Mechanisms by which HIV-1 Nef disrupts the intracellular trafficking of host proteins

by

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To Stephen

Acknowledgements

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Preface

This thesis is the compilation of published and unpublished work on the effects of HIV-1 Nef on cellular trafficking pathways. Nef removes a number of host proteins from the cell surface, interfering with antigen presentation and immune cell activation, resulting in evasion of the host immune system. Nef directly promotes HIV-1 pathogenesis and is therefore an appealing target for therapeutic intervention.

Chapter 1 is a discussion of the biological properties of HIV-1 Nef and Nef's impact on HIV-1 pathogenesis. We compare two competing models for the mechanism by which Nef reduces the surface expression of MHC-I and discuss the current evidence for the downmodulation of a variety of host factors by Nef.

In Chapter 2, we analyze a panel of putative cellular targets of Nef. We compare the relative impact of Nef on expression of each of these targets and assess the contribution of cellular trafficking machinery. This work has been published as Leonard JA, Filzen T, Carter CC, Schaefer M, Collins KL. "HIV-1 Nef Disrupts Intracellular Trafficking of Major Histocompatibility Complex Class I, CD4, CD8, and CD28 by Distinct Pathways That Share Common Elements." *J Virol.* (2011) Jul;85(14):6867-81.

In Chapter 3, we present data assessing the relative contribution of ARF-1 and ARF-6 in Nef-induced downmodulation of MHC-I. Expression of a constitutively GTP-bound ARF-1 mutant revealed a requirement for ARF-1 in Nef-dependant MHC-I trafficking.

Conversely, ARF-6 was found to be dispensable for the effects of Nef on MHC-I, as demonstrated in experiments with ARF-6 mutants or ARF-6 depletion. These data have been submitted for publication and are currently in revision.

In Chapter 4 we describe methods for a live cell flow-cytometric screen to identify chemical inhibitors of Nef-mediated MHC-I downmodulation. We present preliminary findings from a screen of approximately 100,000 small molecules, in which we identified a small pool of Nef inhibitors for further development.

Finally, Chapter 5 is a discussion of impact of the data presented in the previous chapters, as well as proposed future directions for additional mechanistic studies of Nef and for developing small molecule inhibitors of Nef for clinical use.

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ABSTRACT

Mechanisms by which HIV-1 Nef disrupts the intracellular trafficking of host proteins

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The Nef protein is an important HIV virulence factor that promotes the degradation of host proteins to augment virus production and facilitate immune evasion. The best-characterized targets of Nef are major histocompatibility complex class I (MHC-I) and CD4, but Nef also has been reported to target several other proteins, including CD8β, CD28, CD80, CD86, and CD1d. To compare and contrast the effects of Nef on each protein, we constructed a panel of chimeric proteins in which the extracellular and transmembrane regions of the MHC-I allele HLA-A2 were fused to the cytoplasmic tails of CD4, CD28, CD8β, CD80, CD86, and CD1d. We found that Nef coprecipitated with and disrupted the expression of molecules with cytoplasmic tails from MHC-I HLA-A2, CD4, CD8β, and CD28, but Nef did not bind to or alter the expression of molecules with cytoplasmic tails from CD80, CD86, and CD1d. In addition, we used short interfering RNA (siRNA) knockdown and coprecipitation experiments to implicate adaptor protein-1 (AP-1) as a cellular cofactor for Nef in the downmodulation of both CD28 and CD8β. The interaction with AP-1 required for CD28 and CD8β differed from the AP-1 interaction required for MHC-I downmodulation in that it was mediated through the

dileucine motif within Nef ($LL_{164,165}AA$) and did not require the tyrosine binding pocket of the AP-1 μ subunit. In addition, we demonstrated a requirement for COP-I coatomer subunit β-COP as a cellular cofactor for Nef that was necessary for the degradation of targeted molecules HLA-A2, CD4, and CD8. Additionally, we expressed ADP-ribosylation factor (ARF) mutants to demonstrate a requirement for ARF-1, but not ARF-6, in Nef-dependent downmodulation of MHC-I. Finally, we developed cell-based, flow cytometric high throughput screening methods to identify a select group of chemical compounds which may be potent inhibitors of MHC-I downmodulation by Nef. These studies provide important new information on the similarities and differences between the ways in which Nef affects intracellular trafficking of different host proteins and help focus future research on the best potential pharmaceutical targets.

Chapter 1

The effects of HIV-1 Nef on cellular proteins important for immune responses

1.1. Overview

This dissertation primarily pertains to HIV-1 Nef function. Nef removes a variety of host proteins from the cell surface, disregulating immune signaling and activation. However, conflicting reports exist regarding which proteins are targeted by Nef, and what mechanisms are involved. In this chapter, the relevant background and current understanding of Nef function will be discussed. Subsequent chapters will describe the development and employment of tools to directly compare the relative effects of Nef on various putative target proteins, and investigate trafficking factors and adaptor protein involvement. Furthermore, this dissertation will explore methods for identifying chemical inhibitors of Nef, an appealing drug target due to the importance of Nef for HIV-1 pathogenesis.

1.2. Summary of the HIV pandemic

Despite major advances in research and treatment, the human immunodeficiency virus (HIV) continues to persist as a pandemic. In 2009, an estimated 33.4 million people were living with HIV, including 2.1 million children. There were 2.7 million new infections

and 2 million people died of acquired immunodeficiency syndrome (AIDS) (194). While great progress has been made in drug therapies that dramatically decrease mortality and prevent mother to child transmission, a cure for the disease remains an elusive goal and an effective, prophylactic vaccine is not yet in hand.

1.3. Natural history of the untreated disease

Following initial infection by HIV, a partially effective immune response reduces the viral load to an equilibrium level, or set point, the magnitude of which has prognostic significance with respect to how rapidly disease progression occurs (137). During a period of clinical latency, HIV preferentially infects and destroys activated $CD4^+$ T lymphocytes, including those T cells that are HIV-specific, which eventually leads to a defective anti-HIV immune response (54). Once the total $CD4^+$ T cell count reaches <200 cells per microliter of blood, the clinical definition of AIDS, the immune system is functionally impaired and HIV infected individuals become susceptible to opportunistic infections, which are the primary cause of death from AIDS.

1.4. The virus

HIV-I is a retrovirus of the genus *Lentivirus*, a group of viruses that characteristically cause a chronic infection with a long incubation period and have the ability to replicate in nondividing cells. Like all retroviruses, HIV-1 reverse transcribes its single-stranded

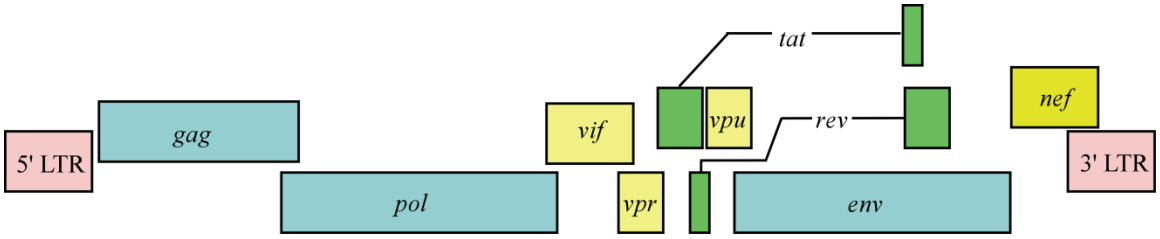


Figure 1.**1. HIV-1 Genome and Nef structure.** Schematic of HIV-1 genome. HIV-1reading frames are shown to reveal HIV-1 genes and their relative genome locations. Open reading frames are shown as rectangular boxes. The spliced reading frames, tat and rev, are shown as boxes connected by lines. Adapted from (205).

RNA (ssRNA) genome into a DNA intermediate prior to integration into the host cell genomic DNA. Also typical of retroviruses, HIV encodes group-specific antigen (*gag*), polymerase (*pol*), and envelope (*env*) (Figure 1.1). (for review see (68), (86)). Gag polyprotein is the major structural component of the viral capsid, and is necessary and sufficient for virus-like particle assembly (71). Gag is processed into matrix, capsid, and nucleocapsid by the viral protease within nascent virions in the process of particle maturation. Pol is also initially expressed as a polyprotein that is cleaved within newly budding virions into functional enzymes reverse transcriptase (RT), integrase, and protease. Envelope (Env) is likewise expressed as a 160 kilodalton (kDa) polyprotein (gp160), which is cleaved by the viral protease into gp41 and gp120 within the Golgi of the infected cell. gp120 directly interacts with CD4, the viral receptor, while gp41 is required for virus fusion with the host cell (115). Additionally, HIV-1 Env has been demonstrated to directly reduce the surface expression of CD4 in order to prevent viral superinfection and enable viral particle assembly and release (17, 88, 113, 167).

1.4.1 HIV-1 replication

The HIV-1 lifecycle begins with binding of Env to the viral receptor, CD4, and co-receptors, typically CCR5 or CXCR4 (Figure 1.2) (3, 44, 48, 64). This ligation event induces a conformation change in Env and allows fusion with the cell membrane and release of the viral capsid into the cytoplasm (172). In the cytoplasm, RT accesses the viral genome and begins transcribing the RNA into double-stranded DNA (11). This DNA genome, along with RT, Vpu, matrix, and integrase form a pre-integration complex

Figure 1.2. HIV Life Cycle. 1. HIV Envelope binds to CD4 and co-receptor CXCR4 or CCR5. The virion fuses with the plasma membrane of the target cell and releases the RNA-containing capsid into the cytoplasm. 2. The viral reverse transcriptase (RT) transcribes the single-stranded RNA (ssRNA) genome into double-stranded DNA (dsDNA). 3. The dsDNA genome is transported into the nucleus through a nuclear pore, where integrase mediates integration of provirus into the cellular genome. The provirus may remain latent indefinitely. 4. Active infection results from the expression of viral mRNA and proteins. 5. Viral proteins, along with two strands of viral ssRNA, assemble at sites of Gag multimerization at the plasma membrane. The nascent virion buds and is then released from the plasma membrane. 6. Protease cleaves HIV polyproteins within the immature virion, inducing maturation into fully infectious HIV.

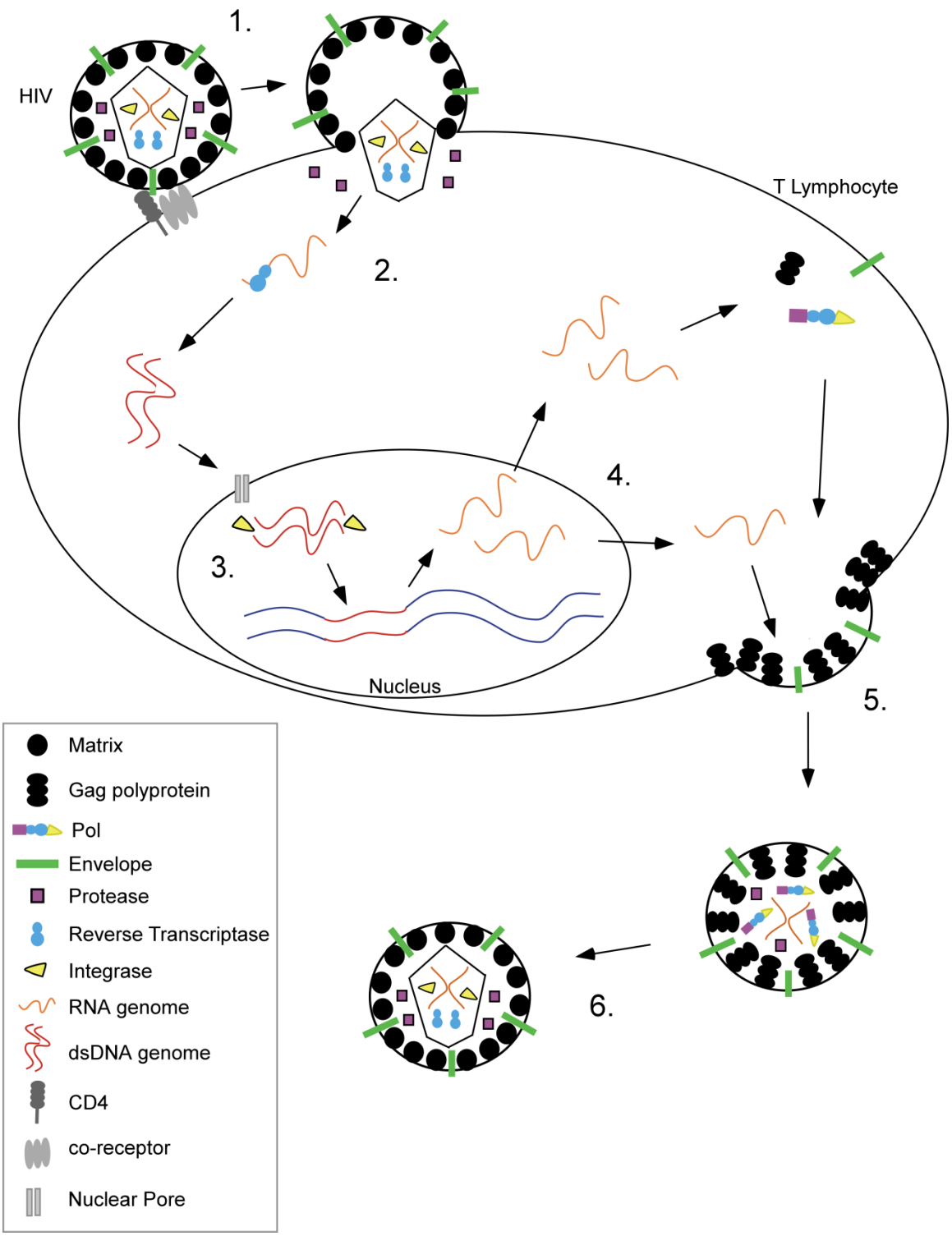


Figure 1.2. HIV Life Cycle

(PIC) (23, 61, 62, 98, 100). The PIC is translocated into the nucleus of the cell through a nuclear pore, where integrase mediates the insertion of viral DNA into the cellular genome, forming a provirus (22, 58). Here, the provirus may remain latent in the absence of viral gene expression, or may engage in active infection. During active infection, viral genes are transcribed into RNA for incorporation into virions as full-length viral genomes, or processed for subsequent translation of viral proteins. Virion assembly occurs through the accumulation of HIV structural proteins Env and Gag at the plasma membrane, where Gag also associates with nascent RNA genomes (34, 95, 121, 170). Gag multimerization induces virion budding from the plasma membrane (191). After scission of immature virions from producer cells, the HIV protease enzyme cleaves polyproteins Gag and Pol, which then reassemble into a mature, fully infectious virion (196).

1.4.2 Accessory genes

HIV is unique among retroviruses in that it has acquired accessory genes *tat, rev, vif, vpr, vpu,* and *nef,* which encode proteins that optimize viral fitness and spread in the host. HIV *tat* and *rev* promote transcription of the viral genome and nuclear export of viral RNA, respectively (for review see (146, 155)). Tat binds to *trans*-activating response element (TAR) in the LTR to promote transcription elongation (99). Rev binds to Rev Response Elements (RRE) in viral RNA transcripts to promote nuclear export and RNA stability and utilization. The Vif protein counteracts the intrinsic antiviral factor apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), a

cellular cytosine deaminase which hypermutates the viral genome during reverse transcription, resulting in attenuated or non-infectious virus. Vif associates with a cellular ubiquitin ligase complex in order to target APOBEC3G for degradation (179). Similarly, Vpu also utilizes a cellular ubiquitin ligase complex to degrade cellular targets, including the viral receptor CD4 and the intrinsic antiviral protein tetherin. Degradation of these targets allows more efficient budding of nascent virions (145). The role of Vpr is not entirely clear, but it is known that Vpr induces a G_2 mitotic arrest in infected cells (12), a state which favors transcription from the HIV-1 LTR (73). Recent data indicate that Vpr associates with a cellular ubiquitin ligase complex to degrade cellular factors that may otherwise inhibit viral infection and/or spread in the host (for review see (55, 110)). Finally, the viral accessory protein Nef is a multifunctional protein that disrupts intracellular signaling and trafficking pathways to favor viral infection and spread. Nef has the well-characterized ability to alter the intracellular trafficking of major histocompatibility complex proteins class I and II (MHC-I and MHC-II) and CD4, and has also been reported to affect the trafficking of a variety of other cellular proteins, such as CD28, and CD8 (69, 120, 178, 183, 184, 187). Nef is important for viral fitness and persistence, and mediates immune evasion by HIV (139, 140).

1.5 HIV-I Nef

The importance of Nef in viral pathogenesis is highlighted by a cohort of blood transfusion recipients exposed to an HIV-I strain that contained a large deletion within the *nef* gene as well as the long terminal repeat (LTR), but not affecting any of the major

promoter elements in the LTR (47, 118). Three decades after exposure, and in the absence of anti-retroviral treatment, half of the patients demonstrated delayed progression to CD4 T cell loss or AIDS (19). These patients are termed long term non-progressors (LTNPs), while the other patients in the cohort maintain undetectable viral loads and are categorized as elite controllers (74, 75). Thus, Nef is required for maximal pathogenic potential.

More direct evidence for the requirement for Nef in HIV-induced immune collapse has been revealed through non-human primate research. Rhesus macaques infected with a Nef-deleted (Δ*nef*) strain of simian immunodeficiency virus (SIV) did not progress to AIDS (105). The combination of non-human primate research and longitudinal patient cohort studies has revealed the requirement for Nef in progression from HIV disease to AIDS.

Nef is a multifunctional adaptor protein which is known to disrupt intracellular trafficking of host proteins, activate cellular kinases, and enhance virion infectivity. As mentioned, one of the primary physiological roles of this protein is the downregulation of cell surface receptors such as MHC-I and CD4 from the cell surface. Nef accomplishes this by interacting with a multitude of endogenous trafficking and signaling proteins. This dissertation will focus on the effects of Nef on the trafficking and expression of a variety of cellular proteins significant to immune recognition and signaling.

1.6 Nef disrupts antigen presentation to cytotoxic T lymphocytes

$CD8^+$ cytotoxic T lymphocytes (CTLs) are important for the control of chronic viral infections. The T cell receptor (TCR) in conjunction with CD8, another reported target of Nef downmodulation, is capable of distinguishing between "self" and "non-self" peptide antigens presented by MHC-I on the cell surface (Figure 1.3). Normal cellular peptides typically do not activate a CTL response. However, in a virally infected cell, MHC-I molecules also present peptides derived from viral proteins ("non-self" peptides). In response to the recognition of a "non-self" signal presented by MHC-I, the CTL releases perforins and granzymes that kill the virally infected cell, preventing further spread of the virus (reviewed in (18)).

There is a great deal of evidence that CTLs play an important role in the control of HIV infection (for review see (38)). For example, individuals mounting a Gag-specific CTL response have improved parameters with regard to controlling disease (70, 106). Despite the efficacy with which CTLs control viral load early in infection, in most individuals, anti-HIV CTLs ultimately fail to prevent progression of disease. There is evidence that antigenic variation, viral effects on CTL differentiation, viability, proliferative capacity and function influence the ability of CTLs to control HIV infection. However, this chapter will focus on the effect of HIV Nef on the trafficking of cellular proteins that influence CTL activation.

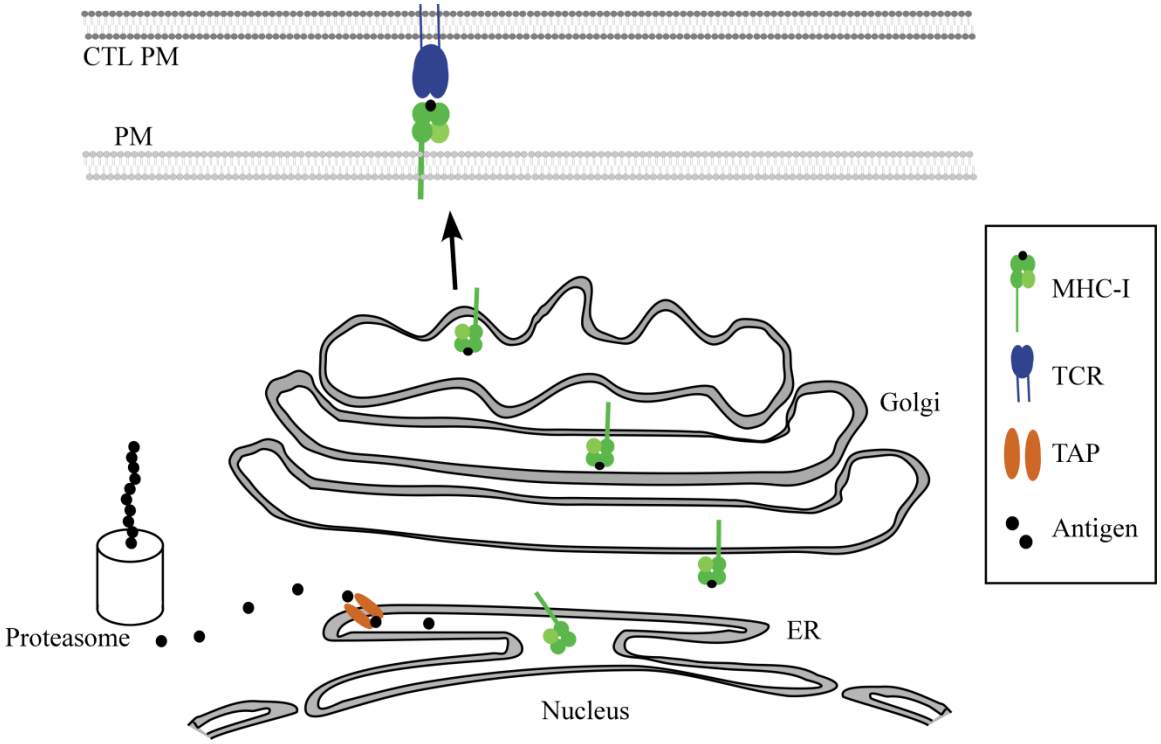


Figure 1.3. Presentation of Antigen to Cytotoxic T Lymphocytes. Intracellular peptide antigens are generated through protealytic cleavage and transported into the endoplasmic reticulum (ER) through transporter associated with antigen processing (TAP). Immature MHC-I is loaded with peptide antigen within the ER and then proceeds through the Golgi to the plasma membrane (PM). There, peptide-loaded MHC-I will bind to the T cell receptor (TCR) of CD8+ cytotoxic T lymphocytes (CTLs) for recognition of self or non-self antigen. Foreign antigen in the context of MHC-I will activate the cognate CTL to lyse the presenting cell. Adapted from (205).

While Nef expression is required for viral spread and pathogenesis *in vivo*, it is not necessary for production of infectious virus *in vitro* and, in fact, is quickly lost in culture. Therefore, it is clear that Nef functions to antagonize the host immune response. It was first observed that MHC-I cell surface expression is reduced in HIV-1-infected cells, and this activity was subsequently attributed to HIV-1 Nef (178). Functional significance was provided by a study which demonstrated that HIV-1-infected peripheral blood mononuclear cells (PBMC) expressing Nef were rescued from anti-HIV CTL lysis as compared to PBMC infected with a Δ*nef* variant of HIV-1 (39). This work was the first to directly demonstrate that Nef downmodulates MHC-I molecules to allow HIV-1-infected cells to evade detection and lysis by CTLs.

Additional studies in which killing of HIV-infected cells was directly compared plus or minus Nef expression have supported the conclusion that Nef protects infected cells from CTL-mediated lysis (37, 122, 189, 207). Nef has been shown to protect HIV-infected primary T cells from CTL lysis using flow cytometric killing assays (37, 122), CTL co-culture assays (207) and chromium release assays (189). Although Nef limits the ability of CTLs to recognize and kill infected cells, it does not appear to abrogate the capacity of CTLs to produce inhibitory cytokines in response to infected cells (189). Recent *in vivo* evidence supports the hypothesis that CTLs may control HIV infection *in vivo* primarily by the elaboration of inhibitory cytokines, but fail to eradicate the infection because the CTLs cannot efficiently lyse the infected cell source of new virions (206).

Based on *in vivo* studies, it is known that progression to AIDS is delayed in the absence of an intact *nef* gene in most humans and monkeys (46, 105, 111). However, Nef has multiple functions including CD4 downmodulation and kinase activation; therefore these studies do not prove an important role for Nef-mediated MHC-I downmodulation specifically *in vivo*. To address this point, several studies have used SIV systems to demonstrate that the capacity to downmodulate MHC-I is selected for *in vivo* (26, 142, 186). In addition, it was recently demonstrated that the ability of *in vivo*-derived Nef to down-regulate MHC-I predicted the resistance of HIV-1 to suppression by CTL *in vitro* (123). Taken together, these data demonstrate that the ability of Nef to down-regulate MHC-I *in vivo* is maintained by the selective pressure for HIV-1 to evade the antiviral CTL response.

1.6.1 Natural killer cells

Many viruses, HIV-1 included, have evolved strategies to reduce MHC-I expression in order to evade CTL recognition by their hosts. As a countermeasure, host natural killer (NK) cells monitor the overall surface levels of MHC-I. Low expression of MHC-I can activate NK cells to lyse target cells. In order to evade NK cell detection, Nef selectively downmodulates some MHC-I allotypes, while allowing others to remain on the cell surface.

There are three classical MHC-I genes expressed by all nucleated cells in humans; HLA-A, HLA-B and HLA-C. These genes are highly polymorphic and hundreds of alleles of

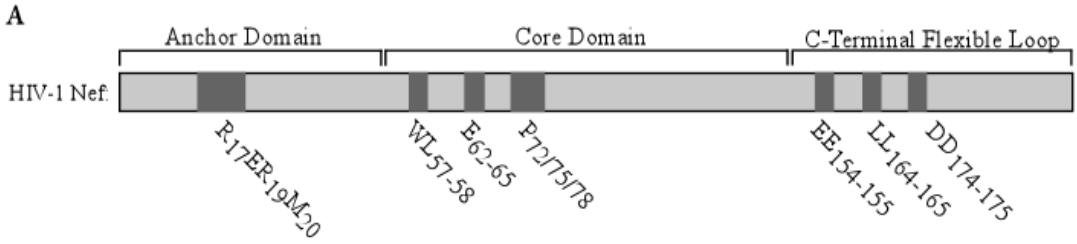
each have been identified. HLA-A and HLA-B are the primary allotypes that present antigens to CTLs, whereas HLA-C may function primarily to regulate NK cell function. In addition, a non-classical MHC-I called HLA-E, which does not commonly present antigens to CTLs also inhibits NK cell function (reviewed in (144)).

Nef has been shown to directly interact with an amino acid sequence ($Y_{320}SQAASS_{326}$) present in the cytoplasmic domain of HLA-A and HLA-B molecules (203). This interaction is necessary for Nef-dependent downmodulation of MHC-I molecules (35, 117, 203). In contrast, HLA-C and HLA-E have amino acid variations within this domain (35, 117, 203) and thus remain unaffected by Nef. It has therefore been proposed that Nef selectively downmodulates a subset of MHC-I molecules to evade CTL killing without activating NK cell lysis. However, recent evidence demonstrating that HLA-C is expressed at very low levels on primary T cells suggests that additional mechanisms may be necessary to fully explain HIV evasion of NK cells (175).

1.6.2. Functional domains required for Nef to downmodulate MHC-I

Though small (25 kDa), Nef is structurally complex, containing numerous protein-protein interaction domains that mediate associations with a variety of host proteins (Figure 1.4). Nef can be divided into the N-terminal anchor domain, the core domain, and the C-terminal flexible loop. The N-terminal anchor domain and C-terminal flexible loop are relatively disordered, making structural analysis of the full length protein difficult. However, structures have been solved separately for the core domain by X-ray

A



B

Domain Name		MHC-I	CD4	CD28	CD8	CD1d	CD80/86
Myristoylation	G_2	+	+	+	+	+	+
Arginine-rich motif	$RR_{17,19}$	+	-	-	ND	ND	ND
M20	M_{20}	+	-	-	-	-	-
CD4-binding site	WL_{57-58}	-	+	+	+	ND	-
Acidic cluster	$EEEE_{62-65}$	+	-	+	-	+	-
Polyproline motif	$P_{72/75/78}$	+	-	-	-	+	-
Diacidic motif	$EE_{154-155}$	-	+	ND	ND	ND	-
Dileucine motif	$LL_{164-165}$	-	+	+	+	+	-
Diacidic motif	$DD_{175-176}$	-	+	+	+	+	-
References		*(2, 60, 77, 133, 202)*	*(31, 41, 60, 63, 80, 124, 133, 156)*	*(14, 120, 183, 187)*	*(120, 183)*	*(32)*	*(27)*

Figure 1.4. Functionally important Nef domains. (A) Domains in Nef that are pertinent to reducing the surface expression of cellular targets. Adapted from (205). (B) Table of Nef domains that are required for the downmodulation of the indicated molecules. A "+" indicates that a domain is required, while a "-" indicates that a domain is dispensible.

crystallography and nuclear magnetic resonance (NMR), and for the N-terminal anchor with NMR spectroscopy (7, 67, 79, 119). These structural data were combined to assemble a model for the conformation of full length Nef. The result predicts that the flexibility of Nef allows for exposure of numerous protein-protein interaction domains, regulated by multiple conformations that may depend on intracellular localization and binding partners (7).

Two sites in Nef are required for most of the functions of Nef. First, Nef is myristoylated at the glycine residue at position 2, which allows Nef to bind the inner leaflet of the plasma membrane (60). In addition, an aspartic acid at position 123 (D_{123}) is required to form homodimers of Nef (126). If either of these sites is mutated (G_2A or $D_{123}G$), Nef is inactive for nearly all of its functions.

The three structural elements of Nef that are required for MHC-I downmodulation are contained within the N terminal anchor or core domains. They are an N-terminal a-helical domain ($R_{17}ERM_{20}RRAEPA_{26}$ and specifically M_{20}) (2, 133), an acidic cluster (E_{62-65}) , and a polyproline repeat ($P_{72/75/78}$). These motifs are required for Nef to bind to the cytoplasmic tail of MHC-I (202) and for Nef to downmodulate MHC-I (77, 133). The C-terminal loop of Nef contains a number of trafficking signals capable of binding adaptor proteins, a coatomer protein, and a vacuolar ATPase (for review see (164)). However, the C-terminal loop of Nef is only active against other Nef targets, such as CD4 (133), suggesting that there are structural constraints that limit the ability of the C-

terminal loop to recruit trafficking factors when Nef is bound to MHC-I with its natural conformation.

