completely attenuated in controllers, one theory was that these rare patients had been infected by a grossly defective virus or replication incompetent virus (reviewed in Lobritz et al., 2011). However, sequencing of viruses in elite controllers found no major genetic defects (Miura et al., 2008a). Indeed, replication competent virus was isolated from multiple EC (Blankson et al., 2007). There were also several cases of HIV+ transmission pair couples, where one individual maintained elite controller while the other progressed to disease (Bailey et al., 2008), suggesting that at least some controllers harbour replication competent virus which must be contained by the individual's host immune response. Analysis of sequences from a cohort of 95 EC found that there were no obvious shared polymorphisms in these patient sequences which affected virus fitness (Miura et al., 2009c); rather there are likely to be a diversity of polymorphisms amongst patients, differing based on HLA and CTL combinations (to be discussed below).

1.6.2. Host genetics in EC

A genome wide association study of elite controllers found that the B*57 HLA gene was strongly associated with control (Fellay et al., 2007), consistent with Carrington's findings for LTNP (Carrington and O'Brien, 2003). One study found that protective HLA alleles are enriched amongst EC compared to chronic progressors (CP): 44% of EC had B*57 (compared to 10% in CP) and 15% had B*27 (compared to 3% in CP); overall 68% of EC had at least one known protective HLA allele compared to 37% of CP (Pereyra et al., 2008). Therefore, having a protective HLA allele is not sufficient to control viral spread; presumably, presence of a strong and specific CTL response restricted by these protective HLA is also necessary.

In the one-third of EC who don't have protective HLA, it is unclear what host genetics are responsible for viral control. Neither CCR5-delta32 deletion (Pereyra et al., 2008), nor induction of broadly neutralizing antibodies seem to be responsible for elite

control (Bailey et al., 2006; Pereyra et al., 2008), just as they do not play a clear role in controlling acute infection as discussed above.

Nevertheless, evidence suggested that the CTL responses are different in EC: they tend to be less broad and more focused on attacking gag, while CP responses were broader and more focused on Env (Pereyra et al., 2008). This is consistent with the observation that, generally, responses that target Gag are most effective (Kiepiela et al., 2007). Indeed, several Gag-specific CTL responses, notably those restricted by HLA-B*57/8 and B*27, are able to cripple viral replication (Migueles et al., 2000; Altfeld et al., 2003), such that the virus must accumulate multiple mutations in order to escape the response (Goulder et al., 1997; Goulder et al., 2001; Kelleher et al., 2001; Feeney et al., 2004).

1.6.3. Acquired escape mutations impact viral fitness in EC

Even though viral replication is low in EC, evidence of HLA-associated mutations was found in 30-40% of expected sites (compared to 50% in CP), suggesting that immune pressure is strong (Miura et al., 2009c). Notably, some of these escape mutations can have an enormous fitness cost on viral replication (Peyerl et al., 2004; Martinez-Picado et al., 2006), which may explain why these specific HLA-restricted CTL responses are protective. In some cases, numerous compensatory mutations must accumulate before fitness can be restored (Kelleher et al., 2001; Peyerl et al., 2004). For example, a study of HLA-B*57+ patients identified immunodominant responses against the TW10 epitope in Gag (Brockman et al., 2007). Briefly, immune pressure crippled viral spread, such that the virus had to develop a T242N mutation to evade this B*57-restricted CTL response; this mutation impaired virus replication capacity; however, over time compensatory mutations in the Gag capsid protein could restore viral replication. Notably, these compensatory mutations were more common in chronic progressors (Brockman et al., 2007), possibly because EC CTL responses target the virus at multiple epitopes in multiple genes at once, thereby slowing viral replication and the kinetics of accumulating compensatory mutations.

Similarly, the HLA-B*27 restricted CTL response against the gag KK10 epitope is immunodominant, cripples viral replication, selects for an escape mutation at R264K which reduces viral fitness, but is compensated by a compensatory mutation at S173A

that can take years to be selected (Schneidewind et al., 2007; Schneidewind et al., 2008).

To investigate more fully the impact of natural sequence variation in EC on protein function, Miura et al. isolated EC *gag* sequences, cloned them into a virus backbone, and assayed *in vitro* viral replication (Miura et al., 2009a). Viruses encoding EC-derived *gag* sequences displayed lower replication capacities than those with CP-derived *gag*; when stratified by HLA allele, Gag proteins from B*57+ individuals were most functionally impaired. Furthermore, impaired function correlated with several HLA-associated polymorphisms including T242N. However, B*57-restricted responses did not account for all impairment in EC *gag* sequences (Miura et al., 2009b), again suggesting that other acquired mutations are important. A follow-up study reported that most *gag* sequences from B*57+ EC had rare mutations in and around the TW10 epitope (Miura et al., 2009b).

In addition to *gag*, other HIV genes from elite controllers were isolated and tested for function by our lab. Viruses encoding reverse transcriptase (RT) and integrase (Int) isolated from EC also had lower replication capacity compared to CP (Brumme et al., 2011). There was a significant association between viral function and HLA-B*57 expression within CP, and a modest association within the EC cohort, suggesting that B*57-restricted CTL responses can also cause attenuating mutations in HIV enzymes RT and Int (Brumme et al., 2011).

As mentioned, the two HIV proteins most highly targeted by CTL in early infection are Gag and Nef (Lichterfeld et al., 2004; Frahm et al., 2004; Kiepiela et al., 2007). A key aim of the present dissertation is to investigate the impact of sequence variation on the function of primary Nef isolates. If elite controllers target Nef more dramatically than do chronic progressors, one might expect to see reduced function in Nef.

1.7. HIV-1 Nef

1.7.1. Nef and HIV pathogenesis

HIV-1 Nef is an adaptor protein that is about 206 amino acids in length with a mass of 27-34 kDa. Its name is an abbreviation of “negative factor,” as initial reports ascribed a negative role for Nef on viral production and replication (Luciw et al., 1987;

Terwilliger et al., 1986). However, a consensus has emerged that Nef has a positive effect on HIV-1 pathogenesis.

Although Nef is not absolutely required for *in vitro* replication, *nef*-defective viruses replicate much more slowly (Mwimanzi et al., 2013a). In fact, Nef is essential for viral pathogenesis *in vivo*. For example, in the 1980s, eight individuals who were infected with *nef*-deleted HIV accidentally during a blood transfusion showed signs of slow disease progression (Deacon et al., 1995; Learmont et al., 1999; Zaunders et al., 1999). There are additional reports of slow disease progression in the context of *nef*-defective HIV (Kirchoff et al., 1995; Corro et al., 2012; Premkumar et al., 1996; Tobiume et al., 2002; Aldrovandi et al., 1998). The discovery of an essential role for Nef in HIV pathogenesis prompted renewed interest in targeting Nef therapeutically (Kirchhoff et al., 1995).

1.7.2. Nef is a multifunctional adapter protein

Nef is one of the earliest and most abundant viral proteins expressed by cells following infection (Kim et al., 1989; Klotman et al., 1991; Robert-Guroff et al., 1990). Its N-terminus is myristoylated, a modification required for attachment to membranes and essential to most functions (Aiken et al., 1994). Nef does not show enzymatic activity, but rather serves as an adaptor protein to interact with host proteins, thereby altering the trafficking of surface receptors or other signaling molecules (Pawlak and Dikeakos, 2015). Nef is thought to have a positive effect on viral replication by promoting virion release through down-regulation of CD4 (Garcia and Miller, 1991; Aiken et al., 1994; Ross et al., 1999) and by enhancing virion infectivity through down-regulating SERINC5 (Rosa et al., 2015; Usami et al., 2015). Nef also plays a role in immune evasion by downregulating HLA-class I (Collins et al., 1998; Schwartz et al., 1996) and up-regulation of HLA class II associated invariant chain (CD74) (Schindler et al., 2003; Stumptner-Cuvelette et al., 2001). Nef is also packed into virions and is exocytosed from infected cells (Baur, 2011). Finally, Nef is has been shown to modulate T cell activation, apoptosis, and various T cell signaling events. Nef domains that have been characterized as being associated with these functions are depicted in **Figure 1.7.1**. All of these Nef functions are reviewed in depth in **section 1.8** below, with an emphasis on their relation to T cell signaling and activation.

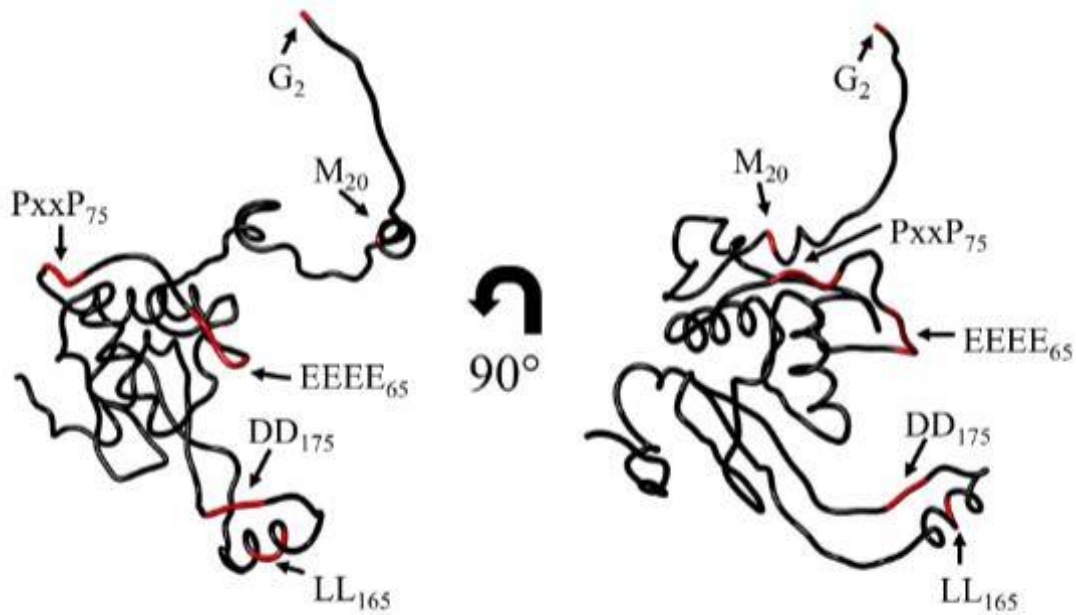


Figure 1.7.1 Nef structure and characterized functional domains.

The indicated domains have been associated with the following Nef functions. G₂: myristoylation and membrane association; M₂₀: HLA-downregulation; EEEE₆₂₋₆₅: PACS-binding and HLA-downregulation; PXXP₇₂₋₇₅: SH3-binding, Lck-internalization, and HLA-downregulation; DD₁₅₄₋₅: B-COP and lysosomal trafficking; LL₁₆₄₋₅: AP-2-binding, CD4-downregulation and SERINC5-downregulation; DD₁₇₄₋₅: ViH-binding and CD4-downregulation (Geyer et al., 2001).

1.8. Nef and T cell activation

1.8.1. Nef: A “negative factor”?

Early studies of HIV-1 “open reading frame b” suggested that it inhibited virion production and viral replication (Luciw et al., 1987; Terwilliger et al., 1986), resulting in it being called “negative factor” or Nef. Subsequent reports indicated that Nef silenced gene expression, supporting the notion that Nef dampens T cell stimulation to prolong viral progeny production (Ahmad and Venkatesan, 1988; Niederman et al., 1993). However, a series of later studies observed that Nef enhanced HIV-1 replication in primary cells. Spina *et al.* demonstrated that Nef-deleted viruses replicated poorly in quiescent CD4⁺ T cells (Spina et al., 1994) and Miller *et al.* highlighted the ability of Nef to enhance virion infectivity (Miller et al., 1994). Therefore, contrary to its name, a consensus has emerged that Nef enhances infection. This model of Nef as a “positive factor” is consistent with *in vivo* observations that Nef-deleted strains of HIV and SIV are less pathogenic (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995).

1.8.2. Relevance of T cell activation for HIV-1 pathogenesis

Antigen-stimulated (“activated”) CD4⁺ T cells are highly permissive to HIV-1 infection (Korin and Zack, 1998; Stevenson et al., 1990), due in part to increased metabolic activity and reduced expression of intrinsic antiviral host restriction factors (Baldauf et al., 2012; Chiu et al., 2005; Ganesh et al., 2003). Maintenance of a “semi-activated” state may be required for efficient production of viral progeny (Li et al., 1996; Song et al., 1996), since over-stimulation results in activation-induced cell death (AICD) (Krammer et al., 2007) that would shorten the life span of infected cells.

In addition to a productive replication cycle, HIV-1 establishes latent infection in resting CD4⁺ T cells, allowing persistence of proviral genomes despite immune responses or initiation of drug therapy (Eisele and Siliciano, 2012). Studies have begun to elucidate the viral and cellular mechanisms involved in establishment and maintenance of latency (Eisele and Siliciano, 2012; Karn, 2011), and differences in T cell activation state are likely to play a role (Kuang and Brockman, 2018). Notably, a recent study found that blocking Nef with a drug improved the ability of CD8⁺ T cells to eliminate latently infected cells *in vitro* (Mujib et al., 2017). An improved understanding of the factors involved in regulating HIV-1 latency could provide new avenues for treatment or lead to strategies capable of eradicating the virus from an infected individual (Eisele and Siliciano, 2012; Kuang and Brockman, 2018).

CD4⁺ T cell activation occurs when its T cell receptor (TCR)/CD3 complex engages a foreign peptide presented by HLA class II on an antigen-presenting cell (APC) (**Figure 3.4.1**). TCR recognition initiates a series of events, including rearrangement of the actin cytoskeleton, stabilization of adhesion molecule binding, and recruitment of new proteins to the plasma membrane (Morris and Allen, 2012). This results in formation of a TCR-APC contact zone, referred to as an “immune synapse”, which further enhances TCR signalling. A cascade of phosphorylation events ensues, involving Lymphocyte-specific Protein Tyrosine Kinase (p56-Lck) and Zeta-chain-Associated Protein kinase 70 (ZAP-70), adapter protein SH2 domain-containing Leukocyte Protein-76 (SLP-76), transmembrane protein Linker Activator of T cells (LAT), and others. These proximal events initiate distal signalling pathways, including Mitogen-Activated Protein Kinases (MAPKs), Protein Kinase C-theta (PKC- θ), and Calcium (Ca²⁺)/Calcineurin, resulting in translocation of three critical transcription factors into the

nucleus: Nuclear Factor of Activated T-Cells (NFAT), Activator Protein-1 (AP-1), and Nuclear Factor-KappaB (NF-κB).

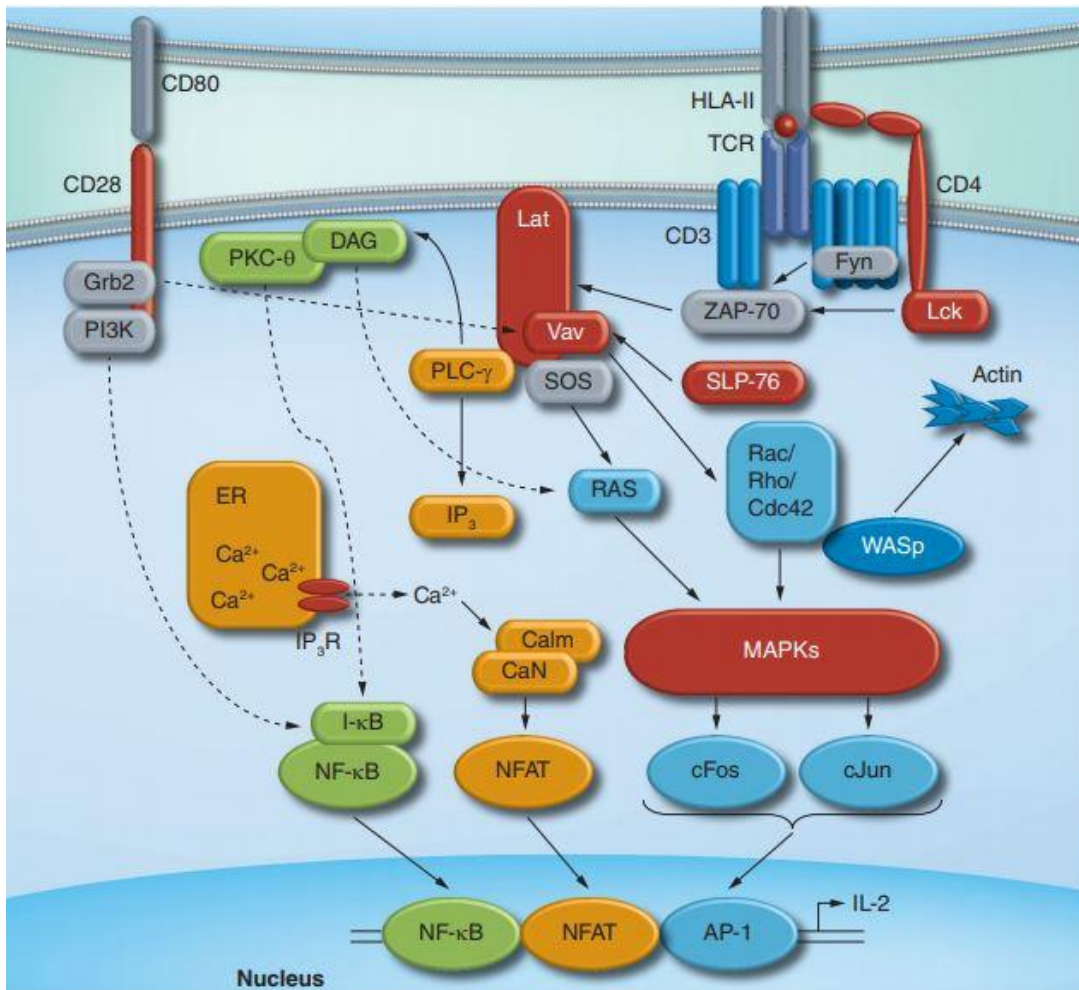


Figure 1.8.1. T cell receptor (TCR) signalling. Recognition of peptide/HLA ligand by TCR leads to cellular activation and IL-2 production.

The TCR/CD3 complex mediates proximal signalling events through recruitment of cellular kinases (Lck, Fyn, ZAP-70), adapter molecules (SLP-76), and the scaffold protein LAT. Distal events result in the activation of three key transcription factors (NFAT, NF-κB, and AP-1) and their translocation into the nucleus. NFAT is triggered by Calcineurin (CaN) through a process that requires Phospholipase C-gamma (PLC-g), IP₃, release of Calcium (Ca²⁺) from the ER, and Calmodulin (Calm) (shown in orange). Activation of NF-κB requires degradation of I-κB, which is mediated by Diacylglycerol (DAG) and Protein Kinase C-theta (PKC-θ) (shown in green) and enhanced by co-stimulatory signals provided by CD28. AP-1, comprised of cFos and cJun, is stimulated by MAP kinase cascades that are triggered by the Ras family GTPases, including Ras, Rac, Rho, and Cdc42 (shown in blue). Wiskott–Aldrich Syndrome Protein (WASp) initiates actin cytoskeletal rearrangement. Proteins whose activities are reported to be modulated by HIV-1 Nef are indicated in red. (Image from Markle, et al, Future Virol 2013)

The affinity and kinetics of TCR binding determines the effectiveness of signalling (Sykulev, 2010). Induction of interleukin-2 (IL-2) expression requires stimulation of NFAT, AP-1, and NF- κ B transcription factors (**Figure 3.4.2A**), and this cytokine is therefore a hallmark of robust CD4⁺ T cell activation. Secreted IL-2 enhances T cell proliferation and is crucial for immunity against infection (Wilson and Livingstone, 2008). Notably, the HIV-1 Long Terminal Repeat (LTR) promoter sequence also encodes binding sites for these transcription factors (**Figure 3.4.2B**) and TCR-mediated signalling events, among others, can reactivate latent proviral genomes (Chan and Greene, 2012).

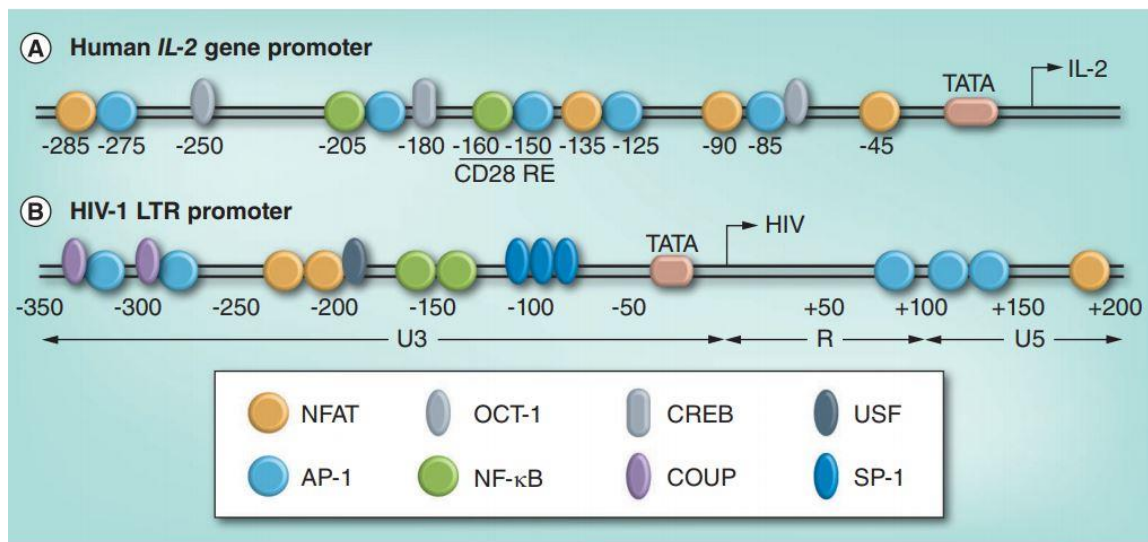


Figure 1.8.2. Human IL-2 and HIV-1 LTR promoters.

The location of critical transcription factor binding sites in (a) the human IL-2 gene promoter and (b) the HIV-1 Long Terminal Repeat (LTR) sequence are indicated. In addition to NFAT, NF- κ B, and AP-1 (discussed in the text), constitutive transcription factors OCT-1 (octamer transcription factor 1), COUP (chicken ovalbumin upstream promoter), and USF-1 (upstream stimulatory factor 1), and SP-1 (Specificity Protein 1), as well as CREB (cAMP response element-binding protein) contribute significantly to promoter activity. (Image from Markle, et al, Future Virol 2013)

1.8.3. Effects of HIV-1 Nef on IL-2 gene expression

To investigate the impact of Nef on T cell activation, its ability to modulate IL-2 has been assessed. Most reports indicate that Nef alters IL-2 expression; however, both positive and negative effects of Nef have been observed using similar experimental approaches.

Initial studies indicated that Nef reduced the ability of Jurkat T cells to induce IL-2 mRNA or to activate an IL-2 promoter-driven gene expression following stimulation with phorbol 12-myristate 13-acetate (PMA)/phytohaemagglutinin (PHA) or CD3 antibody

(Collette et al., 1996a; Luria et al., 1991). However, a series of reports observed the opposite effect, namely that Nef enhanced IL-2 expression. Rhee *et al.* found that Nef augmented CD3-mediated activation of a murine T cell hybridoma to produce IL-2 (Rhee and Marsh, 1994). Nef was subsequently shown to increase IL-2 secretion following CD3/CD28 stimulation using both Jurkat cells and PBMC (Schrager and Marsh, 1999; Wang et al., 2000). Other results using Jurkat cells indicated that Nef protein or HIV-1 infection enhanced IL-2 promoter-driven gene expression (Fenard et al., 2005; Schindler et al., 2006), and indicated that myristoylation, the proline-rich SH3-binding motif, and the PAK2 binding motifs of Nef were required for this function (Olivieri et al., 2011).

Further complicating the story, Nef was shown to enhance IL-2 production in resting CD4⁺ cells following stimulation with PMA-ionomycin, but not CD3/CD28 (Keppler et al., 2006). Arhel *et al.* observed that HIV-1 Nef did not affect IL-2 production in virus-infected primary cells (Arhel et al., 2009). And Thoulouze *et al.* found that although HIV-infected Jurkat cells displayed increased IL-2 production compared to uninfected cells, Nef-deleted viruses resulted in higher levels of IL-2 compared to wild type HIV-1 (Thoulouze et al., 2006).

1.8.4. Regulation of key transcription factors and signalling molecules by Nef

In the context of Nef and T cell activation, the transcription factors NFAT, AP-1, and NF- κ B, the tyrosine kinase p56-Lck, and the cytoskeleton modulator p21-Activated Kinase-2 (PAK2) have received greatest attention. We briefly review key observations for each here.

NFAT

The NFAT family of transcription factors is activated by TCR-mediated events through the Ca²⁺/calmodulin pathway, and is critical for induction of IL-2 (Chow et al., 1999). TCR binding initiates an inositol triphosphate (IP₃) signalling cascade, leading to an influx of calcium into the cytoplasm, which binds Calmodulin (Calm) and in turn activates the Calm-dependent serine/threonine phosphatase Calcineurin (CaN). Dephosphorylation of cytoplasmic NFAT family members NFAT1 and NFAT2 by CaN allows their translocation into the nucleus and binding to promoter sequences (Rao et al., 1997). Since NFAT binding sites are present in the HIV-1 LTR, over-expression of

NFAT induced viral replication in resting T cells (Kinoshita et al., 1998; Kinoshita et al., 1997).

Nef has most often been described to hyper-activate NFAT (Manninen et al., 2001; Manninen et al., 2000). Wang *et al.* observed that Nef localized to membrane lipid rafts and that increased IL-2 secretion following CD3/CD28 stimulation was associated with enhanced NFAT signalling (Wang et al., 2000). NFAT was also determined to contribute to increased Nef-mediated responsiveness of infected CD4⁺ T cells (Fortin et al., 2004). Other groups have observed similar results using NFAT promoter-driven luciferase constructs (Fenard et al., 2005). Nef may also activate NFAT in the absence of TCR signalling through synergistic interactions with the Ras/MAPK pathway (Manninen et al., 2000) or through direct binding to IP₃ receptors (IP₃R), causing release of intracellular calcium stores (Manninen and Saksela, 2002). TCR-independent activation of NFAT appears to be conserved among Nef alleles and is dependent on Nef motifs involved in membrane localization and SH3 binding (Manninen et al., 2001). Other studies suggest that Nef's ability to modulate NFAT following TCR activation may be dependent on the status of the cell, since Nef hyper-induces NFAT and IL-2 in quiescent T cells, but impairs NFAT and IL-2 in sub-optimally activated T cells (Neri et al., 2011).

In contrast to these results, inhibition of NFAT-driven luciferase was observed when Jurkat T cells were transfected with a Nef-CD8 fusion protein and stimulated with peptide-pulsed APC (Tuosto et al., 2003). Of interest, this function was associated with Nef-mediated modulation of Vav, possibly using PAK2 as an intermediary.

Notably, a recent study proposed an alternative mechanism for Nef-mediated modulation of calcium signalling, namely by associating with trafficking proteins MAL and Rab11 to cause Lck to accumulate in vesicles and regulate the formation of calcium membrane territories (Silva et al., 2016).

AP-1

The AP-1 transcription factor is a heterodimer of cFos and cJun proteins activated by Mitogen Activated Protein Kinases (MAPK). There are three major members of the MAPK family: Extracellular signal-Regulated Kinase (ERK), Jun-N-terminal kinase (JNK), and p38. cFos is activated by ERK, while cJun is activated by JNK. MAPK cascades are essential for the regulation of cell differentiation, proliferation and death (Pearson et al., 2001), and AP-1 binding contributes to the expression of

numerous cellular genes, including IL-2 (Hess et al., 2004). The HIV-1 genome encodes three adjacent AP-1 binding sites located within the R5 region of the LTR (**Figure 3.4.2B**) and three intragenic AP-1 sites within the *pol* gene, which contribute to viral replication (Li et al., 1994). Other studies have demonstrated that ERK activity is required for HIV-1 infectivity (Yang and Gabuzda, 1999), reverse transcription (Mettling et al., 2008), as well as virion assembly and release (Hemonnot et al., 2004).

Early studies reported that HIV-1 Nef inhibited AP-1 activity through an effect on TCR-dependent signalling (Bandres and Ratner, 1994; Niederman et al., 1993). Greenway *et al.* also demonstrated that Nef's proline-rich domain bound to MAPK and inhibited its kinase activity (Greenway et al., 1996). These findings contrast with subsequent studies, which have described either little effect of Nef on AP-1 (Yoon and Kim, 1999) or increased activation of MAPK by Nef. Fortin *et al.* reported higher AP-1 activity due to Tat and Nef in virus-infected T cells (Fortin et al., 2004). Schragger *et al.* indicated that Nef increased ERK activity following TCR stimulation (Schragger et al., 2002). These results are consistent with Witte *et al.*, who reported that Nef enhanced Tat-mediated HIV transcription through a p56-Lck-mediated process that required stimulation of ERK by PKC- θ (Witte et al., 2008). Activation of cJun by JNK is also required for AP-1 function, and HIV-1 Nef has been reported to induce the JNK pathway through interaction with the guanine nucleotide exchange factor, Vav (Fackler et al., 1999). However, in other studies, Nef failed to enhance JNK activity in the presence or absence of TCR stimulation (Schragger et al., 2002).

NF- κ B

The NF- κ B transcription factor plays a key role in cellular activation and proliferation. It is a heterodimer comprised of one Ankyrin repeat-containing subunit (p50 or p52) and one transactivation domain-containing subunit (p65/RelA, RelB, or c-Rel). Cytoplasmic NF- κ B is sequestered by Inhibitor of NF- κ B (I- κ B) and activated by various stimuli, including TCR activation coupled with a co-stimulatory signal typically provided by CD28 (Li and Verma, 2002). Binding of CD28 to CD80 (or CD86) on the APC activates Phosphatidylinositol 3-kinase (PI3K), which triggers Akt and then I- κ B kinase to phosphorylate I- κ B, resulting in its ubiquitinylation and degradation, thereby releasing NF- κ B for translocation to the nucleus (Li and Verma, 2002). NF- κ B binding

sites located in the HIV-1 LTR are critical for viral replication (Cook et al., 2003; Levine et al., 1996; Nabel and Baltimore, 1987; Riley et al., 1998).

Consistent with early evidence that Nef inhibited IL-2, initial studies indicated that Nef suppressed NF- κ B by interfering with a TCR-derived signal (Bandres and Ratner, 1994; Niederman et al., 1992). In contrast, other reports suggested that Nef enhanced NF- κ B activity. Wang *et al.* observed that Nef recruitment to membrane lipid rafts promoted NF- κ B (Wang et al., 2000). Fortin *et al.* reported that Nef increased NF- κ B nuclear translocation in Jurkat cells after stimulation with PMA-ionomycin or CD3/CD28 (Fortin et al., 2004). Yet another series of studies indicated that Nef had no effect on NF- κ B. Collette *et al.* observed that CD28 signalling did not contribute to Nef-mediated modulation of IL-2 (Collette et al., 1996c), and Yoon *et al.* reported that there was no influence of Nef on NF- κ B or AP-1 (Yoon and Kim, 1999). Furthermore, Schragger *et al.* found that Nef did not alter I- κ B phosphorylation in CD4⁺ T cells (Schragger et al., 2002). Echoing these results, a report by Witte *et al.* observed that induction of HIV-1 transcription by Nef involved Lck and PKC- θ recruitment to membrane lipid rafts, but resulted to activation of ERK rather than NF- κ B (Witte et al., 2008). In addition, Neri *et al.* demonstrated that Nef had no effect on early substrates in the NF- κ B pathway (including I- κ B α) or phosphorylation of NF- κ B following stimulation with CD3/CD28 (Neri et al., 2011).

p56-Lck

Nef's proline-rich motif binds to the SH3 domain of p56-Lck, a member of the Src family of protein tyrosine kinases (Collette et al., 1996b; Greenway et al., 1995; Saksela et al., 1995). Lck is a "master regulator" of TCR signalling that is recruited to the plasma membrane by CD4. Upon TCR engagement, Lck phosphorylates Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on the TCR/CD3 zeta chain. Phosphorylated TCR recruits ZAP-70 kinase, which is also phosphorylated by Lck. Activated ZAP-70 phosphorylates downstream signalling molecules, including transmembrane protein LAT and adapter protein SLP-76. Of note, Nef is reported to interact with other Src family kinases, including Hck and Fyn (Arold et al., 1997; Lee et al., 1995; Lee et al., 1996), which may also contribute to altered intracellular signalling.

CD4-Lck binding and altered Lck localization. Bandres *et al.* observed that Nef-mediated down-regulation of CD4 was more efficient in the presence of Lck (Bandres et

al., 1995); however, other studies indicated that Nef and Lck interact with the same hydrophobic motif on CD4's tail (Salghetti et al., 1995). Furthermore, Nef's proline-rich motif is dispensable for CD4 down-regulation (Saksela et al., 1995) and Nef's ability to disrupt the CD4-Lck complex and to target CD4 to lysosomes for degradation are genetically separable (Kim et al., 1999; Laguette et al., 2009). Nef inhibits Lck clustering at the TCR and Lck is instead retained in endosomal compartments (Haller et al., 2007; Haller et al., 2006; Thoulouze et al., 2006). Haller *et al.* further demonstrated that Nef uses distinct mechanisms to retain Lck within these compartments and to block Lck recruitment to the TCR (Haller et al., 2007), and Rudolph *et al.* have indicated that this function is conserved among HIV and SIV Nef proteins (Rudolph et al., 2009). These results suggest that Nef displaces Lck from CD4 and redistributes it to an intracellular location.

Modulation of Lck phosphorylation. Several studies have indicated that Nef inhibits Lck phosphorylation and blocks its ability to phosphorylate downstream targets (Baur et al., 1994; Collette et al., 1997; Collette et al., 1996b; Greenway et al., 1995; Greenway et al., 1996). Reduced Lck phosphorylation was associated with Nef's ability to disturb immune synapse formation (Haller et al., 2007; Thoulouze et al., 2006), for which Nef's proline-rich motif appeared to be essential (Haller et al., 2006). However, Abraham *et al.* reported that Nef did not reduce Lck phosphorylation or inhibit its ability to phosphorylate its immediate downstream target, ZAP-70 (Abraham et al., 2012). Rather, they observed that Nef blocked subsequent steps required for TCR signalling: namely LAT recruitment to TCR microclusters, LAT phosphorylation, and the interaction between LAT and SLP-76 (Abraham et al., 2012). This is consistent with previous work by Haller *et al.*, which also found that Nef inhibited the recruitment of phosphorylated LAT to the immune synapse (Haller et al., 2006). In contrast, other studies have concluded that Nef activates Lck, which in turn stimulates PKC- θ and up-regulates ERK signalling (Witte et al., 2008; Wolf et al., 2008).

PAK2

p21-activated kinases (PAKs) are serine/threonine kinases that regulate the small GTPases Cdc42 and Rac. PAKs are involved in early signalling events that lead to MAPK (Trono and Wang, 1997) and function in cytoskeleton remodelling and apoptosis (Chan and Manser, 2012). PAK2 mutants that lack the kinase domain block TCR-induced CD69 up regulation, calcium flux, NFAT activation, and IL-2 production in T cells

(Chu et al., 2004). Sawai *et al.* first reported the presence of a Nef associated kinase (NAK) (Sawai et al., 1994), and Nef binding to NAK was found to depend on Cdc42 and Rac1 (Lu et al., 1996). NAK was identified as a PAK family member (Nunn and Marsh, 1996; Sawai et al., 1997; Sawai et al., 1996), and Renkema *et al.* determined it to be PAK2 (Renkema et al., 1999). Mutational analysis studies have identified amino acids within HIV-1 Nef that are critical in the interaction of Nef with PAK2. The Nef myristoylation residue G2, SH3-binding domain and the highly conserved arginine R105/R106 were shown to be required for Nef interaction with PAK2 (Sawai et al., 1995; Wiskerchen and Cheng-Mayer, 1996). Furthermore Agopian *et al.* further demonstrated that Nef amino acids at positions 85, 89, 187, 188, and 191 (L, H, S, R, and F in the clade B consensus, respectively) are critical for PAK2 association (Agopian et al., 2006). Using primary Nef isolates Foster et al found that Nef mutants F193I and S189R were defective for Nef PAK2 association (Foster et al., 2001). Mutational analysis on PAK2 revealed that an intact Cdc42-Rac1 interactive binding motif was required for Nef PAK2 interaction (Renkema et al., 2001). While the majority of Nef research has been conducted on PAK2, other studies have indicated that NAK was PAK1 (Fackler et al., 2000) and that PAK1 may be important for Nef-mediated enhancement of virion production (Nguyen et al., 2006).

Nef association with PAK2 is a conserved property of HIV-1, HIV-2 and SIV. This interaction occurs in membrane lipid rafts and forms a multi-protein complex comprised of Nef, PAK2, PI3K GTPases Rac and Cdc42, and Vav1 (Linnemann et al., 2002; Rauch et al., 2008), referred to as the Nef-PAK2 signalosome. Through interactions with PI3K, Nef-PAK2 may increase virion production (Linnemann et al., 2002) and prevent apoptosis (Wolf et al., 2001). More recently, it was observed that PAK2 binding is important for Nef-mediated enhancement of cellular activation and viral replication in primary T cells (Olivieri et al., 2011). TCR-mediated remodelling of the actin cytoskeleton is necessary for T cell activation (Billadeau et al., 2007; Huang and Burkhardt, 2007). Nef associates with actin (Fackler et al., 1997) and Nef-PAK2 can inhibit actin rearrangement (Haller et al., 2007; Haller et al., 2006). Nef has been shown to modulate the key actin organizer Wiskott–Aldrich Syndrome protein (WASp) (Haller et al., 2006) and to deregulate the actin-severing factor cofilin (Stolp et al., 2010; Stolp et al., 2009), which may alter immune synapse formation required for early events in TCR activation.

1.8.5. Effects on surface receptors that may contribute to activation

CD3/TCR

HIV-2 and most SIV Nef alleles efficiently down-regulate CD3/TCR (Bell et al., 1998; Howe et al., 1998). This Nef activity, which has been shown to potently suppress T cell activation *in vitro*, was lost in the lentiviral lineage that gave rise to HIV-1 (Schindler et al., 2006). The resulting lack of CD3 down-regulation function by HIV-1 Nef has been suggested to contribute to the high pathogenicity observed with human infection compared to that of natural hosts (Arhel et al., 2009). A recent study demonstrated that HIV-2 Nef-mediated down-regulation of CD3 and CD28 was associated with higher CD4 counts (Khalid et al., 2012), but symptomatic HIV-2 patients also displayed Nef clones able to down-regulate CD3 (Munch et al., 2005), and CD3 down-regulation alone was insufficient to explain SIV virulence (Schindler et al., 2004).

CD4

Nef's ability to down-regulate CD4 is well characterized (Anderson et al., 1993; Garcia et al., 1993; Garcia and Miller, 1991). In addition to its role in viral entry, CD4 enhances TCR signalling by recruiting p56-Lck to the immune synapse (Holdorf et al., 2002). CD4 endocytosis is a normal response to TCR stimulation (Greenway et al., 2003); however, internalization of CD4 by Nef is independent of T cell activation and has been suggested to modulate early events requiring Lck. Indeed several studies indicated that Nef-mediated down-regulation of CD4 may alter TCR signalling (Mariani and Skowronski, 1993; Skowronski et al., 1993), while other reports have determined that Nef-mediated inhibition of TCR signalling is independent of its CD4 down-regulation function (Iafrate et al., 1997; Manninen et al., 2001; Thoulouze et al., 2006).

CD28

HIV and SIV Nef proteins down-regulate cell surface CD28 expression (Bell et al., 2001; Khalid et al., 2012; Swigut et al., 2001). Co-stimulation provided by CD28 is necessary to elicit optimal antigen-specific T cell activation and absence of co-stimulation often results in T cell anergy (Rudd et al., 2009). This Nef activity may contribute to pathogenesis, since *in vivo* reversion of H196Q, a codon important for CD28 down-regulation (Bell et al., 2001), was associated with progression in SIV-infected rhesus macaques (Whatmore et al., 1995).

1.8.6. Nef-mediated activation of uninfected bystander T cells

A series of studies have illuminated mechanisms of Nef secretion. A protein-binding study showed that Nef interacts with multiple cell proteins involved in exocytotic machinery (Mukerji et al., 2012). Nef is thought to hijack this machinery in order to promote exocytosis, including of Nef itself (Muratori et al., 2009; Baur 2011).