1.6.3 Candidate host factors that partner with Nef to downmodulate MHC-I

1.6.3.1. Adaptor protein complexes

Nef is reported to interact with a variety of cellular trafficking factors, including the clathrin-associated adaptor proteins. Clathrin-coated vesicles transport cargo from the *trans*-Golgi network (TGN), plasma membrane, or endosomal network. Clathrin-associated adaptor proteins (APs) are composed of four subunits: two large subunits (β1 or β2 and AP-1γ, AP-2α, or AP-3δ), one medium subunit (μ), and one small subunit (σ) (161, 162, 190). The four subunits combine to function as a heterotetrameric adaptor complex that recognizes Yxxϕ (Y, tyrosine; ϕ, bulky hydrophobic amino acid; x, any amino acid) and [D/E]xxxLL (D, aspartic acid; E, glutamic acid; L, leucine) sorting signals and recruits clathrin coats. AP-1 transports proteins between the *trans*-Golgi network and endosomes (52, 112, 197). AP-2 localizes to the plasma membrane and is necessary for internalization of some types of cargo into endosomes (190). AP-3 localizes to endosomes and is thought to transport proteins into acidic, degradative compartments (151).

Recent structural studies have provided confirmation that clathrin adaptor proteins have physically separate signal-recognition sites for Yxxϕ and [D/E]xxxLL motifs. The μ

subunit contains a tyrosine binding pocket (TBP) and a hydrophobic binding pocket, which recognize Yxxϕ signals (149). In contrast, a hydrophobic pocket in the σ_2 subunit plus a positively charged patch made from residues in both the σ_2 and α subunits combine to form the recognition site for [D/E]xxxLL motifs (104).

Yeast two-hybrid assays initially revealed that HIV Nef's C-terminal dileucine motif ($LL_{164,165}$) interacts with the μ subunit of AP-1 and AP-3 (21, 40, 42, 59, 76, 90, 92, 117, 152). However, consistent with the structural analysis described above, a much more robust interaction occurs between Nef's dileucine motif and hemicomplexes composed of σ and γ, α, or δ subunits (30, 53, 92). Recent data suggest that a robust interaction between the μ subunit of AP-1 and Nef also occurs, but only when Nef is bound to the MHC-I cytoplasmic tail. In this case, the MHC-I cytoplasmic tail provides the tyrosine residue necessary for binding to the AP-1 μ1 subunit tyrosine binding pocket (147, 165, 181, 204).

1.6.3.2. ADP-ribosylation factors

In addition to the clathrin-associated adaptor proteins, the small GTPases, ADP-ribosylation factors (ARFs), are important for cellular control of assembly and disassembly of various intracellular trafficking complexes (10, 97). ARFs are important for clathrin dependent (50, 148, 150, 163) and clathrin independent (188, 198) trafficking pathways. ARF activation and recruitment to cellular membranes is regulated by its guanine nucleotide exchange factors (GEFs), which are required for the recruitment of

GTP to ARF and are necessary for the maintenance of overall Golgi structure ((85, 89) and reviewed in (51)). Conversely, GTPase-activation proteins (GAPs) promote GTP hydrolysis, thus inactivating ARFs (193).

ARF-1 is a clathrin regulatory protein that recruits AP-1 or coat protein complex-I (COP-I) coatomers (9). In studying AP-1 involvement in Nef biology, it was observed that functional ARF-1 is required for recruitment of AP-1 by Nef (36). Additionally, an interaction between Nef and β-COP, which promotes degradation of internalized CD4, also requires ARF-1. Interestingly ARF-1 does not need to be in the activated GTP bound state for this interaction to occur (63).

There is also evidence that activation of ARF-6, a regulator of clathrin independent trafficking, by a Nef-dependent multi-kinase cascade induces MHC-I endocytosis (Figure 1.5) (20, 28, 87). This is consistent with normal ARF-6 biology, as ARF-6 localizes to the plasma membrane and is involved in clathrin-independent endocytosis and recycling (158). ARF-6 is regulated by ARF nucleotide binding site opener (ARNO), an ARF-6 GEF that is activated and recruited to the plasma membrane by PI3-kinase (195). There is evidence that overexpression of ARF-6 and ARNO mutants alters the intracellular localization of MHC-I in Nef-expressing HeLa cells (20). This mechanism is discussed in detail below.

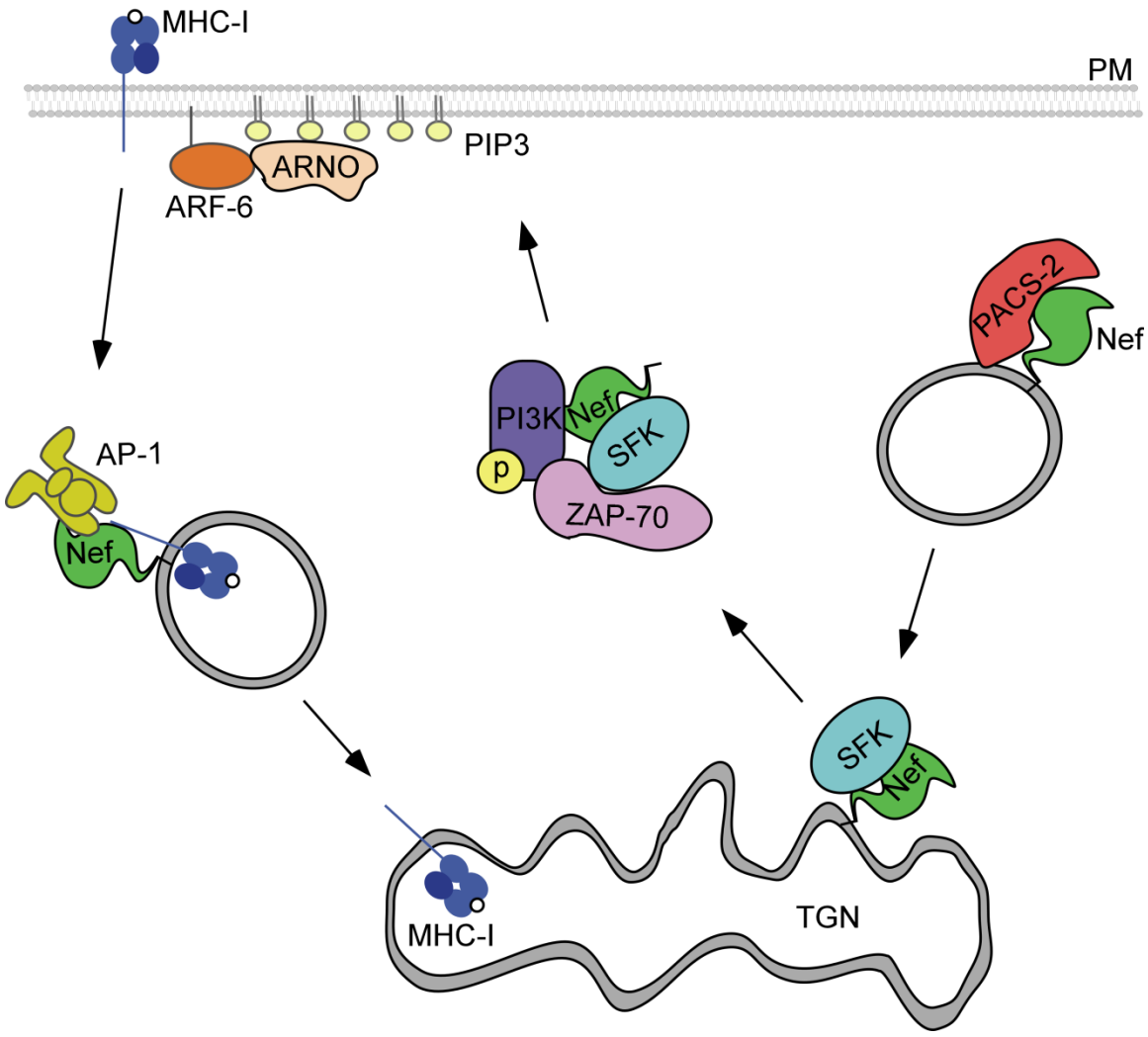


Figure 1.5. Endocytic model of MHC-I downmodulation by Nef. Nef binds to PACS-2 resulting in translocation to the *trans*-Golgi network (TGN). At the TGN, Nef activates a Src family kinase (SFK), which in turn phosphorylates ZAP-70. This signaling event activates phosphoinositide-3-kinase (PI3K), resulting the generation of PIP_3. PIP_3 recruits and activates a guanine exchange factor, ARNO, which subsequently recruits and activates ARF-6, inducing clathrin-independent endocytosis of MHC-I. Nef then binds to AP-1, and this interaction is responsible for transporting MHC-I from endosomes to the TGN. Adapted from (205).

1.6.3.3. β-COP

β-COP is a component of COP-I coatomer complexes. COP-I and COP-2 coatomers are involved in normal protein trafficking within the Golgi and ER (169, 176) and have also been found associated with low pH endosomes in an ARF-1-dependent manner (5, 82). These COP-I coatomers are implicated in recycling endosome function (45) and in transport from the endolysosomal network into multivesicular bodies (81).

The interaction of Nef with β-COP was also discovered using a yeast two-hybrid screen (15). This interaction was found to require the diacidic motif ($EE_{155,\ 156}$) in the C-terminal loop domain of Nef and to result in targeting internalized CD4 to degradative compartments. (63, 153, 174). RNAi directed against β-COP, as well as another member of the COP-I complex, α-COP, was reported to inhibit CD4 downmodulation and degradation by Nef, further supporting the involvement of the COP-I complex in Nef-dependent trafficking (30, 174).

1.6.4. Downmodulation of MHC-I: endocytic mechanism

Although Nef-dependent downmodulation of MHC-I has long been the subject of intense study, the mechanism by which this occurs is not entirely clear. In fact, two competing models have been proposed; in the first, Nef stimulates endocytosis of MHC-I at the plasma membrane. In the second model, Nef inhibits the transport of newly synthesized MHC-I. Both models are evaluated here.

Initial studies examining the effects of Nef on MHC-I trafficking in T cell lines revealed that the rate of MHC-I synthesis and trafficking through the ER and *cis*-Golgi is unaffected by Nef, but that MHC-I stability over time is decreased through lysosomal degradation (cited as data not shown (177). Furthermore, Nef causes an accumulation of MHC-I in juxtanuclear and endosomal compartments and enhances the rate of endocytosis in some cell types (for review see (164)). Normally, T lymphocytes and macrophages spontaneously internalize and recycle MHC-I back to the plasma membrane at high rates in an AP-2 dependent manner (131, 192). However, in Nef-expressing cell lines, over-expression of a dominant negative dynamin, a protein that mediates scission of clathrin vesicles, (29, 116, 185) or a dominant negative mutant subunit of AP-2 (20) did not affect Nef-induced MHC-I endocytosis suggesting this process could be clathrin and AP-2 independent. It has also been demonstrated that Nef does not affect the rate of MHC-I recycling in HeLa cells or CEM SS T cells (101). More recently it was shown that a dominant negative dynamin reduced Nef-induced MHC-I downmodulation in primary T cells from about 50% in this assay system to approximately 25% (208) indicating that clathrin-dependent endocytosis cannot fully account for MHC-1 downmodulation. Furthermore, Greenberg *et al* determined that MHC-I co-localizes with AP-1, but not AP-2 in Nef-expressing cells (77), arguing against an AP-2-dependent internalization pathway.

Alternatively, there is evidence for an ARF-6-dependent, clathrin-independent pathway by which Nef affects MHC-I in some cell types (Figure 1.5). This mechanism relies on an interaction between the acidic cluster E_{62-65} in Nef and phosphofurin acidic cluster sorting proteins (PACS-1 and PACS-2) (154). Antisense to hPACS-1a increases steady state MHC-I surface expression in Nef-expressing cells by about 20% and redistributes the intracellular localization of MHC-I in A7 astrocytic cells. Another group confirmed that knockdown of PACS-1 inhibited Nef-induced MHC-I downmodulation in HeLa cells (but not Jurkat T cells) (208). Based on these data a model was proposed in which Nef physically recruits MHC-I and links it to a PACS-1 based TGN retrieval pathway (154). This model was later modified to indicate that PACS-2 was more important for translocation of Nef to the TGN (8). Once at the TGN, Nef is proposed to bind to and activate a *src* family kinase (SFK), such as Hck, through Nef's polyproline motif, $P_{72/75/78}$, which is a Src homology 3 (SH3)-binding domain. The active SFK then triggers a signaling cascade by binding to and phosphorylating tyrosine kinase ZAP70, which in turn enables ZAP70 to activate phosphoinositide 3-kinase (PI3K) (87). PI3K activity generates phosphatidylinositol (3,4,5)-triphosphate (PIP3), which results in ARNO recruitment to the plasma membrane and activation. There is evidence that overexpression of ARF-6 and ARNO mutants alters the intracellular localization of MHC-I in Nef-expressing HeLa cells and that overexpression of Nef and PACS-1 in A7 cells increases PI3-kinase-dependent GTP loading of ARF (20). A relatively small effect (approximately two fold) of a dominant negative ARF-6 mutant was noted in primary T cells when pan-MHC-I antibodies were used (208). These antibodies recognize all MHC-I allotypes, including those that are unaffected by Nef and thus small effects of Nef

are usually detected. ARF-6 activation is proposed to result in clathrin-independent endocytosis of MHC-I and accumulation in the Golgi in a mechanism that depends upon AP-1 and M_{20} within the alpha helical region of Nef (20, 28).

1.6.5 Evidence against an endocytic mechanism

Arguing against an important role for PACS proteins are data from investigators who reported no effect of knocking down PACS-1 on Nef-induced downregulation of MHC-I HLA-A2, or on the localization of other proteins containing acidic cluster motifs, in HeLa cells (127). Additionally, Baugh *et al* were unable to demonstrate a significant interaction between the acidic cluster in Nef and the PACS-1 furin binding region (13), and mutating three of the four glutamates in the acidic cluster decreased Nef's effects on MHC-I by only 50% (13). Chemical inhibitors of SFKs, Src knockdown, and expression of dominant negative Src all failed to inhibit Nef-dependent downmodulation of MHC-I in U937 monocytic cells, arguing against an important role for SFKs in this pathway (29). Overexpression of a GTP-locked ARF-6 mutant (ARF-6 $Q_{67}L$) is reported to alter the intracellular localization of MHC-I in Nef-expressing HeLa cells (20). However, mutation of an additional residue in ARF-6 (ARF-6 $N_{48}I$,$Q_{67}L$) prevents phospholipase D activation, an effector required for endosomal membrane recycling (96), but had no effect on MHC-I downmodulation in Jurkat T cells (114). Furthermore, inhibition of PI3-kinase, had no effect on the internalization step in U373mg astrocytoma cells (114). Instead, other investigators provided evidence that PI3-kinase inhibitors affected localization of intracellular MHC-I to the TGN in Nef-expressing U373mg astrocytoma

cells (114, 185). Therefore, an alternative mechanism of MHC-1 downregulation by Nef has been proposed, as discussed below.

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1.6.6. Downmodulation of MHC-I: evidence for targeting of newly synthesized protein in the secretory pathway

1.6.6.1. Disruption of MHC-I transport

A dramatic effect of Nef on MHC-I is required for HIV-infected primary T cells to effectively evade anti-HIV CTLs (up to a 300-fold reduction (cited as data not shown (37)). The degree of MHC-I downmodulation in HeLa cells (2-4 fold reduction (20) is small relative to the effect of Nef on an endogenous MHC-I allotype (HLA-A2) in HIV-infected primary T lymphocytes (37). Thus, the internalization pathways described mainly in HeLa cells may not fully explain the intracellular trafficking required for the maximal effect of Nef necessary for HIV immune evasion in T cells. Indeed, direct comparison of Nef activity in HeLa versus T cell lines revealed striking differences in the degree of MHC-I downmodulation (101).

Most viruses that disrupt antigen presentation target newly synthesized MHC-I rather than "old" MHC-I at the cell surface because the newly synthesized molecules harbor viral antigens present at the time of infection. For example, Herpes Simplex Virus, Human Cytomegalovirus, Epstein-Barr Virus, and Adenovirus all encode proteins that block peptide translocation into the ER, target nascent MHC-I for degradation, induce ER

retention of peptide-loaded MHC-I, or prevent transport of MHC-I to the plasma membrane (for review see (83)). Older MHC-I molecules are likely to be presenting cellular antigens, which are present prior to infection, and therefore would not be a threat to the virus. In fact, MHC-I loaded with cellular antigens would be protective against NK cell recognition.

In Nef-expressing cells, reports of MHC-I localizing to the *trans*-Golgi and AP-1-containing vesicles suggested that Nef could be directly disrupting MHC-I trafficking at the *trans*-Golgi network to prevent nascent MHC-1 from presenting new antigens rather than only affecting MHC-I after it had reached the cell surface (Figure 1.6). The first study supporting this model examined the effect of Nef on an HLA-A2-GFP fusion protein in U373mg astrocytoma cells (185). In this series of experiments investigators utilized a temperature block (20^{o}C) to prevent TGN exit and to allow accumulation of MHC-I in the TGN. When cells were subsequently shifted to 37^{o}C, MHC-I could be detected by microscopy at the cell surface within 15 minutes, whereas in Nef-expressing cells MHC-I remained within a juxtanuclear compartment (185). Biochemical experiments examining the transport of newly synthesized MHC-I to the cell surface in T cell lines confirmed that there was a dramatic effect of Nef on the transport of MHC-I to the cell surface. Moreover, the effect of Nef on transport of newly synthesized MHC-I was much greater than its effect on MHC-I internalization (101). An effect of Nef on intracellular transport of endogenous MHC-I HLA-A2 was confirmed in HIV-infected primary T cells (101). PI3-kinase inhibitors did not reduce the ability of Nef to disrupt

Figure 1.6. Newly synthesized MHC-I is targeted for degradation by Nef. Nef binds to the cytoplasmic tail domain of MHC-I in the Golgi network. Nef recruits AP-1 to the complex at the TGN, resulting in routing of MHC-I into endocytic compartments. Subsequent recruitment of β-COP targets MHC-I into endolysosomal compartments. Nef binds to the cytoplasmic tail of CD4 at the plasma membrane and recruits AP-2, inducing rapid CD4 internalization. Subsequent recruitment of β-COP results in the convergence of MHC-I and CD4 into $Rab7^{+}$ late endosomes or multivesicular bodies (MVBs) for eventual lysosomal degradation. Adapted from (205).

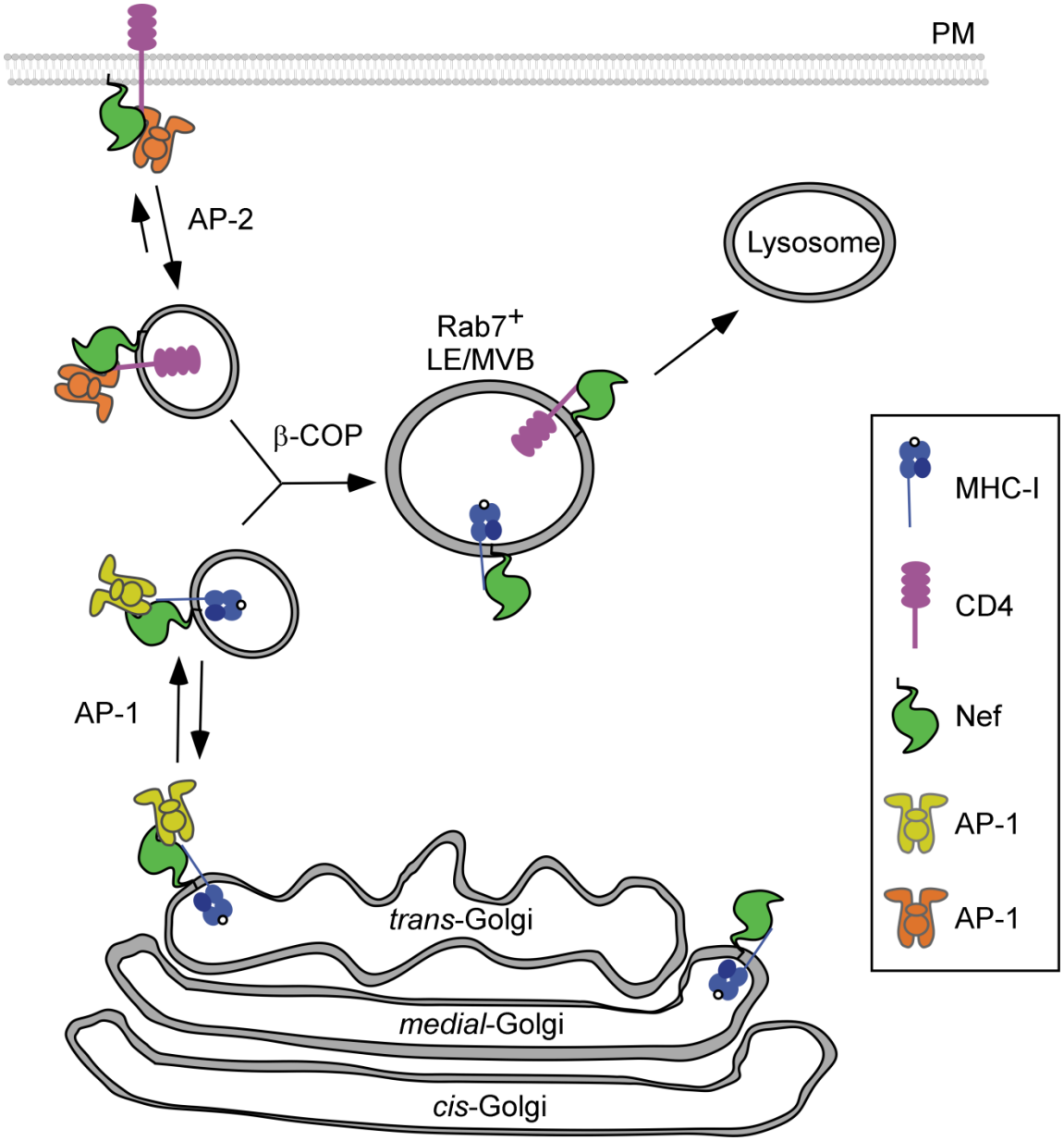


Figure 1.6. Newly synthesized MHC-I is targeted for degradation by Nef

MHC-I transport to the cell surface as measured by a one hour biochemical assay but the investigators could not rule out an effect of PI3-kinase on the intracellular localization of retained MHC-I molecules (101) as was subsequently proposed (114).

Additional evidence in favor of a transport block are studies in which Nef was found to associate with immature forms of MHC-I (102). The HLA-A2 cytoplasmic tail is phosphorylated at specific serines upon exiting the TGN (57). Interestingly, Nef preferentially binds immature, hypophosphorylated forms of HLA-A2 and inhibits phosphorylation of the MHC-I cytoplasmic tail (102). Based on these data it was proposed that Nef binds MHC-I very early in the secretory pathway (102). In support of this model, a recent study was able to observe a Nef-CFP fusion protein in complex with a subset of HLA-A2-Venus in the ER as well as in the Golgi and at the plasma membrane of HeLa cells using two photon two color fluorescence cross correlation spectroscopy (208). However, there was no detectable effect of Nef on MHC-I transport until MHC-I reached the *trans*-Golgi apparatus, thus binding to Nef was not sufficient for disruption of MHC-I trafficking (102, 165) (Figure 1.6).

1.6.6.2. AP-1 is required for disruption of antigen presentation by HIV Nef

Because AP-1 is a clathrin adaptor protein that acts at the TGN and because Nef had been reported to interact with AP-1, it was hypothesized that Nef might disrupt post-TGN transport of MHC-I by promoting an interaction between MHC-I and AP-1. Indeed, RNAi directed against the AP-1 μ1 subunit inhibited downmodulation of endogenous

HLA-A2 in U373mg astrocytoma cells and exogenous HLA-A2 expressed in CEM-SS T cells (120, 165, 174, 204). Furthermore, siRNA directed against AP-1μ1 also inhibited Nef-induced downmodulation of MHC-I in HeLa and Jurkat cell lines, as well as in primary T lymphocytes (208).

Consistent with these observations, AP-1 co-precipitated with Nef and endogenous HLA-A2 from lysates made from HIV infected primary T cells (165). In contrast, complexes of Nef-MHC-I and AP-1 were not detected in HeLa cells unless the cells were incubated at room temperature overnight. Further experiments revealed that temperature reduction decreased the rate of MHC-I trafficking sufficiently to allow the Nef-MHC-I-AP-1 complex to form. For unclear reasons, T cells naturally traffic MHC-I at slower rates and lower incubation temperatures do not change the ability of Nef to form this complex (102). These data help explain why investigators that focused on non-T cell lines did not detect this pathway.

1.6.6.2.1. Nef stabilizes an interaction between the AP-1 tyrosine binding pocket and the MHC-I cytoplasmic tail

Yeast two-hybrid interaction assays and microscopic analyses provided evidence that interactions between Nef and the adaptor proteins AP-1 and AP-3 depend on Nef's dileucine motif (21, 40, 42, 59, 76, 90, 92, 152). In contrast, MHC-I downmodulation and AP-1 recruitment in T cell systems do not require these amino acids (77, 165, 202). Thus, the complex between Nef-MHC-I and AP-1 most likely occurred independently of

the dileucine motif and involved a separate AP-1 binding domain. Indeed, it was demonstrated that the MHC-I cytoplasmic tail mediates a key interaction between the Nef-MHC-I complex and AP-1 (165). The tyrosine in the MHC-I cytoplasmic tail does not form a canonical Yxxϕ AP-1 sorting signal and does not bind AP-1 in T cells in the absence of Nef. Remarkably , Nef binding to the cytoplasmic tail provides the necessary elements for this non-canonical tyrosine signal to function as a potent AP-1 binding motif (165). Providing further support for the model that Nef stabilizes an interaction between the AP-1 tyrosine-binding pocket (TBP) and the tyrosine residue in the MHC-I cytoplasmic tail, it was shown that a dominant negative mutant of AP-1μ1 that had two amino acid substitutions in the tyrosine binding pocket (TBPM) dramatically and specifically inhibited Nef-mediated MHC-I downmodulation (204).

1.6.6.2.2. Nef domains and AP-1-dependent MHC-I trafficking

All of the domains of Nef that are required for MHC-I downmodulation are also required for Nef to interact with the MHC-I cytoplasmic tail (202). To determine whether some of these domains might also be important for recruitment of AP-1, a fusion protein between MHC-I and Nef was examined (165). These studies confirmed that the MHC-I cytoplasmic tail tyrosine was required for AP-1 recruitment and that Nef's dileucine motif was dispensable for this interaction (165, 204). In this system, the acidic cluster (E_{62-65}) and polyproline helix ($P_{72/75/78}$) of Nef were dispensable for AP-1 recruitment as long as a chemical crosslinker was used (165). However, when a digitonin detergent based buffer that lacked crosslinker was substituted, a requirement for these

domains to optimally stabilize the interaction between AP-1 and MHC-I was noted (204). In addition, the N-terminal α-helix and specifically M_{20}, were required for AP-1 recruitment under all conditions tested (165, 204).

Experiments using purified Nef-MHC-I cytoplasmic tail fusion proteins and either whole AP-1 complexes from crude lysates or purified μ1 subunit support the conclusion that Nef stabilizes an interaction between the MHC-I cytoplasmic tail and the AP-1μ1 subunit. Moreover, these experiments provide evidence that the polyproline helix and the acidic domain within Nef are needed for Nef to stabilize the interaction between the AP-1 μ-1 subunit and the MHC-I cytoplasmic tail domain. In the pure protein system, formation of a complex between the Nef-MHC-I cytoplasmic tail fusion protein and the AP-1μ1 subunit also required an intact tyrosine binding pocket in the AP-1μ1 subunit. However, no role for Nef M_{20} was identified and thus this amino acid, although required for Nef-induced MHC-I downmodulation, may not be directly involved in protein-protein interactions but may serve another role in intact cells (181). Therefore, at least three Nef domains are required for AP-1 recruitment and subsequent downmodulation of MHC-I in Nef expressing cells (165, 204).

1.6.6.3. A role for β-COP in disruption of antigen presentation by Nef

Although the experiments described above provide evidence that Nef recruits AP-1 to reroute MHC-I into the endosomal network (165) (Figure 1.6), it remained unclear how

Nef promoted accelerated degradation of MHC-I (165, 177). However, prior reports had determined that Nef accelerated the degradation of internalized CD4 through an interaction between Nef and β-COP. Interestingly, MHC-I and internalized CD4 co-localize in $Rab7^+$ late endosomes in Nef-expressing cells (174, 208) and RNAi against β-COP disrupts Nef-dependent degradation of both MHC-I and CD4 (174). Recent studies have shown that two distinct domains in Nef recruit β-COP, thus clearing up discrepancies in binding data found in previously published literature (63, 91, 124, 153). An arginine rich domain in the N-terminal alpha helix of Nef ($R_{17}XR_{19}$) mediates β-COP binding and MHC-I degradation, whereas a diacidic motif ($EE_{155, 156}$) in the C-terminal flexible loop of Nef mediates β-COP binding and CD4 degradation. (153, 174). The inability of Nef to utilize sequences within the C-terminal loop to affect MHC-I downmodulation or to use sequences within the N-terminal alpha helix to affect CD4 downmodulation support the notion that there are important structural differences between Nef molecules bound to the MHC-I cytoplasmic tail and Nef bound to the CD4 tail.

1.6.7. Summary of MHC-I downmodulation

In sum, a consensus is starting to emerge regarding which host factors are required for Nef to disrupt antigen presentation in HIV infected cells. There is broad agreement among investigators that the cellular clathrin adaptor protein AP-1 is necessary for Nef to disrupt MHC-I trafficking in a wide variety of cell types (49, 127, 165, 174, 204, 208). Additionally, there is agreement that three Nef domains are required (acidic, polyproline and N-terminal alpha helix, including $M_{20}A$) for Nef-induced MHC-I downmodulation

(20, 77, 133, 147, 165, 181, 202, 204). There are data from two separate groups indicating that a three-way complex forms, which contains Nef, MHC-I and AP-1 proteins and that this complex can be detected in lysates from HIV infected primary T cells and in purified protein reactions (165, 181). At least two of the three required Nef domains plus the MHC-I cytoplasmic tail, including the tyrosine at position 320, are directly needed for formation of the Nef-MHC-I-AP-1 complex (147, 165, 181, 204). Moreover, there is a consensus that a functional tyrosine binding pocket in the AP-1 μ1 subunit is needed for formation of the Nef, AP-1, MHC-I complex and for Nef to disrupt MHC-I antigen presentation (181, 204). Finally, a number of groups have noted that PI3-kinase inhibitors reduce the effect of Nef on steady state surface levels of MHC-I, although the exact role of PI3-kinase is debated (20, 87, 114, 185).