HIV-1 Nef can alter the activation of bystander uninfected T cells by modifying protein expression in APCs. In HIV-infected macrophages, a cell type targeted for HIV infection (Gartner et al., 1986; Orenstein et al., 1997), HIV-1 Nef induces the production of two CC-chemokines, macrophage inflammatory proteins 1 α (CCL2) and 1 β (CCL4), resulting in chemotaxis and activation of resting T lymphocytes, and thereby facilitating productive viral infection (Swingler et al., 1999). Furthermore HIV-1 Nef intersects the CD40 signalling pathway in macrophages, inducing the release of the soluble forms of intercellular adhesion molecule ICAM-1 and of the co-activation molecule CD23, which in turn promotes interactions between B cells and T cells that render the T cell permissive to HIV-1 (Swingler et al., 2003). In DC however, HIV-1-infected DCs cannot significantly increase resting CD4⁺ T cell proliferation in DC-T cell co-cultures (St Gelais et al., 2012).

Soluble Nef can be detected in HIV-1 infected patient sera (Fujii et al., 1996), and extracellular Nef may also contribute to T lymphocyte activation. Indeed, recombinant Nef can enter monocytes and macrophages, and induce IL-15 synthesis resulting in activation of PBMC (Quaranta et al., 1999). Furthermore, it has been shown that recombinant Nef triggers a series of phenotypic and functional changes that promotes DC differentiation, enhancing the ability of these cells to activate bystander CD4⁺ T cells (Quaranta et al., 2002).

1.8.7. Role of HIV-1 Nef in T cell apoptosis

HIV-1 Nef is one of several viral proteins that has been described to modulate lymphocyte cell death. The role of Nef in apoptosis remains controversial, with both inhibitory and pro-apoptotic activities reported. The earliest study to assess the effects of HIV-1 Nef on apoptosis was by Fujii *et al.* (Fujii et al., 1996). This group observed that soluble Nef could bind to the surface of CD4⁺ T cells and that cross-linking of these proteins using anti-Nef antibodies was cytotoxic to uninfected cells (Fujii et al., 1996).

These findings were substantiated by other studies reporting that soluble Nef bound not only to CD4⁺ T cells, but also CD8⁺ T cells, B-cells, macrophages and neutrophils, and that cross-linking of membrane-bound Nef led to an apoptosis pathway that was independent of Fas (CD95) (Okada et al., 1997) and that could be blocked by serine/threonine protein kinase inhibitors (Okada et al., 1998). Lenassi *et al.* later described that this soluble Nef exited infected cells via exosomes to cause the apoptosis of bystander CD4⁺ T cells (Lenassi et al., 2010). Various studies have reported enhancement of apoptosis by Nef through up-regulation of cell surface Fas and Fas ligand (Xu et al., 1997; Zauli et al., 1999), which could also serve as a mechanism to evade immune responses by initiating the death of antiviral CD8⁺ T cells (Xu et al., 1997). Another mechanism employed by Nef to modulate apoptosis is by up-regulation of the programmed death 1 (PD-1) surface protein, which appears to be dependent on the activation of p38/MAPK (Muthumani et al., 2008). Nef also reduces the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL as a mechanism to increase apoptosis (Rasola et al., 2001). Finally, soluble Nef is reported to induce apoptosis driven mediated CXCR4 receptor (Huang et al., 2004; James et al., 2004).

HIV-1 Nef has also been reported to inhibit apoptosis. In association with PAK and PI3-kinases Nef phosphorylates the cellular protein Bad to enhance its anti-apoptotic properties (Wolf et al., 2001). In addition, Nef inhibits the function of apoptosis signal-regulating kinase 1 (ASK1) (Geleziunas et al., 2001). This prevents cell death that could result from cis-ligation of Nef-induced Fas and FasL on the surface of virus-infected cells, thereby resulting in enhance bystander cell death through Fas/FasL mechanisms, but simultaneously protecting the virus-infected cells from Fas and TNF- α death signalling pathways. It has also been reported that the survival of productively infected CD4⁺ T lymphocytes requires Nef expression by HIV-1, as well as activation by TNF- α expressed on the surface of macrophages (Mahlknecht et al., 2000). Finally, Nef has been reported to bind the tumour suppressor protein p53 and to inhibit its ability to mediate apoptosis (Greenway et al., 2002).

1.9. Sequence and functional diversity in Nef primary isolates

While critical Nef motifs have been described for many of its known functions, these tend to be highly conserved in natural HIV-1 isolates (Brumme et al., 2009). For example, the dileucine motif at LL₁₆₄₋₅ is required for CD4 and SERINC5 downregulation (Rosa et al., 2015), but these sites are 100% conserved in our patient Nef sequences. A major aim of this thesis is to discover polymorphisms selected *in vivo* that attenuate Nef protein function; therapeutic interventions could aim to select for these mutations.

There have been few studies of patient-derived Nef sequence diversity and/or function. As mentioned, large nef-deletions are associated with slow disease progression (Zaunders et al., 1999; Kirchoff et al., 1995); however, such deletions are rare even among LTNP (Huang et al., 1995; Corro et al., 2012) or elite controllers (Miura et al., 2008). Kirchoff et al. analyzed Nef codon sequences between LTNP/LTS ([N=49] and slow progressors [N=90], identifying five polymorphisms that were more common among nonprogressors (15, N51, H102, L170, and E182) but did not test protein function (Kirchoff et al., 1999). Tobiume et al. isolated primary nef sequences from eight LTNP, and tested them for several functions. They observed that LTNP Nef displayed inefficient CD4 downregulation and infectivity functions compared to progressor Nef. However, the study did not have the power to investigate the relation between sequence polymorphisms and function. Finally, Corro et al. found that in their cohort of 10 LTNP HIV+ infants, three patients harboured Nefs with R22Q mutation (with the the RxRxRR₁₇₋₂₂ alpha-helix) and three displayed L57V, both of which seemed to impair HLA-I downregulation, but not CD4 downregulation (Corro et al. 2012).

1.10. Nef in the context of HIV vaccine design

The first major HIV vaccine trial, AIDSVAX by VaxGen, sought to elicit neutralizing antibodies against the gp120 glycoprotein, but not CTL responses. It was announced in 2003 that AIDSVAX failed to prevent infection and did not slow disease progression in people infected during the trial (McCarthy, 2003).

Subsequent major vaccine trials sought to elicit CTL responses against *gag*, *pol*, and *nef*. The STEP trial used an adenovirus to deliver *gag/pol/nef* (MRKAΔ5) to

participants. Not only did the vaccine fail to prevent infection, but among subjects seropositive for antibodies against the adenovirus vector, the rate of infection was higher in the vaccinated group than the placebo group (Buchbinder et al., 2008). Initial reports suggested that CTL responses were weak and limited in number, with only a median of 2 detected per participant (Buchbinder et al., 2008). After these disappointing results, there was a robust re-examination of the prospect of T cell based vaccines (Robb, 2008; Sekaly, 2008; Pantaleo, 2008; Yang, 2008; Corey et al., 2009; Barouch and Korber, 2010). Follow-up studies found HIV-specific CTL responses in 77% of vaccinated participants, generally targeting 2-3 HIV epitopes, and comprising up to 12% of circulating CTL (McElrath et al., 2008). These results were impressive compared to other T cell vaccine studies, adding to the puzzle as to why the trial failed (Altfeld and Goulder, 2011). Possible explanations include: a broader array of CTL responses were required, including stronger CTL responses, and a failure of CD8⁺ T cells to offer protection in the mucosa (Fitzgerald et al., 2011).

Interestingly, it was observed that in STEP participants who were infected with HIV and who expressed protective HLA alleles B*57, B*27, and B*58, those who received the vaccine had lower viral loads (Fitzgerald et al., 2011). This is consistent with the possibility of a therapeutic vaccine which targets key HIV protein domains to impair protein function and viral spread. A study of Nef-specific CTL responses in the STEP trial found significantly more escape mutations in immunodominant Nef epitopes amongst vaccinated participants (Park et al., 2016). Interestingly, two of most heavily targeted epitopes were Nef₁₇₋₂₅, which overlaps with Nef's N-terminal alpha-helix, and Nef₃₅₋₇₇, which overlaps with the SH3-binding proline-rich region (Park et al. 2016), two domains which we found crucial to Nef's ability to modulate TCR signaling (see **chapters 3-4** below).

Another T cell-oriented trial, HVTN505, boosted the gag/pol/nef Adenovirus vector with DNA plasmids expressing clade Gag, Pol, Nef and Env (DNA/rAd5), but this trial also failed to prevent infection (Hammer et al., 2013).

There was modest success in the RV144 trial which sought to elicit CTL responses with gag/pol/env delivered via canarypox vector (ALVAC-HIV) as well as antibody responses prompted by AIDSVAX gp120 protein boost. The RV144 study showed a 26%-31% efficacy in preventing infection, but did not reduce viral load or improve CD4⁺ cell count in those infected during the trial (Rerks-Ngarm et al., 2009).

However, there were few CTL or neutralizing antibody responses, and protection was thought to be a result of non-neutralizing antibodies that mediated cellular responses against HIV-infected cells (Haynes et al., 2014; Dommaraja et al., 2014).

There are several ongoing HIV vaccine trials. The SAV001 trial, based out of the University of Western Ontario, uses a “whole killed” approach, namely a *nef*-deleted virus that is irradiated. Phase 1 results showed increased CTL responses against gp120 and gag, as well as neutralizing antibody responses (Choi et al., 2016). Another novel approach is to construct immunogens that include a “mosaic” of conserved, functionally constrained regions of the HIV genome in order to better focus the immune response. One example of this strategy is a mosaic vaccine design that includes key epitopes in *gag* and *pol*, but not *nef* (Wee et al., 2016).

It has also been proposed that an important component of HIV T cell-based therapies may be anti-Nef inhibitors. Given that Nef downregulates HLA class I molecules to evade CD8+ T cells responses, inhibiting this Nef function may support the ability of natural or boosted CTL responses to clear infected cells (Trible et al., 2013; Dekaban and Dikeakos, 2017), including latently-infected cells (Mujib et al., 2017).

1.11. Thesis objectives

In this dissertation, I explored the impact of natural sequence variation on Nef function by examining Nef clones isolated from elite controllers (EC) and chronic progressors (CP). I hypothesized that elite controller-derived Nef would display reduced function because of immune escape mutations.

In chapter 2, I cloned Nef isolates from 46 CP and 45 EC to assess their in vitro function. Five well-known Nef functions were assayed, namely viral replication capacity, viral infectivity, CD4 down-regulation, HLA-I down-regulation, and CD74 up-regulation.

In chapter 3, I reviewed Nef’s known impacts on TCR signaling. I attempted to reconcile the many discrepancies in the literature with respect to Nef’s role as either a positive or negative regulator of T cell activation and signaling.

In chapter 4, I developed an assay to assess Nef’s ability to modulate TCR-mediated signaling. Using a panel of canonical site-directed Nef mutants, I identified domains required for Nef’s TCR signaling inhibition function. I also employed confocal

microscopy and Proximity Ligation Assay (PLA) tools to investigate Nef's impact on membrane proximal signaling events.

In chapter 5, the NFAT luciferase reporter assay developed the previous chapter was used to assess the impact of natural sequence variation in EC and CP on Nef's ability to inhibit TCR-mediated signaling.

This is the largest study to date investigating the function of primary Nef isolates from both chronic progressors and elite controllers, and the first study investigating multiple patient isolates for their ability to modulate T cell receptor (TCR) mediated signaling. Notably, increased power allowed me to find novel correlations between amino acid polymorphisms and Nef functions. A better understanding how host immune responses select for polymorphisms that attenuate Nef function can help inform vaccine or immunotherapy designs that seek to mimic or enhance these responses (Altfeld and Allen, 2006), and to "hit Nef where it hurts."

1.12. References

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Chapter 2. Functional analysis of Nef sequences isolated from HIV-1 Chronic progressors and Elite controllers

This chapter is based on two published studies of which I was co-first author:

Mwimanzi, P., Markle, T.J., Ogata, Y., Martin, E., Tokunaga, M., Mahiti, M., Kuang, X.T., Walker, B.D., Brockman, M.A., Brumme, Z.L., Ueno, T., 2013. Dynamic range of Nef functions in chronic HIV-1 infection. *Virology* 439(2), 74-80.

Mwimanzi, P., Markle, T.J., Martin, E., Ogata, Y., Kuang, X.T., Tokunaga, M., Mahiti, M., Pereyra, F., Miura, T., Walker, B.D., Brumme, Z.L., Brockman, M.A., Ueno, T., 2013. Attenuation of multiple Nef functions in HIV-1 elite controllers. *Retrovirology* 10:1.

I isolated Nef clones from 46 chronic progressors (CP) and 45 elite controllers (EC), and assessed them for CD4 down-regulation and HLA class I down-regulation activity. I isolated additional clones from patients whose first clone showed impaired function, and again assessed for these functions. Nef clones were shared with collaborators at the University of Kumamoto, Japan, to undertake viral infectivity and replication capacity measurements.

2.1. Dynamic range of Nef functions in chronic HIV-1 infection

2.1.1. Abstract

HIV-1 Nef is required for efficient viral replication and pathogenesis. However, the extent to which Nef's functions are maintained in natural sequences during chronic infection, and their clinical relevance, remains incompletely characterized. Relative to a control Nef from HIV-1 strain SF2, HLA class I and CD4 down-regulation activities of 46 plasma RNA Nef sequences derived from unique chronic infected individuals were generally high with narrow dynamic ranges, whereas Nef-mediated virion infectivity, PBMC replication and CD74 up-regulation exhibited broader dynamic ranges. 80% of patient-derived Nefs were active for at least three functions examined. Functional co-dependencies were identified, including positive correlations between CD4 down-regulation and virion infectivity, replication, and CD74 up-regulation, and between CD74 up-regulation and PBMC replication. Nef-mediated virion infectivity inversely correlated with patient CD4⁺ T-cell count. Strong functional co-dependencies and the polyfunctional nature of patient-derived Nef sequences suggest a phenotypic requirement to maintain multiple Nef functions during chronic infection.

2.1.2. Introduction

The highly variable HIV-1 Nef protein is required for efficient viral replication and disease progression *in vivo* (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Nef exhibits multiple functions *in vitro*, including enhancement of virion infectivity and replication (Miller et al., 1994; Münch et al., 2007), down-regulation of cell-surface CD4 (Aiken et al., 1994; Garcia and Miller, 1991) and HLA class I (HLA-I) (Collins et al., 1998; Schwartz et al., 1996), up-regulation of HLA class II associated invariant chain (CD74) (Schindler et al., 2003; Stumptner-Cuvelette et al., 2001), and others (Das and Jameel, 2005; Heigele et al., 2012; Kirchhoff et al., 2008). Variation in Nef activity has been demonstrated for laboratory-adapted viral strains (Fackler et al., 2006; Keppler et al., 2006), viral quasispecies within a single individual (Ali et al., 2009; Lewis et al., 2008), and small numbers of clinically isolated sequences (Na et al., 2004; Zuo et al., 2012), including those from long-term nonprogressors (Corro et al., 2012; Premkumar et al., 1996; Tobiume et al., 2002) and patients with advanced infection (Carl et al.,

2001). However, the functional breadth of naturally occurring Nef variants have not been comprehensively assessed using panels of clinically derived sequences. Here, we assessed five key Nef functions (enhancement of virion infectivity and replication capacity in PBMC, down-regulation of cell surface CD4 and HLA-I, and up-regulation of CD74) using 46 clonal *nef* sequences from unique chronic HIV-1-infected individuals. We examined the dynamic ranges, co-dependence, and clinical correlates of these five Nef activities.

2.1.3. Methods

Amplification and cloning of patient-derived Nef isolates: Forty-six untreated chronic subtype B infected individuals (median [IQR] plasma viral load 90850 [28840-231000] copies/ml; CD4⁺ T-cell count 297.5 [72-455] cells/mm³) were recruited in the Boston area with written informed consent (Brumme et al., 2011; Miura et al., 2009). Nef was amplified from plasma HIV-1 RNA by nested RT-PCR as described (Sato et al., 2008) and cloned into the pIRES2-EGFP vector (Clontech). A median of three Nef clones was sequenced per patient; a single clone with an intact Nef reading frame that clustered with the original bulk sequence was selected for analysis (GenBank accession numbers: JX440926-JX440971). *Nef* clones were sub-cloned into a pNL43-ΔNef plasmid as described previously (Ueno et al., 2008). As a control, pNL4.3 harboring *nef* from strain SF2 was used (Ueno et al., 2008). Proviral clones were transfected into HEK-293T cells and culture supernatant containing infectious virions was collected 48 hours later. Nef protein expression was verified by Western blot using two different polyclonal primary antibodies as described previously (Mwimanzi et al., 2011).

Virion infectivity and replication assays: Recombinant virus infectivity was determined by exposing 10⁴ TZM-bl cells (NIH AIDS Research and Reference Reagent Program) to 3ng p24^{Gag} recombinant virus followed by chemiluminescence detection 48hrs later as described (Wei et al., 2002). Infectivity values represented the mean of triplicate experiments, normalized to control strain NL4.3-Nef_{SF2}, such that values >100% and < 100% indicated increased or decreased infectivity, respectively. Recombinant virus replication was assessed by exposing 10⁶ freshly isolated PBMC from four HIV-negative donors to 10ng p24^{Gag} recombinant virus for 8 hours, washing twice, and then resuspending cells in a culture medium (RPMI 1640, 10% FCS) as described (Fackler et al., 2006; Ueno et al., 2008). Three days later, PBMCs were stimulated with

phytohemagglutinin at 5 µg/ml. Culture supernatants were collected and replaced with fresh medium supplemented with human rIL-2 every 3 days. Viral replication was monitored by measuring p24^{Gag} in the culture supernatant using ELISA over 12 days. ELISA values during the initial burst of viral replication (on day 9) were used as our measure of replication capacity. Results were expressed as the mean of quadruplicate assessments in each donor, normalized to control strain NL4.3-Nef_{SF2}.

Analysis of receptor modulation HIV-infected cell surface: To assess Nef-mediated HLA-I down-regulation and CD74 up-regulation, 721.221 cells stably expressing CD4 and HLA-A24:02 (provided by Masafumi Takiguchi, Kumamoto University, Japan) were exposed to 300ng p24^{Gag} recombinant virus for 48hr, followed by staining with anti-HLA-A24-PE (MBL), anti-CD74-Alexa Fluor-647 (BioLegend), 7-amino-actinomycin D (BioLegend), and anti-p24 Gag-FITC (Beckman Coulter), as described (Ueno et al., 2008). Mean fluorescence intensity (MFI) of each receptor in live p24^{Gag} positive and negative subsets was determined by flow cytometry (FACS Canto II; BD Biosciences). Results were expressed as the mean of duplicate experiments, normalized to control strain NL4.3-Nef_{SF2}.

Analysis of Nef-mediated CD4 down-regulation: To assess Nef-mediated CD4 down-regulation, 3 x 10⁶ CEM-SS cells were transfected with 5 µg plasmid DNA encoding Nef protein and GFP by electroporation (BioRad GenePulser MX) and stained 24 hr later with anti-CD4-APC (BD Biosciences). MFI of GFP-negative and GFP-positive (Nef-expressing) subsets was determined by flow cytometry (Guava easyCyte 8HT, Millipore). Results were normalized to plasmid expressing Nef_{SF2}.

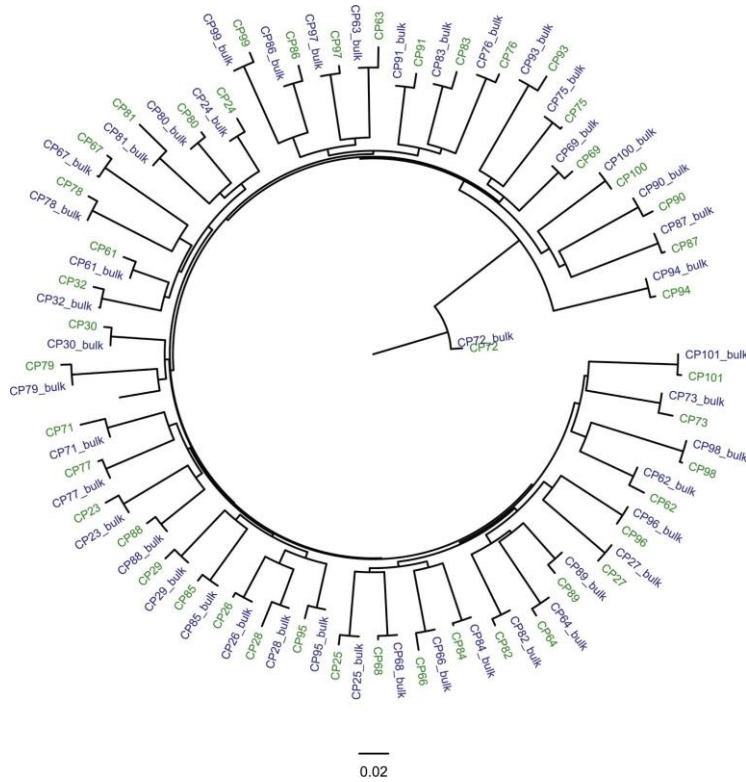
2.1.4. Results and discussion

Genotypic and phenotypic profile of patient-derived Nef sequences

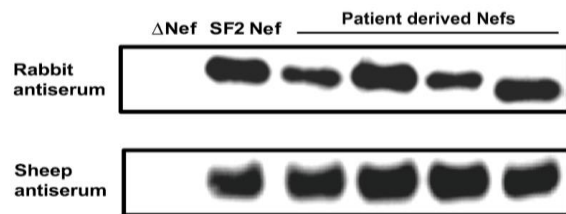
We analyzed plasma HIV-1 RNA sequences, as these represent the current replicating virus better than proviral DNA (Crotti et al., 2006). Patient Nef sequences displayed no major phylogenetic clustering (**Figure 2.1.1A**). Codon-specific Shannon entropy scores of the patient-derived Nef clonal sequences (N=46) correlated significantly with those of 1191 subtype B sequences retrieved from the Los Alamos database (Spearman R=0.92, p<0.0001), suggesting them to be representative of subtype B sequence diversity. All patient-derived and SF2 Nef proteins were examined

by Western blot using two independent anti-Nef primary antibodies (representative data shown in **Figure 2.1.1B**). No major differences in steady-state expression levels were observed among Nef proteins (**Figure 2.1.1C**).

A



B



C

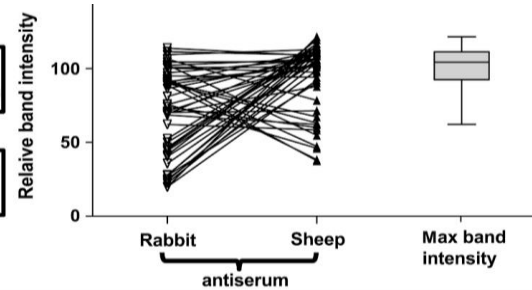


Figure 2.1.1. Analysis of 46 patient-derived Nef sequences and their protein expression.

(A) The phylogeny of 46 *nef* clones derived from plasma viral RNA (one per subject) was analyzed using maximum-likelihood methods. Shown in the tree is the selected Nef clone (Green) in relation to the original bulk Nef sequence (Blue). Control Nef sequence from strain SF2 is indicated in red. (B) Representative blots of Δ Nef, SF2 Nef, and four patient-derived Nef clones are shown. (C) For each patient-derived Nef, band intensities relative to SF2 control, using rabbit vs. sheep primary Nef antisera, are shown. Although the antisera differed in their ability to detect individual patient-derived sequences, all Nef clones were detected readily by at least one of them.

Functional characterization of patient-derived Nef sequences

All 46 patient-derived Nef proteins exhibited at least partial activity for all functions tested (**Figure 2.1.2E**). Relative to a control Nef, derived from HIV-1 strain SF2, patient-derived Nef sequences were in general highly functional with respect to down-regulation of HLA-I and CD4, while dynamic ranges of other Nef functions were broader (**Figure 2.1.2E**). Median [IQR] Nef activities, normalized to those of SF2 control, were: virion infectivity, 116% [88-160]; viral replication capacity, 76% [57-98]; HLA-I down-regulation, 106% [98–112]; CD74 up-regulation, 112% [69–151]; and CD4 down-regulation, 99% [92–102] (**Figure 2.1.2E**).

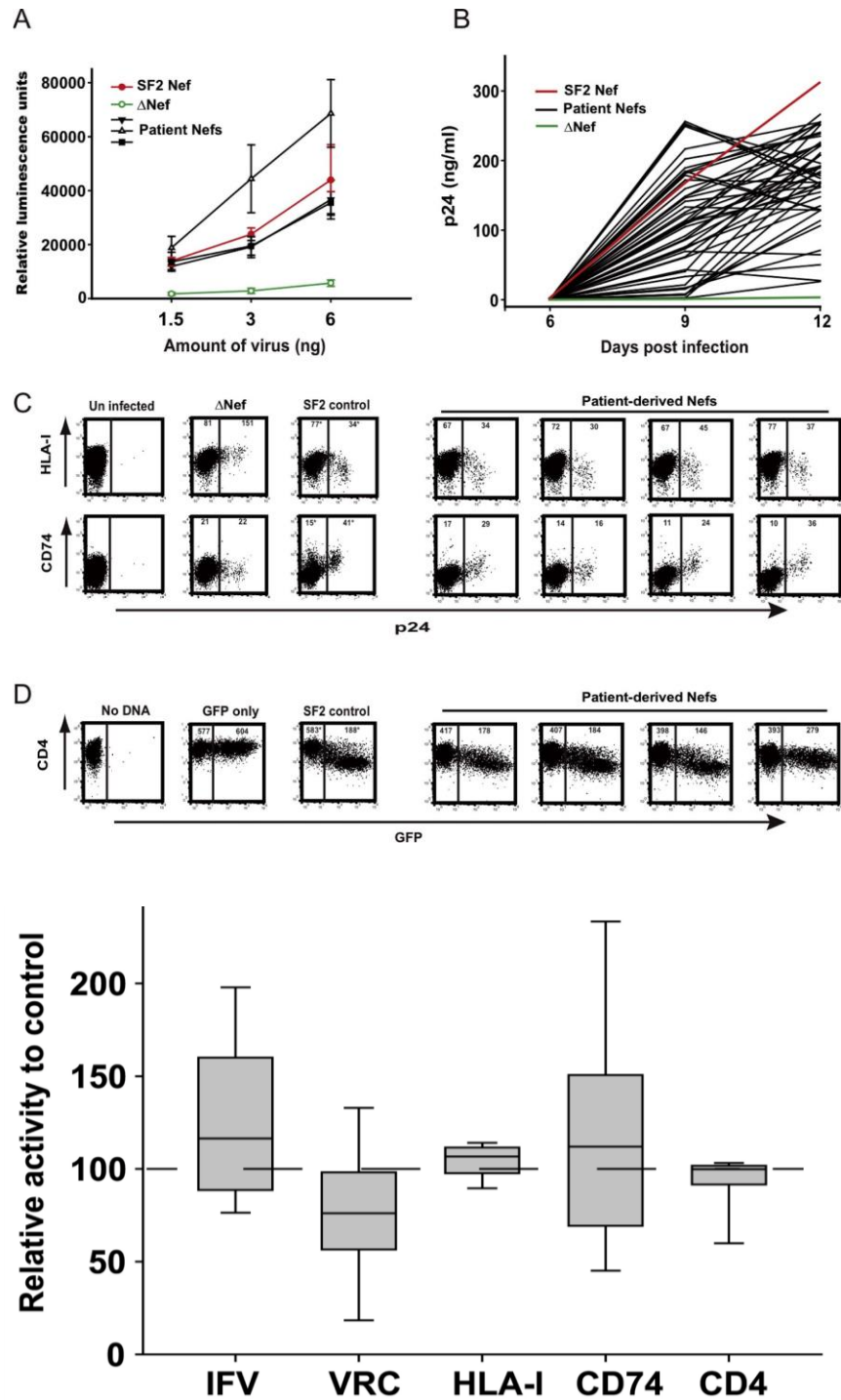


Figure 2.1.2. The *in vitro* dynamic range of five Nef-mediated activities.

Analysis of individual Nef functions: (A) Infectivity of recombinant viruses encoding patient Nef sequences was measured using TZM-bl cells. Cells were exposed to viruses encoding SF2 Nef, Δ Nef, and three unique patient-derived Nefs at the indicated amount of Gag^{p24}. Viral infectivity was determined using chemiluminescence. Data represent mean \pm standard deviation (SD) of three independent experiments. (B) Replication of Nef-containing viral constructs was determined using PBMC from healthy donors. 10^6 cells were infected with virus and culture supernatant was collected on the indicated days. Replication was measured by ELISA to detect Gag^{p24}. Representative replication kinetics of viruses expressing patient-derived Nef clones (black lines),

control SF2 Nef (red line), and Δ Nef (green line) in one PBMC donor is shown. Replication was determined at the peak of virion production (day 9) and values were normalized to that of the control strain encoding SF2 Nef. (C) To analyze the effect of patient-derived Nef sequences on surface expression of HLA-I and CD74 in virus-infected cells, 721.221 cells expressing CD4 and HLA-A*24:02 were infected with recombinant viruses encoding SF2 Nef, Δ Nef, or four unique patient-derived Nef clones. Cells were stained with antibodies to HLA-A*24 (HLA-I) and CD74, followed by intracellular staining for Gag^{p24} and assessed using flow cytometry. Representative data are shown. Median fluorescence intensity (MFI) of HLA-I and CD74 was determined in the Gag^{p24}-positive and negative cell subsets, and changes in MFI were normalized to that of control virus encoding SF2 Nef. (D) To examine the effect of Nef protein on surface expression of CD4, CEM-SS cells were transfected with plasmid DNA encoding GFP only or Nef plus GFP by electroporation. At 24 hours post-transfection, cells were stained with anti-CD4 mAb and MFI was determined by flow cytometry in the GFP-positive and GFP-negative cell subsets. Representative data are shown. CD4 down-regulation activity of patient-derived Nef clones was normalized to that of a control plasmid encoding SF2 Nef. (E) Here we show infectivity (IFV), viral replication (VRC), HLA-I down-regulation, CD74 up-regulation, and CD4 down-regulation. Nef function in each assay was normalized to that of control Nef strain SF2, which was considered as 100 % (dotted line). Box and whisker plots show the median (horizontal line), interquartile range (edges of box) and range (whiskers) of functions for N=46 chronic patient-derived Nef clones.

The relatively conserved CD4 down-regulation function observed in our cohort is consistent with most previous studies of chronic Nef sequences (Agopian et al., 2007; Carl et al., 2001; Zuo et al., 2012). Similar preservation of HLA-I down-regulation function has also been reported by some studies (Noviello et al., 2007; Zuo et al., 2012), however others have observed wider ranges in chronic infection (Lewis et al., 2008) or inefficient Nef-mediated HLA-I down modulation in later infection stages (Carl et al., 2001). Our observation that Nef-mediated enhancement of virion infectivity was relatively well preserved among chronic patient-derived sequences, while Nef-mediated viral replication capacity was on average lower than the SF2 control strain, is perhaps notable since previous studies of these Nef activities have failed to observe consistent associations with clinical status (Carl et al., 2000; Crotti et al., 2006; Tobiume et al., 2002). Nef function can be influenced by the choice of assay systems, cell lines, and control strain used (Kirchhoff et al., 2008; Mwimanzi et al., 2011; Suzu et al., 2005); these factors, combined with the smaller number of patients previously studied, may explain some of these divergent results.

Taken together, our data support CD4 and HLA-I down-regulation as essential *in vivo* functions during chronic HIV-1 infection. In contrast, the broader dynamic ranges of virion infectivity, replication capacity in PBMC, and CD74 up-regulation may suggest differential requirements for these activities in maintaining viral fitness during chronic infection. Alternatively, some functions may serve as surrogates of other Nef activities

not assessed, such as modulation of cellular activation. Indeed, an association between CD74 up-regulation and polyclonal T-cell activation was demonstrated in HIV-infected subjects, suggesting that Nef could mediate this effect directly or indirectly through CD74 up-regulation in virus-infected cells (Ghiglione et al., 2012). Nonetheless, our results extend our understanding of Nef functions that facilitate viral replication and immune evasion in naturally occurring sequences (Brambilla et al., 1999; Casartelli et al., 2003; Crotti et al., 2006; Foster et al., 2001).

Combined functional analyses: Nef polyfunctionality score

To investigate the extent to which individual patient-derived Nef proteins maintained multiple functions simultaneously, we defined a “polyfunctionality” score ranging from 0 (all functions relatively poor) to 5 (all functions adequate) where the 33rd percentile of each function was defined as the cutoff between these two categories (**Figure 2.1.3**). More than half (27 of 46) of patient-derived Nefs exhibited a polyfunctionality score ≥ 4 whereas 19.6% (9 of 46) exhibited a score ≤ 2 . Two Nef clones scored 0 although both had functional activities $>10^{\text{th}}$ percentile for all five activities, indicating that they were not completely defective. These results support the importance of maintaining multiple Nef functions during chronic infection.

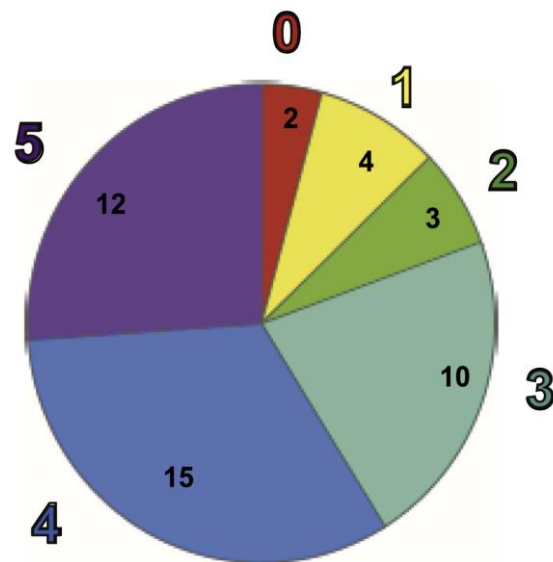


Figure 2.1.3. Nef polyfunctionality score.

To assess combined functional differences of each patient-derived Nef, a polyfunctionality score was developed. For each of the five Nef activities tested, functions above the 33rd percentile of the population were defined as “adequate” while those below this cutoff were defined as “poor”. The number outside the pie chart indicates the “polyfunctionality score”, while the number within each slice indicates the number of patient-derived Nef sequences exhibiting this score.

Correlation of Nef functions with HIV-1 clinical parameters

A significant inverse relationship was observed between Nef-mediated virion infectivity and CD4⁺ T-cell count in our cohort (Spearman's $R=-0.338$, $p=0.02$) (**Figure 2.1.4A**). To our knowledge, this is a novel observation in chronic infected individuals. Nef polyfunctionality score was also inversely related to CD4⁺ T-cell count in our cohort ($R=-0.307$, $p=0.03$) (**Figure 2.1.4B**), although this did not remain significant after removing infectivity from the scoring scheme. Of note, Lewis *et al.* previously reported a relatively broad range of Nef-mediated HLA-I down-regulation function in eleven chronic infected patients and positive correlations with CD4⁺ T-cell counts (Lewis *et al.*, 2008), whereas our results showed no relationship between these two parameters. This difference may be due to the fact that Nef-mediated HLA-I down-regulation activity was relatively highly preserved in our cohort (**Figure 2.1.1C**). No correlation was observed between plasma viral load and any Nef function or the polyfunctionality score. Although further studies will be required to elucidate the underlying mechanism(s) of our observations, these results suggest an important role for Nef-mediated virion infectivity in HIV-1 pathogenesis.

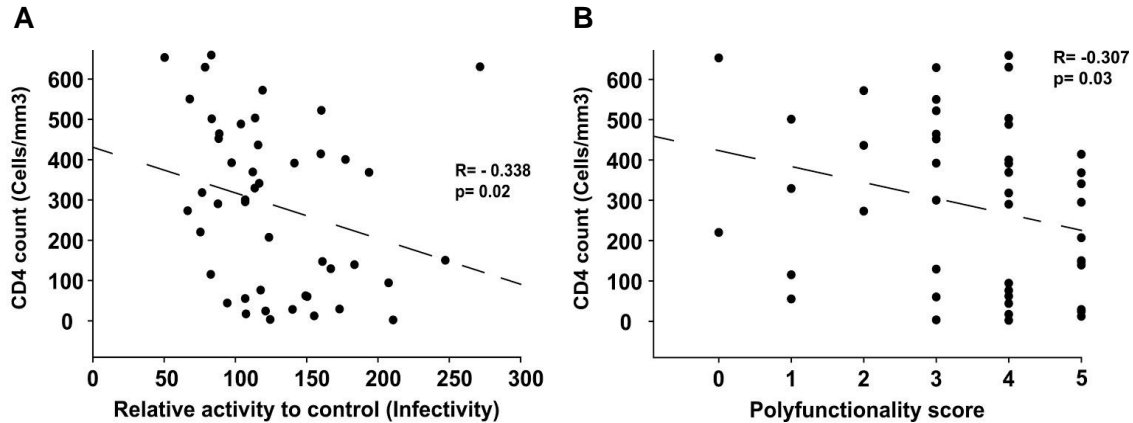


Figure 2.1.4 Correlation of Nef functions with HIV-1 clinical parameters.

Individual Nef functions and the Nef polyfunctionality score were compared to markers of clinical disease in this population of chronic patients. (A) An inverse correlation was observed between Nef-mediated viral infectivity and patient CD4⁺ T cell count ($R=-0.338$, $p=0.02$; Spearman's correlation). (B) An inverse association was also observed between Nef polyfunctionality score and CD4⁺ T cell count ($R=-0.307$, $p=0.03$; Spearman's correlation).

Nef functional co-dependencies

Mutational studies indicate that the genetic determinants of Nef's various functions are largely distinct from one another, and that these functions may therefore be considered largely independent (Dai and Stevenson, 2010). For instance, CD4 down-regulation is determined by the highly conserved Nef motifs LL_{163,164} and DD_{174,175}, while HLA-I down-regulation is mediated by other motifs including PxxP₇₂ and EEEE₆₂₋₆₅ (Geyer et al., 2001). However, the extent to which secondary genetic polymorphisms contribute to Nef function, and thus the extent to which the various activities of patient-derived Nef sequences are functionally independent, remains incompletely known.

Pairwise correlations of Nef functions in our patient-derived sequences revealed positive relationships between CD4 down-regulation and all other activities, except HLA-I down-regulation (**Figure 2.1.5**), suggesting shared molecular mechanisms and/or functional complementarity. Indeed, interaction of Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity (Craig et al., 1998). Nef point mutants impaired in CD4 down-regulation were also most delayed in viral replication (Lundquist et al., 2002). Furthermore, mutations WL_{57,58}AA and LL_{163,164}GG that affected Nef-mediated CD4 down-regulation activity also affected CD74 up-regulation (Stumptner-Cuvelette et al., 2001), an interaction possibly influenced by the sequestration of AP-2 by Nef during CD4 downregulation (Mitchell et al., 2008). Indeed, interactions with these sorting pathways also thought to be crucial for Nef's down-regulation of SERINC, which has been recently identified as a key mechanism responsible for promotion of viral infectivity (Usami et al., 2015; Rosa et al., 2015). Taken together with previous studies, our results suggest that Nef-mediated CD4 down-regulation functions of patient-derived sequences may be, at least in part, mechanistically linked to other Nef functions through common functional motifs and/or interactions with common host proteins *in vivo*.

In contrast, HLA-I down-regulation showed no correlation with any other activity (**Figure 2.1.5**), suggesting that it may be differentially regulated *in vivo*. This observation is consistent with previous studies of site-directed mutants of laboratory-adapted strains (Akari et al., 2000; Lundquist et al., 2002; Stoddart et al., 2003). Also consistent with previous studies using Nef point mutations undertaken in CD4⁺ T-cells, we observed no correlation between Nef-mediated viral infectivity and viral replication enhancement in PBMCs (Lundquist et al., 2002), supporting distinct genetic determinants of these two

functions. One study observed that HIV-1 gp41 enhanced viral infection through activation of the CD74 protein-mediated extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (Zhou et al., 2011), raising an intriguing hypothesis that Nef might enhance viral infection through the same mechanism; however, our data do provide evidence in support of this model. Of note, no inverse relationships were observed between Nef activities, arguing against functional tradeoffs or the existence of particular substitutions or domains that enhance one function at the expense of another. This is consistent with the maintenance of polyfunctionality for most patient-derived Nef sequences.