1.7. Nef downmodulates CD4

In addition to reducing MHC-I surface expression, Nef also targets the HIV-1 receptor CD4 for endocytosis and degradation (69, 135). CD4 downmodulation promotes viral spread, as CD4 on the surface of producer cells would bind to Env and inhibit the release of nascent virions (6, 113, 128, 167). Downmodulation of CD4 also prevents superinfection (199). Interestingly, HIV encodes two accessory proteins to antagonize CD4 expression. Nef is an early viral gene product which downmodulates mature CD4 early in infection (1, 160). In contrast, late viral gene product Vpu reduces newly synthesized CD4 expression through an endoplasmic reticulum associated protein degradation (ERAD) pathway (56, 200, 201). Additionally, the HIV-1 structural protein

Env has also been demonstrated to associate with CD4 in the endoplasmic reticulum (ER) and inhibit CD4 transport (43, 88). That HIV has evolved three proteins to achieve CD4 downmodulation by complimentary mechanisms suggests that this function is essential to HIV-1 pathogenesis.

1.7.1 *In vivo* evidence for the importance of CD4 downmodulation

The existence of a long term non-progressor with a unique Nef mutation provides direct evidence for the importance of CD4 downmodulation in HIV-1 pathogenesis. The virus from this patient had a deletion of amino acids 26-37 within Nef that abrogated MHC-I and CD4 downmodulation, as well as enhancement of virion infectivity. However, a compensatory duplication of amino acids 43-53 restored the ability of this Nef variant to downmodulate MHC-I and enhance virion infectivity, but not to downmodulate CD4. This patient demonstrated relatively high viral loads but no decline in CD4+ T cell counts (25). This single example suggests that HIV-1 requires Nef-induced CD4 downmodulation to maintain maximal pathogenic potential.

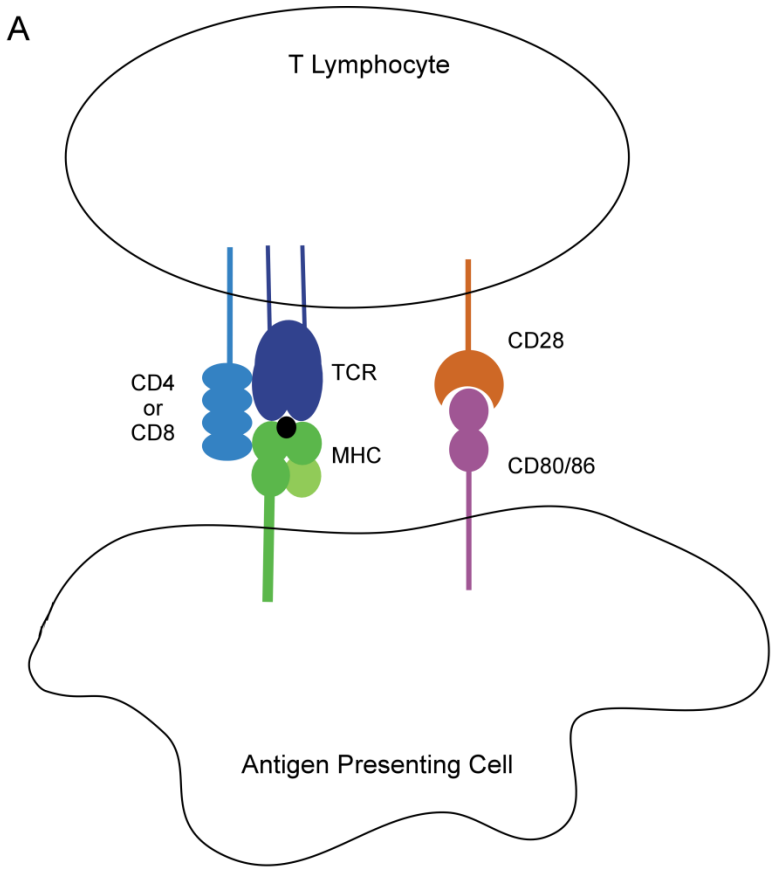
1.7.2. Functional domains required for Nef to downmodulate CD4

A number of motifs within the C-terminal loop of Nef have been implicated in CD4 downmodulation (Figure 1.4). These sites within Nef mediate binding directly to the cytoplasmic domain of CD4, as well as recruitment of cellular adaptor proteins. Nef has been demonstrated to interact directly with the cytoplasmic domain of CD4 by a number

of investigators using a variety of methods including: yeast-2 hybrid (168), in *vitro* binding (84, 156), immunoprecipitation (94, 120), and fluorescence resonance energy transfer (FRET). NMR and fluorescence spectroscopy analyses have identified residues 57-59, 95, 97, 106, and 110 of Nef as forming the binding interface between Nef and CD4 (80, 156). Specifically, mutation of WL_{57-58} abrogates CD4 downmodulation by Nef (133). This Nef interface binds to a membrane proximal sequence (QIKRLLSEKKT) within the CD4 cytoplasmic domain; the two leucines within this sequence are required for binding to Nef (Figure 1.7) (80).

Additional structural motifs within Nef required for CD4 downmodulation are a dileucine motif ($ENNSLL_{160-165}$) and a diacidic motif ($D/ED_{174-175}$), which mediate the association of Nef with cellular adaptor proteins (31, 41, 124). Another motif, $EE_{154-155}$, was reported by some investigators to be required for Nef-induced CD4 trafficking to acidic compartments, while others found this motif to be dispensable for the Nef-dependent reduction in CD4 surface expression (63, 91, 124, 153). This controversy was addressed by a recent study in which it was observed that the di-glutamic acid motif in Nef was dispensable for surface downmodulation and intracellular accumulation of CD4, but was required for subsequent CD4 degradation through a β-COP-dependent pathway (174).

Figure 1.7. Cellular proteins targeted by Nef participate in immune activation and signaling. (A) An interaction between an antigen presenting cell (APC) and a T lymphocyte. The T cell receptor (TCR) and either CD4 or CD8 bind to MHC-II or MHC-I, respectively. Activation of the T cell requires a second signal which is provided through ligation of CD28 by CD80 or CD86. (B) The cytoplasmic tail domains of Nef target proteins do not share significant homology. Sequence comparison of the cytoplasmic regions of cellular proteins targeted by Nef. Regions reported to be involved in Nef-binding or Nef-induced downmodulation are underlined, while critical residues are highlighted in red.



B

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HLA-A2:  RRKSSDRKGGSYSQAASSDSAQGSDVSLTACKV
   CD4:  HRRRQAERMSQIKRLLSEKKTCQCPHRFQKTCSPI
  CD8β:  CCRRRRARLRFMKQFYK
  CD28:  RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS
  CD1d:  TSRFKRQTSYQGVL
  CD80:  RCRERRRNERLRRESVRPV
  CD86:  KWKKKKRPRNSYKCGTNTMEREESEQTKKREKIHIPERSDEAQRVFKSSKTSSCDKSDTCF
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Figure 1.7. Cellular proteins targeted by Nef participate in immune activation and signaling.

1.7.3 Nef downmodulates CD4 by an endocytic mechanism

The mechanism by which Nef affects CD4 surface expression is quite distinct from the mechanism of MHC-I downmodulation by Nef (Figure 1.6). Furthermore, the understanding of Nef-induced CD4 downmodulation within the field is less controversial. It has long been known that Nef associates with CD4 at the plasma membrane, connecting CD4 to cellular endocytic machinery and inducing an increased rate of CD4 endocytosis (1, 66, 72, 78, 101, 124, 132, 138, 152, 153, 183). Consistent with an endocytic model, Nef-induced downmodulation of CD4 was found to be inhibited by dominant negative dynamin expression (93, 116), as well as by chemical inhibitors of clathrin-mediated endocytosis (129). Additionally, microscopic analysis revealed that Nef increases the number of CD4-containing clathrin pits (66), and RNAi knockdown of the clathrin heavy and light chains inhibited Nef-induced downmodulation of CD4 (30). Thus, downmodulation of CD4 by Nef depends on clathrin-associated endocytic machinery.

1.7.4 Host factors that partner with Nef to induce CD4 downmodulation and degradation

1.7.4.1. AP-2 mediates CD4 downmodulation by Nef

Nef links CD4 to cellular endocytic machinery through clathrin associated adaptor protein 2 (AP-2) (Figure 1.6). An early study used fluorescence microscopy to observe colocalization between Nef and the β subunit of AP-2 at the plasma membrane. It was soon noted that Nef contains a canonical dileucine trafficking signal, ($ENNSLL_{160\text{-}165}$),

and that this domain was not only required for CD4 downmodulation but was also capable of interacting with both AP-1 and AP-2 (21, 41, 76). AP-2 is known to mediate clathrin-mediated endocytosis at the plasma membrane and this protein seemed the likeliest candidate for the Nef-induced increase in CD4 turnover. RNAi knockdown of the μ2 subunit of AP-2 provided evidence for AP-2 involvement in CD4 downmodulation. While μ2 knockdown alone did not impair Nef-induced downregulation of CD4, co-expression of the siRNA with a dominant negative mutant of an AP-2 accessory protein involved in clathrin-mediated endocytosis, Eps15 (16, 24), reduced Nef-specific CD4 downmodulation (93). Subsequent reports of AP-2 knockdown have been conflicting, with some investigators observing a partial requirement for AP-2 in CD4 downmodulation by Nef (30, 183), while others have not (120, 166). This may depend in part on which subunit of AP-2 is targeted, as in one study AP-2μ knockdown inhibited downmodulation by approximately 50% in HeLa and S2 insect cells, but AP-2α knockdown did not (30).

Though AP-2 knockdown has been controversial, studies taking a different experimental approach have provided ample evidence of an interaction between Nef and AP-2 *in vitro*. Yeast 3-hybrid analyses identified an interaction between Nef and AP-2 α–σ hemicomplexes that required both the dileucine motif and $D/ED_{174-175}$ diacidic motif within Nef, and this interaction was confirmed with GST pulldown experiments (30, 124). This observation is consistent with a recent study which mapped the dileucine binding site within AP-2 to an interface between the α and σ subunits, as discussed

above (104). Interestingly, the requirement for Nef's $D/ED_{174-175}$ diacidic motif in addition to the dileucine motif appears to represent an extended AP-2 binding site as compared to other AP-2-cargo interactions. Yeast 3-hybrid experiments and mutational analysis of AP-2α recently identified a patch of arginine and lysine residues which are responsible for the interaction with the diacidic motif of Nef. Furthermore, Yeast 4-hybrid analysis provided direct evidence for a tripartite complex between CD4, Nef, and the α−σ hemicomplex (31). Taken together, this evidence supports the conclusion that Nef utilizes AP-2 to connect CD4 to the endocytic machinery

1.7.4.2. β-COP is required for Nef-induced CD4 degradation

In addition to decreasing CD4 surface expression through increased internalization, Nef also induces degradation of CD4 (1, 63, 107, 124, 129, 153, 160, 171). Inhibition of lysosomal acidification prevents Nef-induced CD4 degradation but does not restore CD4 surface expression, indicating that CD4 internalization and degradation are separable functions of Nef (129, 159, 171). CD4 targeting to late endosomal and lysosomal compartments requires an association between $EE_{154-155}$ in Nef and the cellular adaptor protein β-COP, as discussed above (Figure 1.6) (63, 153, 174).

1.8 Nef is reported to disrupt trafficking of a variety of cellular proteins

In addition to the effects of Nef on MHC-I and CD4 trafficking, there are a number of recent reports that Nef downmodulates a variety of other cell surface signaling proteins,

including CD28, CD8, CD80, CD86, and CD1d (Figure 1.7). Relative to MHC-I and CD4 downmodulation, these functions of Nef are not well understood. These proteins are all immunologically important molecules, but their cytoplasmic tail domains share no obvious sequence homology (Figure 1.7). Therefore, it is surprising that Nef would be able to affect the trafficking of so many different targets.

1.8.1. Nef downmodulates CD28

Like MHC-I and CD4, CD28 is a molecule of tremendous importance in T cell biology. Ligation of CD28 in conjunction with T cell Receptor (TCR)-MHC interactions provides a potent co-stimulatory signal that is required for T cell activation (Figure 1.7). Early studies reporting CD28 downmodulation by Nef were primarily performed with SIV Nef, although some investigators observed an effect of HIV-1 Nef as well, albeit of smaller magnitude (14, 187). Both SIV and HIV-1 Nef were found to increase the rate of endocytosis of CD28 in Jurkat and SupT1 T cell lines, similar to the effect of Nef on CD4 surface expression (14, 183, 187). Also similar to CD4, downmodulation of CD28 by Nef was found to require the dileucine motif in Nef (14, 183, 187). Further mutational analyses of Nef also implicated the myristoylation site (G_2), the dileucine motif ($ENNSLL_{160-165}$), the diacidic motif ($E/DD_{174-175}$), $WL_{57-58,}$ and acidic cluster ($EEEE_{62-65}$) of Nef in CD28 downmodulation (183, 187). As discussed above, WL_{57-58} residues are involved in Nef binding to CD4, while the dileucine and $E/DD_{174-175}$ diacidic motifs are required for Nef to interact with AP-2. Interestingly, the $EEEE_{62-65}$ acidic cluster is required for Nef-induced downmodulation of MHC-I, but not CD4 (Figure 1.4). Whether

any these residues contribute to Nef-CD28 binding is unclear, as attempts to detect an interaction between SIV Nef and CD28 by yeast two-hybrid analysis or co-immunoprecipitation were not successful (14). However, fluorescence microscopy experiments in IMR90 fibroblasts did reveal colocalization of Nef and CD28, as well as colocalization of CD28 and AP-2 in cells expressing Nef (187). Therefore, it has been proposed that Nef induces rapid CD28 endocytosis in an AP-2 dependent mechanism, similar to the manner by which Nef induces CD4 downmodulation. However, neither an association of Nef with CD28 nor adaptor protein involvement in Nef-induced CD28 downmodulation has been directly demonstrated.

1.8.2 Evidence for downmodulation of CD8 by Nef

CD8, like CD4, is a T cell co-receptor, but CD8 is expressed on CTLs while CD4 is expressed on helper T lymphocytes. CD8 ligation to MHC I is required for efficient signaling through the TCR, resulting in antigen recognition and increased avidity of TCR-antigen interactions (Figure 1.7). Therefore, a lack of CD8 surface expression would likely impair activation of anti-HIV CTLs. However, as CD4 is the viral receptor, HIV infects CD4-expressing cells, not CTLs. Thus it is not clear under what circumstances HIV-1 Nef might encounter CD8.

CD8 is a dimer classically formed by one α and one β subunit, although α/α homodimers do occur in some cell types, such as NK cells. HIV-1 Nef was reported to downmodulate CD8 in the SupT1 T cell line as well as in primary T lymphocytes. Specifically, Nef downmodulated CD8α/β and CD8β/β homodimers, but not CD8α/α

homodimers. This indicated that Nef downmodulates CD8 by acting on the β subunit (183).

Similar to several other Nef targets, CD8 downmodulation by Nef was determined to be due to an increased rate of internalization. In order to assess the involvement of endocytic machinery, investigators used RNAi to knock down expression of the clathrin heavy chain, dynamin, and AP-2μ in the Daudi B cell lymphoma cell line. Dynamin and AP-2 depletion each inhibited Nef-induced downmodulation of CD8, as well as CD4, by approximately half. Though clathrin heavy chain knockdown had no effect on CD8 or CD4 downmodulation, treatment with ikarugamycin, a chemical inhibitor of clathrin-mediated endocytosis, inhibited Nef-induced internalization of CD8, indicating that Nef-dependent endocytosis of CD8 is likely mediated by clathrin (183). Mutational analysis of Nef revealed that downmodulation of CD8 requires the myristoylation site (G_2), the CD4 binding site (WL_{57-58}) the dileucine motif ($LL_{154-155}$) and diacidic motif ($E/DD_{174-175}$) of Nef (183). Each of these Nef domains has also been implicated in CD4 and CD28 downmodulation, suggesting that common molecular interactions mediate these mechanisms (Figure 1.4). All together, this evidence is consistent with the conclusion that HIV-1 Nef uses a common mechanism by which it downmodulates CD4, CD28, and CD8.

The CD8β cytoplasmic tail is short, only 15 amino acids, and, unlike the CD4 cytoplasmic tail, it contains no known protein-protein interaction motifs. Furthermore,

CD8β shares no obvious sequence homology with the CD4 and CD28 cytoplasmic domains (Figure 1.7). Mutational analysis of the CD8β sequence revealed that amino acids $FMK_{204-206}$ of the cytoplasmic domain were required for Nef-dependent downmodulation (183). This motif does not resemble the dileucine signal that forms the Nef-binding site within CD4, nor does it correspond to any motif within CD28. Therefore, it is unclear how Nef can affect these targets by similar mechanisms, mediated through dissimilar protein domains. Therefore, further studies are needed to clarify the mechanism by which Nef affects CD8 and how CD8 is a physiologically relevant target for Nef.

1.8.3. Evidence for CD1d downmodulation by HIV-1

Antigen-presenting molecule CD1d is structurally similar to MHC-I and presents lipid, as opposed to peptide, antigens to Natural Killer T Cells (NKT cells). CD1d has long been known to be capable of presenting the model antigen α−galactosylceramide (aGalCer), a marine sponge-derived lipid (103, 143). Recently, CD1d has also been found to present lipid antigens from microbial pathogens such as *Sphingomonas, Borrelia burgdorferi, Leishmania, and Mycobacteria* (4, 65, 108, 109, 136, 182). It is not clear whether an HIV-associated lipid antigen exists. It is also unknown whether CD1d-restricted NKT cells have any antiviral activity against HIV and whether CD1d expression influences HIV pathogenesis. The literature regarding CD1d downmodulation by HIV-1 is conflicting. Two groups have reported downmodulation of CD1d by HIV-1 Nef but report conflicting mechanisms (32, 33), while another group attributes this function to

Vpu (141). Other groups reported controversial CD80/86 or CD1a downmodulation, but failed to observe CD1d downmodulation by HIV in primary human APCs (27, 180).

1.8.3.1 Reports of CD1d downmodulation by Nef

CD1d was first observed to be downmodulated in human Jurkat T cells infected with NL4-3 HIV-1. Downmodulation of transiently expressed CD1d was observed when NL4-3 Nef was expressed in human T2 cells. The authors state (as data not shown) that HIV accessory protein Vpu had no effect on CD1d expression. In this study, the magnitude of Nef-induced CD1d downmodulation always exceeded that of MHC-I or CD4. Co-immunprecipitation experiments detected Nef in complex with CD1d, although the interaction was described as weak or transient due to the necessity of using a chemical crosslinker to observe co-precipitation. This interaction was dependent upon the CD1d cytoplasmic domain, but was independent of a tyrosine trafficking motif in CD1d (33).

Another group also demonstrated reduced CD1d expression on Jurkat cells infected with NL4-3 HIV as compared to those infected with NL4-3 HIVΔ*nef*. In contrast to the previous study, these investigators reported moderate effects of Nef on CD1d expression (approximately 30%) as compared to MHC-I (50%) and CD4 (70%). Transient transfection of Nef alone resulted in decreased CD1d expression, although not to the same extent as whole HIV, suggesting the involvement of other viral proteins. This study attributed CD1d downmodulation to an increased rate of endocytosis in the presence of

Nef, though the rate increase, measured as the percent of labeled-CD1d unaffected by an acid wash, was not observed at time points earlier than 45 minutes, at which time recycling defects confound endocytosis measurements. Microscopic analysis of HeLa cells revealed that CD1d colocalized with a Nef-GFP fusion protein and accumulated in the TGN. The investigators observed a requirement for the tyrosine motif within the CD1d cytoplasmic domain for Nef-induced downmodulation (Figure 1.7). Mutational analysis of Nef provided evidence for a requirement for the myristoylation site (G_2) as well as a partial dependence upon CD4-downmodulation domains $LL_{164-165}$ and $DD_{174-175}$, and MHC-I downmodulation domains $PxxP_{72/75}$ and $EEEE_{62-65}$, but not M_{20} (Figure 1.4) (32). Therefore it is not apparent that CD1d downmodulation by Nef occurs by a mechanism shared with MHC-I or CD4 downmodulation.

Interestingly, although differing binding domain requirements were reported, both studies reported that Nef-expressing APCs demonstrated reduced capacity to activate CD1d-restricted NKT cells through αGalCer presentation as compared to cells expressing CD1d in the absence of Nef (32, 33). This observation supports the potential functional significance of HIV-dependent CD1d downmodulation.

1.8.3.2. Evidence for CD1d downmodulation by HIV-1 Vpu

Though in agreement with previous studies that HIV-1 induces downmodulation of CD1d, a recent report has identified Vpu as the HIV accessory protein responsible for this activity. As in previous reports, functional assays demonstrated impaired activation

of CD1d-restricted NKT cells by HIV-1-infected primary human monocyte-derived dendritic cells (MDDC). However, in this system HIV-1 Vpu expression was sufficient to induce CD1d downmodulation (141). Vpu did not affect CD1d internalization between 0 and 30 minutes, but recycling of CD1d was impaired in cells expressing Vpu. Fluorescence microscopy revealed colocalization of CD1d and a Vpu-GFP fusion protein within EEA1-positive early endosomal compartments. CD1d, but not Vpu, could also be found in LAMP-1-positive late endosomes/lysosomes in Vpu-expressing cells (141).

Interestingly, HIV-1 Δ*vpu* or Δ*nef* each demonstrated a reduced ability to affect CD1d surface expression in human MDDCs, while inactivation of both Nef and Vpu synergistically abrogated CD1d downmodulation by HIV (141). This observation indicates that HIV-1 Nef and Vpu may both impair CD1d surface expression by distinct pathways, similar to the redundant abilities of Nef and Vpu to induce CD4 degradation (69, 200). Additionally, HIV-1 Vpu downmodulates viral restriction factor BST-2/tetherin, a function that is performed by Nef in primate lentiviruses which lack Vpu, such as SIVsmm, SIVmac, SIVsyk, and SIVagm (173). Therefore, perhaps Nef and Vpu functions frequently overlap, and both may acquire the ability to downregulate CD1d, depending upon the evolution of each virus strain.

1.8.4. Evidence for downmodulation of CD80 and CD86 by HIV-1 Nef

Like CD1d, CD80 and CD86 are expressed on APCs, though the latter two are not antigen-presenting molecules. Indeed, CD80 and CD86, previously known jointly as B7,

are ligands for CD28, providing the critical co-stimulatory signal for T cell activation (125). It is likely that a Nef-induced reduction in surface expression of these molecules would prevent the activation of anti-HIV T lymphocytes. However, the literature on CD80/86 downmodulation by Nef is conflicting; several groups report no change in expression of these molecules in the presence of Nef (130, 157, 180), while other reports describe a detailed mechanism by which Nef affects these molecules (27, 134).

CD80 and CD86 downmodulation by Nef was first reported in a mouse dendritic cell (DC) line in a study characterizing the effects of Nef on immature DC function (134). However, it is notable that every marker stained for, including CD40 and inter-cellular adhesion molecule-1 (ICAM-I), was similarly downmodulated and the isotype background was also highly reduced in the Nef-expressing cells. It is also important to consider the ramifications of utilizing cell lines that stably express Nef, a cytotoxic protein. Indeed, the authors observed changes in cell morphology such as smaller size and loss of dendrites, as well as reduced antigen presentation and an inability to respond to stimulation (134). An additional caveat is the use of a murine cell line. Nef has reduced ability to downmodulate murine MHC-I allotypes, and Nef-induced downmodulation of human MHC-I is impaired in murine cells, indicating that Nef may not be able to bind to cellular cofactors in murine cells (33).

Other investigators did not observe downmodulation of CD86 or ICAM-I by Nef, but did observe CD4 downmodulation, indicating that Nef was active in this experiment (130).

Additional studies detected Nef-induced downmodulation of MHC-I by Nef in primary human DCs, but failed to detect CD86 or CD40 downmodulation (157, 180). Still other experiments performed in primary monocytes did report Nef-dependent downmodulation of CD80 and CD86 (27). Hence, differences in experimental systems must influence the observed effects of Nef on CD80/86.

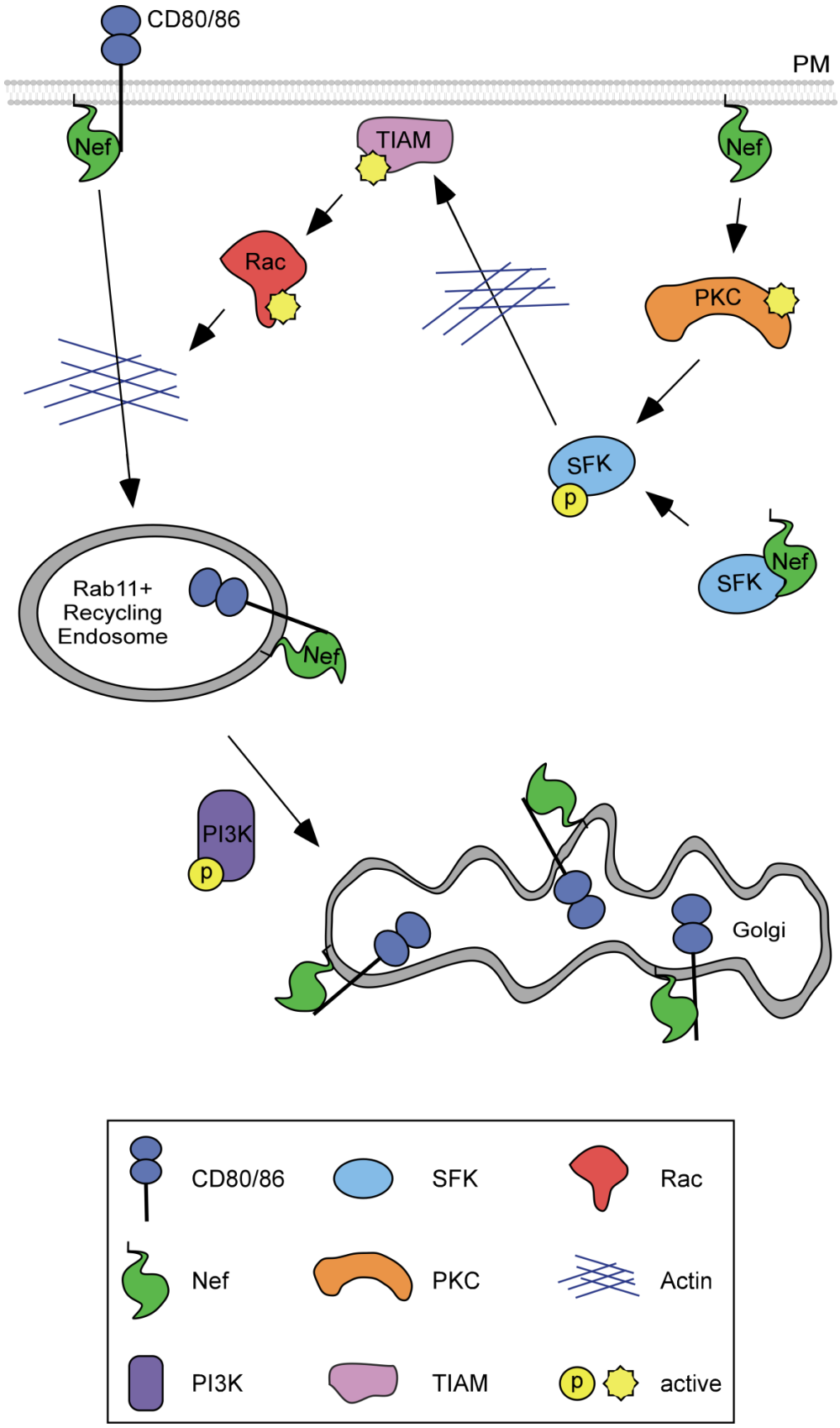
1.8.4.1 Proposed mechanism of CD80/86 downmodulation

Despite the controversy, one group has extensively studied CD80/86 downmodulation by HIV-1 Nef in human APCs, thoroughly detailing a mechanism for this proposed Nef function. They report robust downmodulation of endogenous CD80 and CD86, but not CD1d, in U937 monocytic cells infected with NL43 HIV, as well as in primary human monocytes infected with Ada HIV (27). Transfection of Indian subtype F2 Nef into a mouse myeloid cell line or primary murine DCs and macrophages produced a similar result. Surprisingly, the investigators did not observe degradation of CD80, CD86, or MHC-I in the presence of Nef. Also unexpected was the observation that mutation of a number of functional domains within Nef ($WL_{56,57}$, $E_{62\text{-}65}$, R_{77}, $P_{72/75/78}$, D_{86}, R_{106}, $I_{109,}$ $F_{121,}$ P_{130}, $EE_{154\text{-}155}$, and $LL_{164\text{-}165}$) did not inhibit CD80/86 downmodulation. Only elimination of the myristoylation site in Nef (G_2A) or truncation of Nef at amino acid 100 reduced downmodulation of CD80/86 (27). Despite a failure to identify responsible Nef domains, the authors were able to provide evidence for a direct association of Nef with CD80 and CD86 using microscopy and co-immunoprecipitation assays, as well as yeast-

2-hybrid methods (27). They later reported high affinity interactions between Nef and the cytoplasmic tail domains of CD80 and CD86 (29).

In a subsequent study, the investigators reported that F2 Nef increased the rate of endocytosis of CD80 and CD86, as well as MHC-I, based on measurements collected over 10 hours. This is an unusual time course for an endocytosis assay, as recycling and newly synthesized protein also contribute to surface expression after approximately 15 minutes. The investigators next used a series of chemical inhibitors and dominant negative proteins to propose a cellular mechanism. Like MHC-I downmodulation by Nef, Nef-dependent CD80 and CD86 downmodulation was dynamin and eps15 independent, inconsistent with clathrin-mediated endocytosis. Downmodulation of CD80 and CD86 depended on actin polymerization as well functional WASp, a protein which moderates actin filament formation. Inhibitors of protein kinase C (PKC) and Src-family kinases, as well as Rac1 knockdown, all inhibited CD80/86 downmodulation. TIAM, a Rac1 GEF activated by Src, was found to be phosphorylated in the presence of Nef, and expression of dominant negative TIAM reduced CD80/86 downmodulation. Src knockdown also inhibited downmodulation of CD80 and CD86, but dominant active Rac overcame this block, indicating that Src activation is only required for Rac activation. However, dominant active Rac1 alone is not sufficient for downmodulation of CD80/86 in the absence of Nef (Figure 1.8).

Figure 1.8. An endocytic model for CD80/86 downmodulation by Nef. Nef binds to and activates an SFK, which requires Nef-induced PKC activity. The active SFK then phosphorylates TIAM in an actin-remodeling-dependent manner. Active TIAM in turn activates Rac1, which again induces actin remodeling. Nef binds tightly to the cytoplasmic tails of CD80/86 and increases the rate of internalization from the plasma membrane (PM). Nef then routes CD80/86 to Rab11+ recycling endosomes. Finally, Nef activates PI3-kinase, which results in transport of CD80/86 to the TGN.


CD80/86
PM
Nef
TIAM
Nef
Rac
PKC
SFK
p
Nef
SFK
Rab11+
Recycling
Endosome
Nef
PI3K
p
Golgi
CD80/86
SFK
Rac
Nef
PKC
Actin
PI3K
TIAM
p
active

Finally, microscopic analysis was used to map CD80/86 trafficking in the presence of Nef. CD80 or CD86 were labeled at the plasma membrane, then examined over time for colocalization with markers of various cellular compartments. CD80 and CD86 initially colocalized with Rab11, a key regulator of recycling endosomes. CD80/86 were subsequently found to colocalize with Golgi markers Rab6 and GM130, but were never observed in complex with ARF-6 or late endosome marker Rab7 (Figure 1.8) (28). Therefore, it has been proposed that HIV-1 Nef downmodulates CD80/86 by a unique signaling mechanism that does not rely on known Nef functional domains.

1.8.4.2. Functional importance of CD80/86 downmodulation

In an experimental system where Nef-dependent CD80 and CD86 downmodulation were observed, Nef also reduced the ability of murine macrophages to activate OT-1 CTLs. OT-1 is a transgenic mouse system in which T cells are engineered to express a TCR that recognizes an ovalbumin epitope in the context of the mouse H-2k^b MHC-I molecule. It is expected that MHC-I downmodulation by Nef would abrogate CTL activation. To differentiate between the effects of MHC-I and CD80/86 downmodulation, the investigators expressed a Nef mutant capable of downmodulating CD80 and CD86, but not MHC-I, and found it to behave similarly to wild type Nef in the OT-1 assay, providing evidence that MHC-I antigen presentation is not sufficient for CTL activation, and that Nef inhibits an additional required signal. A G_2A myristoylation mutant of Nef, which is incapable of affecting intracellular trafficking, had no effect on CTL activation. Together, this evidence supports the conclusion that HIV-1 Nef induces downmodulation

of CD80/86 and thereby disrupts CTL activation by macrophages. However, numerous conflicting reports are not in agreement that CD80 and CD86 are downmodulated by Nef, indicating that further investigation is warranted.

1.9. Summary

In conclusion, data from a number of laboratories have contributed to our current understanding of the mechanisms by which HIV-1 Nef affects the trafficking of cellular proteins to disrupt immune cell activation and anti-HIV activity. We currently have a good understanding of the mechanism by which Nef affects CD4 expression, although the importance of this Nef function *in vivo* is not entirely clear. The field is beginning to come to a consensus about the mechanism by which Nef downmodulates MHC-I and an understanding of its role in immune evasion *in vitro* and *in vivo*. However, the extent to which Nef affects other proteins remains ambiguous, with conflicting data originating from various research groups. A greater understanding of which targets are most important for Nef biology is needed. This dissertation describes the development of tools to address this question, insight into the mechanisms involved in Nef-dependent trafficking pathways, and the development of pharmacologic inhibitors of Nef function for future therapeutic use.

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Chapter 2

HIV-1 Nef disrupts intracellular trafficking of MHC-I, CD4, CD8, and CD28 by distinct pathways that share common elements

2.1 Abstract

The Nef protein is an important HIV virulence factor that promotes the degradation of host proteins to augment virus production and facilitate immune evasion. The best characterized targets of Nef are MHC-I and CD4, but Nef has also been reported to target several other proteins, including CD8β, CD28, CD80, CD86, and CD1d. To compare and contrast the effects of Nef on each protein we constructed a panel of chimeric proteins in which the extracellular and transmembrane regions of MHC-I allele HLA-A2 were fused to the cytoplasmic tails of CD4, CD28, CD8β, CD80, CD86, and CD1d. We found that Nef co-precipitated with and disrupted the expression of molecules with cytoplasmic tails from MHC-I HLA-A2, CD4, CD8β, and CD28, but Nef did not bind to or alter the expression of molecules with cytoplasmic tails from CD80, CD86, and CD1d. In addition, we used siRNA knockdown and co-precipitation experiments to implicate AP-1

as a cellular co-factor for Nef in the downmodulation of both CD28 and CD8β. The interaction with AP-1 required for CD28 and CD8β differed from the AP-1 interaction required for MHC-I downmodulation in that it was mediated through the dileucine motif within Nef ($LL_{164,165}AA$) and did not require the tyrosine binding pocket of the AP-1 μ subunit. In addition, we demonstrate a requirement for β-COP as a cellular co-factor for Nef that was necessary for the degradation of targeted molecules HLA-A2, CD4, and CD8. These studies provide important new information on the similarities and differences with which Nef affects intracellular trafficking and help focus future research on the best potential pharmaceutical targets.

2.2 Introduction

Nef is an important virulence factor for human immunodeficiency virus (HIV)-1 pathogenesis which functions to increase viral spread and promote disease progression. The significance of Nef expression on disease progression is highlighted by the delayed progression to AIDS observed in a cohort infected with an HIV with a deletion the *nef* open reading frame and LTR (8, 26, 28, 56, 57). Similarly, rhesus macaques infected with SIVΔNef demonstrated low viral loads and delayed disease progression (50). Nef is a 25-34kDa myristoylated early viral gene product that acts as a multifunctional adaptor protein, containing a number of protein-protein interaction domains. Additionally, Nef has several disordered regions that confer flexibility to facilitate exposure of different interaction domains in response to the local environment ((34) and reviewed in (3)).

Nef has been implicated in signal transduction, intracellular trafficking, and viral infectivity. One of its best studied activities is its role in promoting immune evasion from anti-HIV cytotoxic T lymphocytes (CTL) (22, 58, 80, 87, 95). Nef also downmodulates CD4, the HIV receptor, from the plasma membrane (33) to prevent superinfection (5) and allow more efficient release of budding virus (53, 76). In addition, Nef has been reported to downmodulate a number of other surface molecules, including CD8 (mediated through its β subunit), CD28, CD80, CD86, and CD1d (12-14, 18, 19, 82, 86). Relative to MHC-I and CD4, little is known about the mechanisms and the functional significance of downmodulation of these molecules by Nef.

We have previously demonstrated that Nef disrupts MHC-I trafficking by binding to the cytoplasmic tail of MHC-I in the endoplasmic reticulum or early Golgi, and this complex subsequently recruits adaptor protein (AP)-1 in the *trans*-Golgi network (64, 74, 81, 91-93). AP-1 then directs MHC-I into an endolysosomal pathway as opposed to the cell surface (79, 91-93). In contrast, CD4 anterograde traffic to the plasma membrane is not inhibited in the presence of Nef. Instead, Nef promotes CD4 internalization at the plasma membrane (11, 23, 61, 70, 79). CD4 downmodulation has been shown to require AP-2 and it has been proposed that Nef-dependent AP-2 recruitment to the cytoplasmic domain of CD4 at the plasma membrane promotes clathrin-dependent endocytosis (16, 45, 82). Our lab and others have also demonstrated that Nef utilizes a β-COP-dependent pathway to ultimately target MHC-I and internalized CD4 to lysosomes for degradation (4, 30, 70, 79).

The other putative targets of Nef, CD28, CD8β, CD80, CD86, and CD1d, are expressed in a variety of immune cells, including helper T lymphocytes and CTLs, as well as antigen presenting cells (APCs), such as macrophage and dendritic cells (DC). Though Nef is multifunctional, it is surprising that Nef would affect so many different molecular targets. Therefore, we sought to directly compare the relative effect of Nef on CD28, CD8β, CD80, CD86, and CD1d to assess the relative importance of each target and to provide mechanistic insights. Here we show that HIV-1 Nef dramatically reduces the cell surface expression of MHC-I, CD4, CD8β and, to a lesser degree, CD28. We were

unable to detect significant downmodulation of CD80, CD86, or CD1d by HIV-1 Nef in a variety of cell lines or primary antigen presenting cells (APCs). Downmodulation of CD8β and CD28 was partially dependant on AP-1 expression and we detected a Nef-dependant interaction of AP-1 with the CD8β and CD28 cytoplasmic tails that relied on the dileucine sorting signal within Nef. Additionally, we found that Nef promoted the degradation of CD8β and that this function of Nef required β-COP expression. These new data help define the most relevant cellular targets of HIV-1 Nef and provide insights into the mechanism by which HIV-1 promotes disease.

2.3 Results

2.3.1 Nef targets HLA-A2, CD4, CD8β, and CD28 through their cytoplasmic tail domains

Nef has been reported to downmodulate a large number of host proteins including MHC-I, CD4, CD28, CD8β, CD80, CD86, and CD1d. To assess the relative effects of Nef on these targets, we created a panel of chimeric proteins in which the extracellular and transmembrane regions of hemagglutinin epitope (HA) tagged HLA-A2 were fused to the cytoplasmic tail domain of each protein of interest (Figure 2.1). This allowed us to use the same antibodies and established assays to directly compare the effect of Nef on each cytoplasmic tail domain. We then measured the impact of HIV-1 and SIV Nef on the cell surface expression of each chimeric molecule. We found that HLA-A2, the HLA-A2 chimera with the CD4 cytoplasmic tail (A2/CD4), and the HLA-A2 chimera with the

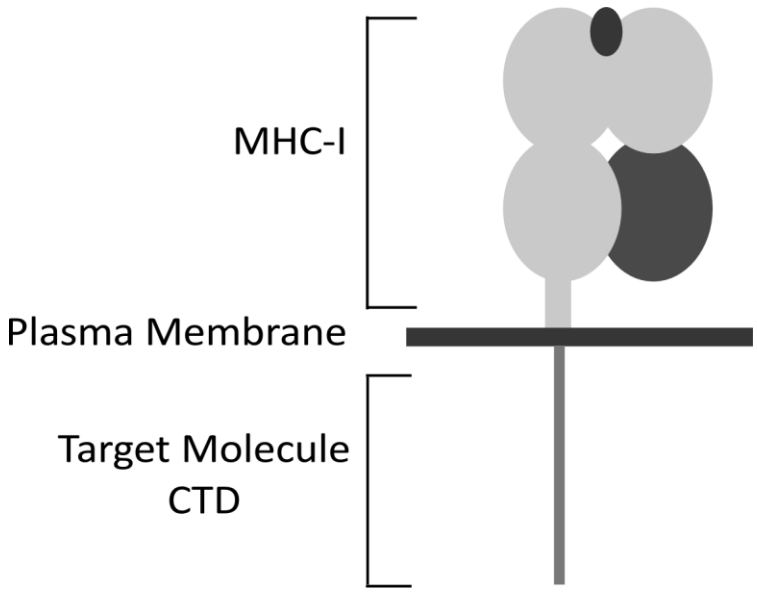


Figure 2.1. Diagrammatic representation of chimeric molecules HLA-A2, A2/CD4, A2/CD8β, and A2/CD28 in CEM T cells.

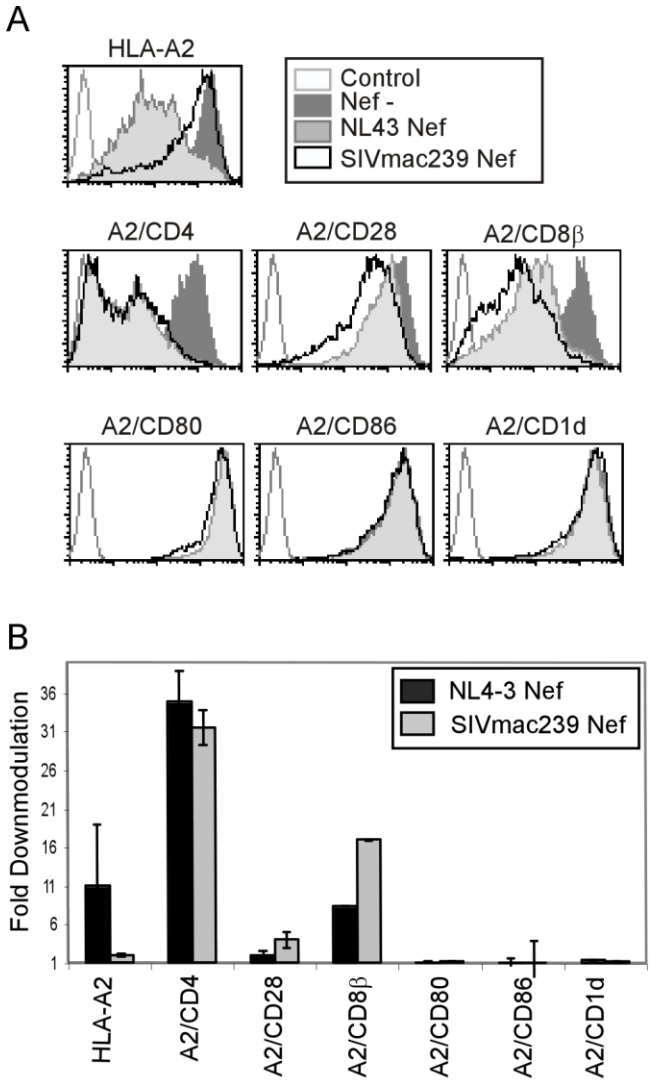


Figure 2.2. Nef downmodulates HLA-A2, A2/CD4, A2/CD8β, and A2/CD28 in CEM T cells. (A) Flow cytometric analysis of CEM SS T cells expressing the indicated chimeric proteins and transduced with the indicated adenoviral vector. Cells were analyzed at 3 days post infection (dpi). (B) Quantitation of flow cytometry experiments from part B. The mean fold downmodulation +/- standard deviation is shown. *n*=3

CD8β cytoplasmic tail (A2/CD8β) were downmodulated by both HIV and SIV Nef expressed using an adenoviral vector system in a $CD4^+$ T cell line, CEM SS (3.7, 8.5, and 4.0-fold, respectively, Figure 2.2). HIV-1 Nef had a smaller (1.8-fold) effect on the HLA-A2 chimera with the CD28 cytoplasmic tail (A2/CD28), whereas SIV Nef downmodulated A2/CD28 to a greater extent (3-fold, Figure 2.2), as previously demonstrated (86). The chimeric molecules with cytoplasmic tails from CD80, CD86, and CD1d (A2/CD80, A2/CD86 and A2/CD1d respectively) were not sufficient for downmodulation by either Nef protein (1.1, 1.1, and 1.3-fold downmodulation, respectively, Figure 2.2).

Similar results were obtained when Nef was expressed using a retroviral vector that also expresses GFP from an internal ribosomal entry site (39). Again we observed strong downmodulation of HLA-A2, A2/CD4, and A2/CD8β, and weak downmodulation of A2/CD28 (Figure 2.3A and quantified in Figure 2.3B). In addition, we again found that HIV-1 Nef did not significantly affect A2/CD80, A2/CD86, or A2/CD1d (Fig. 2.3A-B). Similar results were achieved using another T cell line (SupT1) that expressed wild type, endogenous CD4, CD8 (both α/α and α/β dimers), and CD28 plus exogenous HLA-A2 (Figure 2.3C).

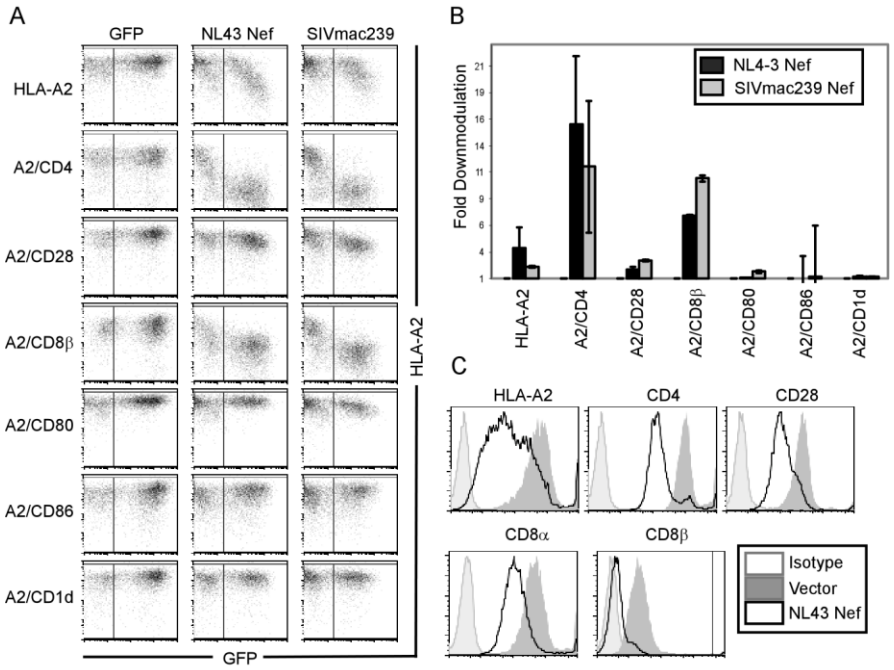


Figure 2.3. Nef downmodulates HLA-A2, A2/CD4, A2/CD8β, and A2/CD28 in T cell lines. (A) Flow cytometric analysis of CEM SS T cells transduced with a retroviral vector expressing GFP alone, or NL43 Nef and GFP reporter. (B) Quantitation of flow cytometry experiments from part D. The mean fold downmodulation of GFP^+ cells +/- standard deviation is shown. *n*=3. (C) Flow cytometric analysis of SupT1 cells transduced with the indicated adenoviral vector. Cells were analyzed at 3 dpi. Sup T1 cells express endogenous CD4, CD28 and CD8.

2.3.2 Endogenous CD80 and CD86 are not downmodulated by Nef in Primary APCs

Because CD80, CD86, and CD1d are normally expressed in antigen presenting cells (APCs), we employed the human monocytic cell lines THP-1 and U937 for further experiments with these molecules. We were unable to detect significant cell surface expression of endogenous CD80 or CD86 in either cell line (data not shown). Therefore we created stable U937 and THP-1 lines expressing the chimeric molecules. Nef was introduced into undifferentiated cells using a retroviral vector and then the cells were stimulated with LPS and PMA to induce macrophage differentiation. Overall, we observed that Nef was less active in the APC lines than in T cells, downmodulating HLA-A2 two to three-fold in APC lines as compared to 17-fold downmodulation in CEM cells (Figure 2.4B). Even so, Nef had minimal effect on the CD80 and CD86 chimeras in all three cell lines (Figure 2.4A-B, 0.8-2.0-fold), indicating that the cytoplasmic tails of CD80 and CD86 were not sufficient for downmodulation by HIV-1 Nef even in macrophage cell lines. In contrast we observed a decrease in MHC-I HLA-A2 surface expression under all conditions. Because PMA also reduces CD4 surface expression (7), we were unable to assess the effect of Nef on CD4 in the PMA-treated cells.

We used the same experimental approach to examine the ability of HIV-1 Nef to downmodulate full length endogenous CD1d in the THP-1 cell line, which also expresses endogenous HLA-A2. We observed minimal downmodulation of CD1d by Nef in either undifferentiated or differentiated THP-1 cells (Figure 2.4C-D).

Figure 2.4. NL43 Nef does not downmodulate CD80, CD86, or CD1d.

(A) Flow cytometric analysis of U937 cells and THP-1 cells transduced with the indicated retroviral vector and treated as indicated with LPS, PMA or DMSO (Undiff.). The cells were analyzed 5 dpi. (B) Quantitation of flow cytometry experiments from part A. The mean fold downmodulation +/- standard deviation is shown. *n*=3. (C) Flow cytometric analysis of THP-1 cells treated as in A. The surface expression of endogenous HLA-A2, CD4, and CD1d expression were measured at 5 dpi. (D) Quantitation of flow cytometry experiments from part C. The mean fold downmodulation +/- standard deviation is shown. *n*=3.

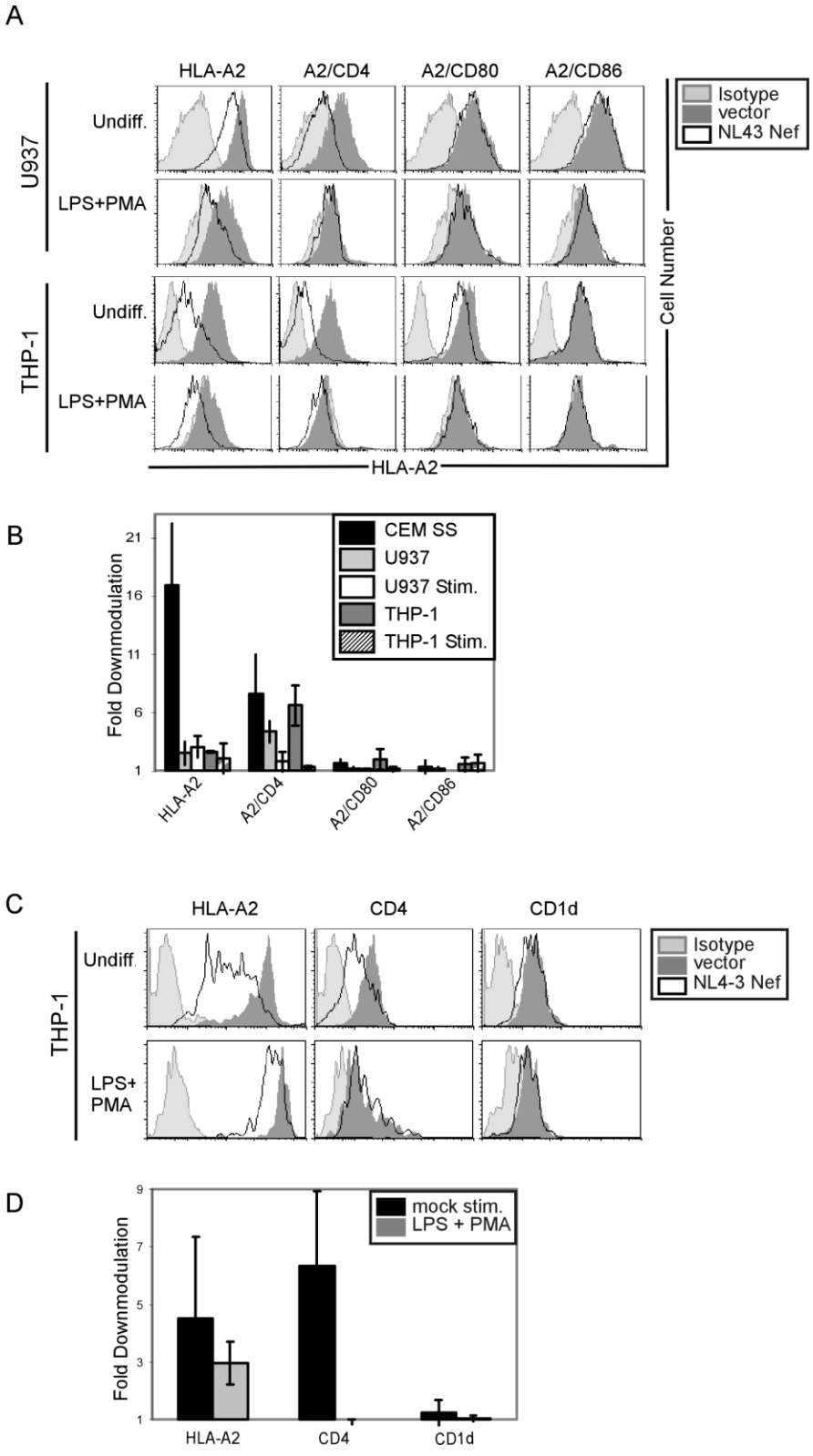


Figure 2.4. NL43 Nef does not downmodulate CD80, CD86, or CD1d.

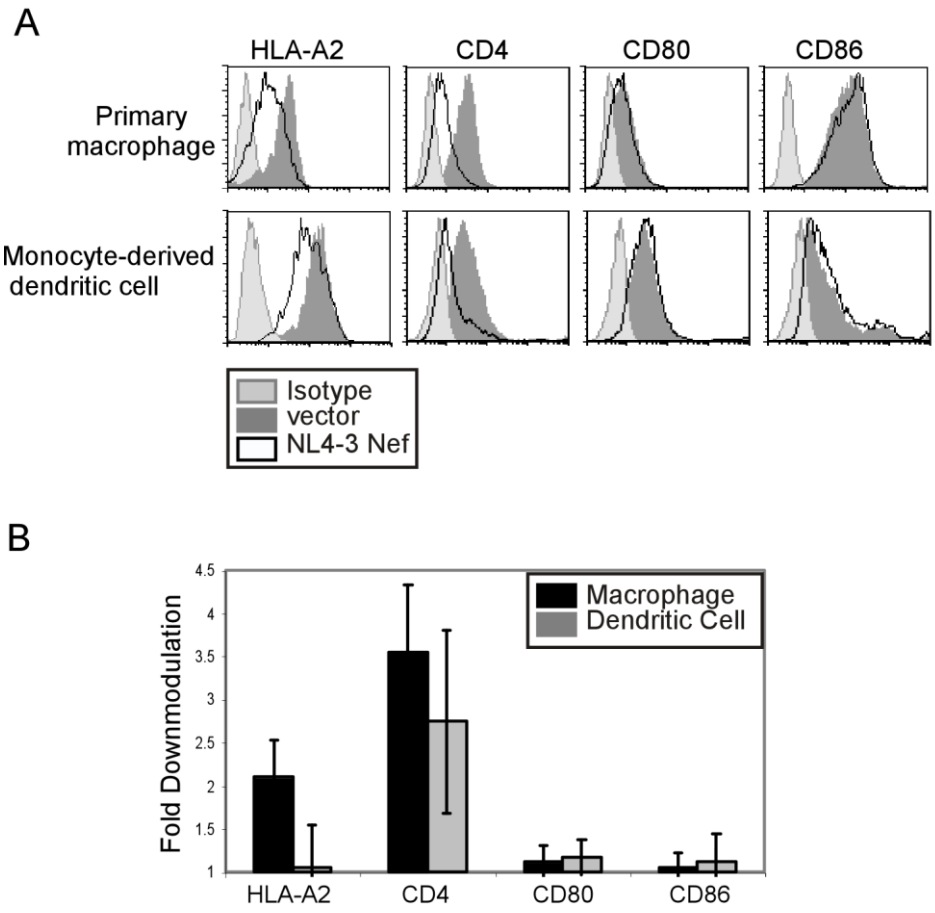


Figure 2.5. NL43 Nef does not downmodulate endogenous CD80 or CD86 in primary antigen presenting cells. (A) Flow cytometric analysis of primary antigen presenting cells that were transduced with the indicated adenoviral vector. Endogenous HLA-A2, CD4, CD80, and CD86 expression were assessed at 3 dpi. Primary macrophages; GM-CSF treated prior to transduction, dendritic cells; GM-CSF, IL-4, and TNF-α treated prior to transduction. (B) Quantitation of flow cytometry experiments from part E. The mean fold downmodulation +/- standard deviation is shown. *n*=3

To examine the ability of Nef to downmodulate full length CD80 and CD86, we obtained primary antigen presenting cells from human peripheral blood mononuclear cells. These cells were stimulated with GM-CSF alone or GM-CSF, IL-4, and TNF-α to induce macrophage or monocyte-derived dendritic cell phenotypes, respectively (78, 96). We observed no significant effect of Nef on the surface expression of endogenous CD80 and CD86 in either cell type (Figure 2.5A, quantified in 2.5B). As controls we demonstrated downmodulation of endogenous MHC-I HLA-A2 and CD4 by Nef, albeit to a lesser extent than in T cells (Figure 2.5A-B).

2.3.3 Multiple HIV-1 Nef variants downmodulate HLA-A2, CD4, CD8β, and CD28

All experiments thus far utilized Nef from the NL4-3 molecular clone of HIV (1). While many of the known functional domains of Nef are conserved, significant sequence variation does occur. Therefore, we examined whether the activity of Nef varied amongst HIV isolates. Although we observed variation in the magnitude of MHC-I HLA-A2 downmodulation, all Nef variants tested (except YU-2) reduced HLA-A2 surface expression dramatically (6-35-fold, Figure 2.6A-B). Similarly, all the Nef proteins downmodulated endogenous CD4, as well as A2/CD8β, and A2/CD28. CD4 was downmodulated to the greatest extent, followed by HLA-A2, then A2/CD8β. A2/CD28 was only moderately affected by all of the Nef variants tested. In contrast,

Figure 2.6. Nef-induced downmodulation of MHC-I, CD4, CD8β, and CD28 is conserved across multiple clades. (A) Quantitation of downmodulation of the indicated molecule in CEM SS cells following transduction with bi-cistronic murine retroviral vectors expressing the indicated Nef. The mean fold downmodulation of each molecule in GFP-positive cells as determined by flow cytometry is shown. *n*=3. The inset displays the data for CD28, CD1d, CD80 and CD86 with an expanded Y axis to highlight differences amongst these molecules that are of a small magnitude. The statistical significance was determined by two-way ANOVA analysis. (B) Quantitation of Nef-dependent downmodulation of the indicated molecule by each Nef variant. The mean fold downmodulation in the GFP positive cells +/- standard deviation is shown. *n*=3. (C) Relative ability of Nef variants to downmodulate each target protein, plotted as fold downmodulation. YU-2 was removed as an outlier.