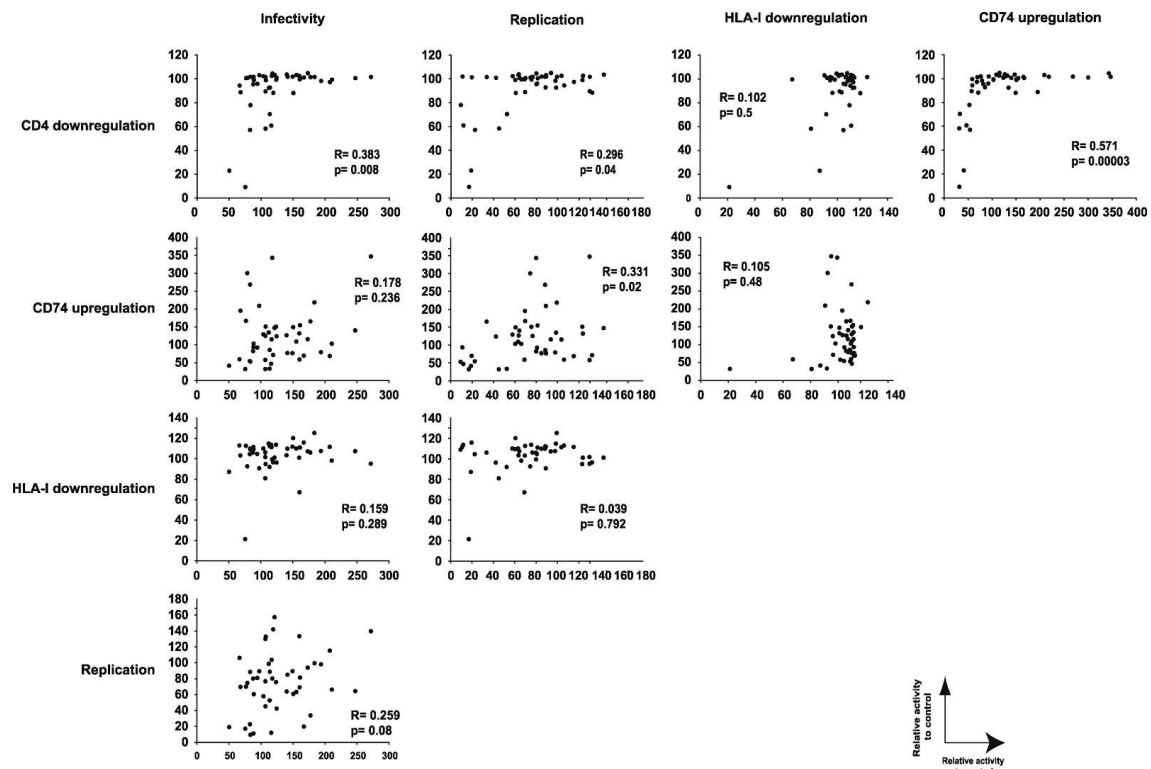


Figure 2.1.5. Co-dependence between *in vitro* Nef activities.

Pairwise associations between each of the five *in vitro* functions were examined for the patient-derived Nef clones. Significant correlations were observed between CD4 down-regulation and infectivity, viral replication, and CD74 up-regulation; and between CD74 up-regulation and viral replication (all $p < 0.05$, Spearman's correlation).

Amino acids associated with Nef functions

Identification of highly conserved Nef residues and domains critically important for Nef's various functions has been performed using mutational studies (Lundquist et al., 2002; Neri et al., 2011; Stumptner-Cuvelette et al., 2001) and limited analyses of patient-derived sequences (Glushakova et al., 2001; Lewis et al., 2012). To investigate the contribution of naturally-occurring polymorphisms at Nef's more variable sites on protein function of patient-derived sequences, we performed an exploratory sequence-function analysis restricted to amino acids observed at a minimum frequency of N=5 in our dataset. Multiple comparisons were addressed using q-values (Storey and Tibshirani, 2003). Eighteen polymorphisms, occurring at 12 unique codons, were associated with at least one Nef function (all $p < 0.05$, $q < 0.25$) (**Table 2.1.1**). No codon was associated with more than two Nef functions, suggesting that, in general, the secondary (variable) residues and domains that mediate Nef's various activities may also be largely genetically separable. No polymorphisms associated with HLA-I down-regulation activity were identified at $q < 0.25$, therefore we are unable to confirm the novel mutations recently identified in chronic infection by Lewis et al (Lewis et al., 2012). However, Y135F, which was previously shown to impair HLA-I down-regulation (Lewis et al., 2012), was associated with higher viral replication in our study. Interestingly, variation at Nef codon 21, (within the highly conserved basic amino acid motif $R_{17}xRxRR_{22}$ involved in membrane targeting of Nef (Fackler et al., 2006) and vesicle secretion (Ali et al., 2010)), was associated with lower Nef-mediated viral infectivity and CD74 up-regulation.

Table 2.1.1. Analysis of Nef Residues associated with functions.

Nef activity	Codon ^a	AA ^b	No. of subjects ^c		Relative Nef activity		p-value	q-value
			AA+	AA-	AA+	AA-		
Viral infectivity	8	R	18	25	107.0	140.1	0.02	0.2
	10	L	5	34	82.8	116.4	0.01	0.2
	10	V	8	31	146.0	107.0	0.005	0.2
	21	R	32	14	131.9	97.8	0.02	0.2
	49	A	36	8	122.4	85.7	0.008	0.2
	85	F	7	39	94.3	121.2	0.02	0.2
	152	Q	5	40	76.7	120.2	0.002	0.2
Viral replication in PBMC	10	M	8	31	104.9	69.5	0.001	0.1
	135	F	8	38	116.8	69.7	0.008	0.2
	135	Y	38	8	69.7	116.8	0.008	0.2
	182	Q	6	40	26.3	80.4	0.001	0.1
	194	M	17	29	60.7	84.9	0.003	0.1
	194	V	21	25	93.8	69.5	0.005	0.2
CD74 up-regulation	12	E	5	41	195.1	102.9	0.01	0.2
	21	Q	6	40	180.9	98.1	0.01	0.2
	94	K	40	6	124.4	52.9	0.004	0.1
	205	D	21	25	150.5	82.1	0.001	0.02
	205	N	25	21	82.1	150.5	0.001	0.02

a) HXB2 numbering; b) AA, amino acid; c) gaps in the alignment are not counted; as such, amino acid totals to not always add up to 46.

Some limitations of our study merit mention. In contrast to previous reports that evaluated specific Nef functions using quasispecies-derived sequences or multiple clones from smaller numbers of patients (Gray et al., 2011; Lewis et al., 2008), we aimed

to evaluate the dynamic range and co-dependencies of a broader array of Nef activities using a larger number of patients. As such, our analysis was limited to a single Nef clone per patient. Although each patient sequence was closely related to the bulk plasma RNA sequence, we cannot rule out selection bias in the clones tested; however, we believe this to be minimal since most clones were polyfunctional. Second, we employed recombinant virus approaches to assess most Nef functions. This method might be limited by incompatibilities between insert and backbone; however, we did not observe significant differences in p24 antigen production among viral stocks. Finally, to eliminate potential confounding effects due to other HIV-1 proteins, we assessed CD4 down-regulation function using transient transfection assays. This approach can be affected by Nef expression or cytotoxicity; however, we saw no significant differences in steady-state protein levels by Western blot or in cell death by propidium iodide staining between clones. Despite these limitations, our study provides an important quantitative assessment of the dynamic range and functional co-dependencies for five of Nef's activities in naturally occurring patient-derived sequences.

2.1.5. Summary: Dynamic range of Nef functions in chronic HIV-1 infection

Nef sequences from chronic HIV-1 infection are in general highly polyfunctional with respect to enhancement of virion infectivity, stimulation of viral replication in PBMC, down-regulation of CD4 and HLA-I, and up-regulation of CD74. The dynamic ranges of CD4 and HLA-I down-regulation function were relatively narrow, whereas those for virion infectivity, stimulation of viral replication in PBMC, and up-regulation of CD74 were broader. An inverse association was observed between Nef-mediated enhancement of virion infectivity and CD4⁺ T-cell count, indicating the potential biological importance of this Nef activity in HIV-1 pathogenesis. Strong functional co-dependencies and the polyfunctional nature of patient-derived Nef sequences suggest a phenotypic requirement to maintain multiple Nef functions *in vivo* during the chronic infection phase.

2.2. Attenuation of multiple Nef functions in HIV-1 elite controllers

2.2.1. Abstract

Impaired HIV-1 Gag, Pol, and Env function has been described in elite controllers (EC) who spontaneously suppress plasma viremia to < 50 RNA copies/mL; however, activity of the accessory protein Nef remains incompletely characterized. We examined the ability of 91 Nef sequences, isolated from plasma of 45 EC and 46 chronic progressors (CP), to down-regulate HLA class I and CD4, up-regulate HLA class II invariant chain (CD74), enhance viral infectivity, and stimulate viral replication in PBMC. In general, EC Nef sequences were functional; however, all five activities were significantly lower in EC compared to CP. Nef from HLA-B*57-expressing EC exhibited poorer CD4 down-regulation function compared to non-B*57 EC, and the number of EC-specific B*57-associated Nef polymorphisms correlated inversely with 4 of 5 Nef functions in these individuals. Results indicate that decreased viral protein function, due in part to host immune selection pressures, may be a hallmark of the EC phenotype.

2.2.2. Introduction

Elite controllers (EC) are rare (<1%) HIV-1 infected individuals who spontaneously suppress plasma viral loads to undetectable levels in the absence of antiviral therapy. Several factors likely contribute to this phenotype, including host genetics (Pereyra et al., 2010), characteristics of HLA-restricted T-cell responses (Chen et al., 2012), immune-mediated reductions in viral protein function and/or replication (Brumme et al., 2011; Miura et al., 2009a), and acquisition of attenuated viruses (Deacon et al., 1995; Kirchhoff et al., 1995). Recombinant viruses expressing *gag* and *pol* sequences from EC exhibit reduced *in vitro* replication capacity, due in part to cytotoxic T lymphocyte (CTL) escape mutations selected by certain HLA class I (HLA-I) alleles (Brumme et al., 2011; Miura et al., 2009a), while EC-derived viral envelopes exhibit impaired entry (Lassen et al., 2009). The *in vitro* function of other viral proteins in EC remains incompletely characterized.

HIV-1 Nef is an accessory protein required for maintenance of high viral loads and progression to AIDS (Kestler et al., 1991), as demonstrated by slow or non-

progressive disease in hosts infected with *nef*-deleted or otherwise *nef*-defective strains (Daniel et al., 1992; Deacon et al., 1995; Kirchhoff et al., 1995; Zaunders et al., 1999). Nef exhibits a variety of *in vitro* functions that may modulate pathogenesis, including CD4 down-regulation (Garcia and Miller, 1991), HLA-I down-regulation (Schwartz et al., 1996), HLA class II invariant chain (CD74) up-regulation (Schindler et al., 2003), enhancement of virion infectivity (Münch et al., 2007), and stimulation of viral replication in PBMC (Miller et al., 1994) (for reviews see (Arhel and Kirchhoff, 2009; Arien and Verhasselt, 2008; Foster et al., 2011)). In our previous study we found that these *in vitro* functions were largely preserved in Nef sequences isolated from patients in chronic infection (Mwimanzi and Markle et al., 2013a; see **section 2.1.1-2.1.5** above). Multiple Nef activities may act together to facilitate immune evasion and enhancement of viral spread *in vivo* (Fackler et al., 2007). However, multi-functional assessments of patient-derived Nef sequences from HIV elite controllers are lacking. Although Nef sequence diversity is highly influenced by host HLA-I selection pressures (Carlson et al., 2012a), the relationship between HLA-associated polymorphisms and Nef function is largely unknown. Assessing multiple *in vitro* Nef functions in EC, a population that is highly enriched for protective HLA-I alleles such as B*57 (Pereyra et al., 2010), provides an opportunity to investigate these questions.

Previous analysis of plasma HIV RNA Nef sequences in our cohort of EC revealed no evidence of gross mutational defects (Miura et al., 2008), suggesting that any impairment in Nef protein function would have a more complex aetiology. In this study we used cloning methods and functional assays used previously (Mwimanzi and Markle et al., 2013a): we generated recombinant viruses encoding a single representative HIV RNA Nef sequence from 45 EC to assess Nef-mediated down-regulation of HLA class I, up-regulation of HLA class II invariant chain (CD74), viral infectivity, and viral replication in PBMC. The same Nef clone was engineered into a GFP-expression vector to assess its ability to down-regulate CD4. Results were compared to the activities of HIV RNA-derived Nef sequences from 46 chronic progressors (CP). Finally, we assessed the role of host immune selection pressures, most notably polymorphisms associated HLA-B*57, on Nef function in EC.

2.2.3. Materials and Methods

Study subjects: 45 EC (median [interquartile range, IQR] pVL 2 [0.2-14] RNA copies/ml (Pereyra et al., 2008); median [IQR] CD4 count 811 [612-1022] cells/mm³) and 46 CP (median [IQR] pVL 80500 [25121-221250] RNA copies/ml); median [IQR] CD4 count 292.5 [72.5-440] cells/mm³) were studied as described previously (Brumme et al., 2011; Miura et al., 2008; Miura et al., 2009a; Pereyra et al., 2008; Mzimanzi and Markle et al., 2013a). All EC and CP were HIV-1 subtype B-infected, untreated at the time of sample collection, recruited from the Boston area, and comparable with respect to ethnicity and date of HIV diagnosis (1985-2006 for EC vs. 1981-2003 for CP). This study was approved by the relevant institutional review boards; all participants provided written informed consent.

Cloning and analysis of nef genes: For EC, HIV RNA was extracted from a starting volume of 4.5 to 35.0 ml of plasma and amplified using nested RT-PCR, as described (Miura et al., 2008). To rule out proviral DNA contamination, all extractions included a DNase treatment step; controls lacking RT enzyme were also performed (Miura et al., 2008). For CP, HIV RNA was extracted from 0.5 ml of plasma and amplified in the same manner. Nef amplicons were cloned into pIRES2-EGFP expression vector (Clontech). A minimum of three Nef clones were sequenced per patient, and a single clone with an intact Nef reading frame that closely resembled the original bulk plasma RNA sequence was selected. Genbank accession numbers for clonal Nef sequences are JX171199-JX171243 (EC) and JX440926-JX440971 (CP). Western blots were performed as reported above in **section 2.1.1-2.1.5** (and published in Mzimanzi and Markle et al., 2013a)

In vitro functional assays: With the exception of CD4 down-regulation activity (see below), all Nef functions were determined using this panel of recombinant viruses. All five functional assays were performed as reported above in **section 2.1.1-2.1.5** (Mzimanzi and Markle et al., 2013a).

Statistical analyses, including identification of B*57-associated polymorphisms in patient-derived Nef sequences: Phylogenetically-informed methods were used to identify Nef amino acids significantly associated with HLA-B*57 allele expression in our EC (Carlson et al., 2012b), implemented at <http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/phylododdsratio/>

default.aspx). Multiple comparisons were addressed using q-values, the p-value analogue of the false discovery rate (FDR) (Storey and Tibshirani, 2003). The FDR is the expected proportion of false positives among results deemed significant at a given p-value threshold; for example, at a $q \leq 0.2$, we expect 20% of identified associations to be false positives.

2.2.4. Results

Nef protein expression and viral production

For each of 45 EC and 46 CP, a single representative plasma HIV RNA-derived Nef sequence with an intact open reading frame (ORF) was cloned into a recombinant NL4.3 virus construct. Consistent with previous analyses of bulk plasma HIV RNA sequences from our EC cohort (Miura et al., 2008), clonal Nef sequences from EC showed no evidence of gross defects or recent shared ancestry (**Figure 2.2.1**).

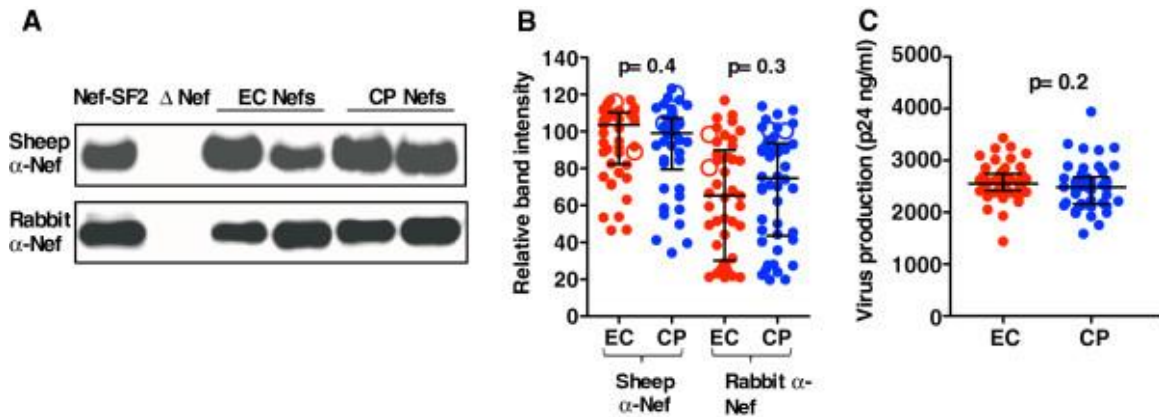


Figure 2.2.2. Nef expression and progeny virus production.

Panel A: Western blot detection of control strains NL4.3-Nef_{SF2} and NL4.3-ΔNef and two representative EC and CP-derived Nefs using two different polyclonal anti-Nef sera. *Panel B:* Relative band intensity of EC (red) and CP (blue)-derived Nefs as detected by Western blot. Open circles identify the representative data shown in panel A. Bars depict median and interquartile ranges. Statistical significance assessed using Mann-Whitney U-Test. *Panel C:* Virus production (measured as p24^{Gag} in culture supernatant) of EC (red) and CP (blue) Nef recombinant viruses.

Nef-mediated enhancement of viral infectivity and replication is impaired in EC

All viruses harboring EC Nef displayed infectivity greater than the negative control NL4.3ΔNef. Relative to control strain NL4.3-Nef_{SF2}, median EC Nef infectivity was 55% (IQR 38-76%), values that were significantly lower than CP-derived Nef (median 116, IQR 88-160%) ($p < 0.001$; **Figure 2.2.3A**). Similarly, all viruses harboring EC Nef displayed higher replication capacities than the negative control NL4.3ΔNef in PBMCs from four HIV-negative donors. Consistent with previous reports (Fackler et al., 2006; Ueno et al., 2008), replication of patient-derived Nef recombinant viruses in PBMC differed to some extent among donors; however, viruses encoding EC Nef displayed consistently poorer ability to replicate in PBMC relative to those harboring CP Nef in all donors ($p \leq 0.01$; **Figure 2.2.3B,C**). Averaged over all four donors, median [IQR] replication capacities were 34 [23-52]% for EC-derived viruses and 76 [57-98]% for CP-derived viruses, respectively ($p < 0.001$).

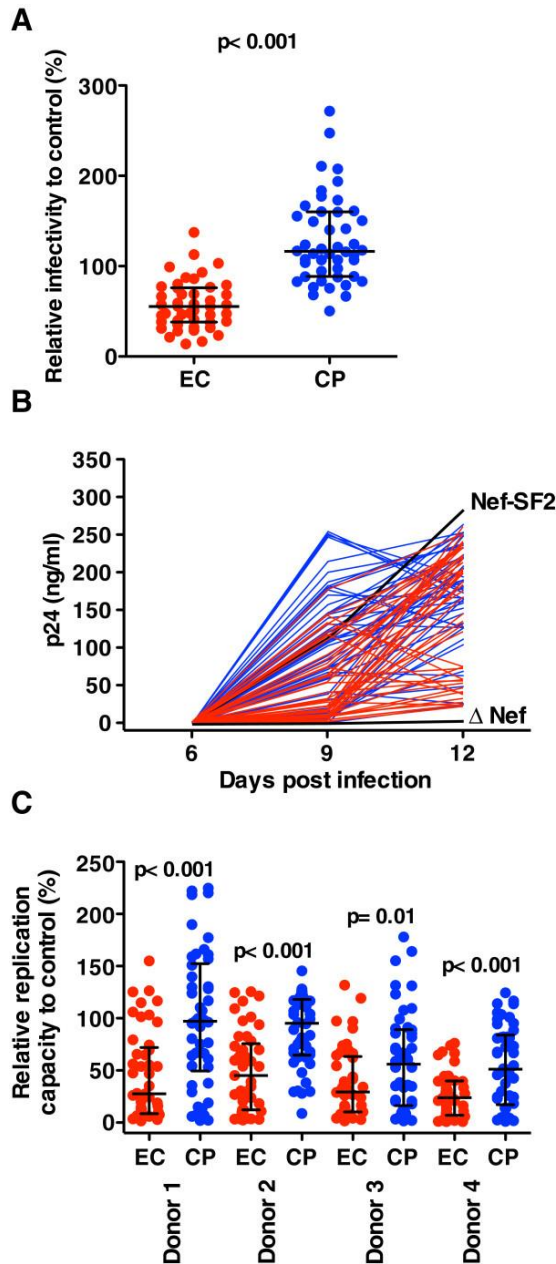


Figure 2.2.3. Ability of EC Nef to enhance viral infectivity and stimulate viral replication in PBMC.

Panel A: Scatterplots depict the ability of EC (red) and CP (blue) Nef recombinant viruses to enhance viral infectivity. Values are normalized to that of control NL4.3-Nef_{SF2}, such that values of 100% indicate replication equal to that of NL4.3-Nef_{SF2}, while values <100% and >100% indicate replication lower than or higher than that of NL4.3-Nef_{SF2}, respectively. Bars represent median and interquartile ranges. The p-value was calculated using Mann-Whitney U-test. *Panel B:* Growth curves of EC (red) and CP (blue) Nef recombinant viruses, plus control viruses NL4.3-Nef_{SF2} and NL4.3- Δ Nef, in PBMCs from donor #1. *Panel C:* Scatterplots depict the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to stimulate viral replication in PBMC from four HIV-negative donors. Values are normalized to that of control NL4.3-Nef_{SF2}. Bars represent median and interquartile ranges. P-values were calculated using Mann-Whitney U-test.

Modulation of surface HLA-I, CD74, and CD4 by EC Nef

All EC Nef sequences displayed greater ability to modulate cell-surface receptors than Δ Nef negative controls. Relative to control strain NL4.3-Nef_{SF2}, EC-derived Nef recombinant viruses maintained considerable HLA-I down-regulation activity (median 95 [IQR 79 -106] %) that was nevertheless significantly lower compared to CP Nef viruses (median 106 [IQR 96-111] %) ($p < 0.001$; **Figure 2.2.4A,B**). The ability of EC Nef viruses to up-regulate CD74 was markedly lower (median 49 [IQR 35-76] %) compared to CP Nef viruses (median 111 [IQR 68-150] %) ($p < 0.001$; Fig. 4C, 4D). HIV-1 Vpu and Env proteins contribute to surface CD4 modulation (Neri et al., 2011); therefore, Nef-mediated CD4 down-regulation activity was assessed using DNA expression plasmids. Relative to control Nef_{SF2}, most EC Nef clones maintained substantial CD4 down-regulation activity (median 91 [IQR 76-95] %) that was nevertheless significantly lower compared to CP Nef clones (median 99 [IQR 89-101] %) ($p = 0.002$) (**Figure 2.2.4E,F**).

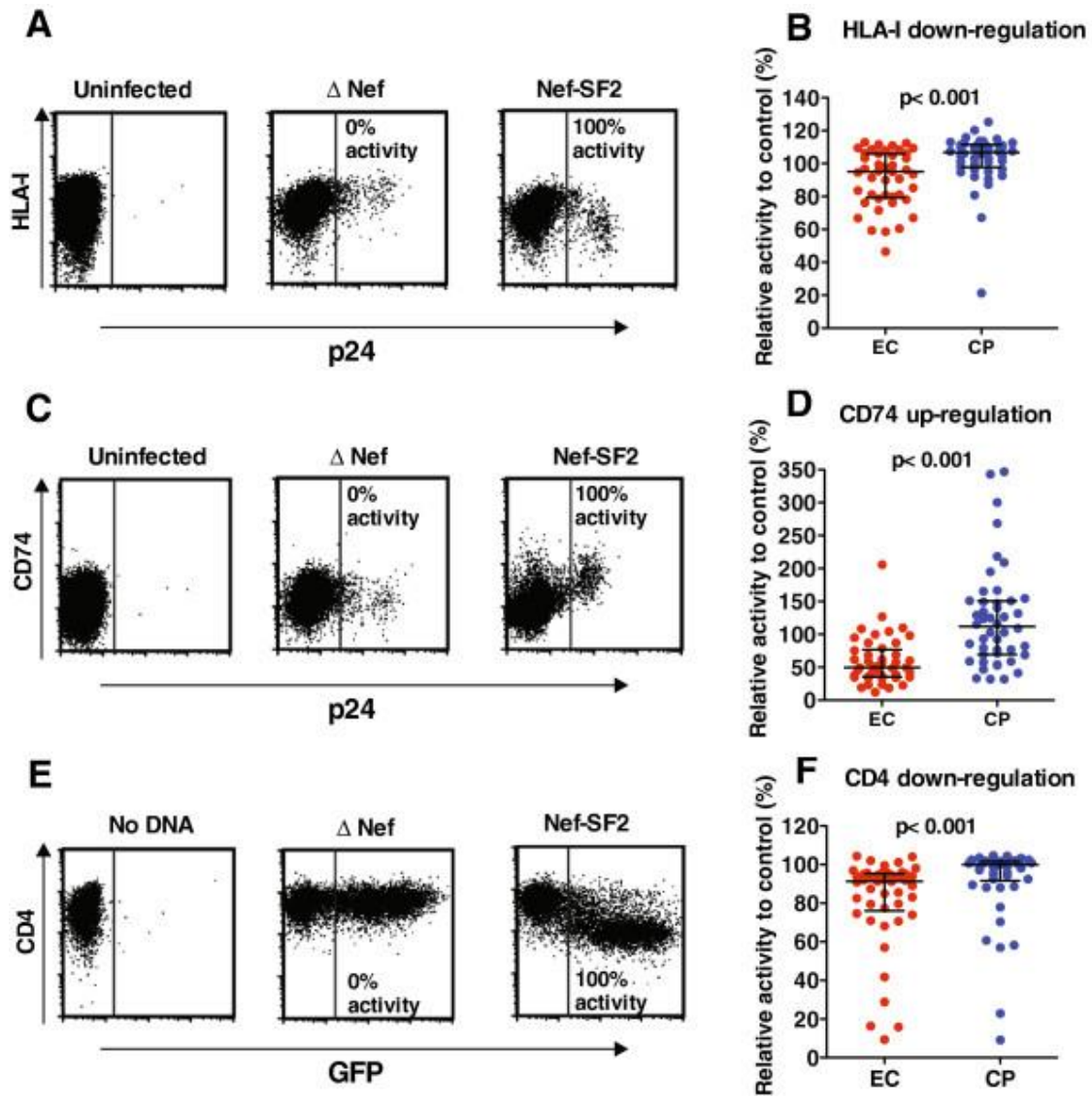


Figure 2.2.4. Ability of EC Nef to modulate cell surface receptor levels.

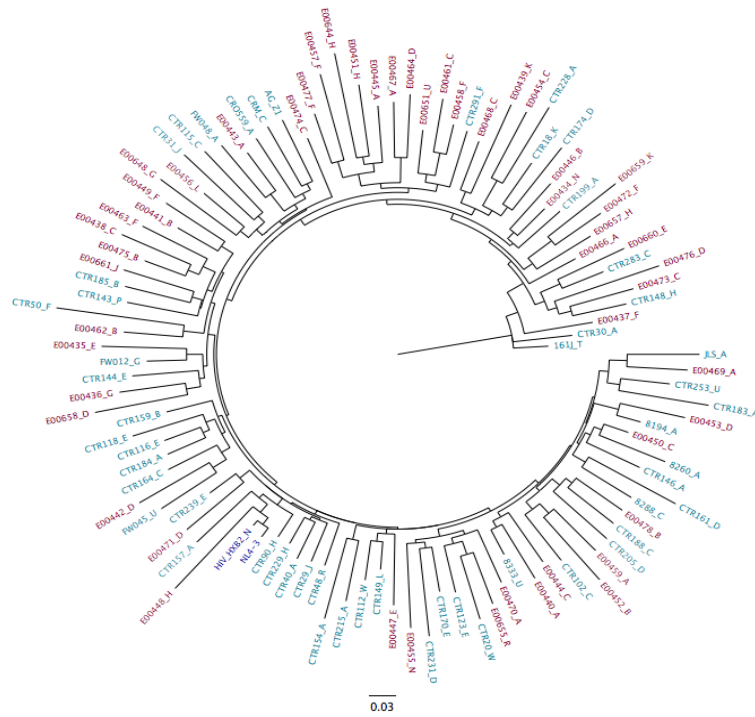
Panel A: Flow cytometry plots depicting representative staining of cell-surface HLA-I (HLA-A*2402; y-axis) vs. intracellular p24^{Gag} (x-axis) for uninfected, NL4.3- Δ Nef and NL4.3-Nef_{SF2} controls. *Panel B:* Scatterplots depicting the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to downregulate HLA-I. Values are normalized to that of control NL4.3-Nef_{SF2}. Bars represent median and interquartile ranges. P-values were calculated using Mann-Whitney U-test. *Panel C:* Flow cytometry plots depicting representative staining of cell-surface CD74 (y-axis) vs. intracellular p24^{Gag} (x-axis) using uninfected and control viruses. *Panel D:* Scatterplots depicting the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to upregulate CD74. *Panel E:* Flow cytometry plots depicting representative staining of cell-surface CD4 (y-axis) vs. GFP (x-axis) after delivery of no-DNA, Δ Nef and SF2-nef vectors. *Panel F:* Scatterplots depicting the ability of EC (red) and CP (blue)-derived Nef to downregulate CD4.

Additional EC clones were also impaired for CD4 down-regulation

There were 22 EC and 9 CP whose whose first Nef clone had a CD4 down-regulation score below 90%. For each of these individuals additional Nefs were cloned and sequenced. A second clone was selected once meeting the criteria of having nucleotide differences resulting in at least one coding change with respect to the first clone. These additional sequences are shown in phylogenetic tree in **Figure 2.2.5A**.

I tested the function of these alternative clones for CD4 down-regulation and compared them to the original clone (**Figure 2.2.5B**). Of CP whose original clones had lower than 90% function, I was able to isolate unique alternative clones from 8 individuals. The second clones had greater function than the first ($p=0.006$; **Figure 2.2.5B, left**), suggesting that while a fraction of clones from CP are impaired, there are functional sequences circulating in most of these individuals. On the other hand, in 22 EC there was remarkable similarity between the first and second clones. There was no significant difference between clone pairs ($p=0.47$, n.s.; **Figure 2.2.5B, right**), suggesting that the majority of Nef circulating in these individuals are indeed non-functional.

A



B

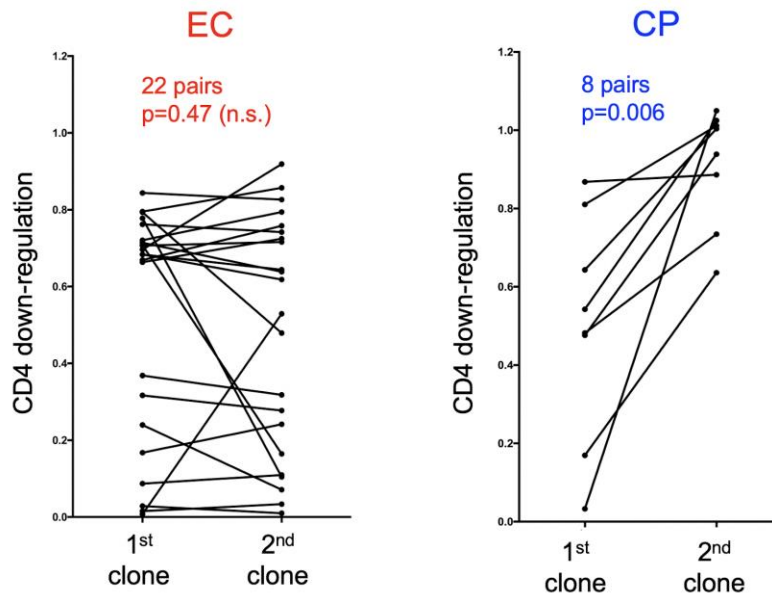


Figure 2.2.5. Alternative Nef clones.

Panel A: Phylogenetic tree of all EC and CP *nef* sequences, including alternative clones. *Panel B:* CD4 down-regulation function of alternative *nef* clones from EC and CP.

Host HLA-I allele expression and Nef function in EC

Protective HLA-I alleles, most notably B*57, are over-represented in EC (Pereyra et al., 2008; Pereyra et al., 2010). To investigate this as a potential confounder in comparisons between EC and CP, we re-analyzed our data excluding individuals who expressed HLA-B*57 (17/45 of EC and 8/46 of CP). Measures for all five Nef functions remained significantly lower among non-B*57 EC compared to non-B*57 CP (all $p < 0.01$). Exclusion of individuals expressing *any* protective allele (defined as B*27, B*57, and B*58:01) yielded similar results (all $p < 0.05$).

Immune selection by protective HLA-I alleles, including B*57, can modulate the in vitro function of certain HIV-1 proteins in EC (Brumme et al., 2011; Miura et al., 2009a). To examine whether this was also true for Nef, we stratified EC Nef sequences by host B*57 expression and observed significantly lower CD4 down-regulation activity in B*57-derived compared to non-B*57-derived EC Nefs (median [IQR] 83 [55-94]% for B*57 vs. 92 [83-97]% for non-B*57 EC, respectively, $p = 0.038$). Significant differences were not seen for the other Nef activities tested (**Figure 2.2.6**). Of 20 HLA-I alleles expressed in a minimum of five EC, correlations with Nef function were also observed for C*06 (in linkage disequilibrium with B*57; median 74 vs 93% CD4 down-regulation activity in C*06 vs. non-C*06 EC) and A*01 (median 83 vs 97% HLA-I down-regulation activity in A*01 vs. non-A*01 EC) (both $p < 0.05$; $q < 0.05$). No HLA-I associations were observed for Nef-mediated infectivity, replication, or CD74 up-regulation activity in EC.

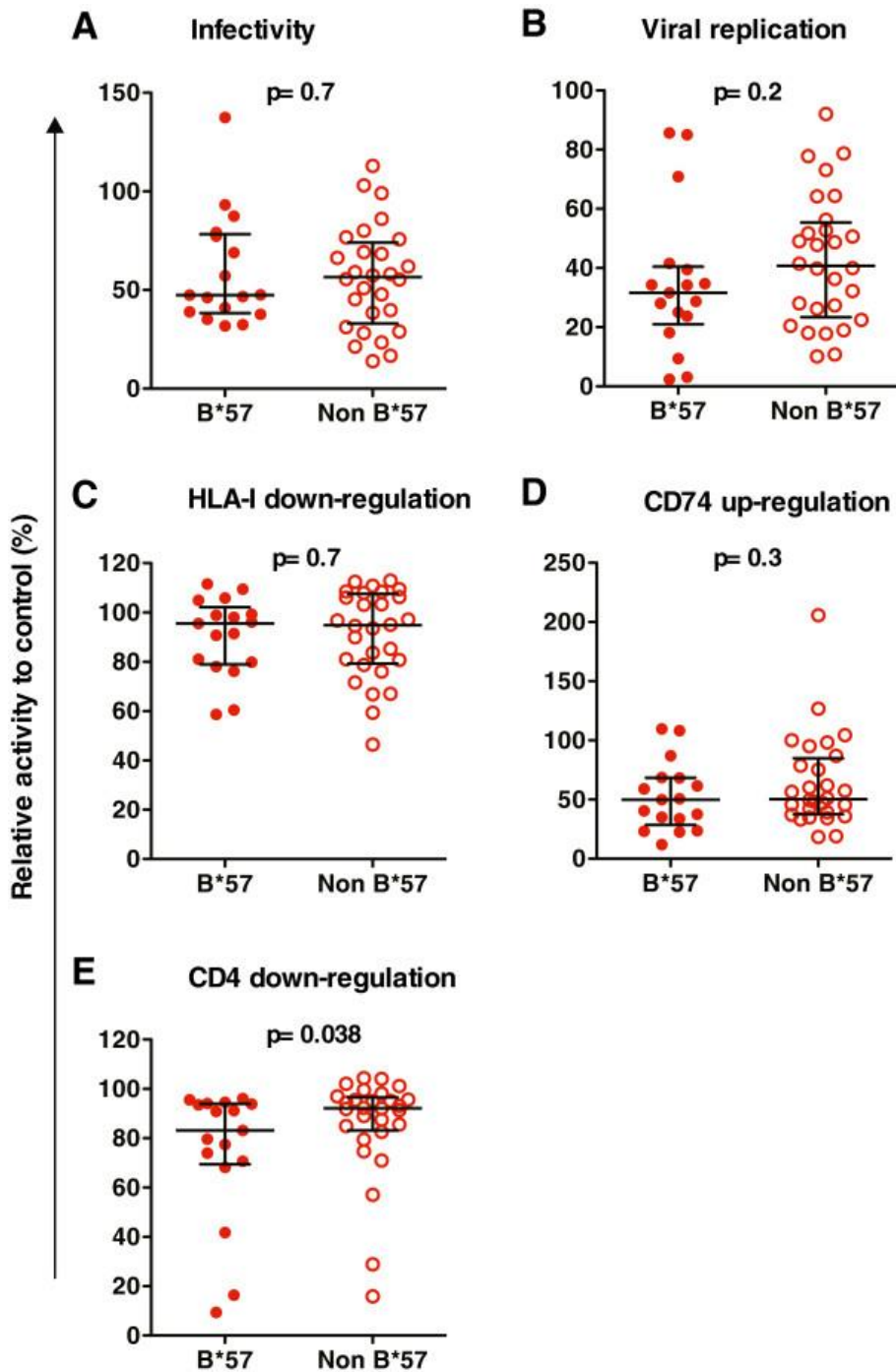


Figure 2.2.6. Relationship between host HLA-B*57 expression and Nef activities in EC.

Scatterplots depicting the ability of EC-derived Nefs from B*57-expressing (closed red circles) and non-B*57 expressing (open red circles) to enhance virion infectivity (*panel A*), enhance viral replication in PBMC (*panel B*; data depict means for all four PBMC donors), downregulate HLA-I (*panel C*), up-regulate CD74 (*panel D*) and down-regulate CD4 (*panel E*). Values are normalized to that of control NL4.3-Nef_{SF2}. Bars represent median and interquartile ranges. P-values were calculated using Mann-Whitney U-test.

Unique HLA-associated polymorphisms and Nef function in EC

Modulation of viral protein function in EC by protective HLA-I alleles may be due to the selection of unconventional HLA-associated polymorphisms (Bailey et al., 2009; Miura et al., 2009b). To examine this, we applied phylogenetically-corrected methods (Carlson et al., 2012b) to identify HLA-B*57-associated Nef polymorphisms in our EC cohort. Nine associations were observed at $p < 0.05$ ($q < 0.4$) in B*57+ EC (**Figure 2.2.7A**). With the exception of V85L, these polymorphisms were distinct from those observed in population-level analyses of B*57 progressors (Brumme et al., 2009; Carlson et al., 2012a), suggesting that they may be largely specific to EC. In contrast, a search for B*57-associated polymorphisms in our CP cohort revealed several expected Nef polymorphisms at $p < 0.05$, including V85L and H116N (Brumme et al., 2009; Carlson et al., 2012a; Frater et al., 2007), supporting our ability to identify HLA-associated polymorphisms in modestly-sized datasets, and indicating that the novel polymorphisms observed in EC merited further attention.

Among B*57-expressing EC (N=17), we observed significant inverse relationships between the number of EC-specific B*57-associated polymorphisms and Nef-mediated replication (Spearman $R = -0.5$, $p = 0.04$), HLA-I down-regulation ($R = -0.57$, $p = 0.02$) and CD74 up-regulation ($R = -0.6$, $p = 0.01$); and a modest, albeit not statistically significant, negative correlation with CD4 down-regulation ($R = -0.37$, $p = 0.1$) (**Figure 2.2.7**). No similar correlations were observed between EC-specific polymorphisms associated with other HLA-I alleles observed at comparable frequencies in our cohort (i.e. A*01, A*02, A*03, A*30, C*06, C*07) and Nef function in EC expressing these alleles.