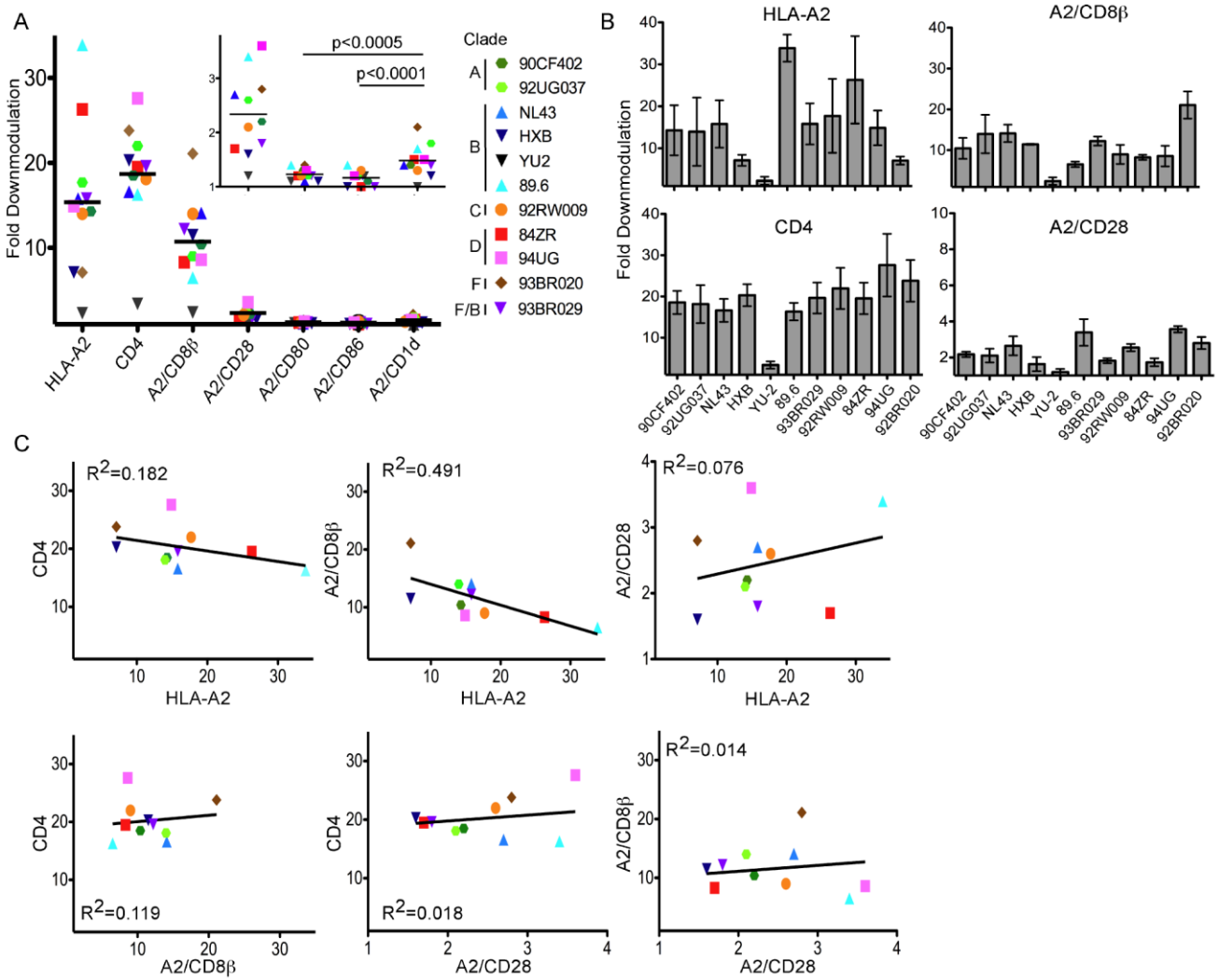


Figure 2.6. Nef-induced downmodulation of MHC-I, CD4, CD8β, and CD28 is conserved across multiple clades.

none of the Nef proteins reduced A2/CD80 or A2/CD86 surface expression more than 1.5-fold (Figure 2.6A and inset).

Interestingly, some of the Nef variants downmodulated A2/CD1d more than 1.5-fold (Figure 2.6A, inset). 93BR020 Nef, a clade F isolate (32), downmodulated A2/CD1d to the greatest extent. Thus, certain HIV subtypes may evolve the ability to significantly affect CD1d trafficking, although the effect was comparatively small.

Notably, Nef proteins which demonstrated higher downmodulation activity on one target protein did not necessarily demonstrate similarly high activity for all other targets examined (Fig. 2.6B). When relative Nef activities were plotted for each target molecule, a number of correlative observations were made (Figure 2.6C). HLA-A2 and A2/CD8β were negatively correlated, indicating that the ability of Nef to downmodulate these proteins did not seem to co-evolve. There is a weak positive correlation between HLA-A2 and A2/CD28, indicating that Nef sequences that contribute to the downmodulation of these targets may be partially shared. Because CD4 and CD8 are analogous molecules, one might hypothesize that CD8β downmodulation is an evolutionary byproduct of CD4 downmodulation. Significantly, however, there was no correlation between CD4 and A2/CD8β downmodulation (Figure 2.6C).

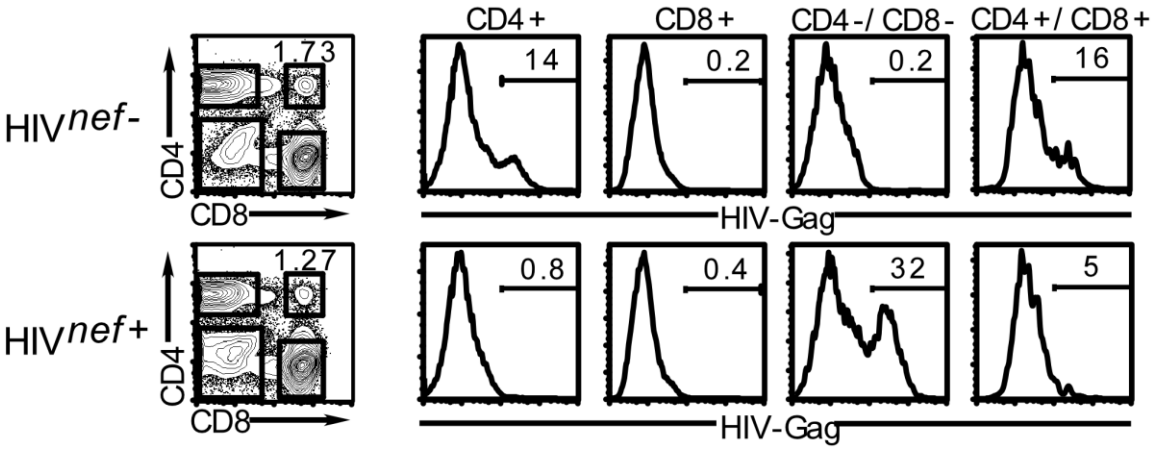


Figure 2.7. CD4 and CD8 are downmodulated by Nef in HIV-1-infected PBMCs.

Flow cytometric analysis of PBMCs infected with HXB ePLAP HIV +/- Nef, pseudotyped with HXB envelope. Surface marker and intracellular Gag stains were performed at 3 dpi.

2.3.4 HIV-1 Nef downmodulates CD4 and CD8 in primary T lymphocytes

CD8 can be expressed as a CD8α/α homodimer or as a CD8α/β heterodimer. Because Nef targets CD8β, only the CD8α/β heterodimer can be affected by Nef (82). T lymphocytes, DCs, and NK cells are all $CD8^+$, but the CD8α/β heterodimer is expressed exclusively in T cells. Typically, T lymphocytes are either $CD4^+$ or $CD8^+$ and only the $CD4^+$ subset can be infected by HIV. Thus, it is unclear how Nef might come in contact with the CD8β molecule. Significantly, a small population of $CD4^+CD8^+$ T lymphocytes circulates in peripheral blood (9, 31, 66, 67, 99) and the frequency of these double positive cells increases in response to infection with a number of viruses, including HIV (42, 54, 60). Additionally, these cells have been reported to be susceptible to infection by HIV (6, 20, 41, 42, 52, 94, 98). While the role of $CD4^+CD8^+$ double positive cells is not entirely clear, they are known to exhibit cytolytic activity as well as antigen-dependant secretion of IFN-γ and IL-2. Moreover, they are enriched for HIV-specific responses in chronically infected patients (40, 66, 84, 97). Importantly, these cells are reported to express the CD8α/β heterodimer (66, 83). Thus, this population represents a potential physiologic target of HIV in which Nef would encounter CD8β.

To investigate the ability of HIV to infect $CD4^+CD8^+$ cells, we infected PBMCs with HIV HXB ePLAP (17) pseudotyped with an HIV envelope (HXB) that lacked Nef expression (HIV*nef* $^-$) so that the expression of cell surface markers would be maintained. As expected, we observed similar rates of infection in $CD4^+CD8^+$ and $CD4^+CD8^-$ cells (16 and 14% respectively, Figure 2.7, upper panel). In contrast, when these cells were

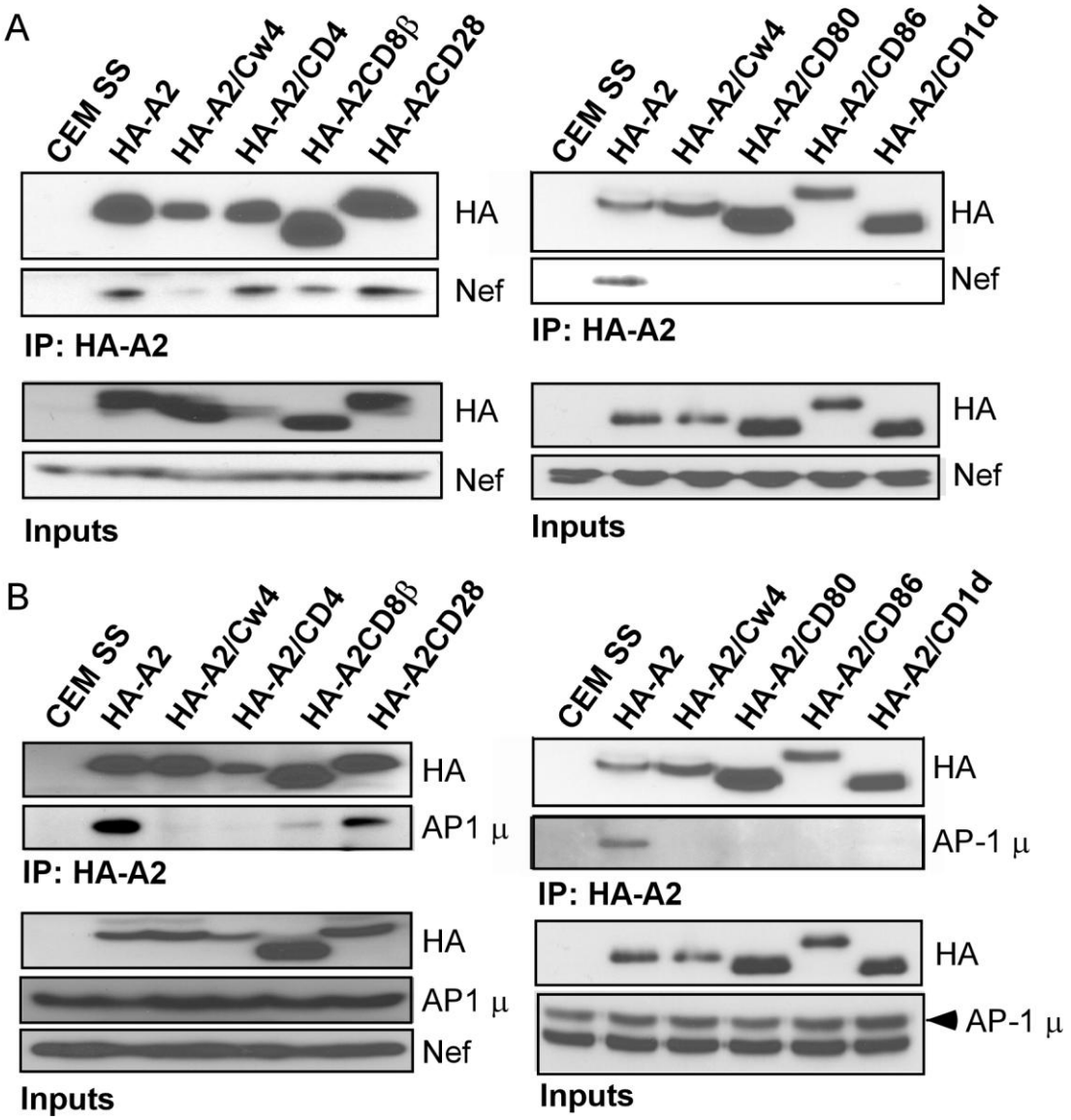
infected with an HIV HXB ePLAP that expressed Nef, most of the infected cells became negative for both CD4 and CD8 (Figure 2.7, lower panel). Therefore, $CD4^+CD8^+$ cells are a physiologically relevant target of HIV-1 and Nef actively downmodulates both CD4 and CD8 in these cells.

2.3.5 Nef recruits AP-1 to the cytoplasmic tail of CD8β and CD28

Prior research has shown that Nef disrupts MHC-I and CD4 host protein trafficking by binding to sequences in their cytoplasmic tail domains (79, 91). Here we confirmed that Nef co-precipitated with HLA-A2 and A2/CD4. Moreover, we observed that Nef co-precipitated with A2/CD8β, and A2/CD28, but not A2/Cw4, A2/CD80, A2/CD86, or A2/CD1d (Figure 2.8A). A2/Cw4 is a negative control in these experiments as Nef does not bind to or downmodulate HLA-C (or E or F) MHC-I allotypes (21, 36, 92). Thus, NL43 Nef physically associates with each of the proteins which it potently downmodulates in T cells.

In Nef expressing cells, HLA-A2 also coprecipitates with the clathrin adaptor protein AP-1 (when degradation is inhibited by ammonium chloride), and this interaction is required for Nef to disrupt MHC-I trafficking (74, 93). Similarly, we observed AP-1 coprecipitation with A2/CD28 and A2/CD8β (Figure 2.8B), although the interaction with A2/CD8β seemed weaker than that observed with HLA-A2 and A2/CD28. In contrast,

Figure 2.8. Nef physically associates with the cytoplasmic domains of HLA-A2, CD4, CD8β, and CD28 and recruits AP-1 to HLA-A2, CD8β, and CD28. Immunoprecipitation and western blot analysis of the indicated chimeric molecule expressed in CEM T cells transduced with the indicated adenoviral vector. The cells were harvested at 3 dpi after an overnight incubation in ammonium chloride to inhibit degradation. Lysates were immunoprecipitated with an antibody directed against HLA-A2 (BB7.2) and the presence of (HA)-HLA-A2, Nef (A) or AP-1 (B) was detected by western blot analysis.


A
CEM SS
HA-A2
HA-A2/Cw4
HA-A2/CD4
HA-A2CD8β
HA-A2CD28
HA
Nef
IP: HA-A2
Inputs
CEM SS
HA-A2
HA-A2/Cw4
HA-A2/CD80
HA-A2/CD86
HA-A2/CD1d
HA
Nef
IP: HA-A2
Inputs
B
CEM SS
HA-A2
HA-A2/Cw4
HA-A2/CD4
HA-A2CD8β
HA-A2CD28
HA
AP1 μ
Nef
IP: HA-A2
Inputs
CEM SS
HA-A2
HA-A2/Cw4
HA-A2/CD80
HA-A2/CD86
HA-A2/CD1d
HA
AP-1 μ
IP: HA-A2
Inputs

we did not observe AP-1 coprecipitating with A2/CD4, A2/Cw4, A2/CD80, A2/CD86, or A2/CD1d.

We also asked whether the other adaptor proteins AP-2 and AP-3 coprecipitated with the chimeras. In two of five experiments we observed AP-2α recruitment to A2/CD28 (Figure 2.9C), which would be consistent with previous reports of AP-2 involvement in CD28 downmodulation (82, 86). However, we were unable to detect coprecipitation of any other chimeras with AP-2 or AP-3 (data not shown).

2.3.6 Nef recruits AP-1 to CD8β and CD28 through its dileucine motif

The AP proteins can utilize either Yxxϕ or (E/D)xxxLL motifs in cargo molecules (x stands for any amino acid, and ϕ indicates a hydrophobic residue). While Nef recruits AP-1 to the tyrosine residue in the MHC-I cytoplasmic tail, such a sequence is not present in the cytoplasmic tail domains of CD28 or CD8β. Interestingly, Nef also contains a conserved canonical dileucine motif (ExxxLL$_{165}$), which has been reported to mediate binding to AP-1, AP-2 and AP-3 (11, 25, 29, 35, 43, 44, 55, 69). Furthermore, Nef LL$_{164,165}$AA is defective at downmodulating CD8β or CD28, yet retains the ability to downmodulate HLA-A2 (Figure 2.9A and (82)). Thus we hypothesized that recruitment of AP-1 to CD8β or CD28 might occur through this dileucine motif. Consistent with our hypothesis, Nef LL$_{164,165}$AA did not support coprecipitation of AP-1

Figure 2.9. **Nef recruits AP-1 for the downmodulation of CD8β and CD28 in a dileucine dependant manner.** (A) Flow cytometric analysis of CEM SS T cells expressing the indicated chimeric protein and transduced with the indicated adenoviral vector. Nef $LL_{164,165}AA$ is indicated as xLL. (B) Immunoprecipitation and western blot analysis of the indicated chimeric molecule expressed in CEM T cells transduced with the indicated adenoviral vector. The cells were harvested at 3 dpi. Lysates were immunoprecipitated with an antibody directed against HLA-A2 (BB7.2) and the presence of Nef, AP-1 subunits and MHC-I (HA) were detected by western blot analysis. Intervening lanes were removed, as indicated by white gap. The data are representative of four independent experiments. (C) Immunoprecipitation and western blot analysis of the indicated chimeric molecule expressed in CEM T cells transduced with the indicated adenoviral vector and treated as described for part B. AP-2 co-precipitation is representative of two of four experiments.

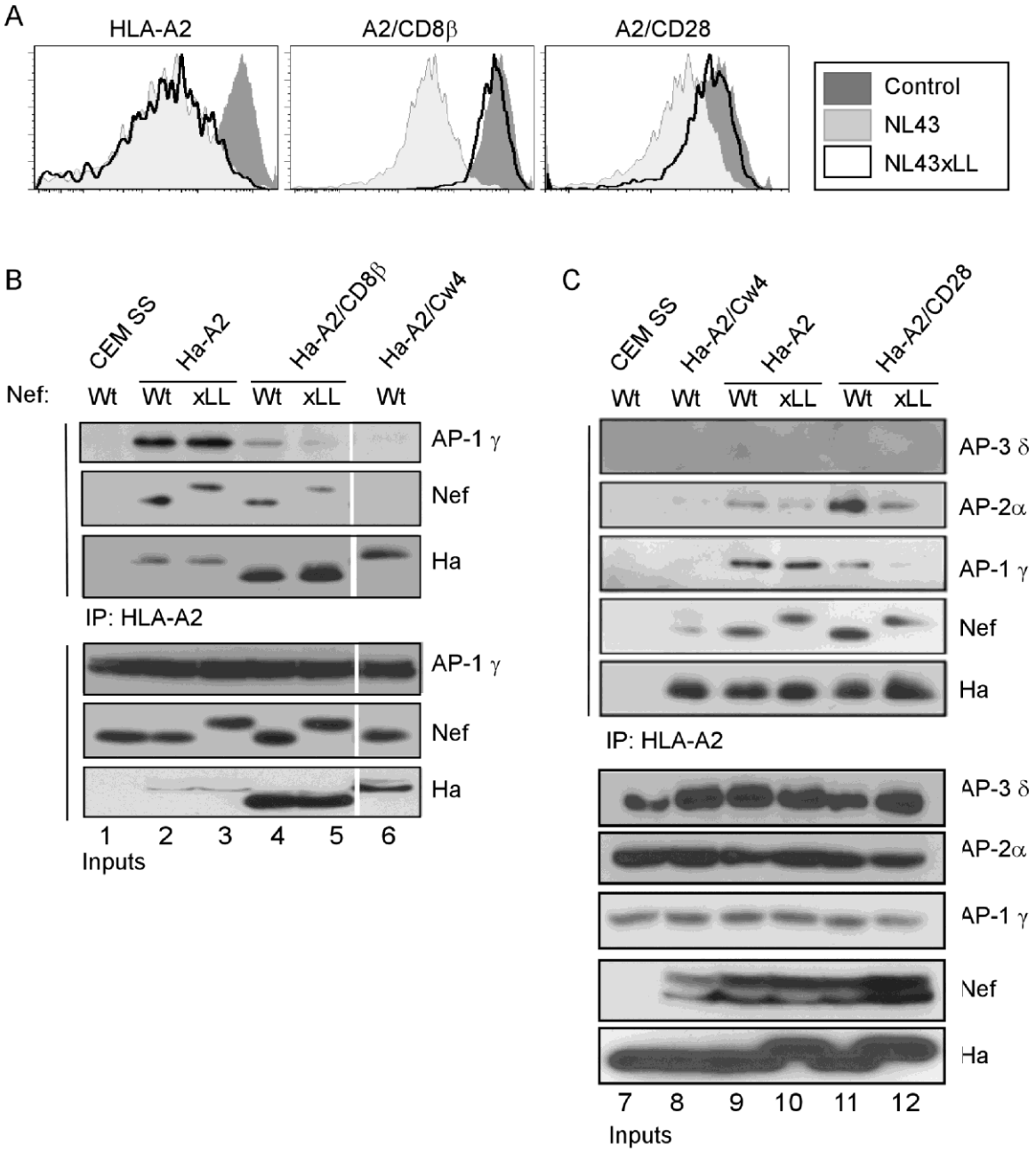


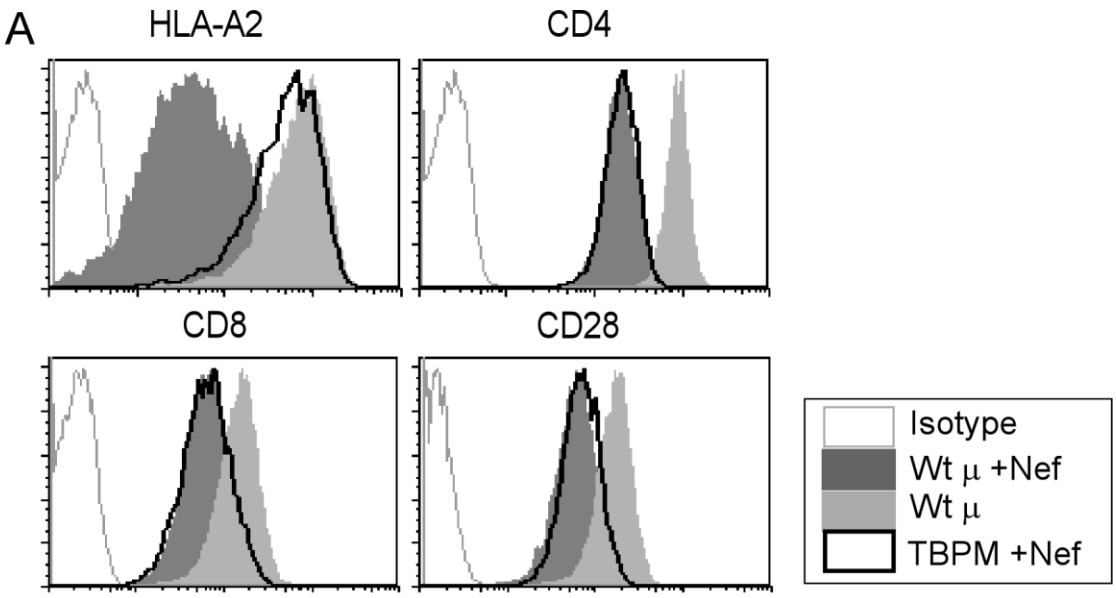
Figure 2.9. Nef recruits AP-1 for the downmodulation of CD8β and CD28 in a dileucine dependant manner.

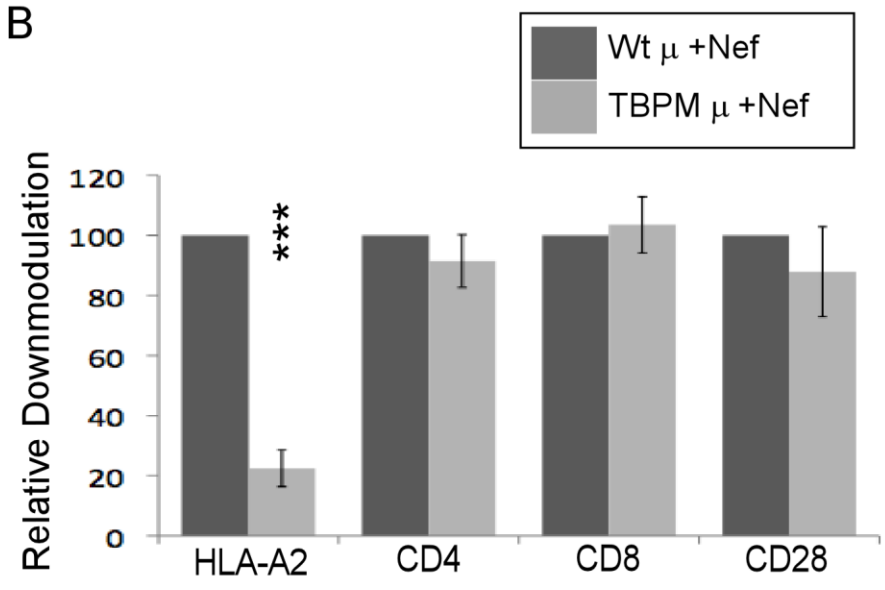
with A2/CD8β and A2/CD28 (Figure 2.9B lanes 4-5 and C lanes 11-12) but did promote co-precipitation of AP-1 with HLA-A2 (Figure 2.9B-C). Therefore, Nef can recruit AP-1 to cytoplasmic tail domains using two different signals, depending upon which cytoplasmic tail domain Nef is bound to.

In experiments where we observed AP-2 coprecipitation with the CD28 cytoplasmic tail, we were able to determine that the AP-2 recruitment also depended upon the dileucine motif in Nef (Figure 2.9C).

The μ subunit of heterotetrameric adaptor protein complexes recognizes tyrosine based motifs (10, 65) whereas an interface between the σ and large subunit (α, δ or γ) recognizes dileucine motifs (15, 27, 48). For MHC-I downmodulation, Nef promotes an interaction between the tyrosine binding motif in the μ1 subunit of AP-1 and a conserved tyrosine in the cytoplasmic tail of MHC-I. An AP-1 mutant with an inactive tyrosine binding pocket acts as a dominant negative inhibitor of MHC-I downmodulation (74, 93). To confirm that downmodulation of CD8β and CD28 did not utilize the AP-1 tyrosine binding pocket, we tested the impact of the tyrosine binding pocket mutant (TBPM) on these pathways. In contrast to its effect on MHC-I, TBPM did not significantly inhibit CD8β or CD28 downmodulation by Nef (Figure 2.10A-B). Similarly, knockdown of the AP-1μ1 subunit had no significant effect on CD8β or CD28 downmodulation by Nef (Figure 2.11A-B). Importantly, depletion of the AP-1μ1 subunit did not significantly

Figure 2.10. **Nef recruits AP-1 for the downmodulation of CD8β and CD28 in a tyrosine independant manner.** (A) Flow cytometric analysis of SupT1 T cells transduced with an adenoviral vector expressing Nef and a retroviral vector expressing the indicated μ subunit of AP-1. TBPM; tyrosine binding pocket mutant μ subunit of AP-1, WT μ; wild type μ subunit of AP-1. The cells were analyzed by flow at 3 dpi. (B) Quantitation of flow data from part A. The relative fold downmodulation, where the fold downmodulation of each molecule in the presence of Wt AP-1 is set to 100% is shown. Error bars represent standard deviation, *** $p<0.00001$, $n=3$.


A
HLA-A2
CD4
CD8
CD28
Isotype
Wt μ +Nef
Wt μ
TBPM +Nef


B
Wt μ +Nef
TBPM μ +Nef
Relative Downmodulation
120
100
80
60
40
20
0
***
HLA-A2
CD4
CD8
CD28

reduce AP1γ expression, and AP-1γ depletion had a partial destabilizing effect on AP-1μ1 (Figure 2.11C). Therefore, AP-1 involvement in CD8β and CD28 downmodulation by Nef is independent of the AP-1 tyrosine-binding activity but could still depend on AP-1 dileucine motif binding.

Based on the requirement for Nef's dileucine motif for CD8β and CD28 downmodulation as well as the lack of an effect of knocking down the AP-1μ subunit, we next examined the effect of knocking down the large subunits of the clathrin associated adaptor proteins, which contain in part the dileucine motif recognition site (15, 27, 48). Knockdown was achieved using lentiviral vectors expressing shRNAs directed against the large subunits of AP-1(γ), AP-2(α), AP-3(δ), and β-COP. Western blot analysis confirmed that we efficiently and specifically reduced expression of each target (Figure 2.11D). We found that knocking down AP-1γ and β-COP significantly reduced the downmodulation of both CD8β and CD28 by Nef but the effect was only partial (Figure 2.11E). The partial effects we observed may be due to incomplete knockdown or Nef utilization of multiple adaptor proteins in redundant pathways to achieve downmodulation of CD8 and CD28. Additionally, AP-2α knockdown had a small but significant effect on CD28 downmodulation, consistent with previous reports and the interaction we observed between CD28 and AP-2 (Figure 2.11E) (82, 86). Surprisingly, knockdown of AP-2 had no significant impact on CD4 downmodulation by Nef, despite a body of evidence tying CD4 downmodulation to this adaptor in other cell systems (16, 45, 82). It is possible

Figure 2.11. Nef requires AP-1 and β-COP for downmodulation of CD8 and CD28.

(A) Flow cytometric analysis of CEM SS cells expressing the indicated chimeric protein and transduced with lentivirus expressing shRNA and GFP and subsequently transduced with the indicated adenoviral vector at 3 days post lentivirus. Flow cytometric analysis was performed at 3 days post adenoviral transduction. Histograms represent GFP-expressing cells. (B) Quantitation of the flow data from part A. Fold downmodulation of the indicated molecule in the presence of shRNA. (C) Western blot analysis confirming specific knockdown of AP-1 subunits in CEM SS cells. (D) Western blot analysis confirming adaptor protein knockdown in SupT1 cells. (E) Relative downmodulation of the indicated molecule in SupT1 T cells transduced with the indicated lentivirus expressing shRNA and GFP plus a bicistronic retrovirus expressing Nef and a PLAP marker gene. shNC; negative control. The cells were analyzed by flow at 3 days post retroviral transduction. The fold downmodulation normalized to the negative control in cells positive for both GFP and PLAP is shown. * $p<0.05$, ** $p< 0.01$, ***$p<0.001$.

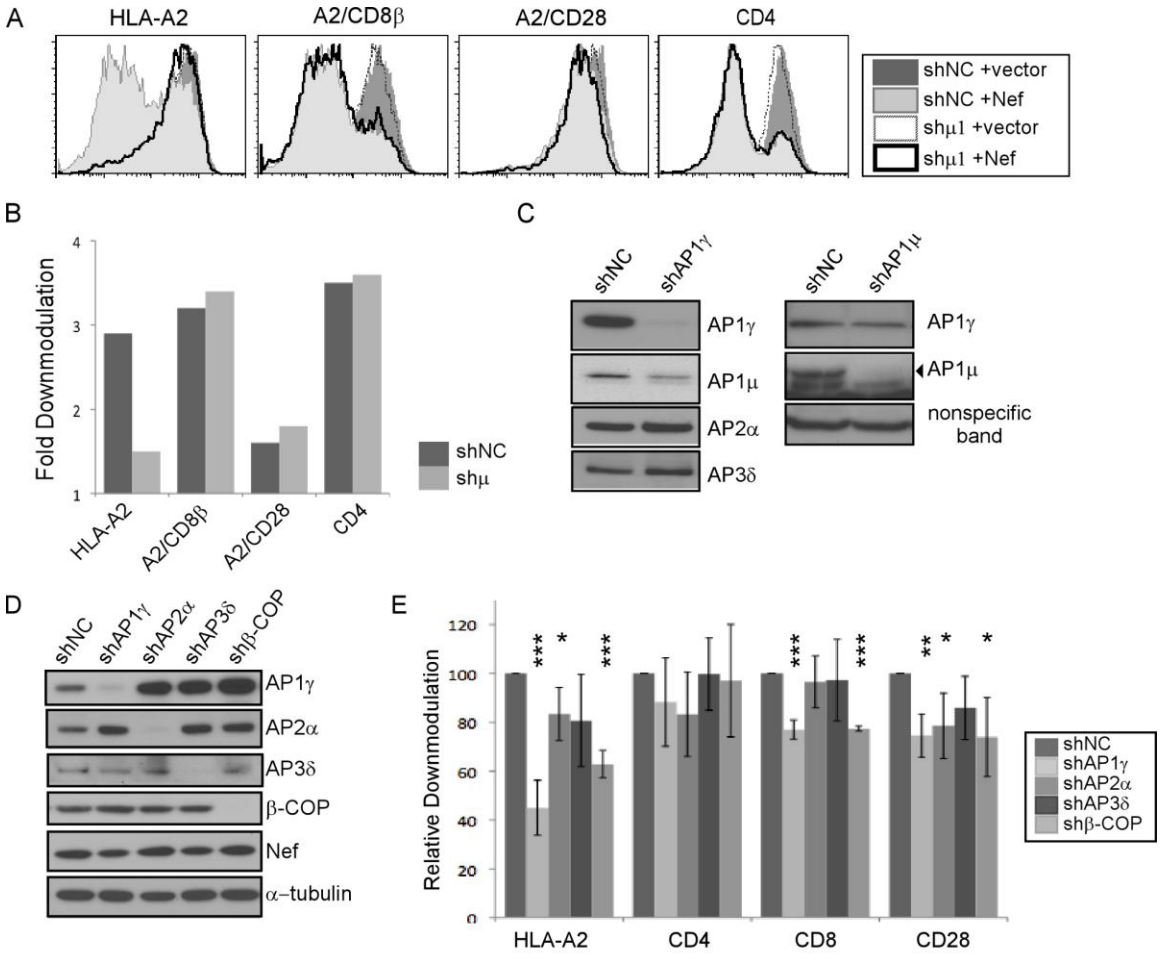


Figure 2.11. Nef requires AP-1 and β-COP for downmodulation of CD8 and CD28.

CD4 downmodulation by Nef can utilize multiple adaptor proteins and the degree to which it does so might vary in different cell systems. However, this possibility remains ambiguous because when we attempted to knockdown more than one adaptor protein large subunits the toxicity was too great to assess Nef function (data not shown).

2.3.7 Nef increases CD28 recycling

We were surprised to observe robust recruitment of adaptor proteins to A2/CD28 in the presence of Nef, because the effect of Nef on steady-state surface expression of A2/CD28 is relatively small. Interestingly, CD28 is internalized at a rate similar to, or even greater than, that of CD4 in the presence of Nef ((82) and data not shown). To address this discrepancy, we performed a recycling assay in which we observed a significant increase in CD28 recycling in the presence of Nef (Figure 2.12). Thus, Nef may initially induce a potent downmodulation of CD28 through recruitment of AP-1 and/or AP-2 in parallel or redundant pathways, and then a subset of CD28 is rapidly recycled to the plasma membrane. Thus, the effect of Nef on CD28 may vary depending on the recycling rates in different cell types.

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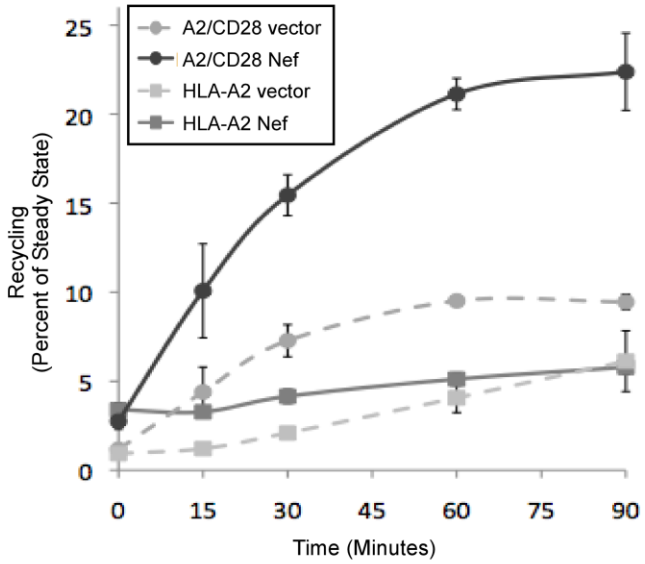

Figure 2.12. CD28 is recycled rapidly in Nef-expressing cells.

Measurement of recycling in CEM T cells expressing HLA-A2 or HLA-A2/CD28 and transduced with the indicated adenovirus. Cells were harvested at 3 dpi, incubated with 150 mg/mL cycloheximide for 2 hours to inhibit protein synthesis, and stripped of stainable HLA-A2 by removing the β2microglobulin with an acid wash (50 mM glycine, 100 mM NaCl pH 3.4). The cells were then incubated at 37°C in culture medium + cycloheximide. Triplicate samples were removed to ice at the indicated time points, stained for surface HLA-A2 expression (BB7.2), and analyzed by flow. Recycling is plotted as percent of steady state, where the mean fluorescence of each time point was divided by the mean fluorescence of cells that were not acid stripped. *n=2*.

2.3.8 Nef utilizes β-COP in CD8β and CD28 downmodulation

We also observed that knockdown of β-COP significantly reduced the effect of Nef on CD8β and CD28 surface expression (Figure 2.11E (SupT1 cells) and 2.13A (CEM SS cells)). This is interesting because β–COP is required for Nef-dependant trafficking of HLA-A2 and CD4 to lysosomes for degradation (79). β–COP knockdown has different effects on cell surface expression of MHC-I and CD4 in Nef expressing cells. Whereas β–COP knockdown increases cell surface levels of MHC-I in Nef-expressing cells, β–COP knockdown does not always alter CD4 surface expression in Nef expressing cells (Figure 2.11E and (79)). Instead, CD4 accumulates in intracellular vesicles and is unable to recycle to the cell surface in some cell types (70, 79).

Nef contains two β-COP binding sites, $RR_{17,19}$ and $EE_{155,156}$ (70, 79). Nef $RR_{17,19}$AA is defective at targeting MHC-I for degradation, while Nef $EE_{155,156}$AA is defective at targeting CD4 for degradation, and both have a partially reduced ability to co-precipitate with β-COP (79). When both domains are mutated (R/E), Nef is unable to efficiently bind β-COP and is defective at promoting the degradation of either MHC-1 or CD4 (79). We assessed the relative contributions of these Nef domains in CD8β and CD28 downmodulation, and found that the R/E double-mutant was defective at downmodulating CD8β and CD28, while each of the single mutant Nef proteins demonstrated intermediate defects (Figure 2.13B-C). Similar results were observed whether we assayed the chimeric panel (Figure 13B) or the full length target molecules (Figure 2.13C).

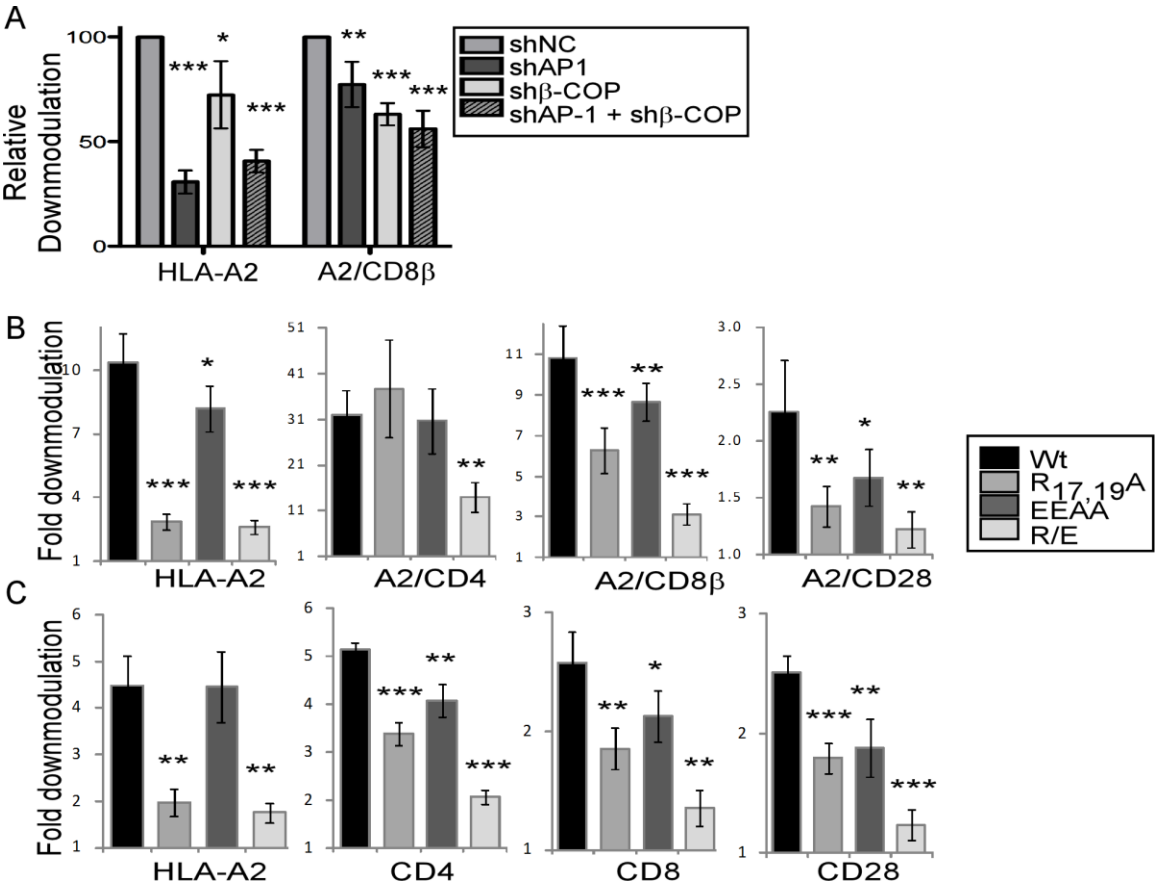

Figure 2.13. **β-COP is required for Nef-mediated downmodulation of HLA-A2/CD8β.** (A) Quantitation of relative downmodulation (normalized to control) of the indicated molecule expressed in CEM SS cells transduced with the indicated lentivirus expressing shRNA and a retroviral vector expressing Nef. The cells were harvested at 3 days post retroviral transduction. *n*=3. (B and C) Quantitation of fold downmodulation of the indicated molecule expressed in (B) CEM SS cells or (C) Sup T1 cells transduced with a retroviral vector expressing Nef or the indicated Nef mutants. Cells with equal GFP expression to control for Nef levels were analyzed at 3dpi. The mean fold downmodulation +/- standard deviation is shown. *n*=3. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.3.9 Combined effects of AP-1 and β-COP

To further investigate the contribution of AP-1 and β-COP to A2/CD8β degradation, we again utilized the shRNA lentivirus system to knockdown AP-1γ and β-COP, both singly and in combination. We observed that knockdown of either AP-1 or β-COP reduced the ability of Nef to downmodulate HLA-A2 and A2/CD8β. However, knocking down both adaptors in combination did not have a significant additional inhibitory effect on downmodulation (Figure 2.13A). Thus, the effect of Nef on CD8β results from effects of at least two cellular trafficking factors and possibly others (82).

To examine whether HIV-1 Nef promoted degradation of CD8β, we performed western blot analysis focusing on the mature, endoglycosidase H (endo H) resistant form of each molecule. As reported previously, Nef expression reduces the amount of endo H resistant HLA-A2 and this degradation is reversed by knocking down AP-1 or β-COP (Figure 2.14A, left panel and quantified in Figure 2.14B). As previously observed, knocking down β-COP also affected the migration of MHC-I, probably by disrupting the trafficking of enzymes required for proper glycosylation. β-COP knockdown does not inhibit trafficking of HLA-A2 through the Golgi, nor does it disrupt gross Golgi structure (79).

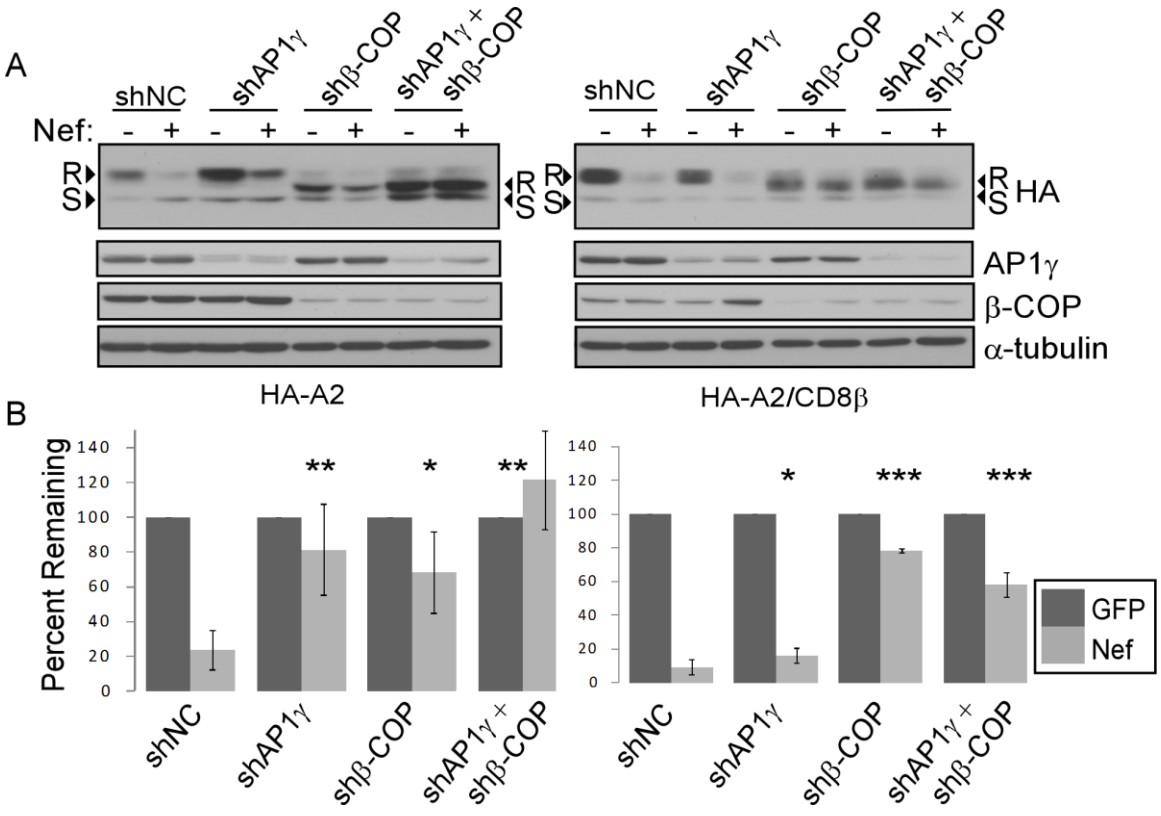

Figure 2.14. **β-COP is required for Nef-mediated degradation of HLA-A2/CD8β.**

(A) Western blot analysis of endonuclease H (endo H)-treated lysates from the cells described in part A. (B) Quantitation of endo H resistant bands. The endoH resistant bands (indicated by "R" in the figure) were quantified, normalized to the loading control, and shNC was set to 100% remaining. *p<0.05, **p<0.01, ***p<0.001, N=3.

In addition, we found that knockdown of β–COP dramatically reduced degradation of A2/CD8β (Figure 2.14A, right panel and quantified in Figure 2.14B). A small but statistically significant effect of knocking down AP-1 on A2/CD8β degradation was also noted (Figure 2.14B).

2.4 Discussion

In summary, we have found that HIV-1 Nef has three major cellular targets: MHC-I, CD4, and CD8. The ability of Nef to downmodulate endogenous CD8 was confirmed in HIV infected primary $CD4^+CD8^+$ T cells. We also report here that the effect of Nef on MHC-I and CD8 depended at least in part on expression of AP-1 and β-COP. In addition, we found that the AP-1 binding motif utilized by Nef to recruit AP-1 to cytoplasmic tails varied depending on which cytoplasmic tail domain Nef was bound to. Finally, we found that mutation of both β-COP binding sites in Nef reduced the activity of Nef against CD8. Thus, one explanation for the capacity of Nef to affect multiple targets is that it has evolved redundant mechanisms to recruit the same adaptor proteins.

Downmodulation of CD8, which participates in the recognition of antigen in association with MHC-1 provides a second Nef-dependent mechanism by which HIV-infected cells could avoid CTL lysis. While $CD8^+$ T cells are not generally thought to be targeted by HIV, there is mounting evidence that a subset of $CD8^+$ T cells upregulate CD4 in response to activation stimuli, and that these cells demonstrate antiviral activity, thus

creating an infectable target cell, the inhibition of which would be beneficial to HIV (31, 51, 52, 83, 94). Nef might also access $CD8^+$ T cells through cell-cell contacts, as was described to occur between infected and bystander T cells (63). Cell-cell conduits have also been described between Nef expressing APCs and bystander B cells. Moreover these nanotubular conduits have been shown to transmit Nef (72). If Nef were transferred when CTLs contacted an infected target, the transmitted Nef could downmodulate CD8 and reduce the efficacy of the anti-HIV-specific CTL attack. Finally, there are reports of CD8-tropic HIV strains providing another potential scenario in which Nef could encounter CD8 (37, 38, 59, 71, 77, 89).

This study is the first to assess the contribution of AP-1 and β-COP to the downmodulation of CD28 and CD8β. Another group has reported a partial requirement for AP-2 in the downmodulation of CD4 and CD8β that we did not observe here (82). However, the literature is conflicting on this point; some groups report a defect in CD4 downmodulation when AP-2 is knocked down, while other groups observe no effect (15, 75, 82). Additionally, co-depletion of Eps15 and AP-2 may be required to observe an inhibition of Nef activity against CD4 (45). Knockdown of adaptor protein subunits is technically difficult, as highly efficient depletion of these proteins can be lethal to cells. For these reasons we may have underestimated the contribution of AP-2 in CD28 and CD8β downmodulation by Nef in our system. AP-1- and AP-2-dependant mechanisms may represent parallel or redundant pathways, which Nef can utilize depending upon cellular localization and availability of cellular adaptors.

Here we have presented direct evidence that Nef can recruit AP-1 by utilizing either the tyrosine motif recognition site or the dileucine recognition site of AP-1, and that this is determined by which cytoplasmic tail domain Nef is bound to (Figure 2.15A-B). Previous studies found that the dileucine motif in Nef is required for glutathione-S-transferase (GST)-Nef pull-down of AP-1 complexes from mammalian cell lysates (11, 44) and for interactions detected in yeast two and three hybrid analyses (11, 24, 25, 29, 35, 43, 44, 55, 69) but the dileucine motif is dispensable for MHC-I downmodulation even though AP-1 is required (62). CD4 downmodulation by Nef requires the dileucine motif, but does not involve AP-1 (35). Thus, the interaction between AP-1 and the dileucine motif has remained unexplained. Here we provide evidence that this interaction is utilized to target CD8 (and CD28).

We have also identified β-COP as a common factor in the downregulation and degradation of all three major targets of Nef: MHC-I, CD4, and CD8β. The evidence presented here is consistent with a model in which Nef utilizes AP-1 or AP-2 to direct cellular targets into endosomal compartments, where Nef subsequently recruits β-COP to route targets to the lysosomes for degradation as previously proposed for MHC-I and CD4 (Figure 2.15F). However, the fact that the degradation of CD8 was far more dependent on β-COP expression than on AP-1 expression indicates that β-COP may also directly target intracellular CD8 for degradation in Nef expressing cells without the need

for additional adaptor proteins (Figure 2.15F). The requirement for β-COP in multiple Nef pathways may represent a good target for therapeutic inhibition of Nef.

CD28 appears to be a unique target of Nef, in that it is downmodulated consistently by a panel of Nef variants, but the effect is small relative to the other targets of Nef. The small effect of Nef on steady-state surface CD28 seems at odds with the observation that CD28 is internalized at a rate similar to that of CD4 in the presence of Nef ((82) and data not shown). This discrepancy may be explained by the observation that CD28 recycling is significantly increased in Nef-expressing cells. SIV Nef downmodulates CD28 more potently than HIV-1 Nef; therefore CD28 downmodulation may be more important for SIV pathogenesis. It is also possible that CD28 recycling may be regulated in some cell systems, and that a physiologically relevant set of conditions exists which would result in less recycling of CD28 and more downmodulation of CD28 by HIV-1 Nef.

We were unable to reproduce data reported by another group that CD80 and CD86 were downmodulated by Nef. There were some differences in the cell lines used in our study compared with the previous report. The previous studies used endogenously expressed CD80 and CD86 in the U937 monocytic cell line. However, even when replicating the published culture conditions, we were able to observe only low levels of CD80 on the cell surface, and no CD86 at all, leading us to believe that our clone of the U937 line is inherently different from that used to report downmodulation of CD80 and CD86. This

may be the cause for the discrepancy between our results and others, and is the reason that we utilized primary macrophages and DCs as a highly relevant system for investigating Nef activity against these potential targets. We were unable to detect an effect of Nef on CD80 or CD86 under established stimulation and culture conditions for primary macrophages and DCs, indicating that Nef targeting of these proteins must only occur under specialized circumstance that we were unable to replicate. CD1d also appears to be a special case, in that all Nef isolates do not retain the ability to reduce surface expression of this molecule, yet one or more Nef variants may be capable of doing so to a limited extent. This may be rare, evolutionarily driven by some infrequently occurring host factor, or may be a feature of certain HIV-1 clades.

In summary, we have identified three major targets of HIV-1 Nef: MHC-I, CD4 and CD8 and these results were generalizable to multiple Nef variants. We also demonstrated that downmodulation of CD8 and CD28 required AP-1 recruitment and was dependent upon Nef's dileucine motif. This observation indicates that Nef can interact with AP-1 through either the tyrosine binding pocket or dileucine recognition domain of AP-1, depending upon which cytoplasmic tail Nef is associated with. Finally, we discovered a role for β-COP in the downregulation and degradation of CD8, as well as MHC-I and CD4, which represents a convergence of the all the trafficking mechanisms utilized by Nef.

Figure 2.15. Schematic representation of the two AP-1 binding sites that Nef utilizes when bound to the MHC-I cytoplasmic domain (A), Nef recruits AP-1 through the tyrosine recognition site in the AP-1μ subunit (93). In this model the three-way complex requires amino acid residues in both MHC-I, especially Y_{320}, as well as in Nef, M_{20} in particular (although assays using purified proteins and a Nef-MHC-I cytoplasmic tail fusion protein did not demonstrate a requirement for M_{20}) (64, 74, 81, 93). When bound to the CD8 or CD28 cytoplasmic tails, Nef recruits AP-1 through the dileucine recognition site at the interface between the γ and σ subunits of AP-1 (49). C-E) Schematic representation of the Nef domains involved in β-COP recruitment. Nef residues $R_{17,19}$ are required for β-COP involvement in MHC-I degradation (C) (79). A diacidic motif in Nef, $EE_{155,156}$, is required for β-COP involvement in CD4 degradation (D) (30, 70, 79). Either or both Nef domains likely participate in β-COP recruitment for the degradation of CD8 by Nef (E). F) Model of the trafficking pathways Nef uses to achieve downmodulation and degradation of target proteins. Nef blocks anterograde transport in an AP-1-dependant pathway (47, 74, 85). Nef also induces internalization of target molecules at the plasma membrane through an association with AP-1 or AP-2 (11, 16, 24, 45, 61, 79, 82, 86). These pathways likely converge at a β-COP-dependant step (79). Because AP-1 knockdown did not block β-COP-dependent degradation of A2/CD8, Nef may be able to direct some targets into endolysosomal compartments by a pathway in which only β-COP recruitment is necessary, and clathrin-associated adaptor proteins are dispensable. PM stands for plasma membrane; LY indicates lysosome; LE/ MVB indicates late endosome or multivesicular body compartments.

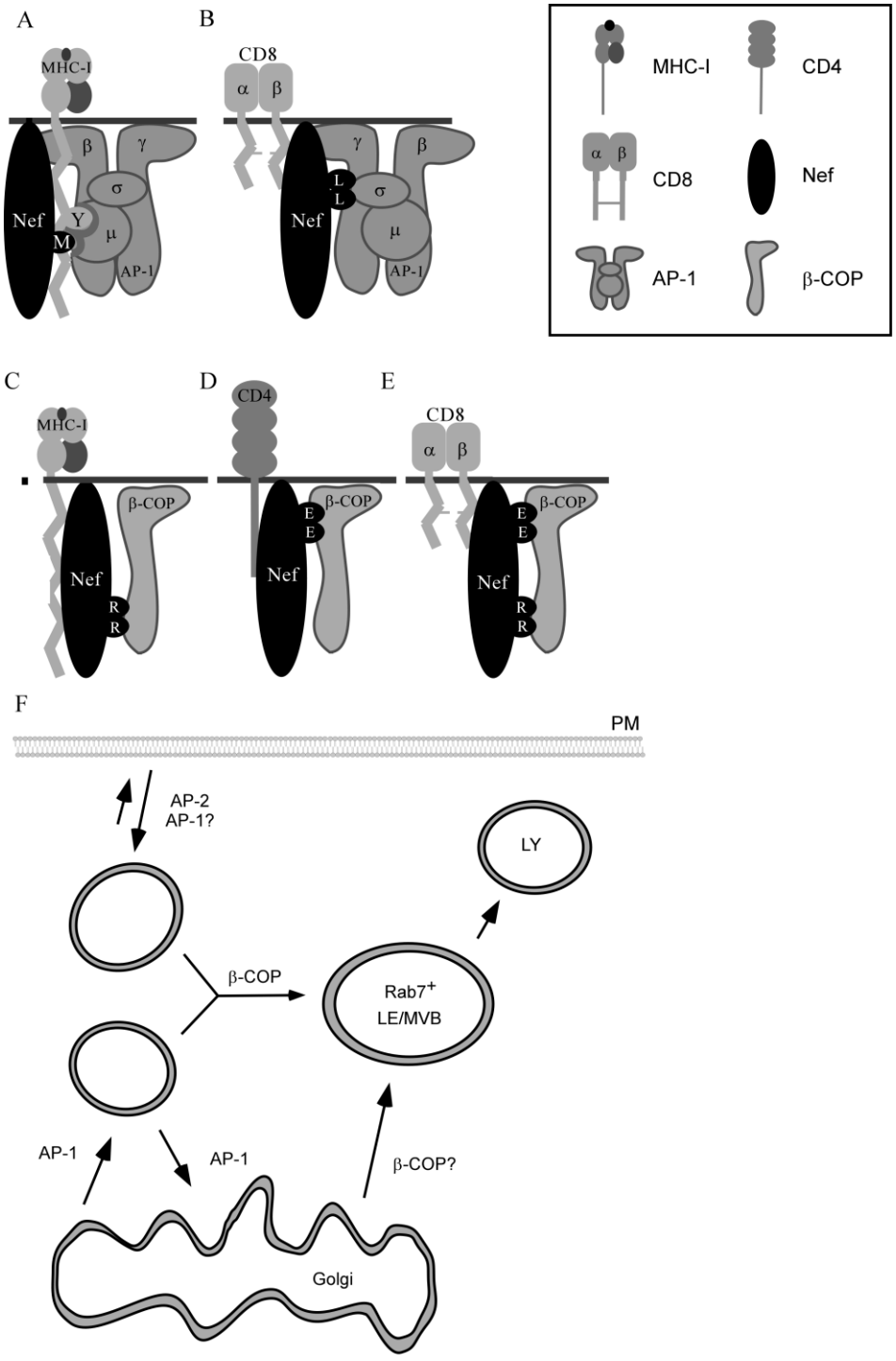

Figure 2.15. Schematic representation of the two AP-1 binding sites that Nef utilizes when bound to the MHC-I cytoplasmic domain

2.5 Experimental methods

2.5.1 Cell culture

Bosc and 293T viral packaging cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM penicillin, streptomycin, and glutamine (PSG). CEM-SS and SupT1 cells lines were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM PSG.