Amino acids associated with EC Nef function

Despite the lack of obvious phylogenetic clustering in EC or CP sequences (**Figure 2.2.1**), nine amino acids were significantly enriched among EC compared to CP clones: 10V, 11V, 14P, 23A, 28D, 33V, 64E, 153I, and 174E ($p < 0.05$, $q \leq 0.05$). Of these, 28D (**Figure 2.2.7A**), and to a lesser extent 10V, 14P, 33V and 174E are due, at least in part, to enrichment among B*57+ EC. To investigate relationships between Nef sequence and function in EC, we performed an exploratory analysis to correlate amino acids with each of the five functions tested, regardless of host HLA. A total of 23 polymorphisms occurring at 14 sites were associated with Nef-mediated modulation of

HLA-I, CD74 and CD4 ($p < 0.05$, $q < 0.4$; Table 2.2.1). No Nef polymorphisms associated with infectivity or replication were observed at this threshold.

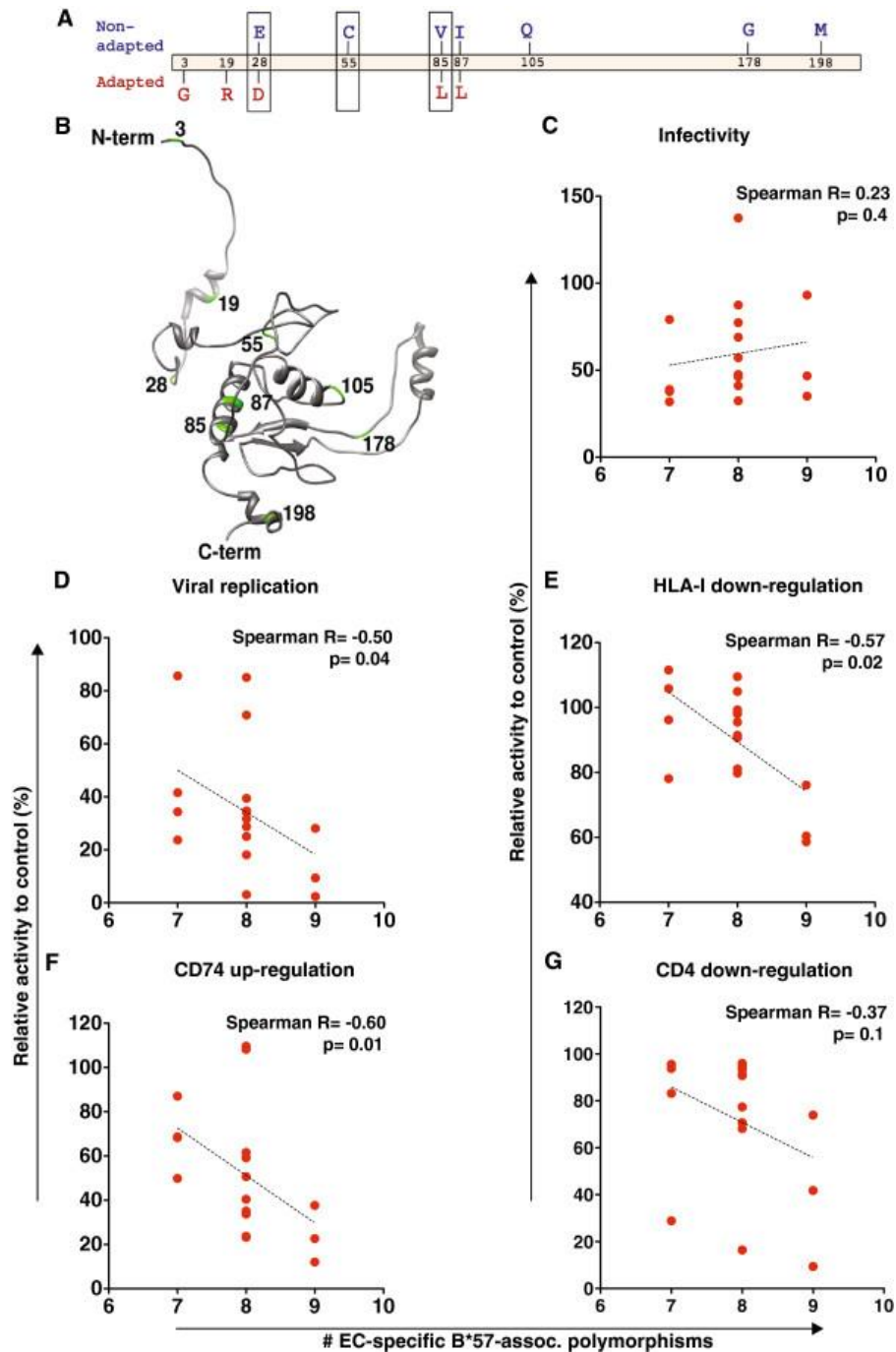


Figure 2.2.7. HLA-B*57-associated polymorphisms in EC, and their relationship with Nef function.

Panel A: Two-dimensional map of B*57-associated polymorphisms identified in an exploratory analysis of the EC cohort using phylogenetically-corrected approaches with $p < 0.05$ (Carlson et al., 2012a). “Nonadapted” forms (those less likely to be observed in the presence of B*57 at a specific location) are shown above the Nef protein in blue; “adapted” forms (those enriched

among B*57-expressing persons) are shown below the Nef protein in red. Due to limited statistical power and other reasons, both nonadapted and adapted forms are not always identified at a given position. Boxed codons indicate those where the amino acid varied in B*57 EC; the remainder were either expressed in 100% of B*57 EC (adapted forms 3G and 19F) or 0% of B*57 EC (nonadapted forms 105Q, 178G, 198M). In subsequent analyses, Nef sequences from B*57-expressing persons were counted as harboring a B*57-associated polymorphism at a given site if that site expressed anything other than the nonadapted form, or the adapted form if no nonadapted form was identified. *Panel B*: The locations of the nine EC-specific B*57-associated polymorphisms are indicated in green on the structure model of the Nef protein (composite crystal structure kindly provided by Art F. Y. Poon, (Poon et al., 2010)) *Panels C-G*: Correlations between the number of B*57-associated polymorphisms in Nef sequences from B*57-expressing EC, and five Nef functions evaluated. Statistical analyses were done using Spearman's correlation.

Table 2.2.1 Analysis of residues associated with EC Nef functions (all N>5 and q<0.4)

Nef activity	Position on HXB2	AA ^a	No. of subjects ^b		Relative Nef activity (%)		p-value	q-value
			With AA	Without AA	With AA	Without AA		
HLA-I down-regulation	8	S	17	27	83.6	97.2	0.01	0.3
	8	R	14	30	106.1	91.1	0.02	0.3
	11	V	20	21	87.2	98.9	0.01	0.3
	14	P	34	11	91.1	108	0.004	0.3
	46	S	36	8	96.7	77.1	0.01	0.3
	108	D	34	11	91.1	105.8	0.02	0.3
	108	E	11	34	105.8	91.1	0.02	0.3
	138	C	5	40	79.8	96.5	0.02	0.3
	138	T	40	5	96.5	79.8	0.02	0.3
	192	H	36	9	97	78.1	0.01	0.3
	192	R	6	39	84	96.8	0.04	0.4
	205	N	22	23	101	83.6	0.02	0.3
205	D	22	23	85.9	98.9	0.03	0.3	
CD74 up-regulation	8	S	17	27	37.7	60	0.004	0.3
	28	E	14	31	73.7	42.4	0.008	0.3
	28	D	29	16	40.4	65.3	0.009	0.3
	135	F	10	35	35	57.5	0.003	0.3
	135	Y	34	11	57.1	35.1	0.01	0.3
CD4 down-regulation	8	S	17	27	84.8	92.6	0.007	0.3
	14	P	34	11	86.1	97.9	0.003	0.1
	14	S	5	40	102.9	87.2	0.02	0.4
	15	A	11	34	76.9	91.3	0.02	0.4
	21	K	11	34	70.7	91.6	0.002	0.1
	21	R	28	17	91.6	76.9	0.02	0.4
	105	R	12	33	76.6	91.9	0.003	0.1
163	C	9	36	98.1	86.6	0.02	0.4	

^a AA, amino acid.

^b Row totals vary depending on the codon position because sequences with gaps in the alignment are considered missing data.

2.2.5. Discussion

We assessed five in vitro Nef functions using clonal plasma HIV RNA sequences from 45 EC and 46 CP. We observed that EC Nef sequences were generally functional,

especially for Nef's most characteristic activities, CD4 and HLA-I down-regulation. Nevertheless, median EC Nef activities were significantly lower for all five functions when compared to those from CP. Median CP Nef activities were consistent with that of HIV-1 strain SF2 used as a normalization control for all assays, indicating that our selection of chronic Nef clones is representative of highly functional Nef isolates examined previously. The range in Nef activities observed here may help to resolve discrepancies between previous studies of HIV long-term non-progressors and controllers that investigated only one or two Nef functions in fewer patients, some of which reported relative preservation of CD4 and/or HLA-down-regulation function (Nou et al., 2009; Zuo et al., 2012), while others reported inefficient Nef-mediated CD4 and/or HLA-down-regulation (Casartelli et al., 2003; Corro et al., 2012; Tobiume et al., 2002) and infectivity (Tobiume et al., 2002) compared to CP. Our data indicate that there is *in vivo* pressure on Nef in EC to maintain CD4 and HLA-I down-regulation functions.

Relative functional impairments between EC and CP are not likely to be due to differences in Nef protein stability or expression levels, nor to recent descent from a defective common ancestor. Similarly, enrichment of protective HLA-I alleles in EC is not likely to be the sole explanation for relative functional attenuation, as differences between groups persisted after excluding persons who expressed protective HLA-I alleles from analysis. Rather, our results are consistent with functional variability of naturally occurring Nef sequences from EC, which may be attributable in part to non-canonical HLA-associated escape mutations in this group. Indeed, analysis of Nef amino acid variation revealed nine residues (10V, 11V, 14P, 23A, 28D, 33V, 64E, 153I, and 174E) significantly enriched in EC compared to CP. Although the underlying mechanism is unclear, our observation that 28D is a B*57-associated polymorphism unique to EC (and that 10V, 14P, 33V and 174E are also enriched, although not significantly so, in B*57+ EC) suggests that some of these mutations represent host immune-driven polymorphisms in this population. Furthermore, 11V, 14P and 28D were also associated with significantly lower Nef activity among EC sequences (**Table 2.2.1**), supporting these as polymorphisms that are both enriched in, and contributing to, EC functional impairments.

Building upon previous studies of recombinant viruses encoding *gag* and *pol* sequences from the same EC cohort (Brumme et al., 2011; Miura et al., 2009a), our results support a complex relationship between B*57-associated immune pressures and

Nef function. Although significantly lower CD4 down-regulation activity was observed in B*57 compared to non-B*57 EC (**Figure 2.2.6E**), this was not true for other Nef functions, indicating that B*57 expression alone does not guarantee Nef attenuation in this group. To assess the influence of HLA-associated viral polymorphisms on HIV-1 protein function, we and others have drawn upon lists of HLA-associated polymorphisms derived from population-level studies of chronically infected individuals (Brumme et al., 2009; Carlson et al., 2012a); however, such lists may not adequately capture rare mutations unique to EC (Bailey et al., 2009; Miura et al., 2009b). Therefore, in this study, we used our EC dataset to identify HLA-associated polymorphisms specific to this population. Statistical power is a major limitation, but HLA-associated polymorphisms can be identified in modestly sized datasets (e.g.: Bhattacharya et al., 2007), especially if analyses are limited to specific alleles. At $p < 0.05$, we identified nine B*57-associated polymorphic sites specific to EC (**Figure 2.2.7A**), most of which differed from B*57-associated polymorphisms commonly identified in population-level analyses (Brumme et al., 2009; Carlson et al., 2012a). In contrast, B*57-associated polymorphisms identified in our CP dataset using the same approach were largely as expected (Brumme et al., 2009; Carlson et al., 2012a; Frater et al., 2007). ELISpot reactivity to overlapping peptides spanning codons 28, 55, 85, 178 and 198 has been documented in B*57+ EC (Bailey et al., 2009) and V85L has been described to function as an escape mutation in the B*57/58-KF9 epitope (O'Connell et al., 2009), further suggesting that these polymorphism may be due to CTL selection pressure in B*57 EC.

Notably, we observed dramatic inverse associations between the number of EC-specific B*57-associated polymorphisms and Nef-mediated replication, HLA-I down-regulation and CD74 up-regulation. CD4 down-regulation, the only function in which host expression of B*57 was in itself significantly associated with poorer function in EC, also displayed a modest, albeit not significant, negative relationship between the burden of B*57-associated escape mutations and function (**Figure 2.2.7**). These remarkable inverse relationships were particular to B*57 and not observed for other HLA alleles with similar frequency in our EC cohort. Taken together, results suggest that HLA-B*57-associated CTL pressures select for non-canonical polymorphisms in EC, which contribute additively to multiple functional impairments in EC Nef.

In an exploratory analysis of Nef amino acid sequences, we identified 23 polymorphisms, located at 14 residues, associated with Nef function in EC. None

overlapped with mutations previously identified in HIV non-progressors (Kirchhoff et al., 1999) or with sites reported to affect HLA-I down-regulation activity in chronic infection (Lewis et al., 2012), a discrepancy that might be due to non-canonical polymorphisms observed in EC. Of interest, 8S was associated with Nef-mediated modulation of cell-surface CD4, HLA-I and CD74, an observation consistent with codon 8's involvement in myristylation (Geyer et al., 2001). Also, 28D and 105X (in this case R), associated with modulation of CD74 and CD4, respectively, are EC-specific B*57-associated polymorphisms identified in the present study.

Viral genetic studies of EC feature numerous challenges and limitations. Although care was taken to choose a Nef clone that reflected each patient's original bulk HIV RNA sequence, and to rule out proviral DNA contamination, potential biases associated with PCR amplification from extremely low copy-number templates must be acknowledged. However, analysis of second clones from a subset of patients (**Figure 2.2.5**) showed that while low-functioning clones in CP could be affected by random mutations or PCR errors, the majority of impaired EC sequences seem to be functionally representative of circulating virus. Furthermore, use of a single sequence per patient alleviates potential biases associated with quasispecies approaches to compare samples with low vs. high genetic diversity. As our goal was to specifically investigate the function of Nef in EC, we employed recombinant virus (and single-protein expression) approaches to eliminate potential confounding effects of other HIV-1 proteins; however, such approaches may not reflect the characteristics of infectious molecular clones or whole-virus isolates recovered from PBMC, procedures that are rarely successful in EC (Julg et al., 2010; Miura et al., 2008). Recombinant virus approaches are also inherently limited by potential incompatibilities between insert and backbone; our choice of a recombinant control strain (NL4.3-Nef_{SF2}) alleviates this to a minor extent. Although we assessed Nef activity using primary PBMCs and immortalized cell lines, Nef's multiple functions (Arhel and Kirchhoff, 2009; Arien and Verhasselt, 2008; Foster et al., 2011; Kirchhoff et al., 2008) may vary in different cell types (Kirchhoff et al., 2008; Mwimanzi et al., 2011; Suzu et al., 2005). Furthermore, the *in vivo* relevance of our observations - in particular, the extent to which these functional differences contribute to the viremia control in EC - remains unclear. Although our results are consistent with the transmission of partially attenuated Nef sequences in at least some EC, and/or further immune-mediated attenuation in others (Brumme et al., 2011; Lobritz et al., 2011; Miura et al., 2009a), it is not possible to disentangle cause and

effect in cross-sectional studies. Furthermore, Nef function may change over the infection course (Carl et al., 2001), therefore longitudinal analysis of Nef function in controllers, is warranted. Finally, although our results suggest that non-canonical polymorphisms in EC may contribute to attenuated Nef function, it will be important to validate these findings in larger EC cohorts. Despite these limitations, our study represents the largest linked analysis of multiple *in vitro* Nef functions in EC to date, and to our knowledge the only study assessing CD74 up-regulation and replication capacity in this group.

In summary, EC Nefs were generally functional; however, all five activities assessed were significantly impaired compared to CP. HLA-I-restricted immune pressure, most notably by B*57, may contribute to the differences observed. Taken together with previous studies of HIV Gag, Pol, and Env function in EC (Brumme et al., 2011; Lassen et al., 2009; Miura et al., 2009a), our results support decreased viral protein function as a hallmark of the EC phenotype and underscore the potential role of immune pressures in modulating viral protein function in this rare group.

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Chapter 3. Mechanisms of Nef mediated modulation of TCR signaling

Section 3.1.2 and Table 3.1.1 in this chapter are adapted from the following published review article, of which I was the first researcher and primary drafter.

Markle, T.J., Mwimanzi, P., Brockman, M.A., 2013. HIV-1 Nef and T cell activation: A history of contradictions. *Future Virology* 8(4). doi: 10.2217/fvl.13.20

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3.1. Introduction

3.1.1. Nef and T cell activation

Anti-retroviral therapy can control HIV replication and delay the onset of AIDS; however, the viral genome remains dormant in cellular reservoirs and discontinuation of drugs results in re-emergence of viremia and disease progression (Eisele & Siliciano 2012). A better understanding of how latent reservoirs are established and maintained will inform therapeutic strategies to eradicate HIV infection, and is therefore a research priority.

The activation state of a CD4⁺ T cell is an important determinant of HIV permissiveness and pathogenesis - and it may also regulate cellular mechanisms that contribute to viral latency (Karn, 2011). HIV replication occurs productively in highly activated CD4⁺ T cells, where appropriate signal transduction factors and metabolic products are readily available. In contrast, “resting” T cells are relatively resistant to viral replication and may be more prone to result in a latent infection (Stevenson et al., 1990).

Nef is reported to modulate CD4⁺ T cell activation (Foster et al., 2011); however, existing literature on this topic is controversial, and results are inconsistent (Greenway et al., 2003). Initial reports indicated that Nef suppresses HIV replication as well as T cell receptor mediated signaling, hence its name “Negative factor” (Luciw et al., 1987; Terwilliger et al., 1986). However, others suggest that Nef enhances T cell signaling, leading to increased HIV replication and higher plasma viral loads (Spina et al., 1994; Miller *et al.* 1994).

3.1.2. Making sense of discrepancies in the literature

Research on Nef and T cell activation has yielded divergent results. On the one hand, substantial data suggests that Nef enhances activation – including increased nuclear translocation of transcription factors and IL-2 expression. On the other hand, Nef appears to disrupt TCR signalling – including reduced Lck activity, LAT phosphorylation, and cytoskeletal reorganization. These contradictions are not new to the field, and several factors have been proposed to contribute to these diverse observations.

Intracellular localization

Baur *et al.* investigated the importance of intracellular localization by fusing Nef to CD8 glycoprotein (Baur *et al.*, 1994). They observed that plasma membrane-targeted Nef activated TCR signalling and induced the NF- κ B pathway, whereas cytoplasmic Nef inhibited both of these events (Baur *et al.*, 1994). The notion that Nef causes differential effects on T cell activity depending upon its intracellular location remains an attractive hypothesis to resolve discrepancies in experimental results. While early work by Kaminchik *et al.* indicated that a naturally occurring N-terminally truncated Nef protein failed to localize to the plasma membrane (Kaminchik *et al.*, 1991), to our knowledge, potential differences in localization of patient-derived Nef alleles has not been examined systematically. In addition, the concentration of Nef or the method used for protein expression (transient or stable) might also affect the relative localization of Nef within a cell, thereby shifting the balance of activating and inhibitory mechanisms.

Alternative signalling pathways

The Fackler research group has reported a series of observations showing that although Nef disrupts classical TCR signalling, this protein also organizes an alternative pathway that can enhance cellular activation. Abraham *et al.* demonstrated that Nef inhibited early events in TCR signalling downstream of ZAP-70, including LAT recruitment to TCR microclusters, SLP-76 activation, and actin reorganization (Abraham *et al.*, 2012). Notably, Nef also redirects Lck from the plasma membrane to the Trans-Golgi Network (TGN) and stimulates Lck-mediated activation of Ras/MAPK signalling (Pan *et al.*, 2012). A review by Abraham and Fackler provides additional discussion of this topic (Abraham and Fackler, 2012).

Cell type and intrinsic activation state

Numerous reports utilizing immortalized Jurkat T cell lines have reported either Nef-mediated enhancement or inhibition of T cell activation (see **Table 3.1.1**), and it remains an important task to systematically evaluate and explain the contradictory results generated using presumably similar cells. Despite their utility as a well-established model for analysis of T cell function, there are pitfalls to using Jurkat cell for Nef studies. Jurkat tumour cells display a constitutive partially-active phenotype that manifest in an idiosyncratic expression profile of several signalling molecules, which has led to mischaracterization of PI3-K-dependent signalling events (Astoul *et al.*, 2001) and

of activation-dependent mechanisms of Nef-mediated HLA Class I down-regulation (Dikeakos et al., 2010; Yi et al., 2010). Therefore, Nef's effect on activation in Jurkat cell lines may not be fully representative of its effect in primary cells. A positive role for Nef in HIV-1 replication was demonstrated in primary CD4+ T cells (Spina et al., 1994) and Nef-mediated enhancement of TCR signalling can contribute to this outcome in primary cells (Schrager et al., 2002; Schrager and Marsh, 1999). Although these and other results using primary cells support an emerging consensus that Nef is a "positive factor", Neri *et al.* recently reported that the intrinsic activation state of primary T cells is important for Nef function, since Nef was able to enhance IL-2 expression in quiescent CD4+ T cells, but not in pre-activated cells (Neri et al., 2011). Thus, it remains too simplistic to argue that Nef's negative effects on T cell signalling are merely artefacts of using Jurkat cell lines, or that Nef always promotes activation in primary cells. Subtle differences in the phenotype of primary T cells, how they are prepared or propagated, or inherent differences between cell lines may affect signalling pathways or protein interactions and tip the balance of Nef function in favour of T cell activation or suppression.

Table 3.1.1. HIV-1 Nef and T cell activation: Contradictory observations *

Cell type	Expression System	Positive effect	No effect	Negative effect
Jurkat cells	Transient	Manninen 2000, 2001 • PMA; NFAT Witte, 2008 • CD3/CD28; ERK	Yoon, 1999 • PHA/PMA; NF-kB, AP-1 Manninen, 2000 • CD3/CD28; NFAT Tuosto, 2003 • APC; NF-kB	Niederman, 1992 • PHA/PMA; NF-kB Liu, 2001 • CD3/CD28; CD69 Tuosto, 2003 • APC; NFAT Haller, 2006, 2007 • CD3/CD28; actin rings • CD3/CD28; Lck
	Stable	Baur, 1994 • CD3; NF-kB • Membrane-targeted Nef Schrager, 1999 • CD3/CD28; IL-2		Luria, • PHA/PMA; IL-2 Niederman, 1992 • PHA/PMA; NF-kB, IL-2, HIV-LTR

		Wang, 2000 • CD3/CD28; IL-2, NFAT, NF-kB Liu, 2001 • CD3/CD28; IL-2 Fenard, 2005 • CD3/CD28; NFAT, NFkB		Bandres/Ratner 1994 • CD3; AP-1, NF-kB Baur, 1994 • CD3; Ca ²⁺ , NF-kB, TCR • Intracellular Nef Collette, 1996 • PMA/CD2; IL-2
	Virus infection	Fortin, 2004 • CD3/28; NFAT, AP1, NF-kB, IL-2 Schindler, 2006 • CD3/CD28; NFAT		Thoulouze, 2006 • APC; IL-2, Lck
Primary T cells	Transient	Keppler, 2006 • CD3/CD28; IL-2		Greenway, 1995 • IL-2; proliferation • PHA; Lck
	Stable	Schrager/Marsh, 1999 • CD3/CD28; IL-2 Schrager, 2002 • CD3; ERK	Schrager, 2002 • CD3/CD28; NF-kB	
	Virus infection	Neri, 2011 • CD3/CD28; IL-2, NFAT Pan, 2012 • APC; ERK	Arhel, 2009 • APC; IL-2 • CD3/28; Zap-70 Neri, 2011 • CD3/28; NFkB, Lck	Thoulouze, 2006 • APC; Lck

* Selected publications describing the effect of HIV-1 Nef on T cell activation, referenced by first author and year. Stimulation reagent(s) and outcome(s) measured are indicated. *Abbreviations:* CD3 (anti-CD3 antibody), CD28 (anti-CD28 antibody), PHA (phytohaemagglutinin), PMA (phorbol 12-myristate 13-acetate), APC (peptide-pulsed antigen presenting cells), IL-2 (interleukin-2), NFAT (nuclear factor of activated T-cells), AP-1 (activator protein 1), NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), Lck (lymphocyte-specific protein tyrosine kinase), ERK (extracellular signal-regulated kinase), TCR (T cell receptor).

Stimulation conditions

Early experiments observing Nef-mediated inhibition stimulated cells using PHA/PMA (Luria et al., 1991; Niederman et al., 1992) or anti-CD3 antibodies alone (Bandres and Ratner, 1994; Baur et al., 1994; Rhee and Marsh, 1994), while one related study found that stimulation with anti-CD28 did not affect Nef's inhibitory role (Collette et

al., 1996c). In contrast, later studies have employed anti-CD3 and anti-CD28 that is either cross-linked (Fenard et al., 2005; Fortin et al., 2004; Keppler et al., 2006; Schragger and Marsh, 1999; Wang et al., 2000), presented on beads (Arhel et al., 2009; Liu et al., 2001; Schragger et al., 2002) or bound to coverglass to mimic the immune synapse (Haller et al., 2007); however, the stimulation dose in these assays may not reflect biological interactions with APC. This limitation may be overcome by activating T cells with peptide-pulsed APCs (Arhel et al., 2009; Thoulouze et al., 2006; Tuosto et al., 2003). It would be of interest to investigate systematically whether reagents or dose can contribute to divergent findings.

Given that early and late signalling events require different kinetics, assay duration may be a complicating factor. Many early studies noting a negative role for Nef on activation looked at transcription factor translocation or IL-2 transcription, requiring stimulation of 4 hours (Bandres and Ratner, 1994; Luria et al., 1991; Niederman et al., 1992) or less (Baur et al., 1994), while those assessing proximal TCR signalling events measured outcome after only minutes (Arhel et al., 2009; Baur et al., 1994; Haller et al., 2007; Neri et al., 2011; Thoulouze et al., 2006). In contrast, many studies noting a positive role for Nef used IL-2 secretion or IL-2/NFAT-driven luciferase as activation markers, and often required longer assay times (Liu et al., 2001; Neri et al., 2011; Schragger and Marsh, 1999; Wang et al., 2000). It would be interesting to investigate whether longer stimulation conditions allow for compensatory processes or feedback loops.

Studies over the past decade have attempted to reconcile a long history of divergent results by proposing more sophisticated models of HIV-1 Nef's ability to modulate T cell activation state. Rather than describing Nef as a "negative" or a "positive" factor, new results indicate that this protein may act in both capacities in virus-infected cells. By inhibiting proximal TCR-mediated signalling events, Nef can prevent AICD that would result in premature death of virus-infected cells. By redirecting Lck and other kinases to intracellular compartments, Nef can stimulate transcription factor activity that is required to maintain viral gene expression and to enhance progeny virion production. Different *in vitro* experimental approaches may favour one of these mechanisms over the other, leading to apparently contradictory observations; however, it is likely that both Nef functions contribute to the ability of this protein to promote *in vivo*

HIV-1 replication and pathogenesis. Additional details of these mechanisms may provide novel targets for antiviral therapy that can counteract Nef's impact on T cell signalling.

3.1.3. Approach of this chapter

In this chapter, I aim to clarify divergent results presented by others in the field by systematically examining the ability of Nef to modulate the activation state of CD4⁺ T cells in the context of varying stimulation conditions. First, I explore the ability of Nef to modulate T cell receptor signaling by stimulating Jurkat T cells' TCR/CD3 antibody and measuring transcription factor activity with promoter-drive luciferase vectors. Next, I attempt to trace the source of Nef's impact on downstream signals to membrane proximal phosphorylation events, including activation of Linker of Activated T cells (LAT) and associated Phospholipase C (PLC)-gamma, which initiates calcium flux and transcription factor activity. To further identify Nef domains that participate in modulation of TCR signaling, I have constructed a large panel of Nef mutants and tested their function using a novel NFAT-luciferase assay. Finally, Nef mutants that were defective for NFAT-inhibition were further examined via microscopy to determine cellular localization, proximity to Lck (employing Proximity Ligation Assay), and ability to internalize Lck to the trans-golgi network (TGN).

3.2. Nef-mediated inhibition of cellular transcription factor activity

3.2.1. Background

The impact of Nef on T cell receptor (TCR) signaling has elicited controversy, with some reporting positive effects and others negative effects. One layer of complexity is that TCR activation triggers multiple cascades and result in activation of various transcription factors including NFAT, AP-1 (Fos-Jun), and NFkB (see **Figure 1.8.1**).

Nef is reported to interact with numerous cell proteins that could affect TCR-mediated signaling, including Lck and CD4, which are associated with the TCR-CD3 complex; signaling enhancers such as the scaffold protein LAT, adapter protein SLP-76 and Rho family GTPase Vav, which contribute broadly to activation of NFAT, AP-1 and NFkB transcription factors; and downstream molecules like MAP kinases, which promote

AP-1 activation. To examine the breadth of T cell signals modulated by Nef, I began by assessing transcription factor activity. Transcription factors are distal players in signaling cascades, and therefore can be considered as a general measure of Nef function that incorporates all upstream signaling events. Another benefit of using transcription factor activation as a readout for TCR signaling is that it can be measured using promoter-driven vectors. When activated, transcription factors translocate into the nucleus, where they can bind DNA promoter regions and drive expression of reporter proteins such as luciferase.

I set out to develop an *in vitro* system in immortalized Jurkat T cell lines that uses promoter-driven luciferase reporter constructs to assess the impact of Nef on multiple T cell receptor/CD3-mediated signaling pathways, including NFAT and AP-1. I tested a different stimulation agents at a range of doses to develop an *in vitro* assay that could approximate, as best we could, biologically relevant triggers and strength of TCR signaling.

3.2.2. Methods: Transcription factor-driven luciferase signaling assay

Jurkat T cells were co-transfected wild type Nef (SF2 strain) or empty vector (No Nef) expression plasmids along with transcription factor luciferase reporter vectors. For each transfection, 10^7 Jurkat T cells were resuspended in 200 μ L Opti-MEM medium without phenol red (Thermo Fisher Scientific), then electroporated with 10 μ g of pSELECT-GFPzeo encoding Nef or no Nef, plus 5 μ g of pNFAT-, AP1-, or NFkB-luciferase (Agilent Biosciences) in 96-well plates using a BioRad GenePulser MXCell™ instrument (square wave protocol: 250 V, 2000 μ F, infinite Ω , 25 milliseconds, single pulse). I used a 2:1 ratio of pSELECT to luciferase-reporter so as to minimize the number of transfectants containing the reporter but not the pSELECT-Nef vector.

Transfected cultures were recovered for 18 hours in 2 ml of RPMI 1640 medium without phenol red, supplemented with 2 mM L-glutamine, 1000 U/ml Penicillin and 1 mg/ml Streptomycin (R10+) (all from Sigma-Aldrich) at 37°C plus 5% CO₂ to allow for Nef expression. After 18 hours, cultures were single cell sorted for GFP (as a marker for Nef transfection). 10^5 cells were resuspended in 100 μ L RPMI (as above) and stimulated for 6 hours on flat-bottomed 96-well culture plates (Greiner CELLSTAR) pre-coated with 0.01-1 μ g/mL of anti-CD3 antibody and/or anti-CD28 (OKT3 clone; eBioscience).

Luciferase activity was detected using the Steady-Glo® Luciferase Assay system (Promega) by mixing substrate/lysis solution (100 µL) with stimulated cells (100 µL) on a flat-bottom, white polystyrene microplate (Corning or Grenier). Luminescence was measured as absolute light units (ALUs) using a Tecan Infinite M200 PRO plate reader (2000 ms integration time; 100 ms settle time). See **Figure 3.2.1** for a schematic of the method.

In parallel, 10⁵ cells were assessed by flow cytometry for transfection efficiency (GFP expression) and viability using annexin V-APC antibody and 7-AAD (both from BioLegend).

To stimulate Jurkat cells with antigen presenting cells (APCs), I employed a reporter T cell assay system that was published previously by our lab (Anmole et al., 2015). Briefly, effector Jurkat cells were co-transfected with NFAT-luciferase and pSELECT-Nef (as described above), plus pSELECT-derived vectors expressing the alpha and beta chain genes (3 µg each) of TCR clone 5B2, which is specific for the A*02-restricted Gag FK10 epitope (FLGKIWPSYK), along with 5 µg of a CD8 alpha expression plasmid pORF9-hCD8A (InvivoGen). For target cells, I used a CEM*SS T cell line (cat #776 from Dr. Peter L. Nara; Nara et al. 1987) that was retrovirally transduced to constitutively express HLA A*02. These CEM-A*02 cells were then peptide-pulsed with titrations of the Gag FK10 epitope. 100,000 effector cells and 20,000 target cells were co-cultured at the optimal 5:1 ratio in 100 µl of Optimem for 6 hours, to which 100 µl luciferase was added and analyzed by plate reader as above. (See Anmole et al., 2015 for more background on effector and target cell construction and detailed co-culture methods).

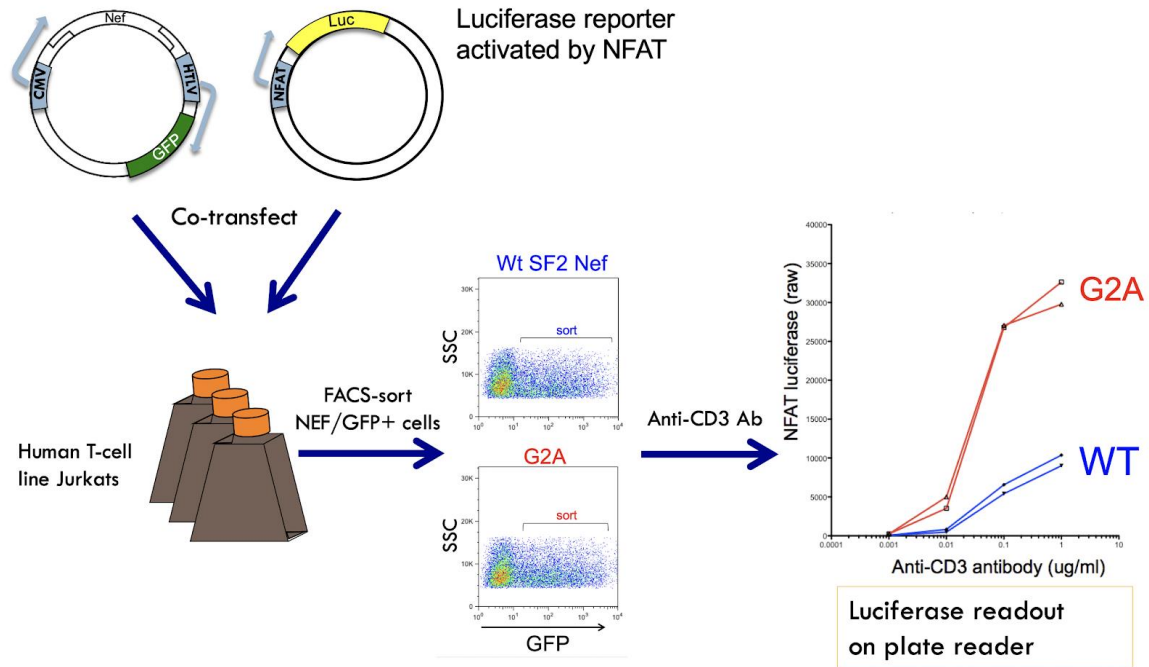


Figure 3.2.1. Methods of promoter-driven luciferase reporter assay.

Jurkat T cells were co-transfected wild type Nef (SF2 strain) or empty vector (No Nef) expression plasmids along with transcription factor luciferase reporter vectors. After 18 hours, cultures were single cell sorted for GFP (as a marker for Nef transfection) and stimulated with anti-CD3 antibody (dose range: 0.001 - 1 $\mu\text{g/ml}$) for 6 hours. Luciferase activity was measured as absolute light units (NFAT activity – raw).

3.2.3. Results

Nef inhibition of NFAT and AP-1 signaling

NFAT and AP-1 activity peaked at around 6 hours (see **Figures 3.2.2** and **3.2.3**). Luciferase readout peaked at doses of anti-CD3 above 100 ng/ml, suggesting that 10-100 ng/ml of antibody was the optimal range for stimulation. For NFAT activity, Nef began to lose its ability to modulate signaling at saturating doses over 1000 ng/ml. At 100 ng/ml anti-CD3, SF2-Nef inhibited TCR-mediated NFAT and AP-1 activity up to 5-fold (**Figure 3.2.2**) and 10-fold (**Figure 3.2.3**), respectively. While Nef inhibited AP-1 activity more profoundly, the relatively high level of basal AP-1 activity in unstimulated cells combined with a lower fold-induction made results more difficult to interpret. NFAT-luciferase was chosen for further experiments because it showed lower background signal and a greater fold-induction.

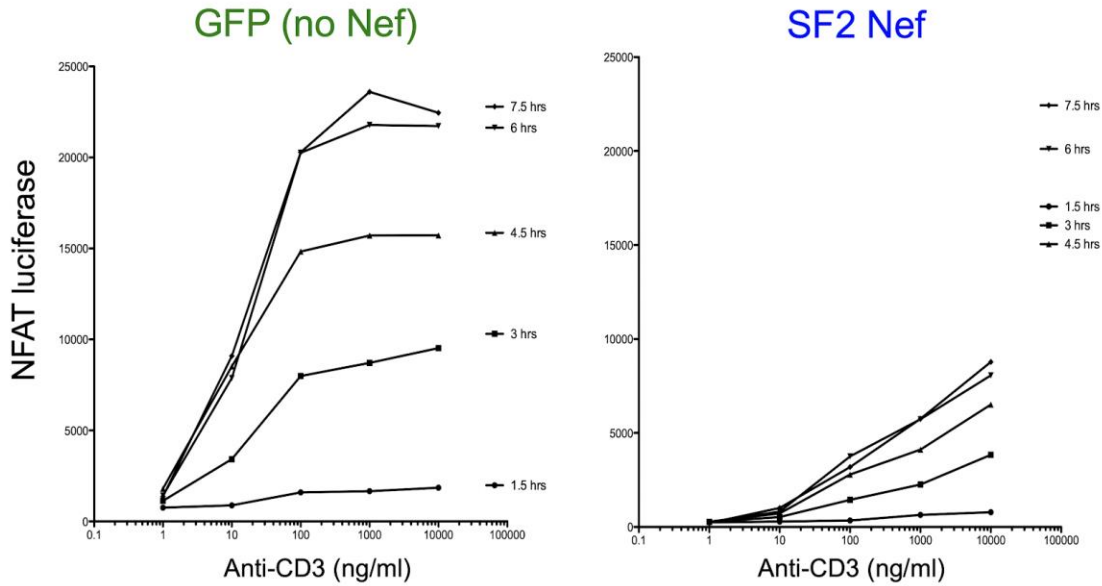


Figure 3.2.2. NFAT-luciferase signal at different timepoints post anti-CD3 stimulation

Jurkat T cells were co-transfected with empty vector (GFP / No Nef) (left) or wild type SF2 Nef (right) expression plasmids along with NFAT-luciferase reporter plasmid. After 18 hours, cultures were single cell sorted for GFP and stimulated with anti-CD3 antibody (dose range: 1 ng/ml - 10 μ g/ml). NFAT-luciferase activity (absolute light units) was measured at 1.5 hrs, 3 hrs, 4.5 hrs, 6 hrs, and 7.5 hrs. Data is representative of at least two experiments.