2.5.2 Preparation of T cell lines expressing HLA-A2-chimeric molecules

Stable cell lines were made as previously described (3). Briefly, each construct was introduced into CEM-SS or SupT1 cells using the Murine Stem Cell Virus (MSCV) retroviral vector pseudotyped with VSV-G. A uniform population was selected by culturing the cells in neomycin.

2.5.3 Primary T cell preparation

Leukopaks were obtained from the New York Blood Center. Peripheral blood mononuclear cells (PBMCs) were purified using ficoll gradients. Non-adherent cells were stimulated with 10μg/mL phytohemaglutinin (PHA, Sigma-Aldrich) overnight and the next day 50 U/mL interleukin(IL)-2 was added.

2.5.4 Preparation of macrophages and dendritic cells

Primary monocytes were obtained from whole PBMCs by positive selection using a magnetic sorting kit (EasySep Human CD14 selection kit, StemCell Technologies). The CD14+ cells were induced to mature into macrophages by culturing in RPMI + 10 ng/mL GM-CSF (R&D Systems) for 5 days. The CD14+ cells were induced to mature into dendritic cells (DC) by culturing in RPMI + 800 U/mL GM-CSF and 500 U/mL IL-4,with the addition of 100 U/mL TNFα on day 3 (all from R&D Systems). DCs were stimulated 9 days in total before infection.

2.5.5 DNA Constructs

The CD80, CD1d, and CD8β cytoplasmic tails were fused to the extracellular and transmembrane regions of HA-tagged HLA-A2 using PCR cloning methods. HA-HLA-A2 (1), was used as the PCR template. The following oligonucleotide primers were used: HA-HLA-A2/CD8β forward primer 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and reverse primer 5'-GTGGTCGCTGCTGTGATGTGGTGCTGCCGGCGGAGGAGAGCCCGGCTTCGTTTCATGAAACAATTTTACAAATAACTCGAGCGG-3'. HA-HLA-A2/CD80 forward primer 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and reverse primer 5'AGCTGTGGTCGCTGCTGTGATGTGGAGATGCAGAGAGAGAAGGAGGAATGAGAGATTGAGAAGGGAAAGTGTACGCCCTGTATAACTCGAGCGG3'. HA-HLA-A2/CD1d forward primer 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and

reverse primer 5’-GCTGCTGTGATGTGGTCCCGGTTTAAGAGGCAAACTTCCTATCAGGGCGTCCTGTGACCGCTCGAGCGG-3’. The CD28 tail was fused to HA-HLA-A2 in a two-step cloning method. First a 3’ fragment was created using two overlapping oligos that span the entire CD28 cytoplasmic tail sequence, along with a forward primer which appended a 15 base pair sequence which overlapped with the transmembrane region of HLA-A2, and a reverse primer to introduce a 3’ XhoI site. The oligo sequences are as follows: 5’ oligo 5’-AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCGCCCCGGGCCCACCCGCAAG-3’, 5’-CGCCGCCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAGCCTATCGCTCCTGA-3’, 5’-GGTCGCTGCTGTGATGTGGAGGAGTAAGAGGAGCAGGCTC-3’ AND 5’-TCGCAGCCTATCGCTCCTGACTCGAGGG-3’. A second round of PCR then generated the HA-HLA-A2/CD28 using HA-HLA-A2 and the CD28 fragment generated in the first PCR reaction as templates. The following primers were used: 5’-CGGGATCCACCATGCGGGTCACGGCG-3’ and

5’-TCGCAGCCTATCGCTCCTGACTCGAGGG-3’. The full length CD86 gene was cloned from the Megaman human cDNA library (Stratagene) into pLITMUS29 (NEB). The CD86 cytoplasmic tail was fused to HA-HLA-A2 in a manner similar to CD28 above. A first round of PCR generated a CD86 cytoplasmic tail fragment with 15 base

pairs 5' to the CD86 tail that overlap with the transmembrane region of HLA-A2 using full length CD86 as the template and the forward primer 5'-gtggtcgctgctgtgatgtggaaatggaagaagaagaagc-3' and reverse primer 5'-aagtgatacatgtttttaactcgagcgg-3'. A second PCR reaction was performed to create HA-HLA-A2 with a 15 base pair 3' region that overlapped with the 5'sequence of the CD86 tail. HA-HLA-A2 was the template and the primer sequences were forward primer 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and reverse primer 5'-GTGGTCGCTGCTGTGATGTGGAAATGGAAGAAGAAGAAGC-3'. A third round of PCR, using the products of the first and second PCR reactions, resulted in the full length HA-HLA-A2/CD86 chimera. The following primers were used: forward primer 5'-CGGGATCCACCATGCGGGTCACGGCG-3' and reverse primer 5'-AAGTGATACATGTTTTTAACTCGAGCGG-3'. The PCR products were digested BamHI to XhoI and then ligated into the BglII and XhoI sites of MSCV2.2 (2). The resultant constructs were sequenced to ensure that no mutations were introduced during cloning.

2.5.6 Cloning of *nef* alleles into pMIG

Nef alleles were cloned into the Bgl II and Eco RI sites of pMIG vector obtained from Dr. Luk Van Parijs (MIT) (28). Nef alleles containing a 5' Bam HI and 3' Eco RI site were PCR-amplified from cloned HIV genomes obtained through the AIDS Research Reference Reagent Program, Division of AIDS, NIAID, NIH (7, 8, 12). The following oligonucleotide primers were used: 5' oligonucleotides: 5' Nef-1 5'-

CGGGATCCACCATGGGTGGCAAGTGGTCAAAA-3’ (All except 92UG037, 93BR029 and 92RW009)

5’Nef-2 5’-CGGGATCCACCATGGGTAACAAGTGGTCAAAG-3’ (92UG037)

5’Nef-4 5’-CGGGATCCACCATGGGTAGCAAGTGGTCAAAA-3’ (93BR029 and 92RW009)

3’ oligonucleotides:

89.6 5’-CGGAATTCTCAGTTCTTGAAGTACTCCGGATG-3’

92UG037 5’-CGGAATTCTCAGCAGTCTTTGTAAAACTCCGG-3’

93BR020 5’-CGGAATTCTCAGTCTTGGTAGTACTCCGGATG-3’

NL4-3/84ZR 5’-CGGAATTCTCAGCAGTTCTTGAAGTACTCCGG-3’

PEYYKDC 5’-CGGAATTCTCAGCAGTCTTTGTAGTACTCCGG-3’

(93BR029, 92RW009, 94UG114)

YU-2 5’-CGGAATTCTCAGTTCTTGTAGTACTCCGGATG-3’

All alleles were sequenced to ensure that no mutations were introduced during cloning.

2.5.7 shRNA constructs

FG12 shRNA lentiviral vectors were constructed as previously described (73, 79). ShNC and construct targeting β-COP were previously described (79). The target sequence for shAP1γ began at position 1201-GATCCCCGCAGACTGTGCATCTGGAATTCAAGAGATTCCAGATGCACAGTCT GCTTTTTGGAAA. The AP2α target sequence was GATCCCCGTCAGAGTTCCGACAGAACTTCAAGAGAGTTCTGTCGGAACTCTG

ACTTTTTGGAAA. The target sequence for shAP3δ began at position 4180-GATCCCCGAGAGGTTGCAGTCAGAACTTCAAGAGAGTTCTGACTGCAACCTCTCTTTTTGGAAA

2.5.8 Viral transductions

Adenovirus was prepared by University of Michigan Gene Vector Core, and infection of CEM-SS cells were performed as previously described (74, 91). Murine retroviral vector preparation and transduction were performed as described (68, 90, 93). shRNA-containing lentivirus preparation and transduction have also been described (73, 79). Infection of PBMCs with HIV constructs was performed as in (22). Primary APCs were infected with adenovirus at MOIs titrated to generate similar Nef expression levels compared to what we typically obtain in CEM cells. In these experiments, CEM cells were infected with an MOI of 50, primary macrophages were infected with an MOI of 250, and primary DCs were infected with an MOI of 500.

2.5.9 Western blot analyses and immunoprecipitations

Cells were lysed in PBS 0.3% CHAPS, 0.1% SDS pH 8, 1 mM PMSF, normalized for total protein and separated by SDS-polyacrylamide gel electrophoresis. Endo H (NEB) digestion was performed according to the manufacturer's protocol. Immunoprecipitations were performed as previously described (93). Briefly, cells were lysed in 1% digitonin (Wako) and normalized for total protein. Lysates were precleared overnight with A/G

beads (Pierce) and isotype control antibody (BD Biosciences). BB7.2-crosslinked A/G beads were used for immunoprecipitation, and were washed five times with 0.1% digitonin buffer prior to elution with 150 mM DTT.

Samples were separated by SDS-PAGE. HA-HLA-A2 and the chimeric proteins were detected using the anti-HA antibody HA.11 (1:5000; Covance). Nef was detected with AG11 (1:5000), and β-COP with M3A5 (2) which were purified as described (46). Adaptor protein subunits were detected with anti-AP-2α, anti-AP1γ, and anti-AP3δ (all 1:500; BD Bioscience) or Ry/1 anti-AP1μ (1:5000) (88). The secondary antibody for anti-Nef, β-COP, HA, AP-2α, and anti-AP3δ was HRP-rat anti-mouse IgG_1 (Zymed), for anti-AP1γ was HRP-rat anti-mouse (nonspecific) (Zymed), and for anti-μ1 was HRP-goat anti-rabbit (Zymed).

2.5.10 Flow cytometry

Intact cells were stained in FACS Buffer (2% FBS, 1% human serum, 1% Hepes, and 0.025% NaN3 in PBS). Primary cells were pre-incubated with 10% Fc-Receptor Blocker (Accurate Chemical and Scientific Corps) prior to primary antibody incubation. Chimeric molecules were detected with HA.11 (1:50; Covance) or BB7.2 (1:500). Endogenous proteins were detected with BB7.2 (1:500), OKT4 (1:500), OKT8 (1:500), all prepared by the University of Michigan Hybridoma Core and purified as previously described (46), or anti-CD8β 5F2 (1:100; Santa Cruz), CD28.2 (1:100; Biolegend), CD80

(1:100; BD Pharmingen), CD86 (1:100; BD Pharmingen), CD1d (1:100; eBioscience). Isotype-specific secondary antibodies conjugated to PE, alexafluor 488, or alexafluor 647 were used at 1:250 (Invitrogen).

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Notes

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Chapter 3

ARF-1 activity is required to recruit AP-1 to the MHC-I cytoplasmic tail and disrupt MHC-I trafficking in HIV-1 infected primary T cells

3.1 Abstract

HIV-1 infected cells are partially resistant to anti-HIV cytotoxic T lymphocytes due to the effects of the HIV Nef protein on antigen presentation by MHC-I and evidence has been accumulating that this function of Nef is important *in vivo*. HIV Nef disrupts normal expression of MHC-I by stabilizing a protein-protein interaction between the clathrin adaptor protein AP-1 and the MHC-I cytoplasmic tail. There is also evidence that Nef activates a PI3-kinase-dependent GTPase (ARF-6) to stimulate MHC-I internalization. However, the relative importance of these two pathways is unclear. Here we report that a GTPase required for AP-1 activity (ARF-1) was needed for Nef to disrupt MHC-I surface levels whereas no significant requirement for ARF-6 was observed in Nef expressing T cell lines and in HIV-infected primary T cells. An ARF-1 inhibitor blocked the ability of Nef to recruit AP-1 to the MHC-I cytoplasmic tail and a dominant active ARF-1 mutant stabilized the Nef-MHC-I-AP-1 complex. These data support a model in which Nef and ARF-1 stabilize an interaction between MHC-I and AP-1 to disrupt presentation of HIV-1 epitopes to CTLs.

3.2 Introduction

$CD8^+$ cytotoxic T lymphocytes (CTLs) are important for the control of chronic viral infections. In a virally infected cell, MHC-I molecules present peptides derived from viral proteins. Once the T cell receptor (TCR) on $CD8^+$ CTLs recognizes a "non-self" signal presented by MHC-I, the CTL releases perforins and granzymes which kill the virally infected cell preventing further spread of the virus (reviewed in (3)). CTLs play an important role in the control of HIV infection (for review see (9)) and recent evidence indicates that individuals mounting a Gag-specific CTL response have improved parameters with regard to controlling disease (16, 24). Despite the efficacy with which CTLs control viral load early in infection, anti-HIV CTLs ultimately fail to prevent progression of disease in most infected people.

Studies performed *in vitro* have revealed that the HIV Nef protein protects infected cells from CTL-mediated lysis (8, 26, 52, 61). Nef has been shown to protect HIV-infected primary T cells from CTL lysis using flow cytometric killing assays (8, 26), CTL co-culture assays (61) and chromium release assays (52). Although Nef limits the ability of CTLs to recognize and kill infected cells, it does not appear to abrogate the capacity of CTLs to produce inhibitory cytokines in response to infected cells (52). Recent *in vivo* evidence supports the hypothesis that CTLs may control HIV infection *in vivo* primarily by the elaboration of inhibitory cytokines, but fail to eradicate the infection because the CTLs cannot efficiently lyse the infected cell source of new virions (60).

Nef binds directly to the cytoplasmic tail of MHC-I allotypes (58) and recruits the clathrin adaptor protein AP-1 to redirect MHC-I into an endolysosomal pathway from the *trans*-Golgi network (TGN) and to prevent its expression at the cell surface (41). There is evidence that MHC-I delivered to endosomes via the activity of AP-1 is subsequently targeted for degradation via Nef binding to the COP-I coatomer, β-COP (44).

Nef has also been implicated in promoting internalization of MHC-I from the cell surface (45). The relative contribution of internalization versus disruption of forward transport to overall MHC-I downmodulation varies depending on the cell type (23) and has been attributed to both ARF-6 (4) and AP-1 (23, 28) dependent pathways. The relative importance of ARF-6 versus AP-1 has not yet been determined in studies that directly compared their roles.

The adaptor protein AP-1 is a heterotetrameric complex that recognizes trafficking signals in cargo and recruits clathrin-sorting machinery to the *trans*-Golgi network. AP-1 is made up of μ1, β1, γ, and σ1 subunits, which collectively sort cargo containing Yxxϕ or [D/E]xxxLL trafficking signals into the endo-lysosomal network (for review see (40)). MHC-I downmodulation can be inhibited by knocking down expression of the AP-1 μ1 subunit (41, 44) or by overexpressing a dominant negative mutant of AP-1 μ1 which lacks a functional tyrosine-binding pocket (47, 59). Mutation of a tyrosine residue in the MHC-I cytoplasmic tail disrupts binding of AP-1 to the Nef-MHC-I complex in cells (41, 59) and in experiments using purified proteins (32, 47). Based on these data, the AP-1

tyrosine-binding pocket has been proposed to interact with the MHC-I cytoplasmic tail tyrosine and this interaction is stabilized by Nef.

Binding of adaptor proteins to trafficking signals is normally regulated by the activity of ADP-ribosylation factors (ARFs), which are small GTPases that control assembly and disassembly of intracellular trafficking complexes. ARF activation and recruitment to cellular membranes is cyclical and regulated by its GTP binding state. Guanine nucleotide exchange factors (GEFs) promote the exchange of GTP for GDP. GTPase-activating proteins (GAPs) support ARF catalysis of GTP and thus are important to inactivate ARF ((20) and reviewed in (13)). There are a number of different ARF proteins expressed by cells. ARF-1 is a clathrin regulatory protein that, upon binding GTP, undergoes a conformational change exposing a myristoyl group that inserts into membranes and subsequently stabilizes AP-1 (1, 48, 54) or COP-I coatomer (46, 54) binding to trafficking signals. The ARF-1 GEF inhibitor brefeldin A (BFA) stabilizes an abortive ARF-GDP-bound complex (35) thus preventing ARF-1 cycling.

ARF-6 is a related GTPase that regulates the recycling of cargo that has been internalized by clathrin-independent endocytosis (10, 38). There is evidence that ARF-6 is required for Nef-dependent MHC-I downmodulation based on experiments with ARF-6 mutants that are "locked" in a GDP-bound, inactive state (ARF-6-$T_{27}N$) or a GTP-bound, constitutively active state (ARF-6-$Q_{67}L$) (4, 10, 38).

Here, we demonstrate that ARF-1 activity was required for Nef-dependent MHC-I trafficking via AP-1. Co-precipitation of AP-1 with the MHC-I-Nef complex was specifically inhibited by BFA and stabilized by constitutively active ARF-1 $Q_{71}L$ (51). In contrast, we were unable to detect a clear requirement for ARF-6. These data help to clarify the relative contributions of the two major pathways implicated in HIV immune evasion and highlight the AP-1/ARF-1 pathway as an important potential target for drug development.

3.3 Results

3.3.1 Functional ARF-1 is required for Nef to disrupt the trafficking of MHC-I

Nef binds directly to the cytoplasmic tail of MHC-I allotypes (58) and recruits the clathrin adaptor protein AP-1 to redirect MHC-I into an endolysosomal pathway from the *trans*-Golgi network (TGN) and to prevent its expression at the cell surface (41). However, it is not known whether Nef-dependent AP-1 binding and clathrin recruitment bypass the normal AP-1 regulatory steps that include the GTPase, ARF-1. To determine if ARF-1 activity was necessary for Nef to disrupt the transport of MHC-I to the cell surface, we utilized two ARF-1 mutants, ARF-1 $T_{31}N$ and ARF-1 $Q_{71}L$. ARF-1 $T_{31}N$ is defective because it does not exchange GDP for GTP (11). ARF-1 $Q_{71}L$ is a dominant active form of the molecule that stably binds GTP (51). We also tested BFA, which inhibits the activity of ARF-1 GTP exchange factors (GEFs).

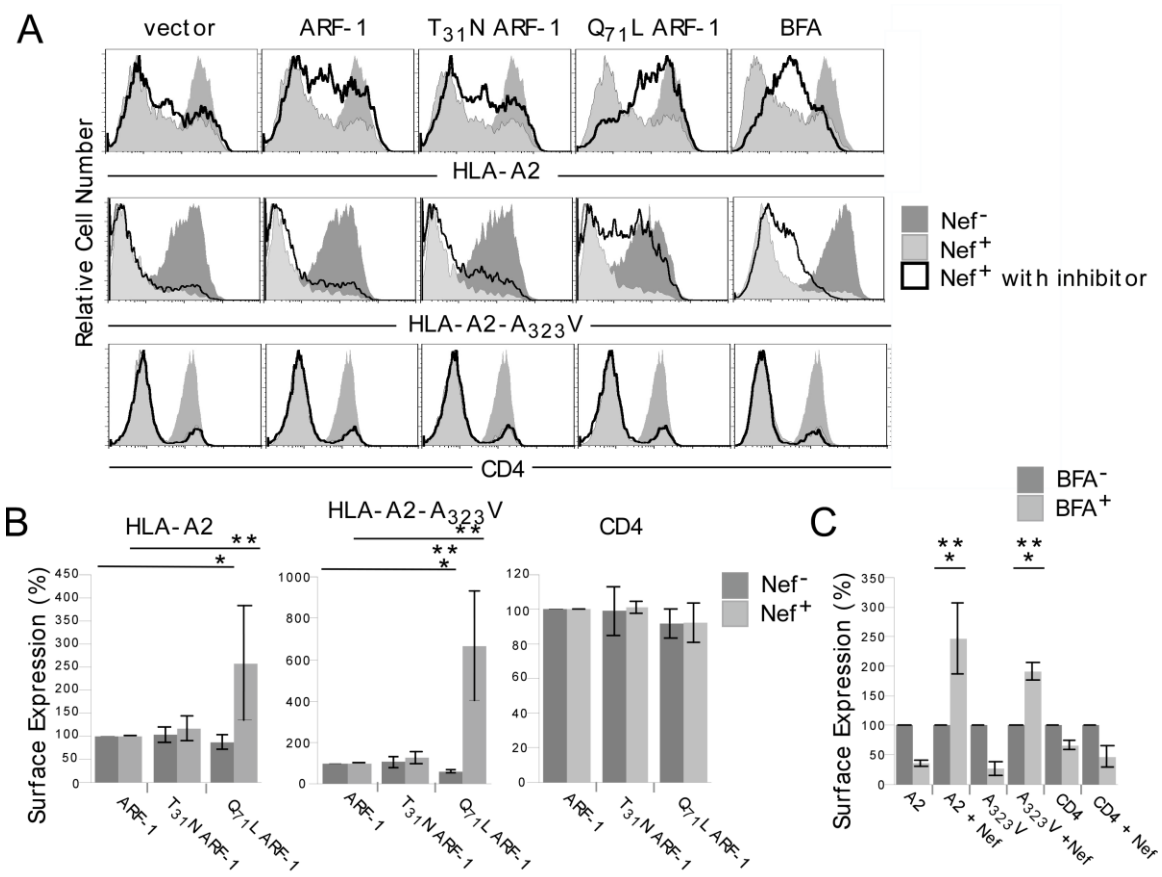


Figure 3.1. ARF-1 activity is required for Nef-induced downmodulation of HLA-A2. (A) Flow cytometric analysis of HLA-A2, HLA-A2 $A_{323}V$, and CD4 surface expression plus or minus Nef in cells expressing ARF-1 constructs as indicated. Cells expressing ARF-1 vector control were GFP^+ and these cells were gated on for this analysis. *Dark gray shaded curve*, vector alone; *Light gray shaded curve*, adeno-Nef; *black curve*, adeno-Nef plus ARF-1 as indicated. BFA, brefeldin A. (B) Western blot analysis of Myc-ARF-1 expression levels in transduced CEM T cells (C and D) Quantitation of part (A). The median fluorescence normalized to wild type ARF-1 is shown ± sd, $n \geq 3$. * denotes a *p*-value of <0.05, ** denotes a *p*-value of <0.01, and *** denotes a *p*-value of <0.001.

We found that overexpression of wild type and $T_{31}N$ ARF-1 did not significantly affect Nef-dependent MHC-I downmodulation (Figure 3.1A and 3.2, quantified in Figure 3.1C-D). However, the ARF-1 inhibitor, BFA, significantly increased MHC-I surface expression in the presence of Nef (Figure 3.1A and quantified in Figure 3.1C-D). The lack of a phenotype with overexpression of MHC-I $T_{31}N$ was not due to poor expression (Figure 3.1B) but likely resulted from the presence of endogenous wild type ARF-1 that was sufficient for Nef activity. CD4 downmodulation by Nef was unaffected by these inhibitors consistent with previously published data showing that internalization and degradation of CD4 are independent of the GTP-bound state of ARF-1 (Figure 3.1A and C) (15).

In addition, we observed a striking inhibitory effect of dominant active ARF-1 ($Q_{71}L$) on Nef-dependent MHC-I downmodulation (Figure 3.1A, quantified in Figure 3.1C). Based on this result, we postulated that this mutant might inhibit MHC-I downmodulation by blocking the cycling of ARF-1 and thus sequestering essential trafficking machinery required to maintain low MHC-I surface levels.

We also observed an inhibitory effect of BFA and ARF-1 ($Q_{71}L$) on MHC-I surface expression in the absence of Nef (quantified in Figure 3.1C-D). This is expected due to the known effects of these factors on protein export form the endoplasmic reticulum (27). Despite the inhibitory effect of these factors on MHC-I surface expression in the absence of Nef, both BFA and ARF-1 ($Q_{71}L$) caused a significant increase in MHC-I surface expression in the presence of Nef (Figure 3.1). The opposing effects observed in the

Figure 3.2. Two-color flow cytometry of HLA-A2 or CD4 in CEM T cells transduced with either control or Nef-expressing adenovirus and ARF-1 or ARF-6 constructs. ARF-1 and ARF-6 open reading frames were inserted into bi-cistronic retroviral vectors and virus produced from these constructs was used to transduce CEM T cells. GFP expression serves as an indicator of transduction efficiency and relative gene expression. The percentage of cells that are GFP^+ is indicated within the plot. The mean fluorescence intensity of MHC-I in the GFP^+ cells is indicated below each plot.

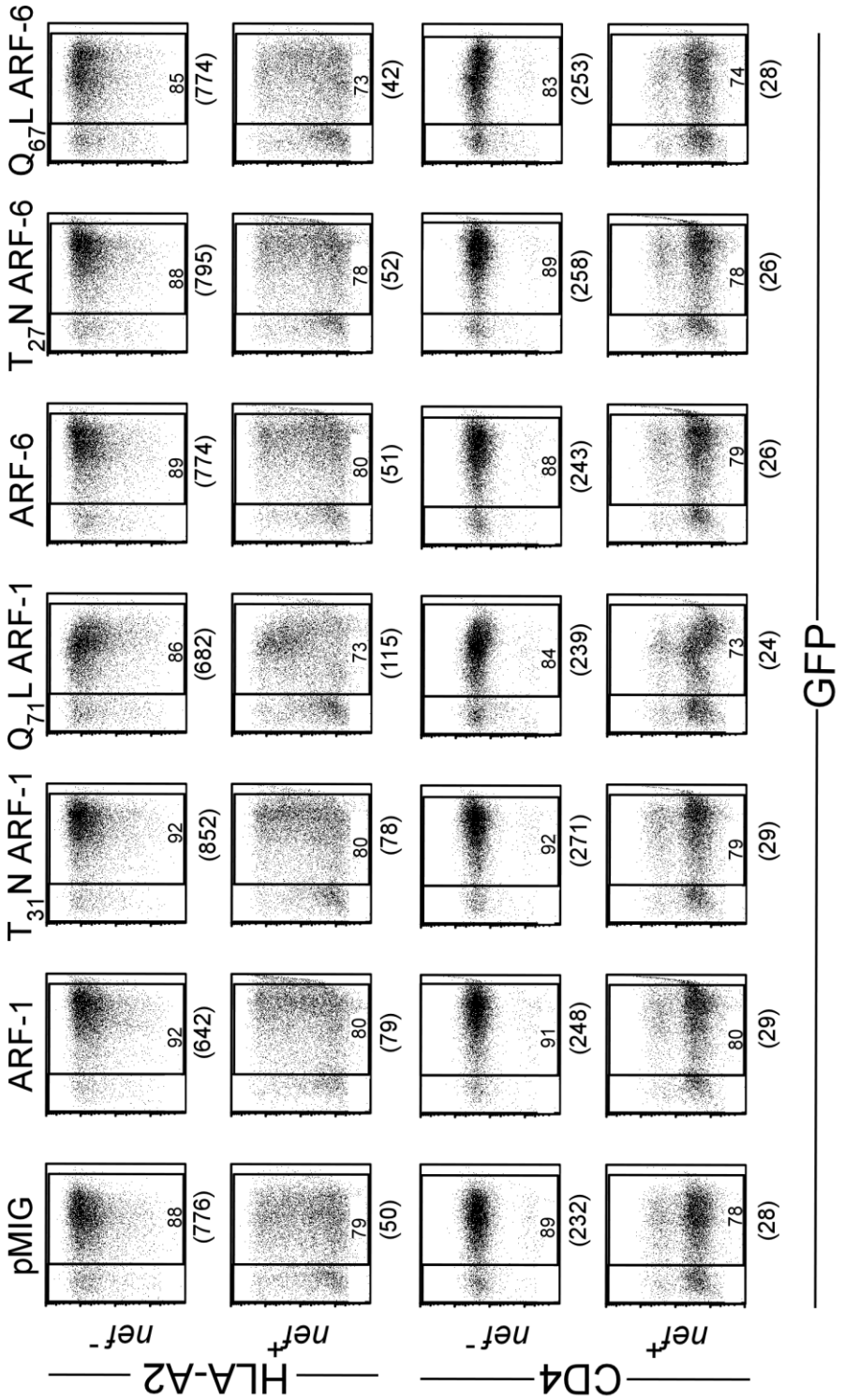


Figure 3.2. Two-color flow cytometry of HLA-A2 or CD4 in CEM T cells transduced with either control or Nef-expressing adenovirus and ARF-1 or ARF-6 constructs.

absence versus the presence of Nef supports the conclusion that these inhibitors are specifically affecting Nef-dependent pathways.

For comparison, we also included a molecule that contained a more natural AP-1 trafficking signal, which was previously shown to bind AP-1 in the absence of Nef. This signal was made by substituting a valine for an alanine in the cytoplasmic tail of MHC-I HLA-A2 to create a Yxxϕ sorting signal [$Y_{320}SQV_{323}$ (HLA-A2 $A_{323}V$)] (59). Compared to wild type MHC-I HLA-A2, this molecule has relatively low surface expression due to its interaction with AP-1 (59). Interestingly, the effect of ARF-1 mutants and BFA on the surface expression of HLA-A2 $A_{323}V$ in the absence of Nef was somewhat different than what occurred in the presence of Nef. The main difference was that, in the absence of Nef, there was a relatively small but statistically significant further reduction of HLA-A2 $A_{323}V$ expression with the dominant active mutant ARF-1 $Q_{71}L$ (Figure 3.1C). Whereas in the presence of Nef, ARF-1 $Q_{71}L$ inhibited the Nef-dependent reduction in surface expression of HLA-A2 molecules (Figure 3.1A and C). Given that HLA-A2 $A_{323}V$ is a model for trafficking due to AP-1, these differences provide suggestive evidence that the effects of Nef on MHC-I HLA-A2 are not due to AP-1 alone and probably involve additional steps that are disrupted by ARF-1 $Q_{71}L$.