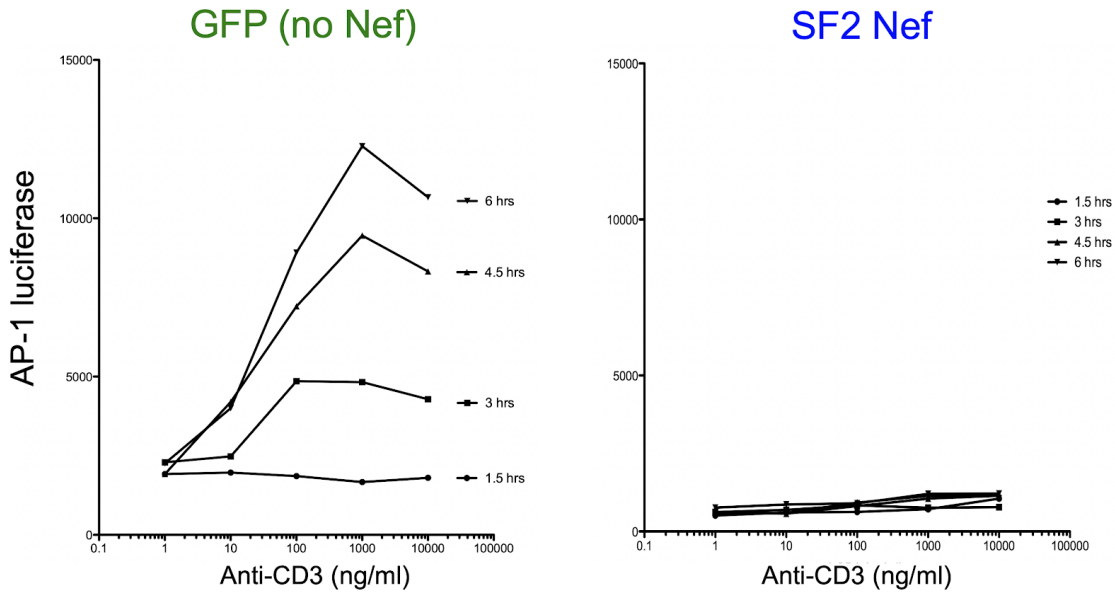


Figure 3.2.3 AP-1-luciferase signal at different timepoints post anti-CD3 stimulation

Jurkat T cells were co-transfected with empty vector (GFP / No Nef) (left) or wild type SF2 Nef (right) expression plasmids along with AP-1-luciferase reporter plasmid. After 18 hours, cultures were single cell sorted for GFP and stimulated with anti-CD3 antibody (dose range: 1 ng/ml - 10 μ g/ml). AP-1-luciferase activity (absolute light units) was measured at 1.5 hrs, 3 hrs, 4.5 hrs, and 6 hrs. Data is representative of at least two experiments.

Induction of NFAT-luciferase activity by peptide-pulsed antigen presenting cells (APCs)

To investigate how my use of anti-CD3 compared to a more biologically relevant stimulation condition, effector Jurkat T cells were co-transfected with NFAT-luciferase, Nef, CD8 alpha, and the alpha and beta chain of TCR clone 5B2, and then co-cultured with antigen-presenting cells (CEM-A*02 cells pulsed with increasing doses of Gag FK10 peptide). WT SF2 Nef demonstrated a suppressive effect at all doses of peptide tested (**Figure 3.2.4**). This result supports the idea that indeed anti-CD3 doses $<1 \mu\text{g/ml}$ are likely to be more representative of *in vivo* conditions than doses $>1 \mu\text{g/ml}$. The “intermediate” function of AXXA is consistent with results in **sections 3.3-3.5**, discussed in detail below.

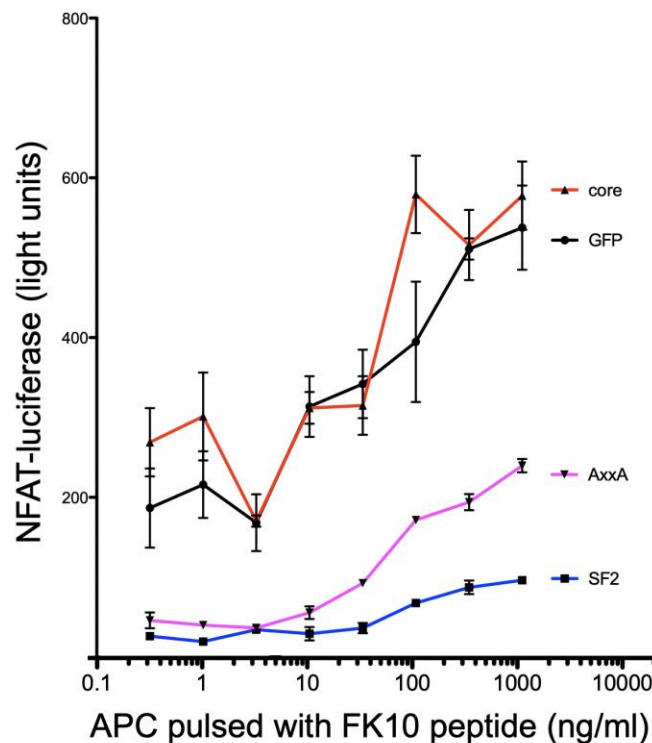


Figure 3.2.4. Induction of NFAT-luciferase activity by peptide-pulsed antigen presenting cells (APCs)

Jurkat T cells were transfected with NFAT-luciferase and pSELECT-GFP dual promoter plasmid expressing WT SF2 Nef (blue), truncated Nef core (red), PXXP-AXXA mutant (pink), and empty vector. After 18 hrs, effector Jurkat cells were co-cultured for 6 hours with target CEM-SS cells, which were pulsed with eight different concentrations of FK10 peptide (peptide concentration ranging from 2.5 ng/ml – 1,000 ng/ml). NFAT-luciferase activity was measured as luminescence (absolute light units). Data represent one transfection and three co-culture technical replicates.

Further validation of NFAT-luciferase assay by single cell sorting

One limitation of the NFAT-luciferase assay is that the final readout is based on bulk signal from a population of T cell transfectants, which may have taken-up varying amounts of Nef and reporter DNA plasmids. Therefore, I wanted to ensure that Nef's effect was not an artifact of overexpression in highly transfected cells. To explore this, I transfected Jurkat T cells with pSELECT-Nef and NFAT-luciferase, then after 18 hours I sorted three populations of cells based on expression of GFP, which is driven from the same dual promoter pSELECT plasmid as Nef, and therefore served as a surrogate marker for Nef. Specifically, cells were sorted into populations that expressed "low", "high", or "all" (both low and high combined) levels of GFP (**Figure 3.2.5A, top**). Next, I stimulated 10^5 sorted T cells with anti-CD3 for 6 hours, and measured luciferase as described in previous experiments. Notably, NFAT activity was inhibited in both low-GFP and high-GFP cells, as well as in both combined (**Figure 3.2.5B**), suggesting that Nef's inhibitory effect on NFAT is consistent across a range of intracellular concentrations. Furthermore, I did not find a marked difference in cell viability between cells transfected with WT Nef or the G2A negative control, indicating that the reduction of NFAT luciferase activity in the presence of WT Nef was not an artifact of cell toxicity (**Figure 3.2.5A, bottom**).

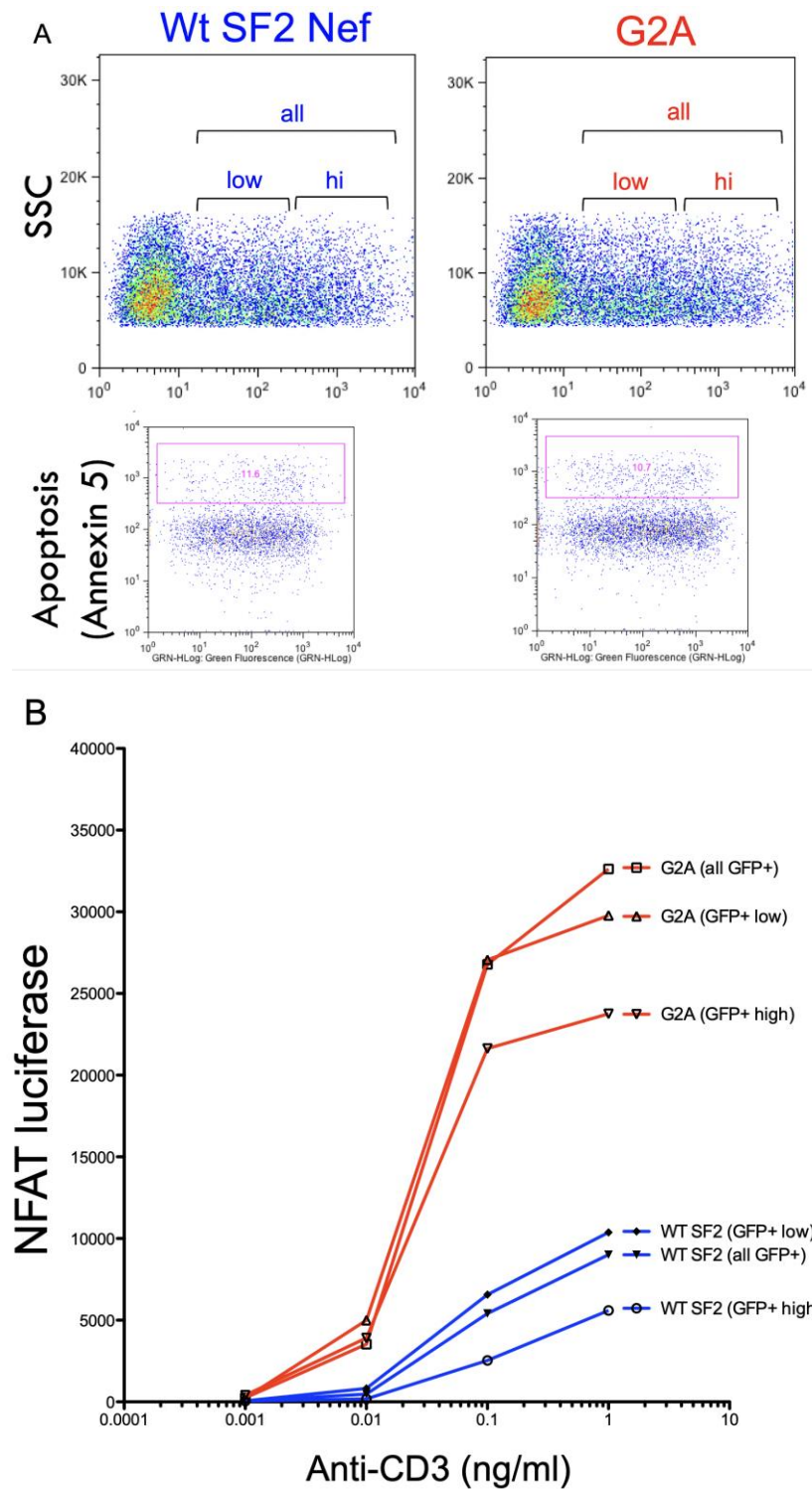


Figure 3.2.5. Further validation of NFAT-luciferase assay by single cell sorting.
 A: Jurkats transfected with pSELECT-GFP/Nef were single cell sorted based on level of GFP expression (low, high, all), and sorted cells were stained for Annexin 5. B: 75,000 cells for each Nef allele (WT and G2A) at each expression level (low, high, all) were measured for NFAT luciferase activity.

3.2.4. Discussion

Results are summarized in **Table 3.2.1**. NFAT-luciferase and AP-1 luciferase were inducible in Jurkat T cells stimulated with anti-CD3 antibody, but anti-CD28 antibody did not induce or enhance signaling. NFkB activity was not detected in Jurkats under any stimulation condition tested, including with TNF-alpha.

Jurkat T cells had low levels of basal NFAT activity, and showed rapid induction of NFAT signaling, translating into a high fold-induction (~10 fold). By contrast, there was a high level of basal AP-1 activity, so although AP-1 was induced by anti-CD3 stimulation, the fold-induction was lower (~5 fold) than was observed with NFAT.

WT Nef inhibited both AP-1 and NFAT signaling. Although inhibition by Nef was more complete in the case of AP-1 (>10 fold inhibition), this was the case for both basal and induced AP-1 signaling, making it difficult to interpret these results in terms of proximal T cell signaling. It is interesting to consider whether a panel of Nef mutants may differentially affect basal vs. induced AP-1 signaling. However, for the purposes of this study subsequent experiments focused on Nef modulation of TCR-induced NFAT signaling.

Table 3.2.1 Summary of results for transcription factor-driven luciferase activity under set of stimulation conditions in the presence or absence of HIV-1 Nef.

Luciferase vector	Anti-CD3	anti-CD3 + anti-CD28	Anti-CD28	Peptide-pulsed APCs	TNF-alpha	Fold NFAT-luciferase induction by anti-CD3	Fold inhibition of NFAT by Nef
NFAT	+	+	-	+	NA	~10	~5
AP-1	+	+	-	NA	NA	~5	~10
NFkB	-	-	-	NA	-	NA	NA

Induction of signaling denoted as "+" and lack of induction as "-". NA = not applicable or not tested.

3.3. Modulation of early TCR-mediated phosphorylation events by Nef

3.3.1. Background

In **section 3.2** I demonstrated that Nef disrupts CD3-initiated NFAT translocation -- a distal signaling event mediated by calcium flux. Next, I wondered which upstream steps in the TCR signaling pathway were affected by Nef. Given that both NFAT and AP-1 signaling were disrupted by Nef, I hypothesized that Nef interfered with proximal TCR signaling at least as far upstream as Linker for Activation of T cells (LAT) and Phospholipase-C gamma 1 (PLC- γ 1), two cellular proteins that are crucial to the induction of calcium flux following TCR engagement (**Figure 3.3.1**). I therefore set out to measure the impact of Nef expression on the phosphorylation LAT and PLC- γ 1.

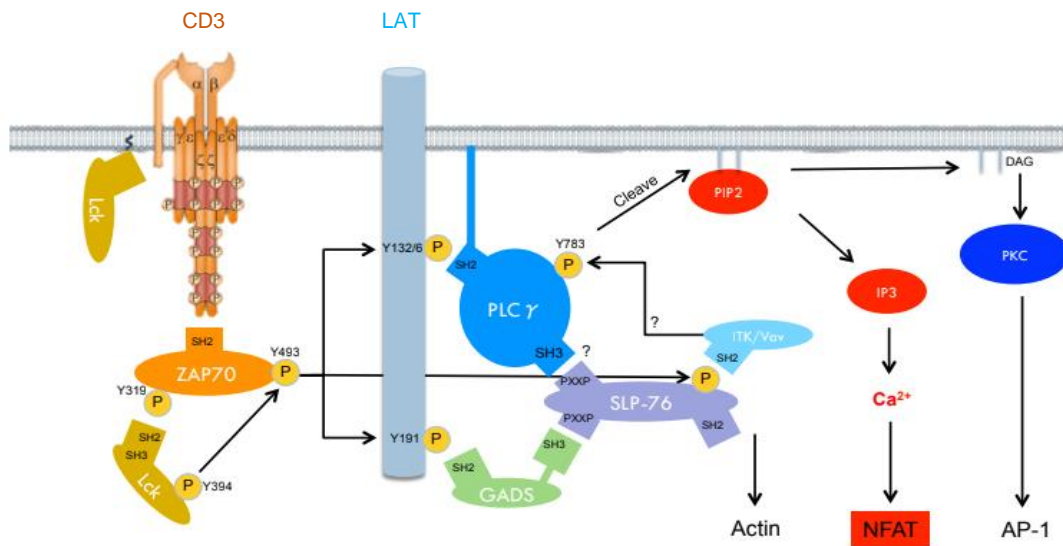


Figure 3.3.1 TCR-mediated phosphorylation events and transcription factor activation

Engagement of the TCR triggers a burst of tyrosine phosphorylation events. First, Lck binds and phosphorylates the CD3-zeta chain. Then CD3 recruits and activates ZAP70, which in turn phosphorylates multiple tyrosines on the adapter protein Linker of Activated T Cells (LAT). LAT forms the core of important multiprotein signaling complexes. These include Phospholipase C-gamma (PLC-gamma), which cleaves PIP2 into DAG and IP3, which lead to AP-1 and calcium flux/NFAT signaling, respectively.

3.3.2. Methods

To assess TCR-mediated signaling events, 10^7 Jurkat T cells were transfected with 10 μ g of empty pSELECT-GFPzeo or vector encoding Nef SF2 by electroporation (electroporation conditions the same as previous experiments). After 18 hours, GFP-positive cells were isolated by FACS and 10^6 cells were stimulated with anti-CD3 antibody (OKT3 clone) at the indicated concentration in R10+ medium for 2 minutes. Cells were immediately chilled on ice, lysed and prepared for Western blot as described previously (Mwimanzi et al., 2011; Mwimanzi et al., 2013a). Linker for Activation of T cells (LAT) was detected using mouse clone 1111 (total protein; BioLegend) (1:2000) and rabbit clone 3584 (pY191; Cell Signaling Technology) (1:500). Phospholipase-C gamma1 (PLC-gamma1) was detected using rabbit clone 2822 (total protein; Cell Signaling Technology) (1:2000) and rabbit clone 2821 (pY783; Cell Signaling Technology) (1:1000). Nef was detected using polyclonal rabbit serum (ARP444; NIBSC Center for AIDS Reagents) (1:2000). Secondary staining for cell signaling proteins or Nef was conducted using donkey anti-mouse or donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare) (1:30000). Proteins were detected using Clarity Western ECL substrate (Bio-Rad) and visualized on an ImageQuant LAS 4000 imager (GE healthcare). Band intensities were quantified using Image Lab™ software (Bio-Rad).

3.3.3. Results

To determine the optimal stimulation kinetics and conditions for measuring early phosphorylation events, Jurkat T cells were electroporated with empty vector (expressing GFP only) or vector expressing SF2 Nef. Transfected (GFP⁺) cells were isolated by FACS at 18 hours and subsequently stimulated from 0-20 minutes with soluble anti-CD3 antibody at concentrations ranging from 0 to 1 μ g/mL. While anti-CD3 coated plates had been used to stimulate Jurkats in the NFAT-luciferase assay, in the present experiment I used soluble anti-CD3 to capture the rapid kinetics of membrane proximal TCR signaling events.

In the absence of Nef, 2 μ g of anti-CD3 antibody induced phosphorylation of LAT (at Y191) and PLC- γ 1 (at Y783) rapidly, peaking at 1-2 minutes and fading by 20 minutes (**Figure 3.3.2**; results consistent with Bilal et al., 2015). Interestingly, LAT

phosphorylation at Y191 peaked slightly later, at 5 minutes, though it is thought to be upstream of PLC-gamma (also consistent with Bilal et al. 2015).

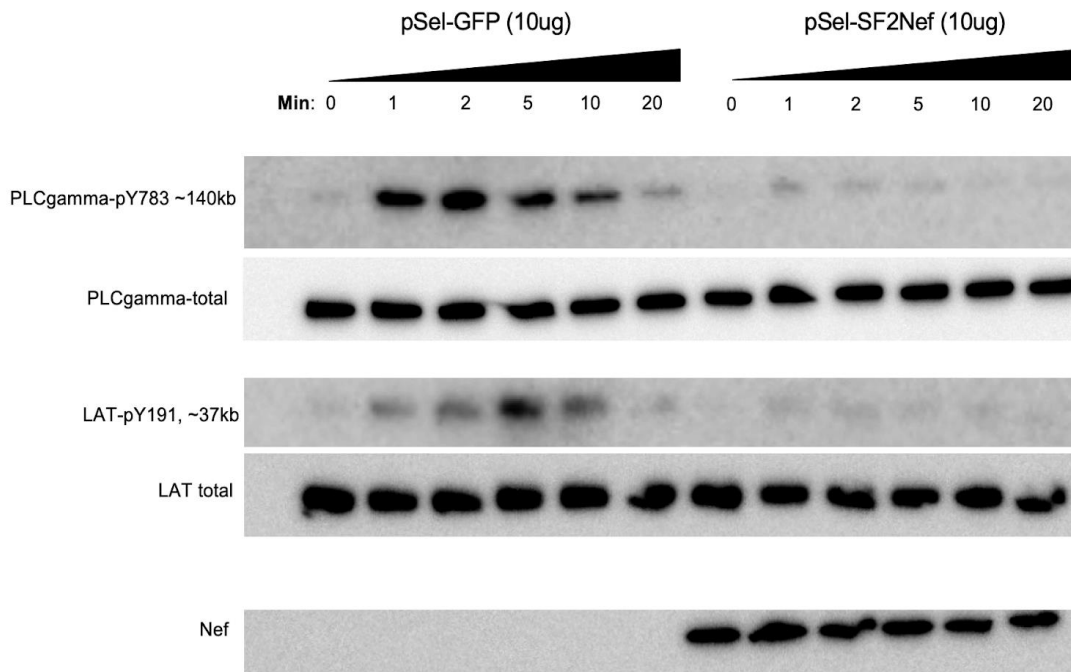


Figure 3.3.2. Kinetics of TCR-mediated phosphorylation events inhibited by Nef. Jurkat T cells were transfected by GFP (left side) or SF2 Nef (right side) and stimulated by soluble anti-CD3 antibody for 0 to 20 minutes. Representative results are shown, based on at least three independent experiments.

Phosphorylation of both PLC- γ 1-pY783 and LAT-pY191 was also dependent upon concentration of anti-CD3 antibody, with optimal signal surging at 0.5 μ g/ml of anti-CD3 antibody and peaking over 1 μ g/ml (**Figure 3.3.3A**). Stimulation conditions were optimized to produce the largest range between Nef and no-Nef cells (0.5 to 1 μ g/ml of soluble anti-CD3 for 2 minutes). In the presence of Nef, phosphorylation of LAT and PLC- γ 1 was inhibited by 59 % and 61 %, respectively (average of three experiments: **Figure 3.3.3B** and **3.3.3C**).

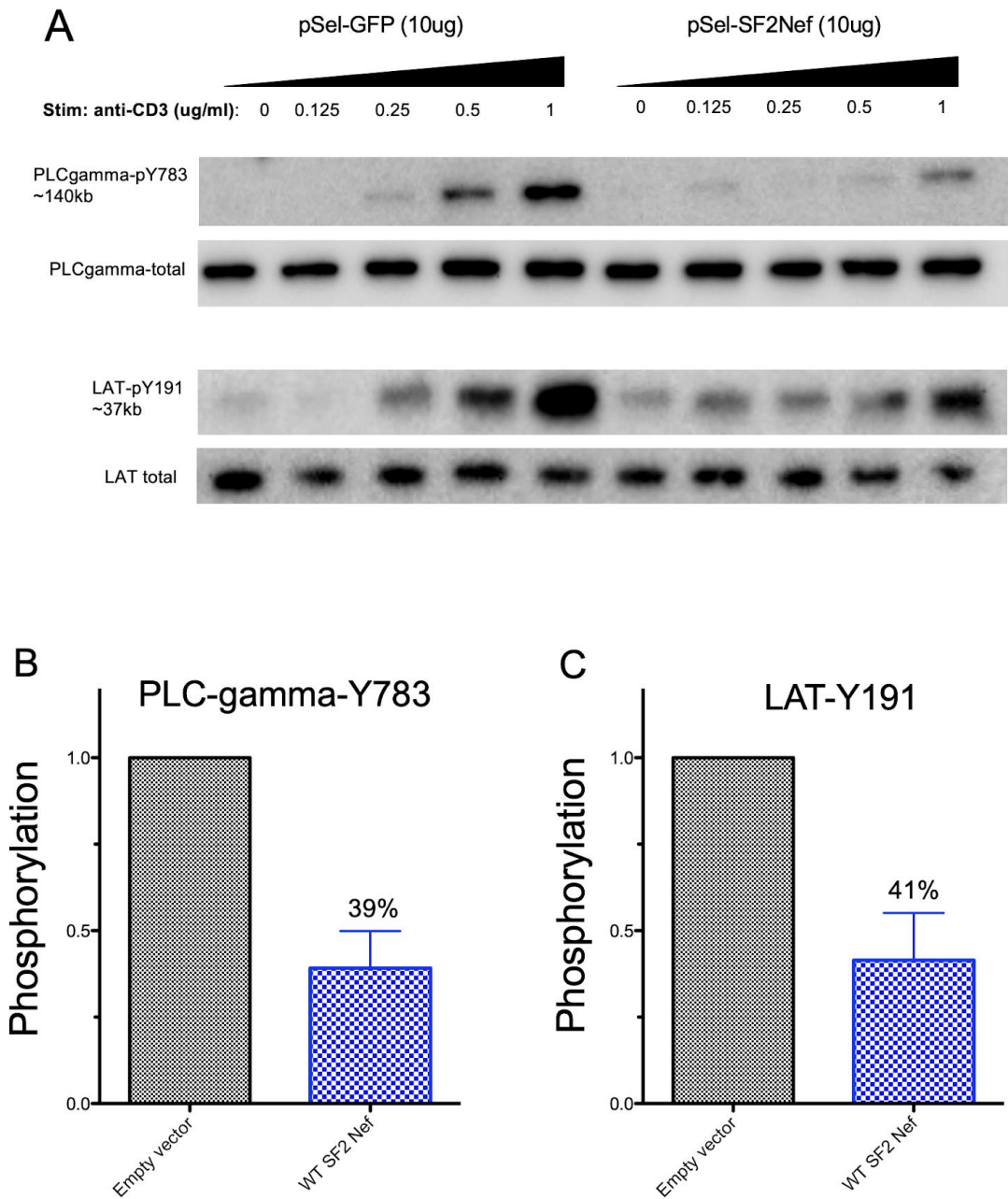


Figure 3.3.3 Nef-inhibition of TCR/CD3-mediated phosphorylation events at different stimulation doses of anti-CD3 antibody.

(A) Jurkat T cells were transfected by GFP (left side) of SF2 Nef (right side) and stimulated for 2 minutes with a range of soluble anti-CD3 antibody (0 to 1 μ g/ml). (B & C) Phosphorylation of PLC-gamma-Y783 or LAT-Y191 after 2 minutes of stimulation with 0.5 and 1 μ g/ml anti-CD3 antibody (averaged for each experiment) in the presence of empty vector or SF2 Nef; bar graphs represent three replicates, each normalized to empty vector.

3.3.4. Discussion

Stimulation of Jurkat T cells with soluble anti-CD3 antibody induced maximal phosphorylation of LAT and PLC-gamma within <5 minutes. Phosphorylation of both proteins was delayed and substantially abrogated in the presence of Nef. These results are consistent with a model whereby Nef's inhibition of NFAT signaling involves disruption of membrane proximal TCR signaling events, upstream of the LAT signaling complex which recruits and activates PLC-gamma to trigger NFAT and AP-1 signaling.

Prior studies in the field have suggest that Nef may modulates TCR-mediated signaling by trafficking p56^{lck}, which acts upstream of LAT and PLC-γ1, away from the plasma membrane. We set out to further explore this hypothesis using microscopy techniques as described in **section 3.5** below.

3.4. Identifying Nef domains required for inhibition of NFAT signaling

Prior studies suggested that Nef can modulate proximal TCR-mediated signaling events by relocalizing the Src family tyrosine kinase p56^{Lck}, which acts upstream of LAT and PLC-γ1, away from the plasma membrane to an intracellular TGN-associated compartment. I therefore set out to explore this hypothesis using microscopy techniques.

3.4.1. Background: Constructing a panel of canonical Nef mutants

The NFAT-luciferase assay developed in **section 3.2** above is a relatively high-throughput system which can be used to screen Nef variants for their ability to modulate TCR-mediated signaling. It is known that Nef's proline-rich region (PXXP at positions 72-75) is required for internalization of Lck (Haller et al., 2007), and several mutations have been identified as necessary for modulation of Pak2 signaling (Olivieri et al., 2011).

To explore which other Nef domains may contribute to the NFAT-inhibition phenotype, I constructed a panel of canonical Nef mutants and measured their effect on NFAT-luciferase signaling.

Table 3.4.1. Panel of canonical Nef mutants in SF2 Nef backbone

AA	Mutant notation	Domain properties	Known effects Nef function(s)
2	G2A	Myristoylation	All functions
12-39	delta12-39	alpha-helix	CD4, infectivity, MHC-I, Pak2 (Fackler et al. 2006)
15-21	R4A4	Alpha-helix	Membrane association (Giese et al. 2006)
20	M20A	Alpha-helix	MHC-I downmodulation
62-5	EEEE-AAAA	Acidic domain	Binds PACS-1 and -2 -> MHC-1 (Aitkins et al. 2008; Dikeakos et al. 2012)
72-5	PXXP-AXXA	SH3-binding	Lck-internalization (Haller et al. 2007)
164-5	LLAA	Dileucine, AP-2 binding	Binds AP-2 -> downregulation of CD4, SERINC -> infectivity (Rosa et al., 2015)
191	F191I	Hydrophobic surface	Binds Pak2 (Agopian et al. 2006)

Nef's N-terminal region and membrane association: G2A, delta12-39, and R4A4

Myristoylation of Nef's N-terminal glycine is required for proper localization of Nef to membranes and to the trans-golgi network (Giese et al., 2006; see also microscopy **section 3.5** below). As a result, alanine mutation of the glycine (**G2A**) impairs receptor downregulation functions (Peng and Robert-Guroff, 2001) and probably all Nef functions. There is some evidence that mutation of the arginines in Nef's N-terminal alpha-helix (**R4A4** incorporates alanine mutations at positions 17, 19, 20 and 21) reduced the fraction of Nef proteins associated with membranes (Bentham et al., 2006) and impairs Nef localization in fibroblast cells (Giese et al., 2006); however, evidence is inconsistent and little is known about the role of the alpha-helix. Similarly, whole-scale deletion of the alpha-helix (**delta12-39**) has affected protein localization and Nef-mediated Lck internalization (Haller et al., 2007). We constructed these three mutants using overlap

extension to investigate the role of Nef's N-terminal region in modulation of TCR signaling.

Receptor downregulation by Nef: LLAA, M20A, and EEEE-AAAA

It has been shown that Nef downregulates CD4 and MHC class I molecules through distinct mechanisms mediated by genetically separable Nef domains (Mangasarian et al., 1999; Akari et al., 2000). Nef's **dileucine motif at amino acids 164/5** is required for downregulation of CD4 but not HLA-I (Riggs et al., 1999); dileucine binds the AP-2 clathrin adaptor and sorts CD4 into clathrin coated pits for degradation (Aiken et al., 1994; Mangasarian et al., 1997; Foti et al., 1997; Craig et al., 1998; Bresnahan et al., 1998; Greenberg et al., 1998). Conversely, residues in Nef's N-terminal alpha-helix, such as **M20**, are required for down-modulation of MHC-I but not CD4 (Mangasarian et al., 1999); Nef is thought to sequester MHC-I in the trans-Golgi (Greenberg et al., 1998; Le Gall et al., 2000) through interaction of Nef's **EEEE65** with Adaptor Protein-1 (Lubben et al., 2007; Baugh et al., 2008; Singh et al., 2009) and/or with PACS-1/2 (Aitkins et al., 2008; Dikeakos et al., 2012).

Nef's SH3-binding domain and cellular kinases: PXXP and F191I

Nef's proline-rich domain from amino acids 69-78 (PXXPXXPXXP) binds to Src family kinases, of which Lck is a member (Saksela et al., 1995); however, it has been difficult to determine whether Nef binds Lck directly. If so, the interaction is likely transient or weak as there is little convincing evidence in of Nef-Lck co-immunoprecipitation in the literature. Indeed, Nef's binding to Lck seems to be an order of magnitude weaker than its binding to Hck, another Src family kinase that is not expressed in T cells (Trible et al., 2006). Nevertheless, the proline rich SH3-binding region has been shown to be absolutely required for Nef-mediated internalization of Lck (Haller et al. 2007). I therefore included the alanine substitution mutant AXXA in my panel. Similarly, Nef has been shown to interact with Pak2 (Arora et al., 2000), which is thought to affect actin rearrangement, including during the formation of the immunological synapse. We constructed the mutant F191I which cannot bind Pak2 (Foster et al., 2001).

3.4.2. Methods

Generation of HIV-1 nef constructs. TCR signaling requires relatively high protein levels (Liu et al., 2001), thus all Nef sequences were re-cloned into pSELECT-GFPzeo (InvivoGen), which features a composite hEF1-HTLV promoter driving nef and an independent CMV promoter driving expression of a GFP:zeocin reporter protein. To do this, our lab modified the multiple cloning site in pSELECT-GFPzeo to incorporate *Ascl* and *SacII* restriction sites. Each nef gene was amplified by PCR using degenerate primers incorporating these restriction sites (Fwd: 5'-AGAGCACCGG **CGCGCCTCCA** CATACCTASA AGAATMAGAC ARG-3', HXB2 nt 8746-8772 underlined, *Ascl* site in bold; Rev: 5'-GCCT**CCGCGG** ATCGATCAGG CCACRCCTCC CTGGAAASKC CC-3', HXB2 nt 9474-9449 underlined, *SacII* site in bold), cloned into the modified pSELECT-GFPzeo vector, and validated by sequencing. Mutations were introduced into SF2 Nef by overlap extension PCR. All mutations and chimeric genes were validated by sequencing.

NFAT reporter assay. NFAT activity was measured using the NFAT-luciferase reporter assay as described above in section 3.1. To compare the NFAT-inhibition function of mutant, luminescence values for each Nef mutant were normalized to positive (pSELECT-nef_{SF2}-GFPzeo) and negative (pSELECT-nef_{G2A}-GFPzeo) controls using the formula: $(ALU_{G2A} - ALU_{patient}) / (ALU_{G2A} - ALU_{SF2}) \times 100\%$, such that function less than or greater than the positive control SF2 Nef is represented by values of <100% or >100%, respectively. The inhibition activity of each Nef clone is reported as the mean \pm standard deviation (SD) based on at least three independent transfection experiments.

Receptor down-regulation: CD4 downregulation and HLA class I downregulation were measured according the protocol published in Mwimanzi et al. 2013. Nef protein levels were measured by Western blot using protocols in the same abovementioned paper.

3.4.3. Results

Mutation of the N-terminal membrane association motifs (G2A) abolished signaling inhibition, highlighting the importance of Nef localization to membranes. Myristoylation of Nef's N-terminus is thought to be required for most, if not all, known Nef

functions, including downregulation of CD4 and MHC-I (Peng and Robert-Guroff, 2001). The G2A mutant was therefore utilized as a negative control for NFAT-signaling assays.

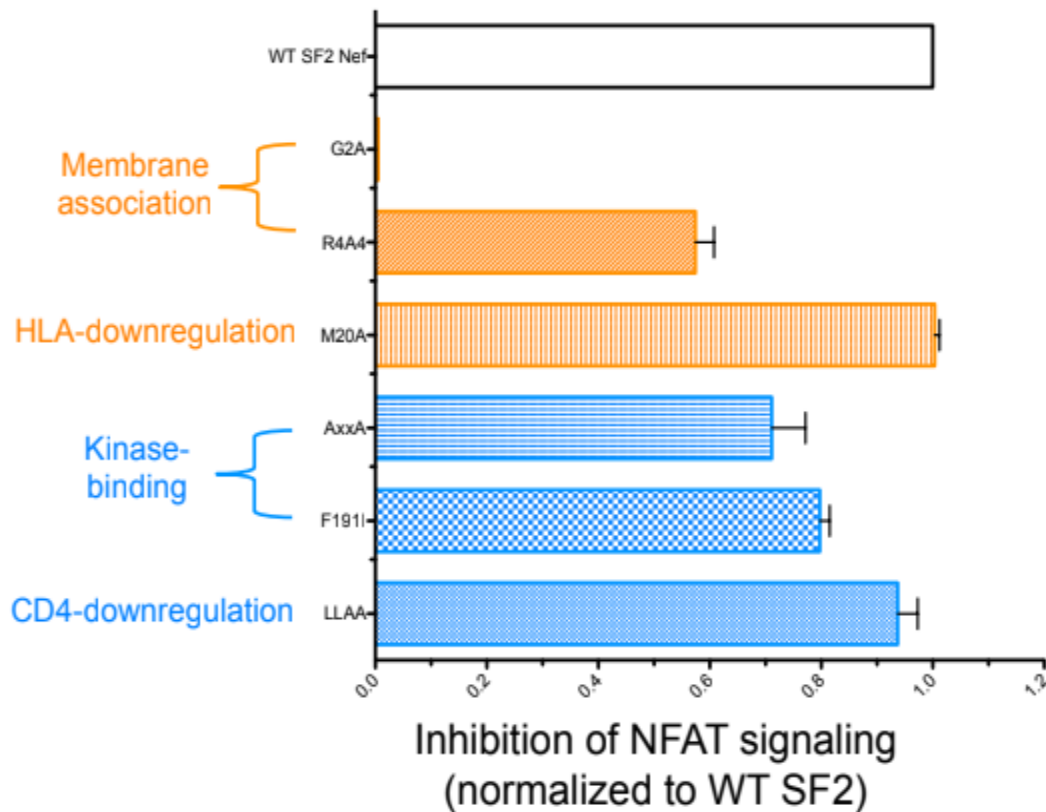


Figure 3.4.1. NFAT-inhibition function of key Nef mutants.

NFAT-inhibition function was normalized to G2A Nef as negative control and WT SF2 Nef as a positive control, such that G2A=0% and WT SF2=100%. Bar graphs represent at least three replicates.

Nef's N-terminal domain and proline-rich region required for NFAT-inhibition

Of the panel of canonical Nef mutants, the myristoylation defective G2A demonstrated the greatest impairment for signaling inhibition (as expected), followed by the R4A4 and AXXA mutants. Mutation of Nef's SH3-binding domain (AXXA) reduced signaling function (71% of WT) (**Figure 3.4.1**). As mentioned, this domain is thought to interact with host cell signaling molecules, including the tyrosine kinase Lck. Interestingly, mutation of the N-terminal arginine-rich α -helix (R4A4) had an even greater impact on NFAT inhibition (57% of WT). This was not an artifact of protein expression or

stability, as the R4A4 mutant showed protein expression levels consistently comparable to wild-type (**Figure 3.4.2**). F191I displayed moderately impaired function.

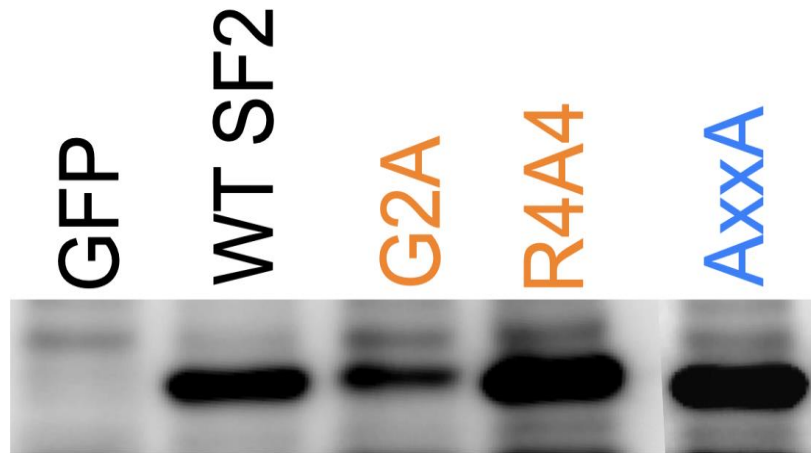


Figure 3.4.2. Protein expression levels of Nef mutants with notably impaired NFAT-inhibition function.

Nef proteins extracted from 10^7 Jurkats cells transfected with 10 μ g pSELECT-GFP/Nef and stained with anti-Nef antibody (rabbit). Representative results are shown, based on at least three independent experiments.

NFAT-inhibition is independent of both CD4- and HLA-downregulation

Our results showed that CD4-downregulation could be abrogated without impairing NFAT-inhibition. Alanine mutagenesis of the dileucine motif (LLAA), which completely abrogates CD4-downregulation function, had no effect on NFAT-inhibition which remained at near wild type levels of 95% (**Figure 3.4.1**). Similarly, I observed that HLA-downregulation could be abrogated without affecting NFAT-inhibition. The M20A Nef mutant, which is defective for HLA-downregulation, showed 100% NFAT-inhibition (**Figure 3.4.1**). This suggests that Nef does not need to bind to MHC-I or AP-1 (or PACS-1 or -2) in order to inhibit NFAT-signaling.

3.4.4. Discussion

As expected, the AXXA mutant showed impaired NFAT-inhibition function. It was however intriguing that it retained some NFAT inhibition function (71% of WT). The residual 71% signal suggested that Lck-recruitment may not be the only mechanism by which Nef inhibits TCR signaling.

It was also intriguing that the R4A4 mutant had an even greater impairment for NFAT-inhibition function than did AXXA. I therefore wondered whether the N-terminal alpha helix was involved in NFAT-inhibition precisely through a second “Lck-independent” mechanism.

It may seem counterintuitive that CD4-downregulation is independent of NFAT-inhibition, given that CD4 plays a key role in TCR signaling. CD4 binds the target cell MHC Class I on target cells, helps to stabilize the TCR complex, and its intracellular tail recruits Lck to phosphorylate the CD3 zeta chain, thereby triggering downstream signaling. However, my results are consistent with literature which has generally found little link between Nef’s ability to down-regulate CD4 and modulation of TCR signaling (Iafrate et al., 1997).

Although it has been controversial whether Nef has a positive or negative effect on T cell signaling, my observations that Nef inhibits TCR signaling were consistent with studies by the Fackler group in Heidelberg, Germany. Whereas I initially observed inhibition of distal signaling events (NFAT translocation), the Fackler group has employed advanced microscopy methods to show that Nef disturbs immunological synapse formation (Haller et al., 2006), Lck internalization (Haller et al., 2007), and early TCR-signaling events such as formation of the LAT signaling complex (Abraham et al., 2012a). These latter findings are also consistent with my phospho-Western blot data reported above in **section 3.3**.

3.5. Nef Localization by confocal microscopy

3.5.1. Background

I wondered whether differences in the function of Nef mutants could be related to their (re-)localization. WT Nef had been shown to localize not only to the plasma membrane, but also to accumulate in an intracellular compartment thought to be the trans-golgi network (TGN) (Haller et al., 2007). It has been predicted that the arginine-rich α -helix may stabilize membrane association (Bentham et al. 2006). Indeed, when the first ~50 amino acids of Nef were fused to a GFP reporter, the fusion construct localized to the plasma membrane, suggesting that the N-terminal arm of Nef is sufficient for membrane association; however this construct did not localize to the TGN (Giese et al., 2006), possibly because the Nef core region is required to bind trafficking,

signaling, and other host proteins. This same study by Giese et al. found that the R4A4 mutant did not localize properly to membranes or to the TGN in Hek cells (Ibid, see their figure 3), raising the possibility that R4A4's abrogated NFAT-inhibition was a function of Nef mis-localization. The PXXP domain was not thought to be required for Nef's localization, but rather for its ability to cause internalization of Lck. To examine the intracellular localization in Jurkat cells, I made a panel of Nef.GFP fusion constructs, including R4A4-GFP and AXXA-GFP, to analyze by confocal microscopy.

3.5.2. Methods

Nef.GFP fusion constructs were assembled using PCR overlap, and cloned into pFLAG-CMV2 (Addgene). Using similar electroporation conditions as above, Jurkat T cells were transfected with 20 - 25 µg of pFLAG-Nef.GFP plasmid. Expression of fusion proteins by pFLAG was slower than expression of Nef proteins by pSelect, likely due to differences in both promoter activity and protein size. At 48 hours, 10⁵ GFP+ cells were sorted by FACS (BD Jazz), fixed with 4% PFA, permeabilized with 0.01% Triton X-100, blocked, stained with primary 1:1000 TGN antibody overnight (TGN46, rabbit, AbCam), stained with anti-rabbit TexasRed, mounted with ProLong Gold (Thermo Fisher), and analyzed by confocal microscopy (Nikon A1R laser scanning confocal system). Maximum intensity projection images are based on 10 z-planes per cell (1 µm/image). To quantify the intracellular accumulation of Nef, gates were then drawn around the 1) the TGN and 2) the entire cell, and "Nef accumulation in the TGN" was calculated as a ratio:

$$\frac{(\text{Nef-GFP fluorescence inside TGN gate})}{(\text{Nef-GFP fluorescence outside the TGN})}$$

3.5.3. Results

WT Nef.GFP showed a ~3-fold enrichment the trans-golgi network (TGN) and recycling endosomes (**Figure 3.5.1**). G2A.GFP did not localize specifically to membranes or the TGN, but rather was distributed relatively evenly/randomly throughout the cell, including in the nucleus. Both the AXXA and R4A4 mutants localized properly to the plasma membrane and to the TGN/RE (see representative images in **Figure 3.5.2**).

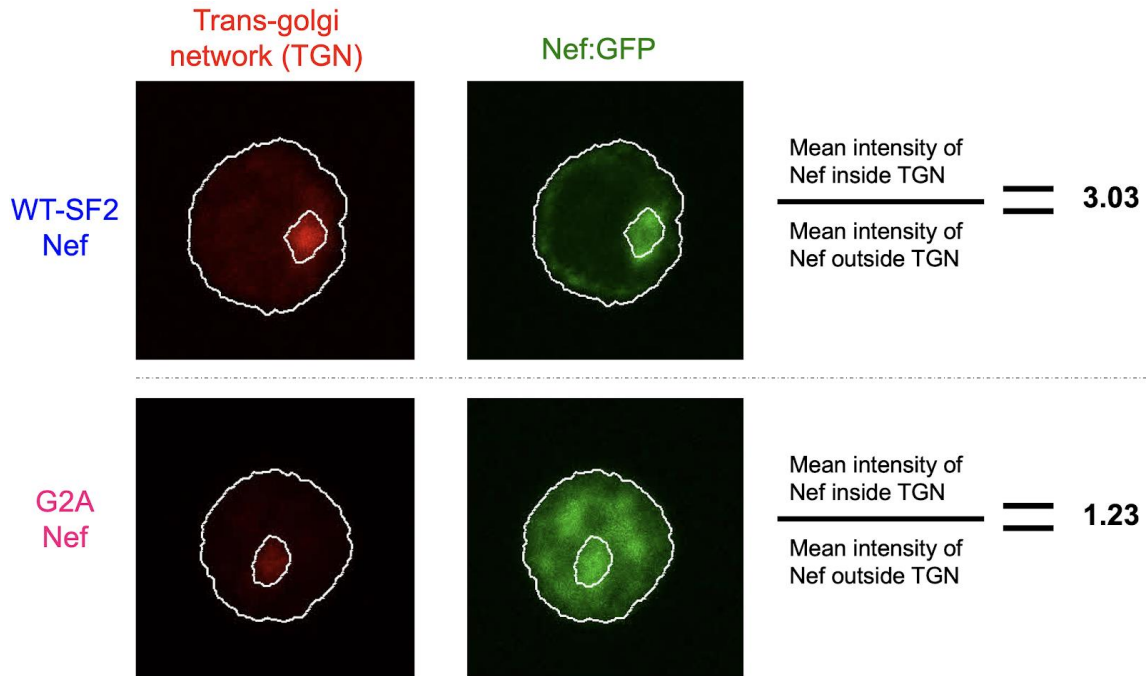


Figure 3.5.1. Accumulation of Nef.GFP or G2A.GFP in the trans-golgi network. Panels on the left show TGN staining, which was used to draw the TGN gate. Panels on the right show Nef:GFP fusion proteins, with WT-Nef enriched in the TGN (top) and G2A-Nef distributed evenly throughout the cell (bottom). Representative results are shown, based on at least 5 cells from two independent experiments.

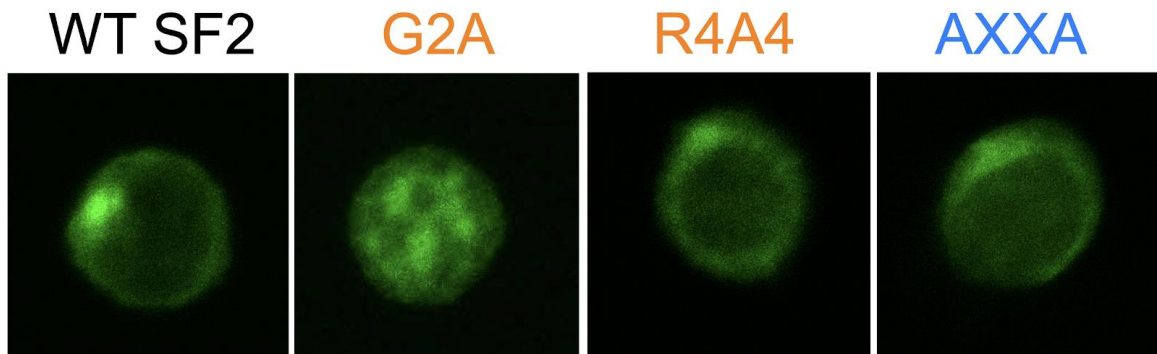


Figure 3.5.2. Maximum intensity projections of the intracellular localization of key Nef.GFP mutants.

Panels show representative staining, based on at least two independent experiments.

3.5.4. Discussion

Both WT Nef.GFP and the AXXA.GFP mutant localized properly to TGN/RE, consistent with Haller et al. 2007. AXXA's loss of NFAT-inhibition function was likely

related to abrogation of PXXP interaction with enzymes such as Lck. Interestingly, in our Jurkat cell system the R4A4 mutant also localized to the TGN. This suggested that if the N-terminal alpha-helix controls localization, it may be through more subtle mechanisms, such as localization within a microenvironment, or affecting Nef conformation and Lck-binding.

3.6. Recruitment of Lck to the TGN by various Nef mutants:

3.6.1. Background

The HIV-1 Nef protein modulates CD4 T cell activation by interacting with host signalling proteins. Nef associates with the tyrosine kinase Lck, which promotes proximal TCR signaling events, and sequesters it near the TGN (Haller et al., 2007). We previously showed that Nef inhibits NFAT transcription factor activity following TCR stimulation in Jurkat cells, and that this function is dependent upon Nef's N-terminal arginine-rich and SH3-binding proline-rich domains. I therefore hypothesized that Nef-mediated internalization of Lck contributes to NFAT-inhibition by sequestering Lck away from the immunological synapse at the plasma membrane. To investigate the relationship between NFAT-inhibition and Lck internalization, I examined a panel of Nef mutants for their ability to relocalize Lck to the TGN.

3.6.2. Methods

10^7 Jurkat T cells were transiently transfected with 10 μ g of pSELECT-Nef (or Nef mutants), using same electroporation conditions as described for the NFAT-luciferase assay. After 18 hours, GFP+ cells were single cell sorted by FACS (BD Jazz), placed on poly-L-lysine-coated slides, fixed (4% PFA) for 10 min, permeabilized (0.01% Triton-X) for <10 min, stained overnight with primary antibodies (1:500 mouse anti-Lck, Cell Signaling; 1:500 rabbit TGN-46, AbCam), then stained for 30 mins with secondary fluorescent antibodies (1:2000 anti-mouse APC, Cell Signaling; 1:2000 anti-rabbit Texas Red, ADD). Coverglass was mounted with ProLong Gold (Thermo Fisher) (see **Figure 3.6.1**).

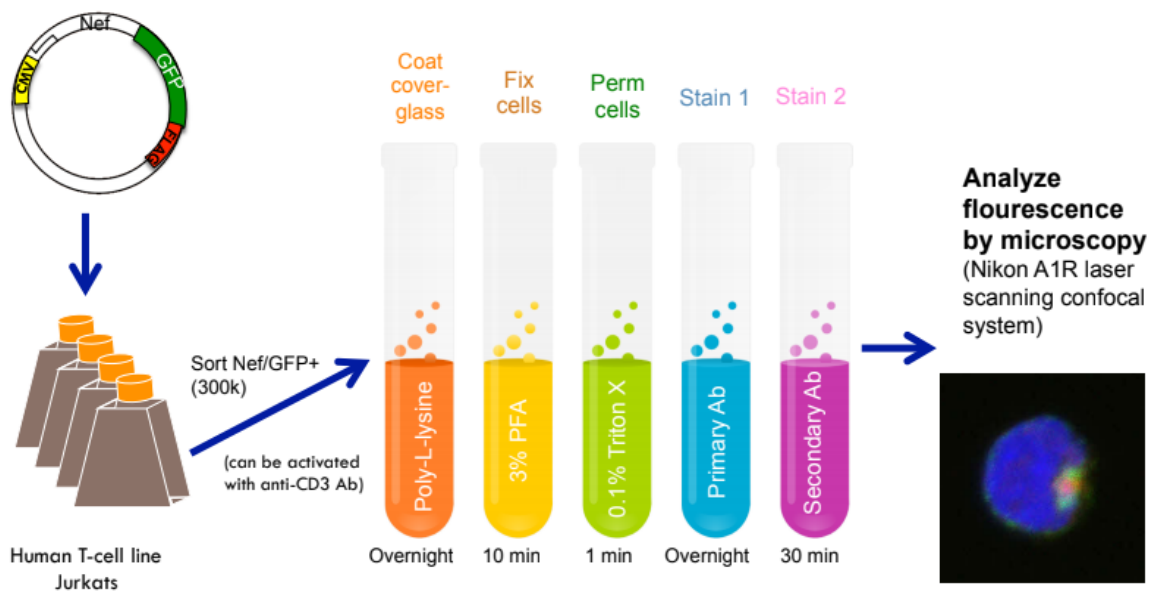


Figure 3.6.1. Cell preparation for Lck internalization assay.

Flow diagram depicts the steps involved in assessing the intraellular localization of endogenous Lck in the presence of Nef.

Slides were analyzed by confocal microscopy (Nikon A1R laser scanning confocal). Individual cells were selected manually based on visually high GFP/Nef fluorescence as well as and intact nucleus (DAPI); TGN (Red) and Lck (Far Red) channels were turned off during selection to reduce bias. For selected cells, the cross-section with the largest TGN gate was chosen, then gates were then drawn around the 1) the TGN and 2) the entire cell. Then “*Lck recruitment to the TGN*” was calculated as the ratio:

$$\frac{(\text{Lck fluorescence inside TGN gate})}{(\text{Lck fluorescence outside the TGN})}$$

This calculation was carried out cell-by-cell, for an average of 57 cells per mutant. Here are some representative images: the five cells with “median” Lck internalization values in the presence of WT Nef (**Figure 3.6.2**) or in the presence of AXXA (**Figure 3.6.3**):

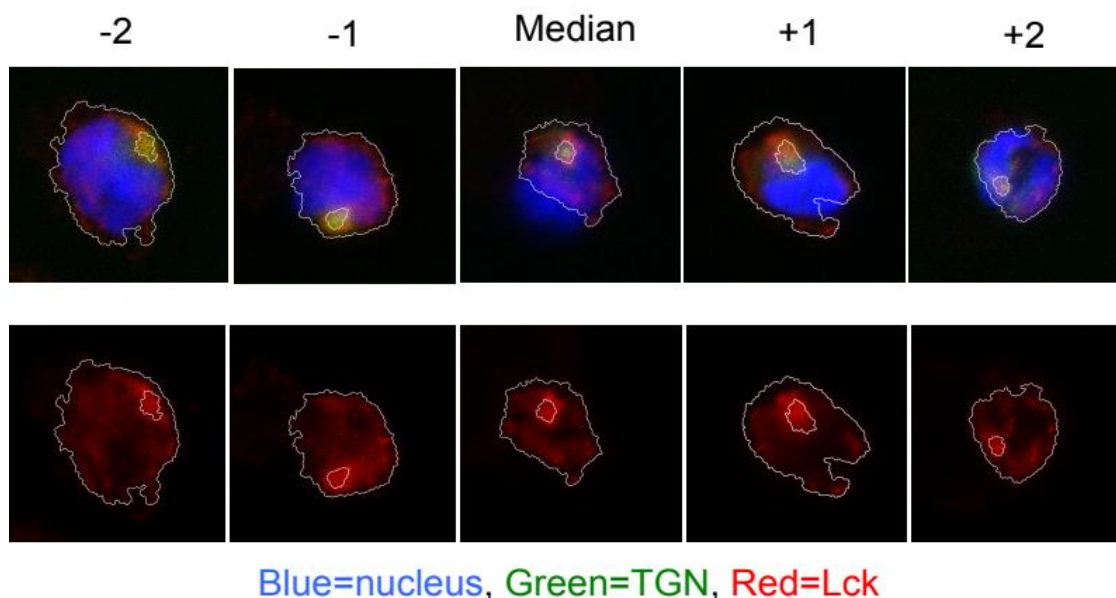


Figure 3.6.2. In presence of WT Nef, the five cells with median Lck internalization values.

Confocal microscopy images (cross sections) are shown. Top panels display staining for cellular DNA (DAPI/blue), Lck (red), and TGN (green), which was used to define the small “inside TGN” gate. Bottom panels display only the Lck channel (red) for same five cells, which is enriched inside the TGN gate in the presence of WT Nef. N=89 cells transfected with WT Nef were analyzed; these are the five cells that ranked around the median Lck-internalization value for WT Nef, such that cell “-1” ranked immediately below the cell with the median value, and “+1” ranked immediately above the median.

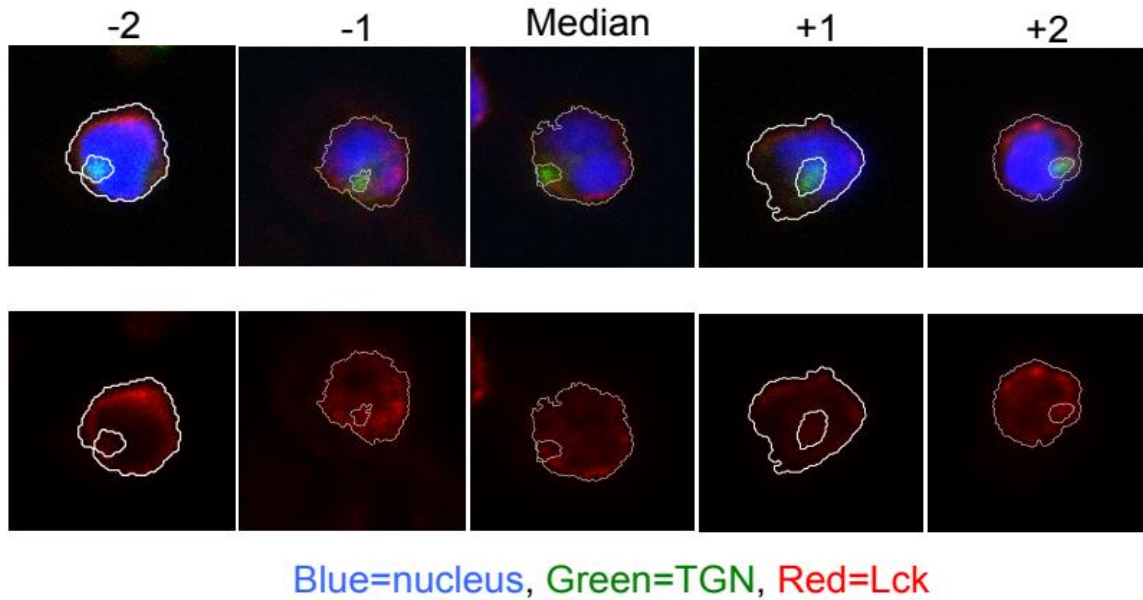


Figure 3.6.3. In presence of G2A, the five cells with median Lck internalization values.

Confocal microscopy images (cross sections) are shown. Top panels display staining for cellular DNA (DAPI/blue), Lck (red), and TGN (green), which was used to define the small “inside TGN” gate. Bottom panels show only the Lck channel (red) for the same five cells, which is not enriched inside the TGN gate in the presence of G2A Nef. N=26 cells transfected with G2A Nef were analyzed; these are the five cells ranked around the median Lck-internalization value for G2A Nef, such that cell “-1” ranked immediately below the cell with the median value, and “+1” ranked immediately above the median.

3.6.3. Results

WT Nef recruited Lck away from the plasma membrane to the TGN, causing a 2.6-fold enrichment of Lck in the TGN compared to outside of the TGN (Mean \pm SEM: 2.59 \pm 0.1). Even for WT Nef, here was a range of values from slightly below 1 (same amount of Lck in TGN as outside the TGN) to a 5-fold enrichment. There are several reasons accounting for this range of values, including:

- *Variability in Lck staining:* Some cells have bright Lck spots at regions of the plasma membrane, which can vary from cell-to-cell and section-to-section (see **Figures 3.6.2** and **3.6.3** above for examples)
- *Cell viability/integrity:* Cell viability was improved though FACS sorting on forward/side scatter and GFP+ cells, fixing, and by selecting cells with intact nuclei; however, integrity of the plasma membrane / cytoplasm could vary from cell-to-cell
- *Transfection efficiency:* Although cells were single cell sorted for GFP, the GFP gate captured a range of cells containing varying amounts of GFP and, therefore, Nef.

The M20A Nef mutant, which is defective for HLA Class I downregulation but retained the ability to inhibit NFAT signaling, was used as an additional positive control. M20A mediated near WT-levels of Lck-internalization (Mean fold-enrichment in TGN: 2.46 +/- 0.12 [N=77]; p=n.s compared to WT). Expectedly, the SH3-binding Nef mutant AXXA failed to recruit Lck (Mean enrichment: 1.24 +/- 0.05 [N=61]; p<0.0005), as did the G2A negative control. The R4A4 Nef mutant showed intermediate Lck enrichment in TGN, only 36% of WT (Mean: 1.58 +/- 0.04 [N=77]; p<0.0005).

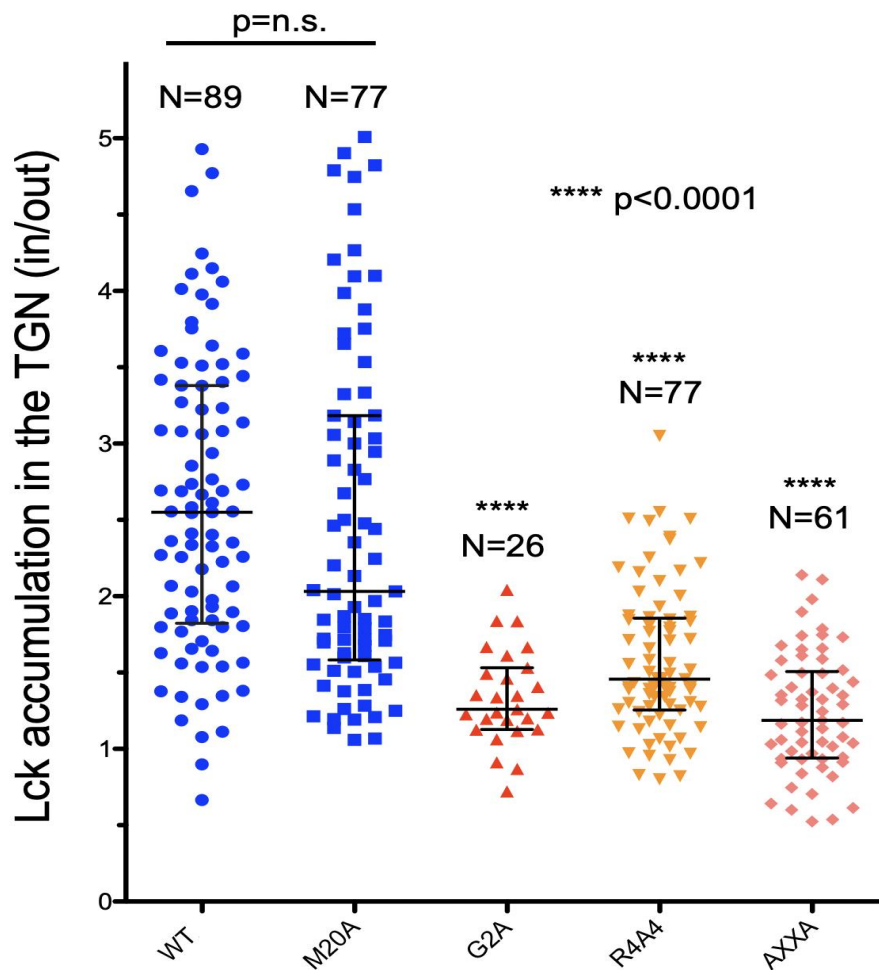


Figure 3.6.4. Recruitment of Lck to the TGN by various Nef mutants.

Each data point represents the fold-enrichment of Lck in the TGN in one Jurkat cell (MFI of Lck in TGN / MFI of Lck outside TGN). There was no significant difference between WT SF2 Nef and the M20A positive control, but there was a significant difference between WT and each of G2A, R4A4, and AXXA (T-test, all p<0.0001). For each Nef mutant, cells were analyzed from three independent transfection/immune-staining experiments.

3.6.4. Discussion

While the AXXA Nef mutant displayed a reduced ability to inhibit NFAT-inhibition, it did nevertheless allow residual signaling (71% of WT); at the same time, Lck-internalization was completely abrogated. Together these results suggested that Lck-internalization may not be the sole mechanism of NFAT inhibition.

Indeed, this Lck-internalization assay does not demonstrate a causal relationship between Lck-internalization and NFAT-inhibition. AXXA results do recapitulate the observation of Haller et al. (2007) that Nef's proline-rich region is required for Lck-internalization. Furthermore, I have shown previously that the PXXP domain is also required for WT NFAT-inhibition (responsible for approx. 30% of NFAT-inhibition). However, it is possible that the PXXP region is important for NFAT-inhibition due to interactions with cellular proteins other than Lck. There is literature showing that Nef may not bind strongly to Lck through its PXXP domain (Trible et al., 2006), even if the domain is required for Lck internalization. For example, a recent paper suggested a more complex mechanism for Nef impairment of the immunological synapse (Silva et al., 2016).

As mentioned, the R4A4 Nef mutant was even more impaired for NFAT inhibition (59% of WT) than AXXA (71% of WT). However, the R4A4 mutant allowed more residual Lck-internalization (36% of WT) than AXXA (15%), suggesting that the arginine-rich helix may disrupt signaling in an at least partly Lck-independent manner.

3.7. Nef-Lck association: Proximity ligation Assay and microscopy

3.7.1. Background

The NFAT-luciferase assay (**section 3.2**) provided evidence that both Nef's N-terminal alpha-helix and its PXXP SH3-binding domain contribute to NFAT inhibition. The Lck internalization assay (**section 3.6**) confirmed that the PXXP domain is absolutely required for recruitment of Lck away from the plasma membrane to the TGN. However, mutating the R4 alpha-helix only partly interfered with Lck internalization. Given the literature suggesting that Nef's alpha-helix mediates membrane association (Bentham et al., 2006), I hypothesized that the R4A4 mutant's defective phenotype may

be related to mislocalization within signaling microenvironments at the plasma membrane. In particular, I asked whether the alpha-helix may be required for Nef to localize in close proximity to Lck. I therefore used the Proximity Ligation Assay technology (PLA, Millipore-Sigma) to investigate the proximity of Nef mutants to Lck in transfected Jurkat cells. The PLA technology amplifies signal when the two target proteins (in this case Nef and Lck) are found within a maximum distance of 40 nm of each other.

3.7.2. Methods

PLA was conducted using the Duolink® In Situ Orange Mouse/Rabbit kit (Millipore-Sigma). Briefly, 10^7 Jurkat T cells were transfected with 10 µg of pSELECT-GFPzeo encoding selected Nef mutants as described above. After 18 hours, 100,000 GFP-positive cells were isolated by FACS, treated with BD Cytofix-Cytoperm™ solution (BD Biosciences), blocked and co-stained using rabbit anti-Nef (ARP444) (1:2000) and mouse anti-Lck (clone 3A5; Santa Cruz Biotechnology) (1:2000) antibodies. Secondary incubation with anti-rabbit PLUS and anti-mouse MINUS probes, ligation, amplification and wash steps were completed in solution as directed by the manufacturer for flow cytometry applications. Stained cells were transferred to polyL-lysine-coated slides and mounted using DuoLink® In Situ mounting medium with DAPI reagent. Maximum intensity projection images were collected using a Nikon A1R laser scanning confocal microscope based on 10 z-planes per cell (1 µm/section). Orange PLA signal was detected using the Cy3 channel (λ_{ex} 554 nm; λ_{em} 576 nm). PLA fluorescence intensity (in arbitrary pixel units) was calculated for each cell using NIS-Elements software (Nikon) after background subtraction in the orange channel based on control cells treated in parallel that lacked one or both primary antibodies (representative controls in **Figure 3.6.1**).

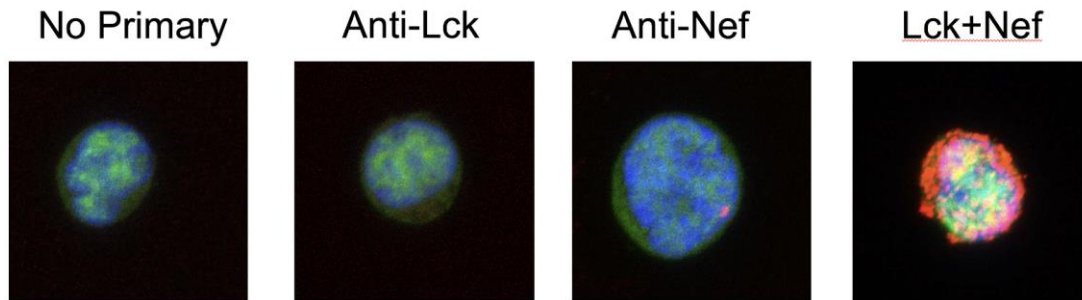


Figure 3.7.1. Representative controls for PLA analysis.

Confocal microscopy images (maximum projection) are shown. Single Jurkat T cells display cellular DNA (DAPI/blue), GFP (from pSELECT-GFP, marker for transfection) (green), and Nef-Lck association (orange/red).

3.7.3. Results

Each PLA image was taken as 10 z-planes per cell, and puncta and MFI were calculated for each image (see examples with raw data in **Figure 3.6.2**). Five images were taken per Nef mutant, which translated into an average of 154 cells per mutant. The median puncta and median MFI were compiled, as presented **Figure 3.6.3**. Results with background subtracted are summarized in **Table 3.6.1**.

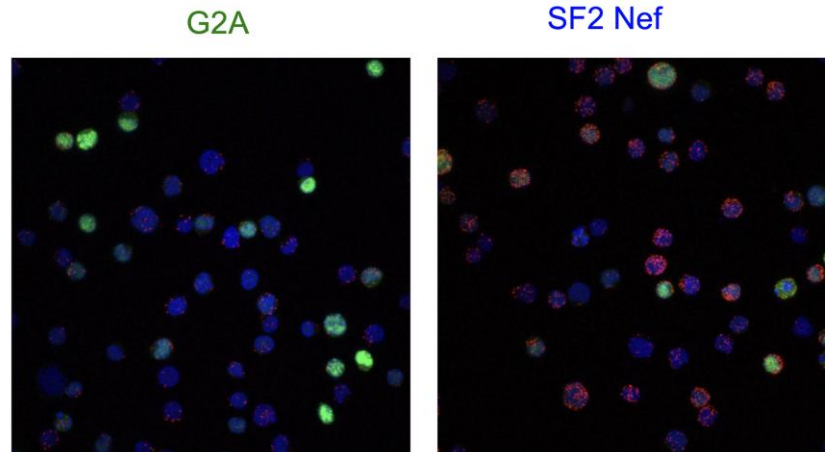


Figure 3.7.2. Representative PLA images collected for negative (G2A) and positive (WT Nef) controls.

One representative image of Jurkat T cells transfected with G2A Nef (left) or WT Nef (right). Colours: Green is GFP expression from pSELECT-GFP (marker for Nef transfection); blue is DAPI-stained genomic DNA, and red/orange is Nef-Lck association (probes binding DNA generated by PLA rolling-circle amplification). Five images were analyzed per Nef mutant.

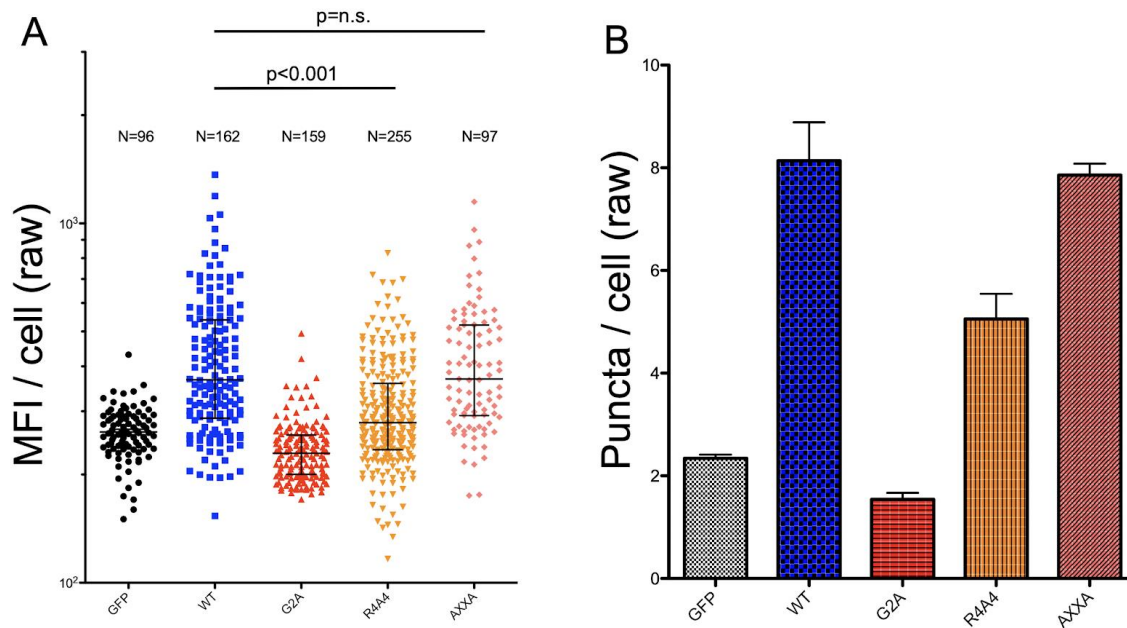


Figure 3.7.3. Proximity Ligation Assay showing Nef-Lck association for key Nef mutants.

Composite data from five confocal microscopy images (see Figure 3.7.2) was analyzed for each Nef mutant. Nef-Lck interaction was measured in two ways: (A) The total MFI of individual cells (raw arbitrary light units); and (B) the average number of puncta (representing one Nef-Lck interaction) per cell over five microscopy images (error bars show variation between the five images). In the MFI analysis (A), R4A4 Nef displayed significantly reduced interaction with Lck, as compared to WT Nef (T-test, $p < 0.001$). Data representative of two independent experiments.

Table 3.7.1. Summary of Proximity Ligation Assay results

	Median # of puncta per cell (with background subtracted)	Median MFI per cell (with background subtracted)	# cells analyzed
GFP (No Nef)	0	0	96
WT	6.6	197	162
G2A	0	0	159
R4A4	3.5	75	255
AXXA	6.4	184	97

Number of puncta per cell and MFI per cell with background subtracted.

Myristoylation defective Nef (G2A) did not interact with Lck. WT Nef interacted with Lck to cause a median of 6.6 amplification reactions per cell above background, the median cell showed an MFI of 197 light units above background [N=162].

Interestingly, the AXXA Nef mutant associated with Lck (6.4 puncta per cell and a median MFI of 184 [N=97]; p=ns compared to WT). The R4A4 Nef mutant had intermediate ability to associate with Lck (3.5 puncta per cell and a median MFI of 75 [N=255], significantly less than WT: p<0.0001).

3.7.4. Discussion

The PLA results indicate that Nef's SH3-binding domain is not required to localize within 40 nm of Lck. The AXXA mutant was able to localize in close proximity with Lck, despite being unable to internalize Lck. This may be consistent with the observation that Nef's binding to Lck is weaker than had previously been expected (Trible et al., 2006), perhaps with insufficient binding affinity to govern Nef localization.

In contrast, these PLA results suggest that Nef's N-terminal alpha-helix plays a significant role in its ability to associate with Lck. R4A4's "intermediate" phenotype for Lck-association is consistent with its intermediate phenotype for Lck-internalization, pointing toward a model whereby Nef's N-terminal arm controls localization within signaling microenvironments.

3.8. Conclusions for Chapter 3

Although Nef's effect on T cell signaling has been controversial, the results presented in this chapter provide evidence in favour of a model whereby WT Nef inhibits early T cell receptor signaling. Both NFAT and AP-1 transcription factor activity were inhibited for at least the first 6 hours of stimulation (**section 3.2**). Using phospho-Western blots, I showed that this disruption of signaling traced back to the early membrane proximal signaling. Whereas phosphorylation of LAT and PLC-gamma peaked within 5 minutes of anti-CD3 stimulation, this phosphorylation burst was strongly inhibited in the presence of WT Nef (**section 3.3**).

Using the NFAT-luciferase assay to screen a panel of canonical Nef mutants, I found that PXXP domain contributed to Nef's NFAT-inhibition function, but alanine mutation of this region did not completely knock-out function. This was the first clue that Lck-internalization is not the sole mechanism of NFAT-inhibition. Interestingly, Nef's N-terminal arginine-rich alpha-helix was even more crucial for NFAT-inhibition function -- through an unknown mechanism (**section 3.4**).

AXXA.GFP and R4A4.GFP mutants seemed to localize properly to the plasma membrane and the the trans golgi network (**section 3.5**). However, R4A4's had an intermediate phenotype for Lck-internalization (**section 3.6**). This latter result suggested that, on the one hand, Nef's N-terminal region plays a role in Lck-interaction possibly by governing membrane association and localization within signaling microenvironments; indeed, the PLA data in **section 3.7** was consistent with this hypothesis. On the other hand, while R4A4 was only partially defective for Lck-internalization, its ability to inhibit NFAT signaling was even more severely impaired than AXXA's -- a second clue that Lck-internalization cannot be the sole mechanism for NFAT-inhibition.

In summary, Nef's ability to inhibit NFAT signaling may be dependent upon these two distinct mechanisms: localization through the R4 domain and Lck-internalization through the PXXP domain. Disrupting Nef's R4 and PXXP domains may have therapeutic implications for latency reversal.

3.9. References

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Chapter 4. Modulation of TCR-dependent NFAT signaling is impaired in HIV-1 Nef isolates from elite controllers

This chapter is adapted from the following published article of which I am second author.

Jin, S.W., Markle, T.J., Anmole, G., Rahimi, A., Kuang, X.T., Brumme, Z.L., Brockman M.A.. Modulation of TCR-dependent NFAT signaling is impaired in HIV-1 Nef isolates from elite controllers. *Virology* 2019. 530: 39-50.

I use the plural pronouns “we” and “our” throughout this chapter to indicate that results reflect the efforts of a team. I isolated the 45 EC and 46 CP Nef isolates that were examined for modulation of TCR-mediated signaling (see **chapter 2**). I developed and optimize the NFAT-luciferase assay (reported in **chapter 3**). I trained Mr. Steven Jin (undergraduate student) to conduct the functional assays and collect data for patient Nef sequences, mentored the construction of Nef chimeras and site-directed mutants, supervised data analysis and assisted with drafting of the manuscript. Furthermore, I carried out all confocal microscopy and Proximity Ligation Assay (PLA) assessments.

4.1. Abstract

HIV-1 Nef modulates the activation state of CD4⁺ T cells by inhibiting signaling events elicited by the T cell receptor (TCR). Primary *nef* sequences exhibit extensive inter-individual diversity that influences their ability to downregulate CD4 and HLA class I; however, the impact of *nef* variation on modulation of T cell signaling is poorly characterized. Here, we measured TCR-mediated activation of NFAT transcription factor in the presence of *nef* alleles isolated from 45 elite controllers (EC) and 46 chronic progressors (CP). EC Nef clones displayed lower ability to inhibit NFAT signaling (median 87 [IQR 75-93]% relative to SF2 Nef) compared to CP clones (94 [IQR 89-98]%) ($p < 0.001$). Polymorphisms in Nef's N-terminal domain impaired its ability to inhibit NFAT signaling. Results indicate that primary *nef* alleles exhibit a range of abilities to modulate TCR-dependent NFAT signaling, implicating natural variation in this function as a potential contributor to differential HIV-1 pathogenesis.

4.2. Introduction

HIV-1 Nef is a 27 kDa myristoylated accessory protein that is critical for viral pathogenesis (Dyer et al., 1999; Kestler et al., 1991; Zaunders et al., 2011). Nef displays multiple functions *in vitro*, including the ability to downregulate CD4 (Garcia and Miller, 1991), HLA class I (Collins et al., 1998; Le Gall et al., 1998; Schwartz et al., 1996), chemokine coreceptors (Michel et al., 2005; Toyoda et al., 2015; Venzke et al., 2006), and the restriction factor SERINC5 from the surface of infected cells (Rosa et al., 2015; Usami et al., 2015), which are thought to enhance viral infectivity and replication (Miller et al., 1994; Trautz et al., 2016; Vermeire et al., 2011). At least some of these activities facilitate viral evasion of host immune responses and promote viral persistence *in vivo* (Alsaifi et al., 2015; Arhel and Kirchhoff, 2009; Chen et al., 2012a; Veillette et al., 2014; Yang et al., 2002). HIV-1 *nef* sequences exhibit substantial genetic and functional diversity (Brumme et al., 2008; Corro et al., 2012; Gray et al., 2011; Kirchhoff et al., 1999; Kuang et al., 2014; Mann et al., 2013; Meribe et al., 2015; Mwimanzi et al., 2013a; Mwimanzi et al., 2013b; Zuo et al., 2012), which may have important implications for disease progression.

One line of evidence supporting Nef genotype/phenotype diversity as a correlate of HIV-1 pathogenesis comes from studies of elite controllers (EC), rare infected individuals (<1%) who spontaneously maintain plasma viral loads below 50 RNA copies/mL without antiretroviral treatment (Blankson and Siliciano, 2008; Walker and Yu, 2013). It is well established that the EC phenotype is due at least in part to favorable host genetics (Carrington et al., 1999; Carrington and O'Brien, 2003; Carrington and Walker, 2012; International-HIV-Controllers-Study et al., 2010), namely possession of protective HLA class I alleles such as B*57:01 or B*27:05 that restrict effective antiviral CD8⁺ T cell responses (Betts et al., 2006; Chen et al., 2012b; Chen et al., 2009; Ladell et al., 2013; Migueles et al., 2003; Migueles et al., 2000). However, viral genetics can also play a major role (Casado et al., 2013; Chen et al., 2015; Kikuchi et al., 2015; Lassen et al., 2009; Lobritz et al., 2011), for example through acquisition of an attenuated HIV-1 strain or within-host selection of fitness-reducing viral mutations under strong immune pressure (Alsaifi et al., 2015; Brumme et al., 2011; Kuang et al., 2014; Miura et al., 2009a; Miura et al., 2009b; Miura et al., 2010; Mwimanzi et al., 2013a; Salgado et al., 2014; Troyer et al., 2009). We and others have demonstrated that while *nef* sequences isolated from EC are generally intact (Miura et al., 2008; Salgado et al., 2010), they nevertheless displayed lower function for a range of *in vitro* activities that contribute to viral pathogenesis (Alsaifi et al., 2015; Kuang et al., 2014; Mwimanzi et al., 2013a; Toyoda et al., 2015), supporting impaired Nef function as a feature of spontaneous HIV-1 control. Further characterization of Nef genotype/phenotype relationships, particularly in EC populations, could inform the discovery of new therapeutic or vaccine strategies.

Nef is reported to inhibit T cell receptor (TCR)-mediated signaling events in CD4⁺ T cells by re-localizing the crucial Src family kinase p56^{lck} to an intracellular compartment (Collette et al., 1996; Greenway et al., 1996; lafrate et al., 1997) and by disrupting actin cytoskeleton remodeling that is crucial to generate a stable immunological synapse with antigen presenting cells (Rudolph et al., 2009; Thoulouze et al., 2006). Lck is the most proximal protein to be activated following TCR stimulation and it directs multiple downstream events, including phosphorylation of signaling mediators Zeta-chain-associated protein kinase 70 (ZAP-70), Linker of Activated T cells (LAT) and Phospholipase C-gamma 1 (PLC-gamma1) that lead to calcium flux and activation of various transcription factors (Denny et al., 2000; Lo et al., 2018; Lovatt et al., 2006). Actin cytoskeletal remodeling mediates cell polarization and establishment of cell-cell contacts that are required to form a stable immunological synapse (Yu et al., 2013). By

dampening antigen-specific TCR signaling, Nef may reduce activation-induced cell death and thereby increase progeny virion production (Abraham et al., 2012; Abraham and Fackler, 2012; Fackler et al., 2007). Residues in Nef's N-terminal domain (Baur et al., 1997; Wolf et al., 2008) and Proline-rich motif (P₇₂XXP₇₅) (Fackler et al., 2001; Haller et al., 2007; Triple et al., 2006) contribute to its ability to alter T cell signaling; however, past studies were conducted mainly using Nef isolates derived from reference strains and relatively few reports have examined primary *nef* alleles. Therefore, the degree to which natural sequence variation influences Nef's ability to modulate TCR signaling has not been fully addressed.

Based on our observations that primary *nef* alleles isolated from EC displayed relative functional impairments compared to those from chronic progressors (CP) (Kuang et al., 2014; Mwimanzi et al., 2013a; Toyoda et al., 2015), we hypothesized that EC-derived Nef clones would also display a reduced ability to modulate TCR-mediated signaling events. To assess this, we used a nuclear factor of activated T cells (NFAT)-dependent luciferase reporter assay in Jurkat T cells to compare the *in vitro* function of 45 Nef clones isolated from EC and 46 clones isolated from untreated CP. Consistent with some prior studies (Ahmad and Venkatesan, 1988; Niederman et al., 1992; Niederman et al., 1993; Niederman et al., 1989), we observed that most primary *nef* alleles were able to dampen TCR-mediated NFAT signaling. Notably, the median activity of EC-derived clones was lower than that of CP-derived clones. Analyses of three poorly functional EC Nef clones identified polymorphisms in the N-terminal domain that contributed to their impairment. Our results highlight the impact of natural sequence variation on Nef's ability to modulate T cell signaling and suggest that differences in this Nef function may contribute to differential clinical outcomes following HIV-1 infection in some individuals.

4.3. Materials and Methods

Study participants. HIV-1 subtype B-infected elite controllers (EC) and chronic progressors (CP) have been described previously (Mwimanzi et al., 2013a). Briefly, the EC cohort comprised 45 untreated individuals with a median plasma viral load (pVL) of 2 RNA copies/mL (interquartile range [IQR] 0.2 – 14) and a median CD4 count of 811 cells/mm³ (IQR 612 – 1022). The CP cohort comprised 46 untreated individuals with a

median pVL of 80,500 RNA copies/mL (IQR 25,121 – 221,250) and a median CD4 count of 293 cells/mm³ (IQR 73 – 440). All participants were recruited from the Boston area, provided written informed consent, and were not receiving antiretroviral therapy at the time of sample collection. This study was approved by the Research Ethics Board at Simon Fraser University (Burnaby, BC Canada) and the Massachusetts General Hospital (Boston, MA USA).

Generation of HIV-1 *nef* constructs. The HIV-1 *nef* gene was amplified from plasma viral RNA and a single phylogenetically representative *nef* sequence was cloned into pIRES2-EGFP (Clontech) for a previous study (Mwimanzi et al., 2013a). The same Nef clones were evaluated here; however, because modulation of TCR signaling requires relatively higher protein levels (Liu et al., 2001), all sequences were re-cloned into pSELECT-GFPzeo (InvivoGen), which features a composite hEF1-HTLV promoter driving *nef* and an independent CMV promoter driving expression of a GFP:zeocin reporter protein. To do this, we modified the multiple cloning site in pSELECT-GFPzeo to incorporate *Ascl* and *SacII* restriction sites. Each *nef* gene was amplified by PCR using degenerate primers incorporating these restriction sites (Fwd: 5'-AGAGCACCGG **CGCGCCTCCA** CATACCTASA AGAATMAGAC ARG-3', HXB2 nt 8746-8772 underlined, *Ascl* site in bold; Rev: 5'-GCCT**CCGCGG** ATCGATCAGG CCACRCCTCC CTGGAAASKC CC-3', HXB2 nt 9474-9449 underlined, *SacII* site in bold), cloned into the modified pSELECT-GFPzeo vector, and validated by sequencing. *Nef* sequences were deposited into Genbank previously; accession numbers are JX171199-JX171243 (EC) and JX440926-JX440971 (CP).

The same strategy was used to clone *nef* from the HIV-1 subtype B reference strain SF2, which served as the positive control in all experiments. A glycine (G) to alanine (A) substitution was introduced into SF2 Nef at residue 2 (G2A) by overlap extension PCR, which served as a negative control. The G2A substitution prevents myristoylation of the Nef protein, thus rendering it defective for most functions, including modulation of T cell signaling (Djordjevic et al., 2004). Overlap extension PCR was also used to generate other mutations in SF2 or primary *nef* sequences and to construct chimeric constructs encoding various regions of primary *nef* and SF2 *nef*, as described in the text. All mutations and chimeric genes were validated by sequencing.

Nef polymorphisms are reported using the HXB2 numbering convention. Sequences were pairwise-aligned to the reference strain HXB2 (GenBank accession

number K03055) and insertions with respect to HXB2 were removed using an in-house alignment algorithm based on the HyPhy platform (Kosakovsky Pond et al., 2005).

NFAT-luciferase signaling assay. To assess TCR signaling, 10 μg of pSELECT-GFPzeo encoding each *nef* allele plus 5 μg of pNFAT-luciferase (Agilent Biosciences) were used to co-transfect 5×10^6 Jurkat T cells resuspended in 200 μL Opti-MEM medium, without phenol red (Thermo Fisher Scientific). Cells were electroporated in 96-well plates using a BioRad GenePulser MXCellTM instrument (square wave protocol: 250 V, 2000 μF , infinite Ω , 25 milliseconds, single pulse). Transfected cultures were recovered for 18 hours in 800 μL of RPMI 1640 medium without phenol red, supplemented with 2 mM L-glutamine, 1000 U/ml Penicillin and 1 mg/ml Streptomycin (R10+) (all from Sigma-Aldrich) at 37°C plus 5% CO_2 to allow for Nef expression. Following this, 50 μL of culture (2.5×10^5 cells) was assessed by flow cytometry for transfection efficiency (GFP expression) and viability using annexin V-APC antibody and 7-AAD (both from BioLegend). In parallel, 100 μL of the culture (5.0×10^5 cells) was stimulated for 6 hours in triplicate on flat-bottomed 96-well culture plates (Greiner CELLSTAR) pre-coated with 0.01-1 $\mu\text{g}/\text{mL}$ of anti-CD3 antibody (OKT3 clone; eBioscience), allowing mobilization of cytoplasmic Ca^{2+} and activation of NFAT-mediated transcription of the luciferase reporter gene (Ledbetter et al., 1987a; Ledbetter et al., 1987b). Luciferase activity was detected using the Steady-Glo® Luciferase Assay system (Promega) by mixing substrate/lysis solution (50 μL) with stimulated cells (50 μL) on a flat-bottom, white polystyrene, half-area microplate (Corning or Grenier). Luminescence was measured as absolute light units (ALUs) using a Tecan Infinite M200 PRO plate reader (2000 ms integration time; 100 ms settle time). Luminescence values for each Nef clone were normalized to positive (pSELECT-*nef*_{SF2}-GFPzeo) and negative (pSELECT-*nef*_{G2A}-GFPzeo) controls using the formula: $(\text{ALU}_{\text{G2A}} - \text{ALU}_{\text{patient}})/(\text{ALU}_{\text{G2A}} - \text{ALU}_{\text{SF2}}) \times 100\%$, such that function less than or greater than the positive control SF2 Nef is represented by values of <100% or >100%, respectively. The inhibition activity of each Nef clone is reported as the mean \pm standard deviation (SD) based on at least three independent transfection experiments.

Downregulation of CD4 and HLA class I. Selected Nef mutants and chimeras were evaluated for their ability to downregulate CD4 and HLA-A*02 (as a representative HLA class I molecule), as described previously (Kuang et al., 2014; Mwimanzi et al., 2013a), with minor modifications. Briefly, 1×10^6 CEM-A*02⁺ T cells were transfected

with 4 µg of pSELECT-GFPzeo encoding each *nef* allele by electroporation (as described above) and stained 20 hours later with anti-human CD4-APC and anti-human HLA-A*02-PE antibodies (BD Biosciences). The median fluorescence intensities (MFI) of surface CD4 and HLA-A*02 expression in GFP-negative and GFP-positive cell subsets were determined by flow cytometry.

Western blot. To assess TCR-mediated phosphorylation, 5×10^6 Jurkat T cells were transfected with 10 µg of empty pSELECT-GFPzeo or vector encoding Nef SF2 by electroporation (as described above). After 20 hours, GFP-positive cells were isolated by FACS and 1×10^6 cells were stimulated with anti-CD3 antibody (OKT3 clone) at the indicated concentration in R10+ medium for 2 minutes. Cells were immediately chilled on ice, lysed and prepared for Western blot as described previously (Mwimanzi et al., 2011; Mwimanzi et al., 2013a). Linker for Activation of T cells (LAT) was detected using mouse clone 1111 (total protein; BioLegend) (1:2000) and rabbit clone 3584 (pY191; Cell Signaling Technology) (1:500). Phospholipase-C gamma1 (PLC-gamma1) was detected using rabbit clone 2822 (total; Cell Signaling Technology) (1:2000) and rabbit clone 2821 (pY783; Cell Signaling Technology) (1:1000). To assess steady-state Nef expression, 5×10^6 Jurkat T cells were transfected with 10 µg of pSELECT-GFPzeo encoding a selected *nef* allele or chimera by electroporation as described above. After 18 hours, cells were pelleted, lysed and prepared as described. Nef was detected using polyclonal rabbit serum (ARP444; NIBSC Center for AIDS Reagents) (1:2,000). Secondary staining was conducted using donkey anti-mouse or donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare) (1:30,000). Proteins were detected using Clarity Western ECL substrate (Bio-Rad) and visualized on an ImageQuant LAS 4000 imager (GE healthcare). Band intensities were quantified using Image Lab™ software (Bio-Rad).

Statistical analysis. Statistical analyses were performed using Prism v.7 (Graphpad). Mann-Whitney U test was used to compare differences in median Nef function between EC and CP cohorts. One-sample t-test was used to compare the function of individual Nef clones, chimeras or mutants to that of the positive control SF2 Nef (whose function was set to 100%). Spearman test was used to assess correlations between study results. Students T-test was used to compare differences in mean PLA fluorescence intensity between Nef mutants in microscopy studies. For all analyses, tests were two-tailed and significance was defined as $p < 0.05$. The Mann-Whitney U test was also used in pair-wise genotype/phenotype analyses to identify natural Nef

polymorphisms associated with differential *in vitro* activity. Briefly, for all Nef polymorphisms observed at least five times in the dataset, the function of all Nef clones encoding the specific polymorphism was compared to that of clones lacking it. Here, multiple comparisons were addressed using q-values, the p-value analogue of the false discovery rate (FDR). The FDR is the expected proportion of false positives among results deemed significant at a given p-value threshold (e.g. at $q < 0.2$, we expect less than 20% of identified associations to be false positives).

4.4. Results

4.4.1. Inhibition of TCR-mediated NFAT signaling by HIV-1 Nef

To examine TCR-mediated signaling in the presence of HIV-1 Nef, we employed an NFAT luciferase reporter assay in Jurkat T cells stimulated with anti-CD3 antibody. NFAT transcription factor activity is induced by calcium flux following TCR engagement and contributes to expression of key cytokines and other activation-induced proteins (Muller and Rao, 2010). In control experiments, cells were transfected with wild type SF2 Nef, myristoylation-defective Nef G2A mutant, or empty vector and then stimulated with anti-CD3 at concentrations ranging from 0 to 1 $\mu\text{g}/\text{mL}$. We observed that NFAT-dependent luminescence was reduced approximately 4-fold in the presence of SF2 Nef compared to Nef G2A under most stimulation condition tested (**Figure 4.4.1A**). Higher luminescence values were consistently seen for cells transfected with Nef G2A compared to empty vector, particularly following stimulation with 1 $\mu\text{g}/\text{mL}$ anti-CD3. The reason for this is unclear; however, we speculate that it may be due to Nef's ability to inhibit cell death following antigen stimulation by interfering with the pro-apoptotic protein Bad (Wolf et al., 2001), which does not necessarily depend on its localization to the plasma membrane. Also, while NFAT signaling was minimal in the absence of stimulation, luminescence values were consistently lower in the presence of SF2 Nef compared to Nef G2A or empty vector controls (**Figure 4.4.1A**, inset), indicating that wild type Nef dampened basal NFAT signaling in Jurkat cells.

To further characterize our *in vitro* system, we examined cytotoxicity and transfection efficiency by flow cytometry. Representative data are shown in **Figures 4.4.1B,C**. We assessed toxicity by measuring induction of the apoptotic marker annexin

V. Following mock electroporation (no DNA), 25% of Jurkat cells displayed annexin V surface staining (**Figure 4.4.1B**). While higher proportions of cells were annexin V-positive following co-transfection with Nef and NFAT-luciferase reporter plasmids, this staining was comparable between SF2 Nef (49%), Nef G2A (55%), and empty vector (52%). Similar results were observed using 7-AAD labeling as an indicator of cell death, which indicated 12% positive cells in mock treated cells compared to 28-31 % positive cells following transfection. Together, these results suggest that cytotoxicity was due to electroporation with plasmid DNA, rather than expression of Nef protein itself. Next, we assessed transfection efficiency by measuring the proportion of viable (annexin-V low) cells that were positive for GFP, which is expressed from an independent promoter on pSELECT-GFPzeo. A similar high percentage of cells were observed to express GFP following transfection with SF2 Nef (51%), Nef G2A (49%), or empty vector (55%) (**Figure 4.4.1C**), indicating that DNA transfection was efficient and comparable between constructs. Based on these results, we concluded that reductions in NFAT signaling observed in cells transfected with SF2 Nef were not likely to be due to differences in toxicity or transfection efficiency. Despite overall toxicity being relatively high following electroporation, this was similar among Nef clones and was not considered to be a major confounder to interpreting assay results. We did not examine cytotoxicity following stimulation with anti-CD3, which might contribute to observed differences in luminescence values between Nef G2A and the empty vector control, as discussed above. For subsequent experiments, we stimulated cells using an intermediate concentration of anti-CD3 antibody (0.1 $\mu\text{g}/\text{mL}$) and selected SF2 Nef and the Nef G2A mutant as positive and negative controls, respectively, since they provided the broadest range to assess relative *in vitro* function.

To confirm the impact of Nef on proximal TCR-mediated signaling events, we also examined the phosphorylation of two cellular proteins that are crucial to the induction of calcium flux following TCR engagement, namely Linker for Activation of T cells (LAT) and Phospholipase-C gamma 1 (PLC- γ 1). Jurkat cells were electroporated with empty vector (expressing GFP only) or vector expressing SF2 Nef. Transfected (GFP⁺) cells were isolated by FACS at 20 hours and subsequently stimulated for 2 minutes with anti-CD3 antibody at concentrations ranging from 0 to 1 $\mu\text{g}/\text{mL}$. In the absence of Nef, phosphorylation of LAT (at Y191) and PLC- γ 1 (at Y783) was rapid and increased with stimulation dose (**Figure 4.4.1D**). In the presence of Nef, phosphorylation of LAT and PLC- γ 1 was inhibited by 59 % and 61 %, respectively

(Figure 4.4.1E). A modest increase in LAT phosphorylation was seen at lower concentrations of anti-CD3 antibody in some experiments (Figure 4.4.1D), but this observation was inconsistent and not assessed further in our studies. Overall, these results are consistent with prior studies indicating that Nef modulates TCR-mediated signaling by trafficking p56^{Lck}, which acts upstream of LAT and PLC- γ 1, away from the plasma membrane. Phosphorylation of PLC- γ 1 is crucial for induction of calcium flux and subsequent activation of NFAT, providing a plausible mechanism to explain Nef's impact on NFAT-mediated signaling.

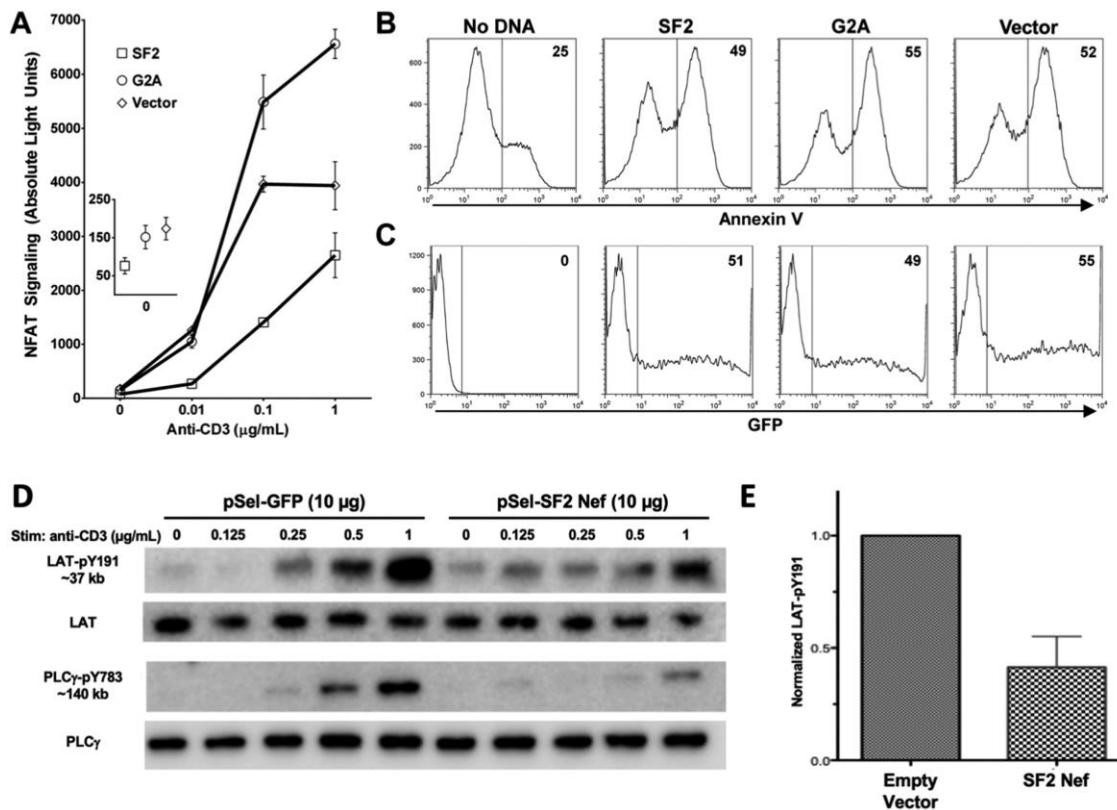


Figure 4.4.1. *In vitro* assay to measure Nef-mediated inhibition of NFAT signaling.

(A) NFAT-driven luciferase activity was quantified using luminescence (y-axis) in Nef-transfected Jurkat T cells following stimulation with anti-CD3 antibody at various concentrations (x-axis). Absolute light units detected from cells expressing either wild type SF2 Nef, myristoylation-defective Nef G2A mutant, or empty vector are shown at 6 hours post-stimulation. The inset figure displays basal NFAT-driven luciferase activity in Jurkat cells in the absence of stimulation. (B) Cellular toxicity was assessed by flow cytometry. Histograms show the proportion of Jurkat cells that were positive for the early apoptosis marker Annexin V (x-axis) following electroporation with no DNA or with pNFAT-luciferase plasmid plus SF2 Nef, Nef G2A, or empty vector. (C) DNA transfection efficiency was measured using flow cytometry to detect the proportion of Jurkat cells producing GFP, which is expressed from an independent promoter present in the pSELECT-GFPzeo plasmid. Histograms show the proportion of annexin-negative cells (from panel B) that expressed GFP (x-axis) following electroporation with no DNA or with pNFAT-luciferase plasmid plus SF2 Nef, Nef G2A, or empty vector (Δ Nef). (D) Nef-mediated inhibition of proximal TCR

signaling events was confirmed using Western blot to detect phosphorylation of LAT (pY191) and PLCgamma (pY783). Jurkat T cells were transfected with empty pSELECT-GFPzeo or vector encoding SF2 Nef. GFP+ (transfected) cells were isolated by FACS, stimulated with anti-CD3 antibody at various doses, and phosphorylation events measured at 2 minutes post-stimulation. **(E)** Nef-mediated inhibition of LAT phosphorylation (pY191) was quantified based on results from three independent experiments following stimulation with anti-CD3 antibody at 0.5 µg/mL.

4.4.2. **Modulation of TCR-mediated NFAT signaling is attenuated in EC Nef clones**

We next used this *in vitro* assay to examine the ability of 91 primary Nef clones to modulate TCR-mediated NFAT signaling. Of these, 45 clones were obtained from untreated elite controllers (EC) and 46 clones were obtained from untreated chronic progressors (CP). Representative luminescence data for three EC- and three CP-derived Nef clones, plus controls, are shown in **Figure 4.4.2A**. Raw luminescence values were subsequently normalized to those of the positive (SF2) and negative (G2A) controls such that Nef inhibition activity less than, equal to or greater than SF2 Nef was represented as <100%, 100% or >100%, respectively; and the activity of G2A Nef was represented as 0% (**Figure 4.4.2B**). For each EC- and CP-derived Nef clone, we then calculated an average normalized value based on triplicate data from at least three independent transfection experiments. All EC and CP Nef clones displayed at least partial function in this assay, in that their ability to inhibit TCR-mediated NFAT signaling exceeded that of the negative control, Nef G2A (**Figure 4.4.2C**). Nevertheless, as a group, the EC-derived Nef clones displayed a significantly lower ability to inhibit signaling (median 87 [IQR 75-93] %) than the CP-derived Nef clones (median 94 [IQR 89-98] %) ($p < 0.001$).

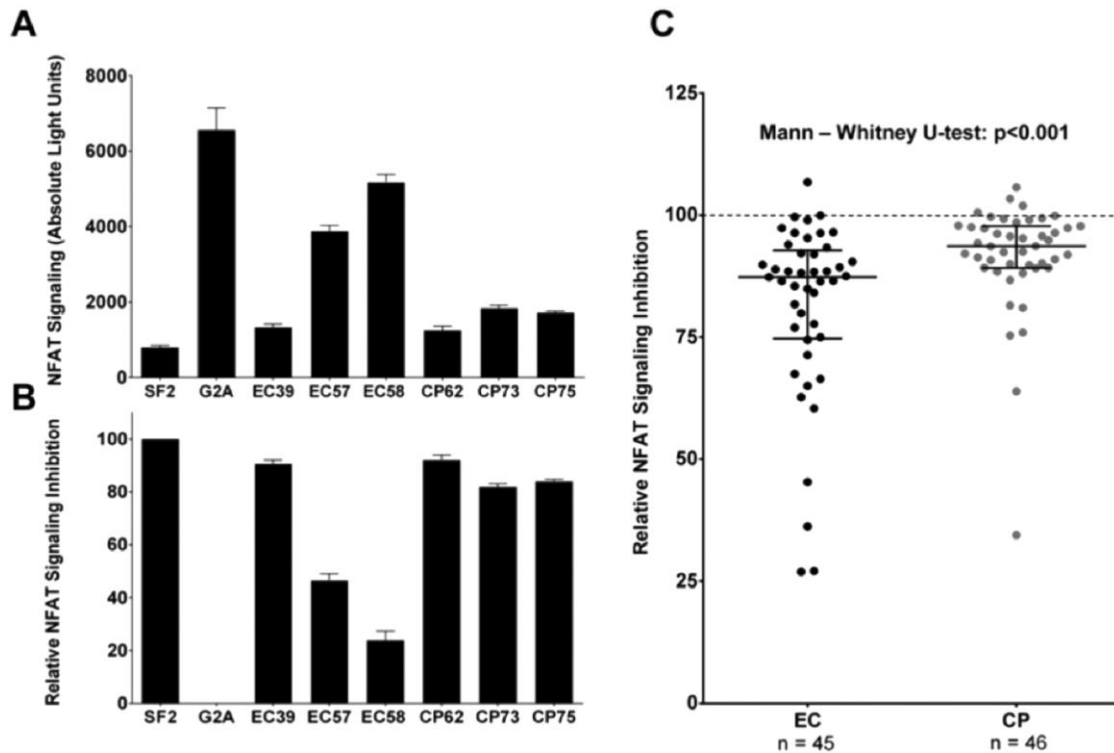


Figure 4.4.2. Nef-mediated inhibition of TCR signaling is attenuated in HIV-1 elite controllers

(A) Representative NFAT-luciferase results (absolute light units, y-axis) are shown for three elite controller (EC)- and three chronic progress (CP)-derived Nef clones, compared to wild type SF2 Nef (positive control) and myristoylation-defective G2A Nef mutant (negative control). (B) Results shown in panel A were normalized to the positive control (set to 100 %) and the negative control (set to 0 %), as described in the Methods. Values greater than or less than SF2 Nef are represented as >100 % or <100 %, respectively. (C) The relative ability of 45 EC-derived (black) and 46 CP-derived (grey) Nef clones to inhibit NFAT signaling is shown. Individual circles represent the mean value for each patient Nef clone, based on triplicate values obtained from at least three independent experiments. Bars represent the median and interquartile ranges for each population. The p-value was calculated using the Mann-Whitney U-test.

4.4.3. Associations between *in vitro* Nef functions and protein expression

We next assessed whether *in vitro* inhibition of NFAT signaling correlated with other Nef functions. The 91 Nef clones used in this study were characterized previously for their ability to downregulate CD4 and HLA class I (Mwimanzi et al., 2013a). While we observed statistically significant associations between Nef-mediated NFAT inhibition and downregulation of CD4 (Spearman $R=0.31$, $p=0.003$) and HLA ($R=0.44$, $p<0.001$) (Figures 4.4.3A and 5.4.3B), we noted that all three activities also correlated with steady-state Nef expression levels as determined by Western blot (NFAT: Spearman $R=0.30$, $p=0.003$; CD4: $R=0.31$, $p=0.003$; HLA: $R=0.18$, $p=0.09$) (Figure 4.4.3C). The

observation that modulation of signaling correlated most strongly with HLA down-regulation is consistent with reports of a link between these functions (Hung et al., 2007; Atkins et al., 2008), if only through shared motifs such as the PXXP domain (Iafrate et al. 1997). Prior work also indicated that Nef's ability to modulate TCR signaling is distinct from CD4 downregulation (Iafrate et al., 1997), consistent with our observation that intrinsic differences in the expression or stability of natural isolates are also likely to affect this function, which requires a relatively high intracellular concentration of Nef (Liu et al., 2001).

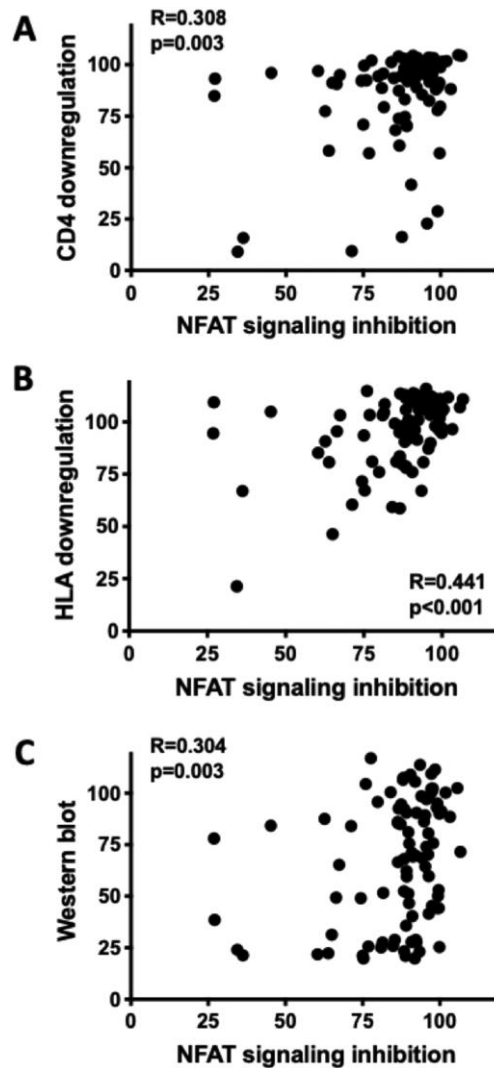


Figure 4.4.3. Associations between *in vitro* Nef functions and protein expression. The ability of each primary Nef clone to inhibit NFAT signaling was correlated with its ability to downregulate CD4 (**A**) or HLA class I (**B**), as well as protein stability as detected by Western blot (**C**) using the Spearman's rank sum test. These data were reported previously (Mwimanzi et al., 2013a).

4.4.4. Natural variation at Nef residue 21 influences modulation of TCR signaling

To investigate potential determinants of Nef's ability to modulate TCR signaling, we conducted genotype/phenotype analyses using data from all 91 primary Nef clones. We identified 12 amino acids located at 9 Nef residues that were significantly associated with this *in vitro* function at $p < 0.05$ and $q < 0.2$ (**Table 4.4.1**). The strongest correlation was seen for lysine at Nef residue 21 (R21K), which was more common in EC clones (24 %; 11 of 45) compared to CP clones (7 %, 3 of 46) (Fisher's exact test, $p = 0.02$). Nef isolates encoding R21K displayed a median activity of 75 [IQR 60-86] % compared to a median activity of 92 [IQR 88-97] % for those harboring the consensus R at this site ($p < 0.001$). To confirm this observation, we substituted the consensus R₂₁ in SF2 Nef with each of the five alternative amino acids observed in at least one EC or CP sequence, namely glutamic acid (E), lysine (K), leucine (L), glutamine (Q) or threonine (T) (**Figure 4.4.4**). All five mutants were able to downregulate CD4 and HLA-A*02 to similar levels as wild type SF2 Nef (**Figure 4.4.4A**), however all mutants were impaired in their ability to inhibit NFAT signaling, displaying normalized functions of 77 %, 81 %, 74 %, 68 %, and 80 % relative to SF2 Nef, respectively (all $p < 0.05$) (**Figure 4.4.4B**). Steady-state Nef levels for all mutants were found to be comparable to SF2 by Western blot (**Figure 4.4.4C**), suggesting that these differences were not due to changes in protein expression or stability. These results demonstrate that natural sequence variation at Nef residue R₂₁ can impact its ability to modulate cellular signaling without significantly compromising its other major functions.

Table 4.4.1. Polymorphisms associated with NFAT inhibition ($p < 0.05$ and $q < 0.2$)

HXB2 Residue	Amino Acid	Impact	Relative Activity (%)		# Nef Clones		p-value	q-value
			With AA	Without AA	With AA	Without AA		
11	V	-	86.4	92.5	27	54	0.0002	0.02
21	K	-	75.5	92.0	14	77	0.0001	0.02
26	A	-	89.8	99.7	84	7	0.0073	0.13
28	E	+	95.3	89.2	39	52	0.0118	0.19
157	N	-	89.1	96.4	72	17	0.0013	0.05
157	T	+	96.3	89.2	14	75	0.0062	0.12
163	C	+	95.2	89.1	27	64	0.0051	0.12
170	L	-	88.5	95.1	55	36	0.0007	0.05
170	Q	+	95.1	88.5	34	57	0.0012	0.05
174	D	+	92.5	88.4	53	37	0.0058	0.12
174	E	-	88.4	92.5	37	53	0.0058	0.12
182	Q	+	96.5	89.3	10	81	0.0059	0.12

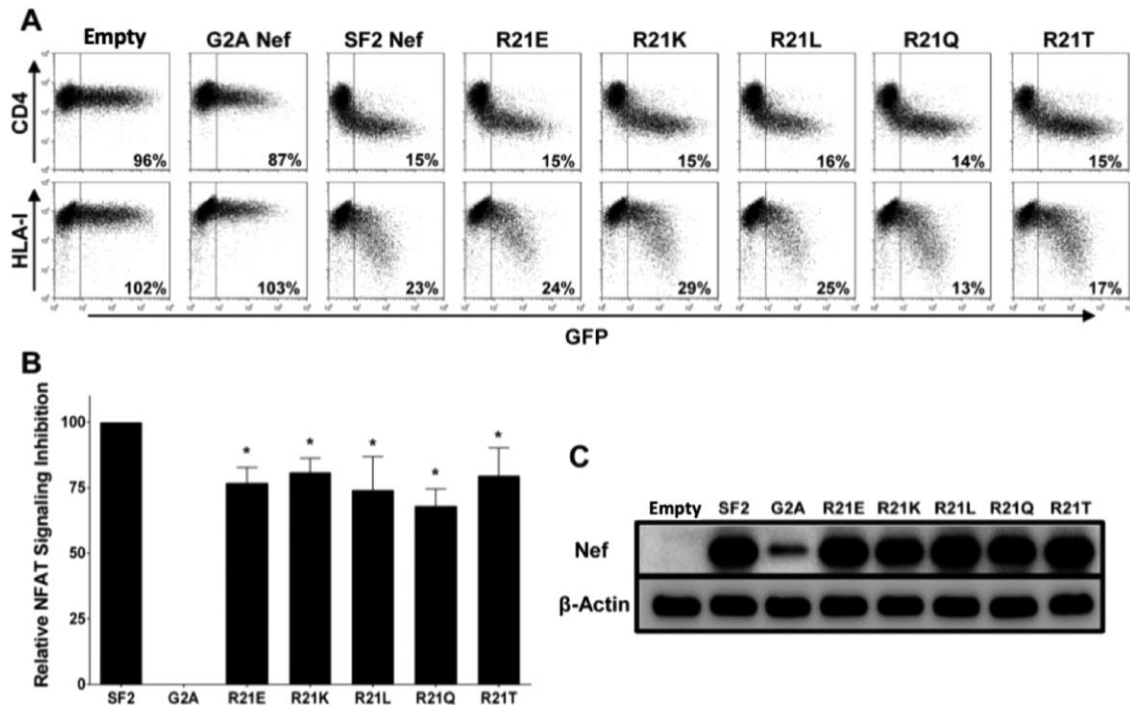


Figure 4.4.4. Polymorphisms at residue R21 impair Nef signaling inhibition function.

(A) The impact of site-directed mutations at residue 21 of wild type SF2 Nef (R21E, R21K, R21L, R21Q, and R21T) on Nef's ability to downregulate CD4 or HLA class I was assessed by flow cytometry. The proportion of CD4 or HLA-A*02 (y-axis) remaining on the cell surface is indicated in the lower right corner of each panel, which was determined by dividing the median fluorescence intensity (MFI) of GFP-positive cells by that of the GFP-negative subset (x-axis). Empty vector (Δ Nef) and myristoylation-defective G2A Nef were included as negative controls. Results are representative of at least three independent experiments. (B) The impact of the R21 mutations shown in panel A on Nef's ability to inhibit NFAT signaling was assessed in Jurkat cells as describe in the Methods. Results were normalized to SF2 Nef (set as 100 %) and the myristoylation-defective Nef G2A mutant (set as 0 %). Bars represent the mean and standard deviation for each mutant based on at least three independent experiments. Asterisks indicate results that were significantly different compared to SF2 Nef (one sample T-test; $p < 0.05$). (C) Western blot analyses were used to assess the steady-state protein expression of R21 mutants (upper blot). Empty vector (Δ Nef), SF2 Nef, and Nef G2A mutant were included as controls. Blots were probed for the β -actin protein as a cellular loading control (lower blot).

4.4.5. Chimeric Nef constructs highlight a critical role for the N-terminal domain

Variation at residue 21 did not fully account for the range of function observed in patient *nef* sequences. To identify additional genetic determinants of activity, we generated a series of chimeric proteins that combined wild type SF2 Nef with various domains from three Nef clones that displayed impaired TCR signaling inhibition (EC36, EC57 and EC58; exhibiting activities of 27 %, 45 % and 27 %, respectively) (Figure 4.4.5A). The least functional CP clone (CP32) and another highly impaired EC clone

(EC51) were excluded from this analysis since they also displayed substantially lower CD4 and HLA-A*02 downregulation activities, suggesting that they encoded mutations that severely affected protein expression or stability.

For EC36, a chimera encoding clone-derived residues 1-103 and SF2-derived residues 104-206 (EC36₁₋₁₀₃) displayed impaired signaling inhibition function (28%, relative to SF2), while function of the reciprocal chimera (EC36₁₀₄₋₂₀₆) was similar to wild type SF2 Nef (102%). Another chimera encoding clone-derived residues 1-32 (EC36₁₋₃₂) remained significantly impaired (15%), while the function of a chimera encoding only EC36-derived residues 33-103 was comparable to wild type Nef (99%). Together, these data indicated that a major determinant of attenuated function for the EC36 Nef clone was located within the first 32 amino acids. Analogous methods were used to study chimeric Nef products generated using EC57 and EC58 clones. Results demonstrated similarly that polymorphism(s) located within the N-terminal 32 amino acids of Nef were likely to be responsible for the impaired function of these clones.

To examine differences in protein expression or stability as a potential explanation for impaired function, we assessed steady-state Nef levels by Western blot (**Figure 4.4.5B**). Notably, while the rabbit polyclonal antibody used for these assays detected only weak bands for the three native EC-derived clones, all of the functionally impaired EC-SF2 chimeras were readily detected (except EC57₁₋₁₀₃ and EC58₁₀₄₋₂₀₆, which nevertheless retained ~70% and ~95% activity respectively). Overall, these results indicate that naturally occurring polymorphisms located in the N-terminal 32 amino acids of Nef significantly alter its ability to modulate NFAT signaling following TCR stimulation.

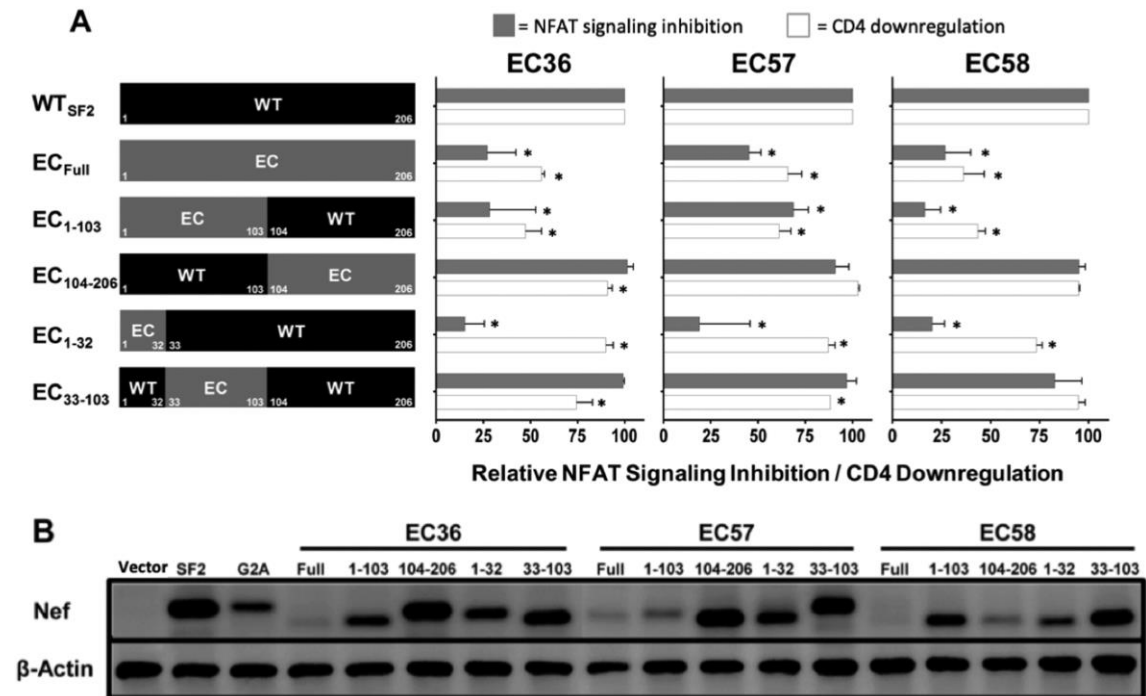


Figure 4.4.5. Determinants of reduced EC Nef function map to the N-terminal domain.

(A) Chimeric Nef clones were constructed between EC36, EC57 and EC58 isolates and wild type SF2 Nef, and their relative ability to modulate NFAT signaling (shaded bars, x-axis) or CD4 downregulation (open bars, x-axis) was assessed as described in the Methods. Data represent the mean and standard deviation for each construct based on three independent experiments. Asterisks indicate results that were significantly different compared to the SF2 Nef control (one sample T-test; $p < 0.05$). (B) Western blot analyses of native EC36, EC57 and EC58 and chimeras were used to assess steady-state protein expression for all constructs (upper blot). Empty vector, SF2 Nef, and Nef G2A mutant were included as controls. Blots were probed for the β -actin protein as a cellular loading control (lower blot).

4.4.6. Mapping N-terminal determinants of Nef-mediated TCR signaling modulation

We next sought to identify specific polymorphisms responsible for the reduced *in vitro* function of EC36, EC57 and EC58 Nef clones. The N-terminal sequences of these clones were aligned to SF2 Nef (using HXB2 as a numbering reference) and the resulting alignments inspected for differences (Figure 4.4.6A). EC36 and EC57 harbored a leucine (L) and lysine (K) respectively at Nef residue 21, which likely contributed in part to their functional impairment (see Figure 4.4.4), while EC58 harbored the consensus arginine at this residue. For all three EC clones, substitutions with respect to SF2 Nef tended to cluster into groups of three residues, suggesting that the predominately alpha-helical structure of Nef's N-terminal domain may constrain amino acid diversity. Introduction of the triple mutations G₈KA₁₀ or H₁₄KV₁₆ from EC36

Nef into SF2 Nef substantially reduced its *in vitro* function (to 58 % or 76 %, respectively) (**Figure 4.4.6B**). While this difference was statistically significant for G₈KA₁₀ (p=0.01), the residual function of this mutant was still higher than that of native EC36 Nef (27 %). Introduction of both G₈KA₁₀ and L₂₁ into SF2 Nef reduced its function to 16 %, whereas combining H₁₄KV₁₆ with L₂₁ did not result in further impairment. Together, these results indicate that the combination mutation, G₈KA₁₀ plus L₂₁, was a major determinant of impaired function for EC36 Nef.

In the case of EC57, introduction of Δ₉VV₁₁ into SF2 Nef resulted in no impairment, while introduction of P₁₄TV₁₆ reduced its function to 68 %. Of interest, while the Δ₉VV₁₁ polymorphism improved function of the K₂₁ mutant (from 81 % to 97 %), introduction of P₁₄TV₁₆ plus K₂₁ reduced SF2 Nef function to 46 %, which was similar to that of the native EC57 clone (45 %). Together, these results suggest that the combination mutation, P₁₄TV₁₆ plus K₂₁, was a major determinant of impaired function for the EC57 Nef clone. Notably, the CD4 downregulation activity of SF2 Nef mutants encoding either G₈KA₁₀ plus L₂₁ or P₁₄TV₁₆ plus K₂₁ remained largely intact (76 % and 99 %, respectively, compared to wild type SF2 Nef), suggesting that these polymorphisms did not have major effects on protein stability or tertiary structure.

Lastly, the EC58 Nef clone harbored a rare proline (P) to threonine (T) substitution at residue 25. Reverting this mutation to consensus proline in the EC58 sequence restored function from 27 % to 75 % (**Figure 4.4.6C**), demonstrating that this mutation was a major determinant of impaired activity for this clone. Other polymorphisms that are present in the N-terminal sequence of EC58 Nef, including P₁₄TV₁₆, may contribute to the remaining 25 % deficit in function.

The impact of these polymorphisms on Nef expression and stability were also assessed by Western blot (**Figure 4.4.6D**). While the G₈KA₁₀-containing mutants displayed lower steady-state protein levels compared to SF2 Nef, detection of the P₁₄TV₁₆-containing mutants was similar to wild type Nef. Notably, reversion of T25P in EC58 Nef significantly enhanced detection of this protein, suggesting that improved function was due at least in part to increased protein stability. Overall, we observed a weak correlation between higher protein expression levels and greater ability to inhibit NFAT signaling for polymorphisms identified in EC36 and EC58 Nef clones, but not for EC57 Nef. Additional studies will be necessary to examine this using a larger number of primary Nef isolates and chimeric proteins. Interestingly, we consistently observed lower

Western blot band intensity for the Nef G2A mutant compared to SF2 Nef (see **Figures 4.4.4C, 4.4.5B, and 4.4.6D**), suggesting that myristoylation may help to enhance the stability of wild type Nef. If so, differences in the N-terminal sequence of primary Nef isolates may alter their intracellular localization, thus affecting protein turnover and indirectly reducing Nef function.

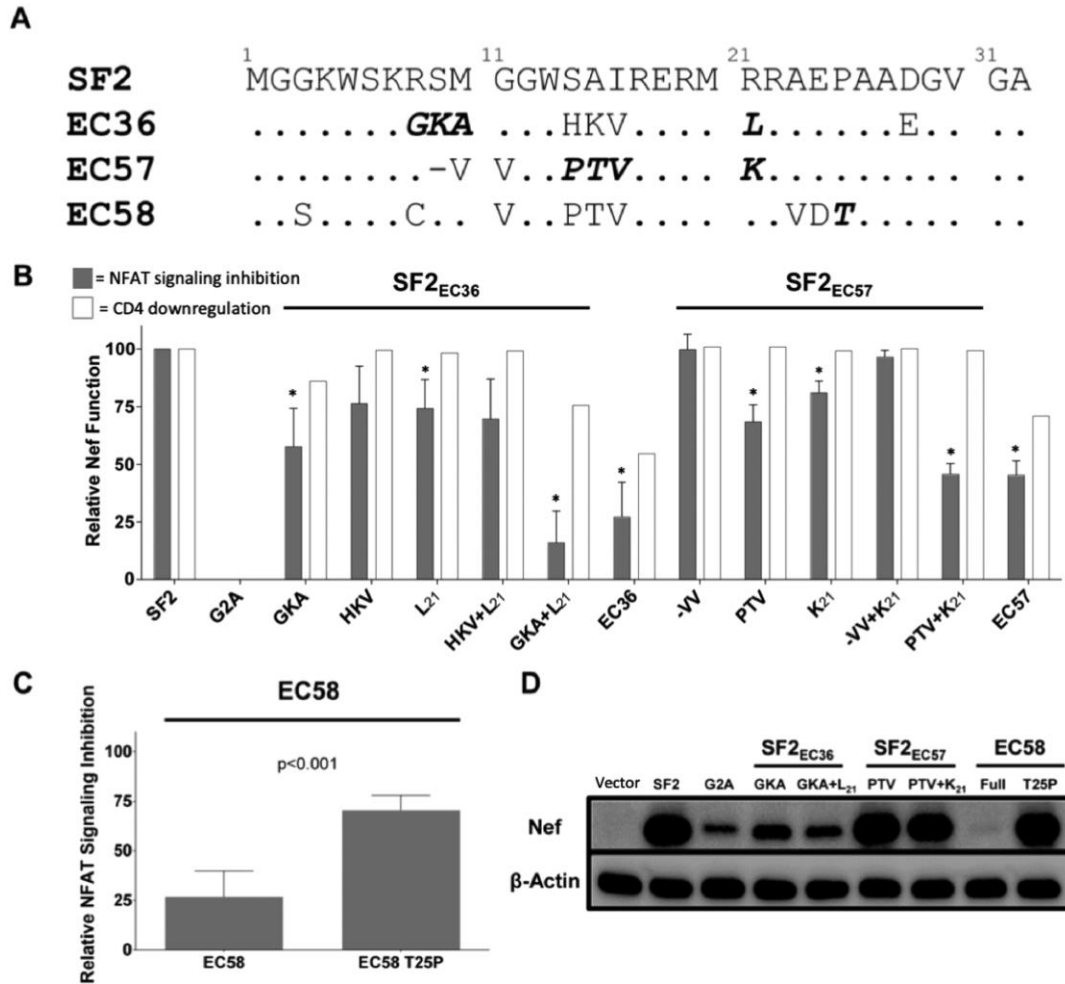


Figure 4.4.6. Identification of polymorphisms responsible for reduced EC Nef function.

(**A**) HXB2-aligned sequences for the first 32 amino acid residues of EC36, EC57, EC58 and SF2 Nef are shown. Identities are indicated by a period (.), deletions by a dash (—) and residues that were identified to contribute to Nef function by bold/italicized text. (**B**) Selected polymorphisms and combinations identified in EC36 and EC57 were introduced into SF2 Nef and their relative impact on NFAT signaling inhibition function (shaded bars, y-axis) or CD4 downregulation (open bars, y-axis) was assessed as described in the Methods. Normalized results for SF2 Nef (100 %) and Nef G2A (0 %) are shown for reference. Data for NFAT signaling inhibition represent the mean and standard deviation for each construct, based on at least three independent experiments. Data for CD4 downregulation represent the mean of two independent experiments, precluding formal statistical analyses. Asterisks indicate results that were significantly different compared to SF2 Nef (one sample T-test; $p < 0.05$). (**C**) A rare polymorphism (T25P) in the EC58 Nef clone was reverted to consensus proline using site-directed mutagenesis and NFAT signaling

inhibition function was assessed. Bars represent the mean and standard deviation of each clone, based on triplicate values obtained from at least three independent experiments. The p-value was determined using the student's T-test. (**D**) Western blot analyses were used to assess the steady-state protein expression of native Nef clones and mutants described in this figure (upper blot). Empty vector (Δ Nef), SF2 Nef, and Nef G2A were included as controls. Blots were probed for the β -actin protein as a cellular loading control (lower blot).

4.4.7. N-terminal Nef mutations disrupt co-localization with p56^{Lck}

To begin to assess potential mechanisms responsible for impaired TCR signaling inhibition by SF2-derived Nef mutants encoding G₈KA₁₀/L₂₁ and P₁₄TV₁₆/K₂₁, we examined their ability to co-localize with endogenous p56^{Lck} using the proximity ligation assay (PLA). Briefly, Jurkat cells were electroporated with pSELECT-GFPzeo vectors expressing each Nef mutant and transfected (GFP⁺) cells were isolated by FACS 20 hours later. SF2 Nef and the G2A mutant were included as positive and negative controls, respectively. Unstimulated cells were co-stained with rabbit anti-Nef and mouse anti-Lck antibodies and co-localization of these proteins was detected using PLA and confocal microscopy, as described in Methods. For a PLA signal to occur under these conditions, the distance between target proteins must be less than 40 nm. In preliminary experiments using SF2 Nef, PLA provided excellent sensitivity to detect Nef/Lck co-localization; however, PLA signal was absent when cells expressed the G2A mutant or if one or both of the primary antibodies was excluded during the staining procedure (**Figure 4.4.7A**). Notably, PLA signal in cells expressing the combination Nef mutants G₈KA₁₀/L₂₁ or P₁₄TV₁₆/K₂₁ was significantly lower than that seen for SF2 Nef (both $p < 0.0001$). Indeed, the signal observed for the G₈KA₁₀/L₂₁ mutant was similar to that seen for Nef G2A, while signal for the P₁₄TV₁₆/K₂₁ mutant was intermediate between Nef G2A and SF2 Nef. These results indicate that the G₈KA₁₀/L₂₁ mutant displayed a more profound impairment for Lck interaction compared to the P₁₄TV₁₆/K₂₁ mutant, consistent with its lower ability to inhibit TCR-mediated signaling events. Additional studies will be necessary to fully examine the intracellular distribution and residual function of these Nef mutants; however, the fact that they largely maintain their ability to downregulate CD4 suggests that the impact of these EC-derived N-terminal mutations on Nef function will be more subtle compared to G2A.

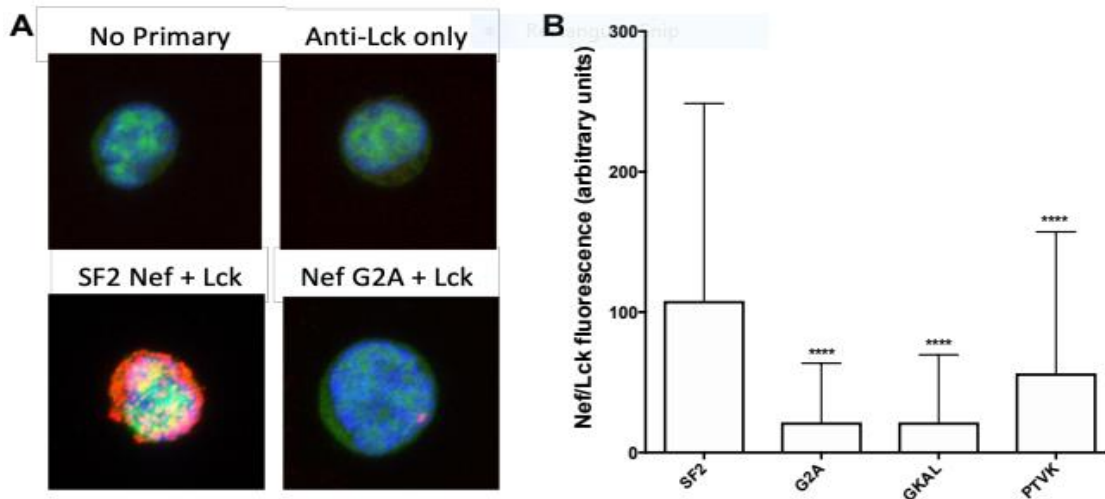


Figure 4.4.7. N-terminal Nef polymorphisms impair Lck interaction.

(A) Co-localization of Nef and endogenous Lck was examined using the Proximity Ligation Assay (PLA). Jurkat T cells were transfected with pSELECT-GFPzeo encoding wild type Nef SF2 and PLA was conducted using rabbit anti-Nef and mouse anti-Lck primary antibodies, as described in the Methods. Representative confocal microscopy maximum projection images illustrate detection of Nef/Lck co-localization in the presence of SF2 Nef, but not Nef G2A mutant. PLA signal was not detected in control reactions lacking one or both primary antibodies. (B) The fluorescence intensity of PLA staining, representing Nef/Lck co-localization, was calculated using confocal microscopy in Jurkat cells transfected with Nef SF2 or SF2 mutants G2A (negative control), GKAL (EC36), or PTVK (EC57). Mean fluorescence intensities per cell (arbitrary units, plus standard deviation) after background correction are shown, based on an average of 200 cells per Nef variant collected in two independent experiments. ****; $p < 0.0001$

4.5. Discussion

In this report, we examined the ability of HIV-1 Nef to modulate TCR-dependent signaling events by measuring NFAT transcription factor activity following anti-CD3 stimulation. Consistent with prior studies using similar transfection-based methods (Bandres and Ratner, 1994; Iafrate et al., 1997; Markle et al., 2013), we found that reference strain SF2 Nef and 91 primary Nef clones inhibited NFAT signaling in Jurkat cells, while the myristoylation-defective Nef G2A mutant did not. Most primary clones displayed relatively good *in vitro* function (73 of 91 showed >80 % function, relative to SF2 Nef), suggesting that there is selective pressure to retain this activity *in vivo*. Nevertheless, substantial differences were observed among natural isolates, including five Nef clones (4 from EC and 1 from a CP) that displayed <50 % function. Overall, the median NFAT inhibition activity of EC-derived clones (87 %) was lower than that of CP-derived clones (94 %) ($p < 0.001$). While the difference between these groups was

modest, the observed variability among clones suggests that moderate to severe impairment of this Nef function may contribute to the EC phenotype in at least some cases.

We identified 12 Nef polymorphisms located at 9 residues that were associated with differential *in vitro* NFAT inhibition activity. Four sites were located in Nef's N-terminal domain (residues 11, 21, 26, and 28) and five sites were located in Nef's core domain (157, 163, 170, 174 and 182). The strongest association, at residue 21, was confirmed by substituting the consensus arginine in SF2 Nef with five naturally occurring variants at this residue, which resulted in 19-32 % reductions in NFAT inhibition activity without appreciably altering Nef's ability to downregulate CD4 or HLA class I. Notably, R₂₁ is one of four positively charged arginine residues comprising the R₁₇xRxRR₂₂ motif, which is located in an amphipathic helix that is thought to stabilize Nef interaction with the plasma membrane (Gerlach et al., 2010; Welker et al., 1998), suggesting that membrane interaction by this domain is an important factor in Nef's ability to modulate TCR signaling. More modest impairments in Nef function were associated with polymorphisms at residues 11, 26, and 28, which could act similarly to affect the charge or helical structure of Nef's N-terminal domain. Prior studies have also shown that Nef's N-terminal domain can stabilize a multi-protein complex that includes Lck and protein kinase C family kinases (Baur et al., 1997; Wolf et al., 2008), which might be disrupted by polymorphisms in this region. Additional studies will be necessary to explore these potential mechanisms.

A critical role for Nef's N-terminal domain (specifically its first 32 amino acids) was also observed in analyses of chimeric proteins constructed using three Nef clones that were impaired for NFAT inhibition activity. Specific polymorphisms in this region were identified to be major determinants of reduced Nef function in each of these cases. Specifically, incorporation of G₈KA₁₀/L₂₁ (analogous to EC36) or P₁₄TV₁₆/K₂₁ (analogous to EC57) into SF2 Nef reduced its ability to inhibit NFAT signaling by 84% and 54 %, respectively, while having only a modest effect on its ability to downregulate CD4.

We also identified polymorphisms at five sites located in the core domain of Nef that were associated with NFAT signaling inhibition function. We did not explore these further in this study, so their impact on Nef function remains to be determined, but it is interesting to note that four of these sites (residues 157, 163, 170 and 182) were reported previously to be associated with clinical outcome in an independent cohort of

41 non-progressors and 50 progressors (Kirchhoff et al., 1999). The fifth site (residue 174) is located within a diacidic motif (D₁₇₄D₁₇₅) that is required for Nef to interact with the AP-2 clathrin adaptor complex and to downregulate CD4 (Janvier et al., 2001; Lindwasser et al., 2008). The importance of this interaction for Nef's ability to modulate T cell signaling is uncertain, since these activities are genetically separable (Iafrate et al., 1997), and the polymorphism identified in our study (E₁₇₄) is frequently observed in natural isolates and E₁₇₄ has been shown to maintain wild type ability to downregulate CD4 (Lindwasser et al., 2008). Notably, no associations were observed in canonical domains that are required for Nef to interact with cellular kinases, including the proline-rich SH3-binding motif (P₇₂xxP₇₅) that allows Nef to re-localize p56^{lck} to an intracellular compartment (Thoulouze et al., 2006) or the phenylalanine-containing motif (F194 in SF2) that binds Pak2 and disrupts actin cytoskeletal remodeling (Agopian et al., 2006; Stolp et al., 2010). While this was expected since these critical motifs are highly conserved in natural isolates (Renkema and Saksela, 2000; Stolp et al., 2010), variation at one or more of the identified residues may affect the function of these domains through alteration of Nef structure or stability. Additional studies will be necessary to elucidate the impact of these natural Nef polymorphisms.

A limitation of this study is that it relied on overexpression of Nef clones in an immortalized T cell line, which may not fully reflect the role played by Nef in primary HIV-infected T cells. Indeed, several reports have indicated that Nef can enhance NFAT signaling through association with Src-family kinases, protein kinase C-theta, RAS/MAP kinase pathways and inositol trisphosphate receptor (Fortin et al., 2004; Manninen et al., 2001; Manninen et al., 2000; Manninen and Saksela, 2002; Neri et al., 2011; Pan et al., 2012). In most of these reports, T cells were stimulated using a combination of anti-CD3 plus anti-CD28 antibodies, which elicits both TCR-dependent and -independent signaling pathways that might alter the impact of Nef on cellular responses. Notably, Neri et al. (Neri et al., 2011) demonstrated that Nef displayed a “dual role” that depended on the T cell's status and the strength of stimulation — resulting in either super-induction of NFAT in freshly isolated quiescent T cells or suppression of NFAT in sub-optimally activated T cells. The results of our study are consistent with the latter scenario and should be interpreted in this context. Additional research will be necessary to confirm our observations in primary T cell models.

Productive infection of a CD4⁺ T-cell by HIV-1 is influenced by the cell's activation state. Our results contribute to a model wherein Nef acts to modulate the intracellular signaling events in virus-infected T cells in order to optimize replication (Abraham and Fackler, 2012). By blocking proximal TCR signaling mediated by NFAT and potentially other transcription factors, Nef may prevent activation-induced cell death and thus enhance progeny virion production. This would be particularly advantageous in the context of high antigen load, such as that seen during chronic infection. We speculate that even a modest impairment in Nef's ability to inhibit TCR signaling could result in premature death of infected T cells and a substantial reduction in viral burst size, which may contribute to lower plasma viremia observed in EC.

In conclusion, this study provides new insight into HIV-1 polymorphisms in primary *nef* sequences that influence its ability to modulate TCR-dependent signaling events. As a group, controller-derived Nef clones displayed modestly lower ability to inhibit NFAT signaling compared to those isolated from progressors, but larger defects were found in a subset of clones. Notably, the data demonstrate that polymorphisms located in the Nef's N-terminal domain, particularly at residue 21 in the arginine-rich (R₁₇xRxRR₂₂) motif, contributed to impaired function. Our results highlight the impact of natural sequence variation on Nef's ability to modulate TCR-mediated signaling, which may contribute to clinical outcome in some cases of spontaneous HIV-1 control.

4.6. References

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Chapter 5. Summary and conclusions

In this dissertation, I explored the impact of natural sequence variation on Nef function by examining primary Nef clones isolated from elite controllers and chronic progressors. My work represents the largest study to date investigating the function of EC Nef sequences, and the first study investigating a large panel of primary Nef isolates for their ability to modulate T cell receptor (TCR) mediated signaling. Results provide evidence that impaired Nef function is a hallmark of spontaneous viral control, that naturally occurring polymorphisms in Nef affect its protein function, that viral adaptation to CTL responses restricted by protective HLA alleles contribute significantly to Nef impairment, and that immune pressure focused on Nef's N-terminal alpha-helix may have the greatest impact on the viral protein's ability to modulate TCR-mediated signaling.

5.1. Summary of chapter 2

In **chapter 2**, I cloned Nef isolates from 45 elite controllers and 46 chronic progressors to assess their *in vitro* function. Five well-established Nef activities were examined, namely enhancement of viral replication capacity, enhancement of viral infectivity, CD4 down-regulation, HLA-I down-regulation, and CD74 up-regulation. Nef clones isolated from chronic progressors generally maintained higher functionality compared to those from elite controllers. Furthermore, 80% of progressor-derived clones were highly active for at least three of the functions tested, suggesting a phenotypic requirement to maintain multiple Nef functions during chronic infection *in vivo*.

Statistical analysis was used to investigate the association between functions, and between function and clinical parameters such as CD4⁺ T cell count and HIV plasma viral load. A significant inverse relationship was observed between Nef-mediated virion infectivity and CD4⁺ T-cell count in chronic progressors. While it was previously known that Nef is required for HIV pathogenesis and disease progression *in vivo*, my results provide evidence that this single Nef function, viral infectivity, may have a significant bearing on disease progression.

Examining statistical associations between the multiple functions in primary Nef sequences helped me to formulate hypotheses about the mechanistic links between functions. Pairwise correlations revealed positive relationships between CD4 down-regulation and all other activities except HLA-I down-regulation. These results suggest, for example, that CD4 down-regulation and CD74 up-regulation may be mediated by shared Nef motifs and cellular interactions. Furthermore, my results are consistent with the hypothesis that Nef motifs and cellular interactions involved in CD4 down-regulation also contribute to promotion of virion release and virion infectivity, either through down-regulation of CD4 itself, which can assist virion budding (Ross et al., 1999), or the recently-discovered down-regulation of SERINC family host restriction factors, which increases the ability of virions to infect target cells (Rosa et al., 2015; Usami et al., 2015). Indeed, recent studies demonstrated that internalization of CD4 and SERINC require interaction between Nef's dileucine motif (ExxxLL₁₆₅) and cellular adapter protein complex AP-2, and that both cell surface proteins are shuttled to Rab5⁺ early, Rab7⁺ late, and Rab11⁺ recycling endosomes (Shi et al., 2018). In my work, I also identified new associations between Nef function and polymorphisms located in the protein's N-terminal domain. Interestingly, variation at Nef codon 21 (within a highly conserved basic amino acid motif R₁₇xRxRR₂₂ that is involved in membrane targeting (Fackler et al., 2006) and vesicle secretion (Ali et al., 2010)) was associated with lower Nef-mediated viral infectivity and CD74 up-regulation. My results presented in **chapters 3-4** provide further evidence that this domain plays a major role in modulation of TCR-mediated signaling.

In contrast, although Nef clones isolated from elite controllers were generally functional, I observed that their activity for all five Nef functions tested was significantly lower compared to clones isolated from chronic progressors. The relatively large size of our elite controller cohort improved my ability to assess the range of activities: while these Nef clones displayed reduced median functionality, some controller-derived clones displayed WT activity for functions. These observations may help to resolve discrepancies between previous studies of HIV long-term non-progressors and controllers that investigated only one or two Nef functions using isolates collected from fewer individuals, some of which reported relative preservation of CD4 and/or HLA-down-regulation function (Nou et al., 2009; Zuo et al., 2012), while others reported inefficient Nef-mediated CD4 and/or HLA-down-regulation (Casartelli et al., 2003; Corro

et al., 2012; Tobiume et al., 2002) and infectivity (Tobiume et al., 2002) compared to clones from progressors.

Results also provided clues as to why controller-derived Nef clones were relatively impaired. One hypothesis is that controllers were infected by a virus that encoded a defective *nef* sequence. However, several pieces of evidence point away from this hypothesis. First, controller Nef clones did not appear to descend from a recent common ancestor, suggesting that genetic variability was not inherited. Second, within the controller cohort, there was a significant association between possession of the protective HLA-B*57 alleles and CD4 down-regulation. Furthermore, accumulation of B*57-associated polymorphisms correlated with impairment in 4 of 5 functions. These observations suggest that within-host immune pressure can cause attenuating mutations in *nef*. My results are consistent with previous studies of recombinant viruses encoding *gag* and *pol* sequences from the same elite controller cohort (Brumme et al., 2011; Miura et al., 2009a), which also found a relationship between B*57-associated immune pressures and viral protein function.

Notably, these results point toward a significant role for cytotoxic T lymphocytes (CTL) in placing strong immune pressure on Nef in elite controllers. Although protective HLA alleles are enriched in controllers, they are not sufficient or necessary for viral control. For example, in our cohort, 17/45 (38%) of ECs expressed HLA B*57, but so too did 8/46 (17%) of progressors. Furthermore, while progressors who expressed B*57 harboured mutations that are commonly associated with expression of this allele, controllers who expressed B*57 accumulated rare or atypical mutations in Nef, which could be the result of a relatively unique and idiosyncratic B*57-restricted CTL response. This is consistent with previous observations that controllers display rare mutations in *gag* that are associated with a greater impact on viral fitness (Bailey et al., 2009; Miura et al., 2009b). In short, not all B*57+ individuals elicit exactly the same CTL response, although the characteristics of more effective CTL responses remain to be fully determined. In light of this, a combination of a protective HLA allele plus the “right” CTL response may be necessary to induce deleterious Nef mutations. Taken together, my results suggest HLA-B*57-associated CTL pressure selects for non-canonical Nef polymorphisms in controllers, which contribute additively to multiple functional impairments. It is notoriously challenging to isolate and identify CTL clones specifically

responsible for protection and viral control, but significant work in this direction has begun in our lab (Ogunshola et al., 2018).

5.2. Summary of chapter 3

In my initial studies, I did not measure the ability of Nef sequences to modulate T cell receptor (TCR)-mediated signaling. Although there was overwhelming evidence that Nef affected signaling, the prior literature did not present a consensus on which signaling events were modulated, nor whether Nef had a positive or negative effect on these events. As a result, it was not immediately apparent which signaling event to measure. Following an extensive review of the literature, I set out in **chapter 3** to develop an *in vitro* assay to measure Nef's ability to modulate TCR-mediated signaling.

My primary objective was for the assay to be relatively high throughput and reproducible so as to allow screening of a panel of canonical Nef mutants and ultimately primary Nef isolates from HIV controllers and progressors (the latter being investigated in **chapter 4**). In preliminary experiments, transient expression of Nef consistently displayed a strongly inhibitory effect on transcription factor activity, consistent with more recent and contemporary findings (Haller et al., 2007; Abraham et al., 2012; Pan et al., 2012). Influenced by these observations, I hypothesized that Nef disrupts proximal TCR-mediated signaling, possibly to increase the threshold for activation of infected T cells. This could function to guard against activation induced cell death in high stimulation environments, or even promote latency in low stimulation environments. However, this scenario may not preclude Nef having a positive effect on cell activation state by organizing alternative signaling pathways (Wolf et al., 2001; Haller et al., 2007) or differentially affecting signaling based on the intrinsic activation state of the cell (Neri et al., 2011). Furthermore, Nef is thought to have a positive effect on viral replication by promoting virion release through down-regulation of CD4 (Ross et al., 1999) and by enhancing virion infectivity through down-regulation of SERINC5 (Rosa et al., 2015; Usami et al., 2015). In sum, despite its name, Nef can act as both “negative factor” and a “positive factor” in order to optimize viral pathogenesis.

I found that wild type SF2 Nef inhibits early TCR signaling events in Jurkat T cells stimulated using anti-CD3 antibody. Both NFAT and AP-1 transcription factor activity were inhibited over the first 6 hours. The NFAT luciferase reporter system was chosen for subsequent experiments because basal NFAT signaling was minimal,

induction of NFAT activity was robust, and inhibition by Nef was reproducibly strong (~5-fold inhibition). Nef also inhibited membrane proximal phosphorylation of LAT and PLC-gamma within minutes of stimulation by soluble anti-CD3, supporting my hypothesis that NFAT activity is an appropriate measure of inhibition of proximal TCR signaling.

Using the NFAT luciferase reporter assay to screen a panel of canonical Nef mutants, I found that Nef's SH3-binding domain (PXXP) contributed ~30% to Nef's NFAT-inhibition function, possibly because of its role in trafficking Lck away from the plasma membrane. Indeed, although Proximity Ligation Assay (PLA) experiments found that the AXXA mutant was surprisingly able to co-localize within <40 nm of Lck, it could not internalize Lck (consistent with the results of Haller et al., 2007). Interestingly, Nef's N-terminal arginine-rich alpha-helix (R4) was even more crucial for NFAT-inhibition, contributing ~40% of the function -- through an unknown mechanism. Alanine mutation of R4 (to A4) caused an "intermediate" defect in Nef's ability to co-localize within 40 nm of Lck and, similarly, an intermediate defect in its ability to internalize Lck.

Together, my results are consistent with a model wherein Nef inhibits TCR-mediated signaling partly through internalization of Lck, but where this is not the sole mechanism. Nef's N-terminal anchor region may play a crucial role in Nef's conformation and/or its ability to properly localize within signaling microdomains, affecting its interaction with Lck and possibly other partners affecting NFAT inhibition. Further studies will be required to tease apart these mechanisms in more detail. While another group reported, in a series of studies, that a chimeric Nef N-terminal anchor domain (fused to a CD8 transmembrane protein) organizes a complex alternative signaling pathway (Wolf et al., 2001; Witte et al., 2004; Wolf et al., 2008; Witte et al. 2008), my findings here are, to my knowledge, the first evidence supporting a significant role for Nef's N-terminal alpha-helix in suppressing TCR-mediated signaling.

5.3. Summary of chapter 4

In **chapter 4**, the NFAT luciferase reporter assay was employed to assess the impact of natural sequence variation on Nef's ability to inhibit TCR-mediated signaling. To do this, we screened a panel of 45 controller- and 46 progressor-derived Nef clones (assembled in **chapter 2**). Jurkat T cells were transfected with NFAT-luciferase and expression vectors encoding controller or progressor Nef clones, then after 18 hours stimulated with anti-CD3 antibody for 6 hours. Note that in this "scaled-up" assay,

transfectants were not single cell sorted; while the NFAT reporter assay had been verified by single cell sorting, results were comparable and reproducible without sorting.

Overall, the median NFAT inhibition activity of controller-derived clones (87 %) was lower than that of progressor-derived clones (94 %) ($p < 0.001$). I observed statistically significant associations between Nef-mediated NFAT inhibition and downregulation of CD4 ($p = 0.003$) and HLA ($p < 0.001$). The observation that modulation of signaling correlated most strongly with HLA downregulation is consistent with prior reports of a link between these functions (Hung et al., 2007; Atkins et al., 2008), which may be mediated by a shared requirement for Nef's PXXP domain (Iafrate et al. 1997). However, in my mutagenesis studies (**chapter 3**), I found HLA down-regulation and NFAT inhibition were separable functions of Nef. I also noted that NFAT and CD4 activities correlated significantly with steady-state Nef expression levels as determined by Western blot, helping to reconcile my data with previous work indicating that Nef's ability to modulate TCR signaling is distinct from CD4 down-regulation (Iafrate et al., 1997).

The strongest correlation between any natural sequence variation and poor NFAT inhibition function was found at Nef residue 21 (R21K), which was more common in controller-derived clones. This result was confirmed by site-directed mutagenesis in the SF2 Nef backbone. Furthermore, in three controller-derived Nef clones with poor NFAT inhibition function, major determinants of attenuated function were located within the first 32 amino acids, including R21K as well as several other positions within or flanking Nef's N-terminal alpha-helix (especially amino acids 8-10 and 14-16). SF2 Nef sequences incorporating these natural N-terminal mutations were also defective for Lck association, as measured by Proximity Ligation Assay. These results were remarkably consistent with my findings in **chapter 3** that mutation of the four positively charged arginines in the N-terminal alpha-helix (R₁₇XRxRR₂₂, which includes R21) had the most dramatic impact on NFAT signaling of all canonical Nef mutants in my panel.

In my published review on Nef's role in modulating T cell signaling (**Markle et al., 2013**), I postulated that Nef can act as a "negative factor" with respect to inhibiting proximal TCR signaling, while at the same time serving as a "positive factor" for viral replication and spread. One model that has been proposed to explain these apparent contradictions is that Nef inhibits proximal TCR signaling, but organizes an alternative distal signaling complex within the TGN involving Ras (Haller et al., 2007; Abraham and

Fackler, 2012). However, there are other ways to explain how Nef acts to modulate the intracellular signaling events in virus-infected T cells in order to optimize replication. For example, by blocking proximal TCR signaling mediated by NFAT and potentially other transcription factors, such as AP-1, Nef may prevent activation-induced cell death and thus enhance progeny virion production. This would be particularly advantageous in the context of high antigen load, such as that seen during chronic infection. Even a modest impairment in Nef's ability to inhibit TCR signaling could result in premature death of infected T cells and a substantial reduction in viral burst size, which may contribute to lower plasma viremia observed in EC. Similarly, Nef could employ the same mechanism to promote HIV latency. Nef is the first protein to be expressed in the viral life cycle, and in the highest quantities. By blocking proximal signaling Nef may increase the threshold for TCR-mediated activation in CD4+ T cells. It is conceivable that Nef could thereby promote reversion of infected cells to a resting memory state thought to be characteristic of latent cells (Siliciano and Greene, 2011) or maintenance of this state in the context of suboptimal stimulation conditions.

At the same time, it is important to emphasize that Nef does also act as a positive factor in various stages of the viral life cycle, including Gag assembly in the context of viral reactivation (XT Kuang, unpublished PhD thesis 2018), virion release (Ross et al., 1999), and virion infectivity (Rosa et al. 2015), and that Nef may organize alternative signaling complexes (Haller et al., 2007; Witte et al. 2008), perhaps to carry out unknown transcriptional programs.

5.4. Conclusions

In conclusion, my thesis has investigated the impact of natural sequence variation in primary *nef* sequences. I have identified naturally-occurring Nef mutations that impact 6 different functions. In particular, analysis of Nef clones isolated from elite controllers has provided evidence that immune responses restricted by protective HLA-B*57 alleles impair Nef function and that rare CTL responses targeting Nef may contribute to viral control. By measuring Nef's ability to modulate TCR-mediated signaling, my results have further elucidated mechanisms underlying this function, as well as the relation between this Nef activity and other more widely studied functions of Nef. TCR signaling inhibition does not require CD4 or HLA downregulation; however, signaling inhibition function does require the PXXP domain which contributes to HLA

downregulation. Notably, Nef's N-terminal alpha-helix anchor plays a crucial role in modulation of TCR signaling, but is dispensable for CD4 downregulation. In primary *nef* sequences, the naturally-occurring polymorphisms that correlated most strongly with signaling inhibition function were also found in the N-terminal alpha-helix, and introduction of these mutations into an SF2 Nef backbone had a severe impact on function.

These results may inform HIV vaccine design. Several ongoing vaccine trials do not incorporate Nef as an immunogen. For example, the SAV001 trial employs a *nef*-deleted live-attenuated virus (Gao et al., 2018) and “mosaic vaccine” approaches strategically target conserved regions of Gag and Pol, but not Nef (Wee et al., 2017). The rationale for excluding Nef is based, in part, on the theory that Nef may act as a “decoy”, leading the host CTL response to shift its focus away from more desirable targets (Daniel et al., 1992). However, the data presented in the current thesis suggest that elite controllers do mount robust CTL responses against Nef, selecting for escape mutations affecting protein function and viral spread – without compromising the “controller” phenotype. Indeed, it may be possible for vaccines to elicit CTL responses against critical Nef domains: for example, follow-up studies to the STEP trial found that there were powerful CTL responses against Nef, and that the two of the most common and dominant responses were directed against epitopes found within the N-terminal alpha helix (Nef₁₇₋₂₅) and SH3-binding domain (Nef₆₅₋₇₆) (Park et al., 2016). Immune pressure against these Nef regions may attenuate TCR-modulation and/or HLA-downregulation function, thereby promoting CTL killing of infected cells.

Furthermore, these Nef domains may be ideal targets for blockade of Nef by small molecule inhibitors. There are currently no safe classes of anti-retroviral drugs that target Nef (Dekeban and Dikeakos, 2017). However, given that Nef downregulates HLA, blocking this activity could also help CTL to clear infected cells more efficiently (Trible et al., 2013). Given that HLA-downregulation and TCR-inhibition function rely on similar Nef domains, drugs could target both functions simultaneously. In particular, the NFAT-inhibition assay could be used to screen libraries of compounds for their ability to blockade Nef's ability to modulate TCR signaling. This “Nef blockade” approach could be used as part of a cocktail, possibly in combination with a vaccine regimen (to boost responses) or with T cell therapies.

Taken together, by inhibiting proximal TCR signaling, Nef may guard against activation induced cell death (AICD) and/or promote latency. In the context of viral control, CTL responses restricted by protective HLA molecules may be responsible for attenuation of this Nef function, particularly by selecting for escape mutations in Nef's N-terminal alpha-helix. As a result, Nef proteins in these individuals may have reduced ability to block AICD or to promote latency. Vaccines or immunotherapies which seek to mimic EC protective immune responses, or drug therapies that target these Nef domains, may contribute to HIV eradication strategies.

5.5. References

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