3.3.2 Functional ARF-6 is dispensable for Nef-dependent MHC-I downmodulation

ARF-6 may also be important for Nef-dependent internalization of MHC-I based on evidence that dominant active ARF-6 ($Q_{67}L$) inhibited internalization of MHC-I by Nef in HeLa cells (4). To examine the relative effect of ARF-1 and ARF-6 mutants on Nef

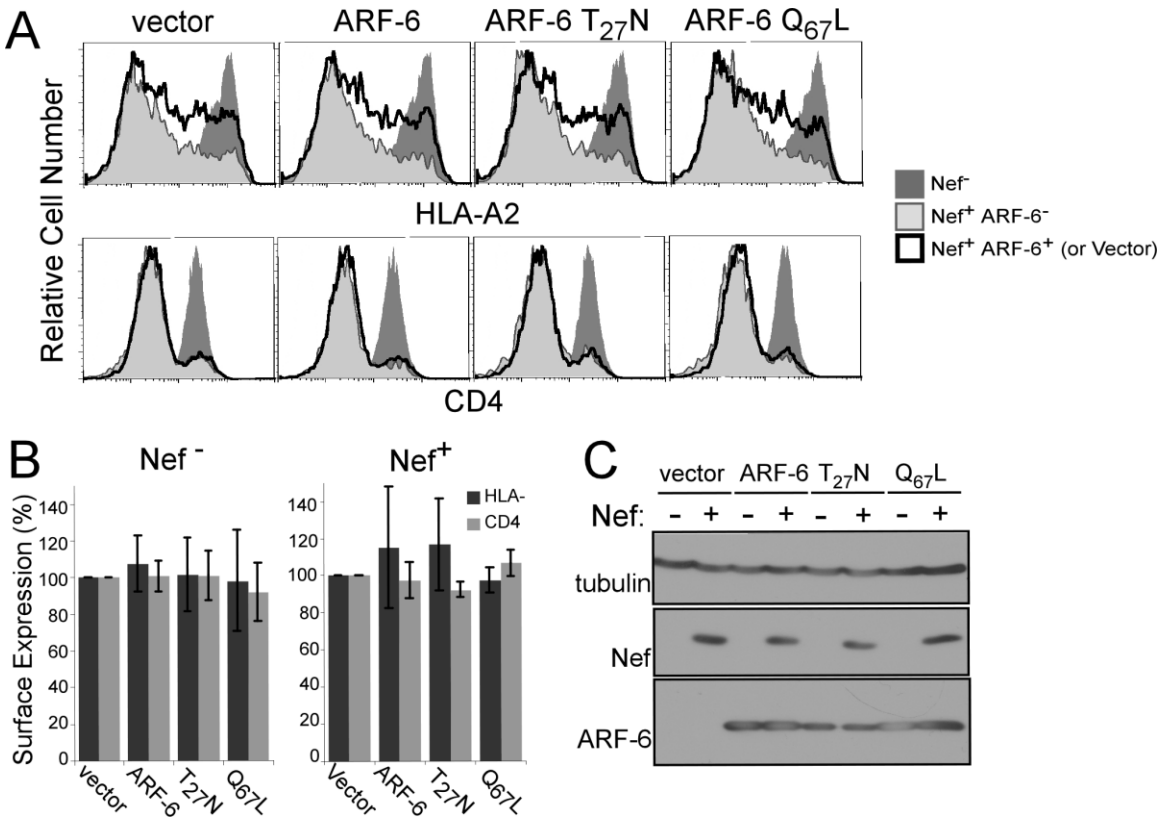


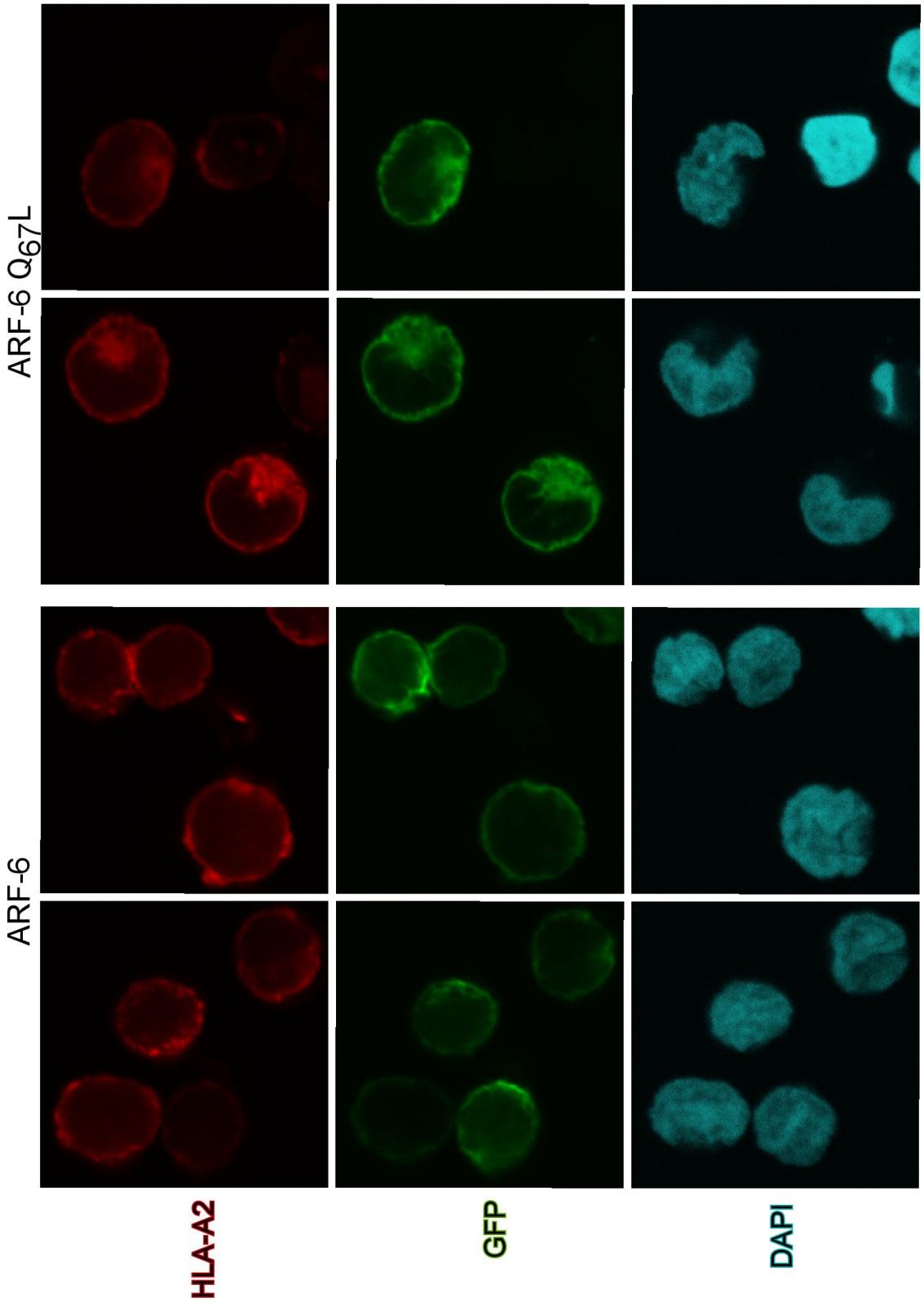
Figure 3.3. ARF-1 but not ARF-6 activity is required for Nef-dependent HLA-A2 and CD4 downmodulation in T cell lines. (A) Flow cytometric analysis of HLA-A2 or CD4 surface expression in CEM-SS cells transduced with control or Nef-expressing adenovirus plus the indicated ARF-6 construct. ARF-6 proteins were expressed via bi-cistronic murine retroviral vectors that also expressed GFP. Similar GFP^+ populations based on FL-1 mean were analyzed for HLA-A2 or CD4. *Gray shaded curve*, control vector; *gray outlined curve*, adeno-Nef; *black curve*, adeno-Nef plus the indicated ARF-6 protein. (B) Quantitation of part (A). The median fluorescence normalized to wild type ARF-6 is shown ± sd, *n*=3. (C) Western blot of ARF-6 and Nef expression.

activity in our system, we generated ARF-6 constructs analogous to the ARF-1 constructs tested in Figure 3.1. We found that, in contrast to the effect of ARF-1 mutants, there was no significant effect of ARF-6 mutants on Nef activity (Figure 3.3A-B). As controls, we demonstrated that the ARF-6 mutants were expressed in the cells (Figure 3.3C). Additionally, we verified that the ARF-1 and ARF-6 constructs transduced the cells with similar efficiency (Figure 3.2). For these experiments the ARF-1 and ARF-6 constructs were made identically as bi-cistronic elements that co-expressed GFP from an internal ribosome entry site (Figure 3.2) allowing GFP to serve both as a measure of transduction efficiency and as a measure of relative expression levels.

ARF-6 $Q_{67}L$ was active in our experimental system based on its effect on MHC-I surface expression in the absence of Nef. Microscopic analysis revealed intracellular accumulation of HLA-A2 in CEM-SS cells expressing ARF-6 $Q_{67}L$, but not in cells expressing wild type ARF-6 or ARF-6 $T_{27}N$ (Figure 3.4). This phenotype is consistent with previous reports that proteins that normally traffic through ARF-6 compartments are trapped in the presence of ARF-6 $Q_{67}L$ (5, 30).

Previous studies have suggested that Nef activates ARF-6 via a PI3-kinase-dependent step. Thus, levels of the PI3-kinase inhibitor, phosphatase and tensin homolog (PTEN), could influence the relative amount of ARF-6 dependent activity we observed. Indeed, it has been suggested that lower expression level of PTEN in the CEM-SS cell line used in some of our studies may influence PI3K-induced ARF-6-dependent effects on MHC-I and limit our ability to detect this pathway (19). To address this possibility, we used

Figure 3.4 ARF-6 $Q_{67}L$ disrupts HLA-A2 localization in a Nef-independent manner in T cell lines. Visualization of HLA-A2 expression in CEM-SS cells transduced with the indicated ARF-6 construct. ARF-6 proteins were expressed via bi-cistronic murine retroviral vectors that also expressed GFP. GFP and HLA-A2 were detected by confocal microscopy.


ARF-6 Q67L
ARF-6
HLA-A2
GFP
DAPI

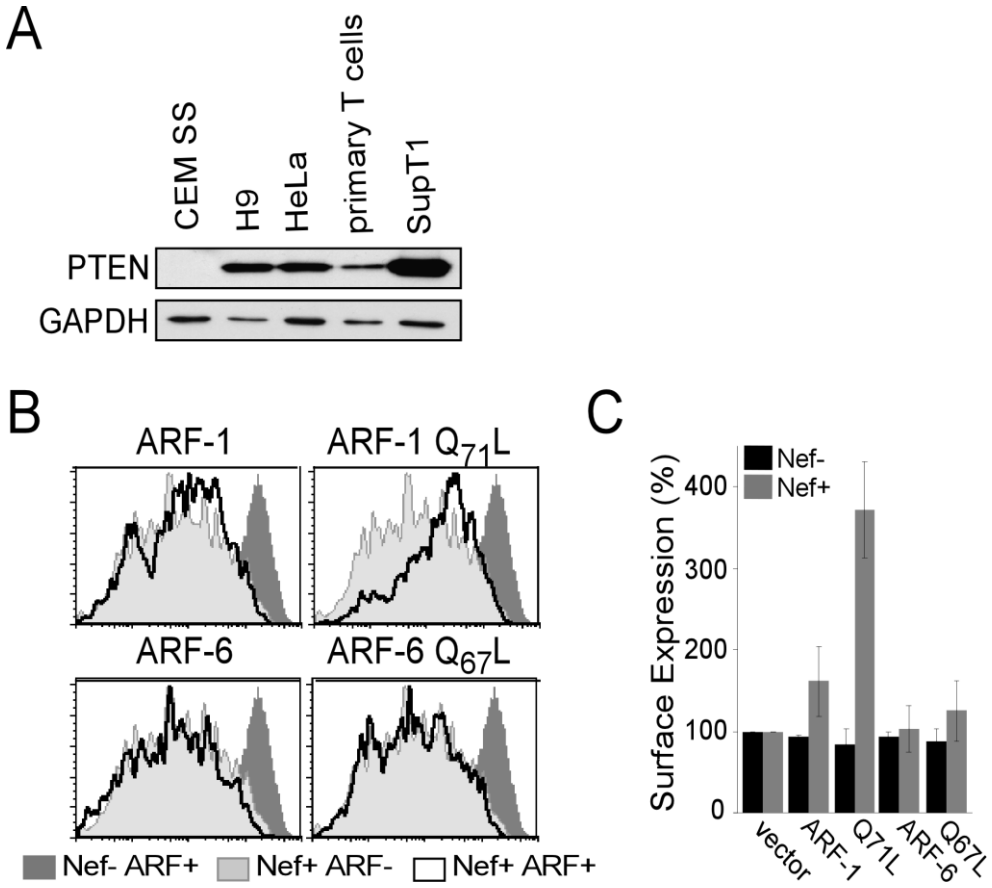


Figure 3.5. ARF-1 but not ARF-6 activity is required for Nef-dependent HLA-A2 and CD4 downmodulation in PTEN-expressing T cell lines. (A) Western blot of PTEN or control (GAPDH) levels in the indicated cell type. (B) Flow cytometric analysis of HLA-A2 surface expression in SupT1 cells transduced with control or Nef-expressing adenovirus plus the indicated ARF-6 construct and analyzed as described in part A. *Dark gray shaded curve*, control adenovirus plus ARF as indicated; *Light gray shaded curve*, adeno-Nef plus vector; *black curve*, adeno-Nef plus ARF as indicated. (C) Quantitation of part (B). The median fluorescence normalized to empty vector treatment is shown ± sd n=3. Values obtained with wild type ARF-1 were significantly different than those with ARF-1 $Q_{71}L$ ($p< 0.01$). Values obtained with wild type ARF-6 were not significantly different than those with ARF-6 $Q_{67}L$ ($p= 0.46$).

western blot analysis to examine the levels of PTEN expression in several cell lines, as well as in primary $CD4^+$ T lymphocytes. We confirmed that PTEN expression varied dramatically amongst cell lines and that CEM-SS cells did not express much, if any, PTEN (Figure 3.5A). We also performed experiments in a cell line (the SupT1 lymphoblastoid line) with very high PTEN expression to determine whether PTEN levels might be affecting our ability to detect a requirement for ARF-6 activity. Similar to our observations in CEM-SS cells, we found that expression of ARF-1 $Q_{71}L$ significantly reduced HLA-A2 downmodulation by Nef, while expression of ARF-6 $Q_{67}L$ did not (Figure 3.5B-C). As a control we again demonstrated that the ARF-1 and ARF-6 constructs transduced the cells and expressed the bi-cistronic message to similar degrees (Figure 3.6).

Finally, we compared the effects of ARF-1 and ARF-6 mutants in HIV-infected primary T lymphocytes expressing endogenous HLA-A2. For these experiments, wild type ARF or its dominant active mutant was inserted directly into the envelope open reading frame of a GFP-expressing HIV molecular clone (Figure 3.7A, NL-GI*arf*). Primary T cells were infected with the virus and then assayed 36 hours post-infection. We reproducibly observed a two-to-three fold inhibition of Nef-dependent MHC-I downmodulation when the infected cells expressed the ARF-1 mutant $Q_{71}L$, but not when the infected cells expressed the corresponding ARF-6 mutant $Q_{67}L$ (Figure 3.7B). These data support a model in which an ARF-1-dependent pathway rather than an ARF-6-dependent pathway is needed for Nef-dependent MHC-I downmodulation in HIV-infected primary T cells.

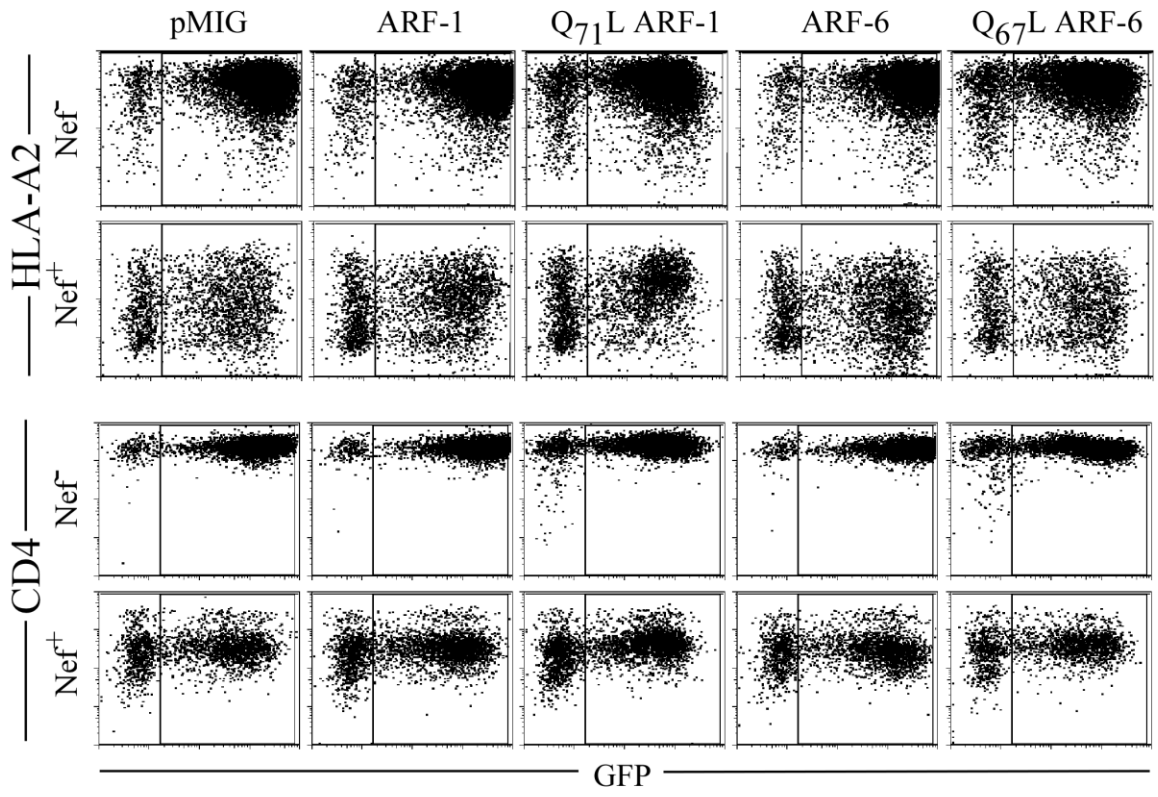


Figure 3.6. T**wo color flow cytometry of GFP and HLA-A2 or CD4 staining in cells transduced with control or adeno-Nef.** The ARF-1 and ARF-6 constructs used in these experiments were made identically by inserting the open reading frames into bi-cistronic retroviral vectors. Virus produced from these constructs was used to transduce Sup T1 cells. GFP expression serves as an indicator of transduction efficiency and relative gene expression.

Further evidence that ARF-6 $Q_{67}L$ was active in this experimental system is its effect on MHC-I surface expression in the absence of Nef. For HLA-A2, Bw4 and Bw6 MHC-I allotypes we observed an 18% (Figure 3.7B), 31% and 29% (data not shown) reduction in MHC-I expression respectively. Over three independent experiments in which HLA-A2 expression was measured in primary T cells expressing ARF-6 $Q_{67}L$ the average reduction in surface expression was 13% ($p< 0.002$). These data are consistent with the inhibitory effect of ARF-6 $Q_{67}L$ on MHC-I recycling (5, 30).

To further examine the contribution of ARF-6 to Nef-dependent MHC-I downmodulation, we used a lentiviral system expressing shRNA to knock down ARF-6 expression in CEM-SS cells. Knockdown of ARF-6 has previously been demonstrated to inhibit clathrin-independent endocytosis of G-protein coupled receptors (18). We found that depletion of ARF-6, as confirmed by western blot, had no significant impact on HLA-A2 or endogenous CD4 downmodulation by Nef, as compared to negative control shRNA (Figure 3.8). This observation lends additional support to the conclusion that ARF-6 is dispensable for Nef-induced MHC-I downmodulation in T cells. Similar analysis was not possible for ARF-1, as knockdown of single Golgi ARFs does not affect cellular trafficking (56).

3.3.3 Dominant active ARF-1 increases AP-1 recruitment to HLA-A2 $A_{323}V$ and the HLA-A2-Nef complex

To determine the mechanism by which ARF-1 mutants affect MHC-I surface expression, we used co-immunoprecipitation assays to measure AP-1 binding to cargo plus or minus

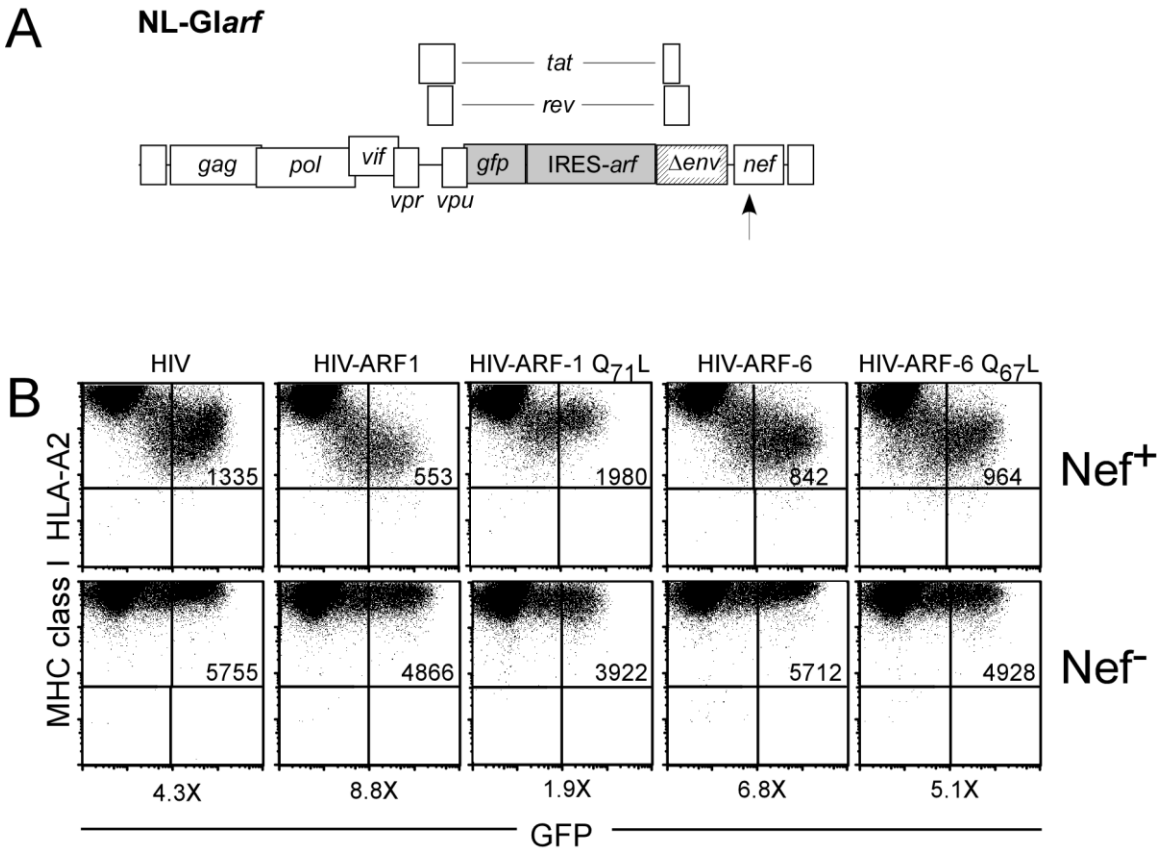


Figure 3.7. ARF-1 but not ARF-6 activity is required for Nef-dependent endogenous HLA-A2 and CD4 downmodulation in HIV infected primary T cells. (A) Map of HIV engineered to express ARF-1, ARF-1 $Q_{71}L$, ARF-6, or ARF-6 $Q_{67}L$ and GFP plus or minus Nef. (B) Flow cytometric analysis of endogenous HLA-A2 surface expression in primary $CD4^+$ T cells infected with the indicated HIV. Cells were harvested 36 hours post infection. Numbers inside each flow plot reflect the mean fluorescence intensity of MHC class I in the GFP^+ population. Fold downmodulation of MHC class I surface expression derived from the *nef*+ and *nef*- populations of each HIV construct is shown under the bottoms panels. Results are representative of three independent experiments.

expression of ARF-1 mutants. As a positive control, we first examined AP-1 binding to a natural Yxxϕ sorting signal using HLA-A2 $A_{323}V$ in the absence of Nef. As seen in Figure 3.9A lanes 2, 3, 4, and 5, and as previously reported (59), AP-1 co-precipitated with HLA-A2 $A_{323}V$ in the absence of Nef. Expression of wild type (lane 3) or $T_{31}N$ ARF-1 (lane 5) did not affect AP-1 recruitment by this assay. However expression of dominant active ARF-1 $Q_{71}L$ (lane 4) dramatically increased co-precipitation of AP-1 with HLA-A2 $A_{323}V$.

Next, we used the same assay system to determine whether ARF-1 activity affected formation of the Nef-MHC-I-AP-1 complex. Similar to what was observed with HLA-A2 $A_{323}V$, we found that dominant active ARF-1 $Q_{71}L$ dramatically increased co-precipitation of AP-1 with HLA-A2 in Nef expressing cells. Conversely, we found that treatment with BFA disrupted complex formation (Figure 3.9B lanes 10 and 11). The effect of BFA was specific because it could be rescued by overexpression of ARF-1 $Q_{71}L$ as previously reported (Figure 3.9B lane 12) (62). Similar to what we observed with HLA-A2 $A_{323}V$, expression of ARF-1 $T_{31}N$ had little effect on AP-1 co-precipitation (Figure 3.9, lane 9), presumably because endogenous ARF-1 was sufficient for this purpose.

We were also able to detect the presence of ARF-1 in the Nef-MHC-I-AP-1 complex (Figure 3.9B). ARF-1 $Q_{71}L$ co-precipitated readily, but both wild type and ARF-1 $T_{31}N$ could also be detected, albeit at lower efficiency. Of note, expression of ARF-1 mutants and BFA treatment had no significant effect on Nef co-precipitation with HLA-A2,

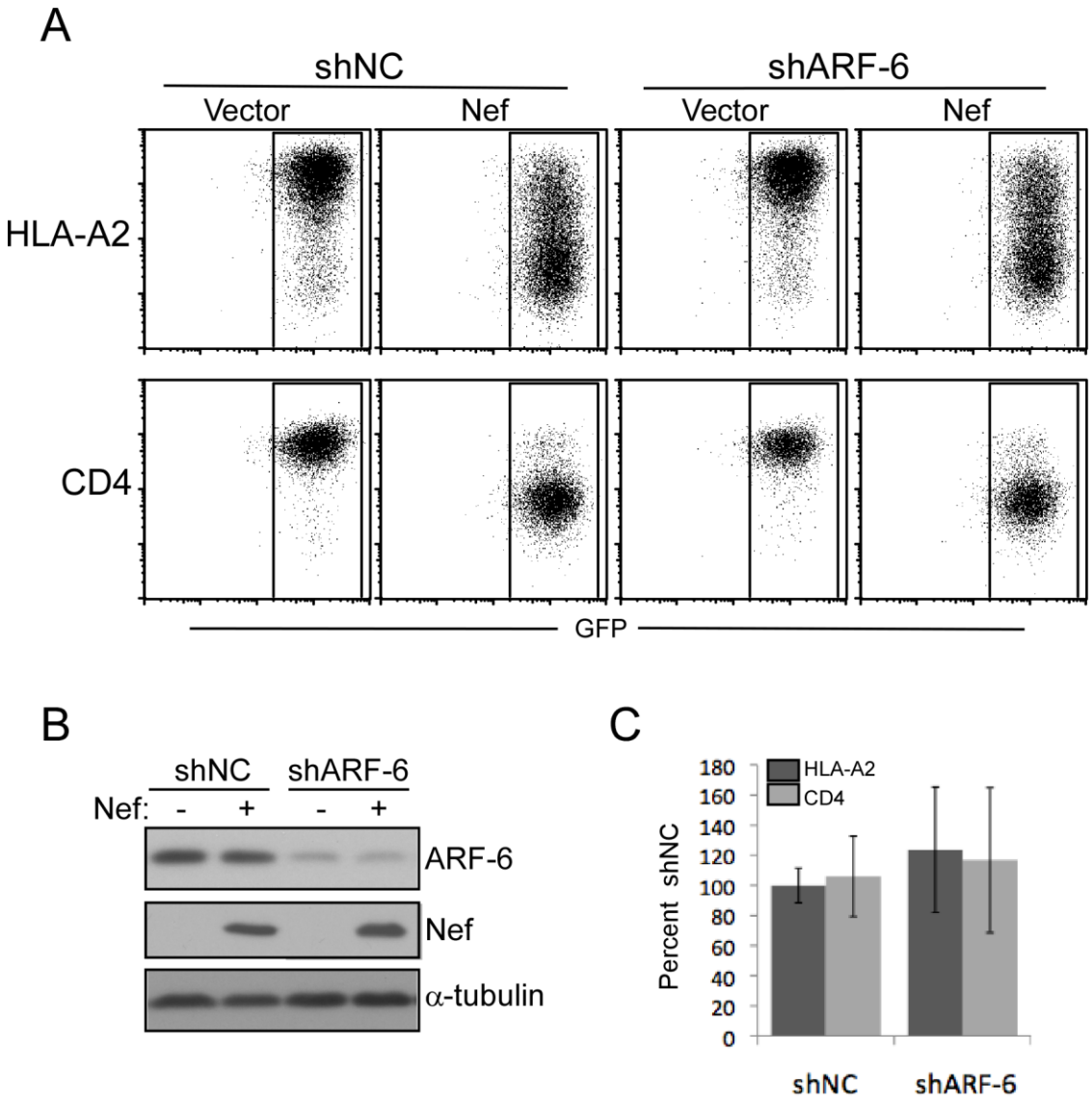


Figure 3.8. ARF-6 knockdown does not inhibit Nef-induced MHC-I downmodulation. A) Flow cytometric analysis of CEM SS cells transduced with lentivirus expressing shRNA and GFP and subsequently transduced with the indicated adenoviral vector at 3 days post lentivirus. Flow cytometric analysis was performed at 3 days post adenoviral transduction. B) Western blot analysis confirming specific knockdown of ARF-6. C) Quantitation of the flow data from part A. Relative downmodulation of the indicated molecule. Errors bars represent standard deviation.

Figure 3.9. Dominant active ARF-1 stabilizes AP-1 binding to the Nef-MHC-I complex. (A) Immunoprecipitation of HLA-A2 $A_{323}V$ with western blot analysis of associated AP-1. HLA-A2 $A_{323}V$ was immunoprecipitated from CEM-SS cells transduced with a bicistronic murine retroviral vector expressing GFP and ARF-1 proteins as indicated. Based on GFP expression, 50-70% of the cells were transduced. (*n*=4) (B) Immunoprecipitation of HLA-A2 with western blot analysis of associated proteins. HLA-A2 was immunoprecipitated from CEM-SS cells transduced with control or adeno-Nef and a bi-cistronic murine retroviral vector expressing GFP and ARF-1 proteins as indicated. Based on GFP expression 50-70% of the cells were transduced *n*=4)

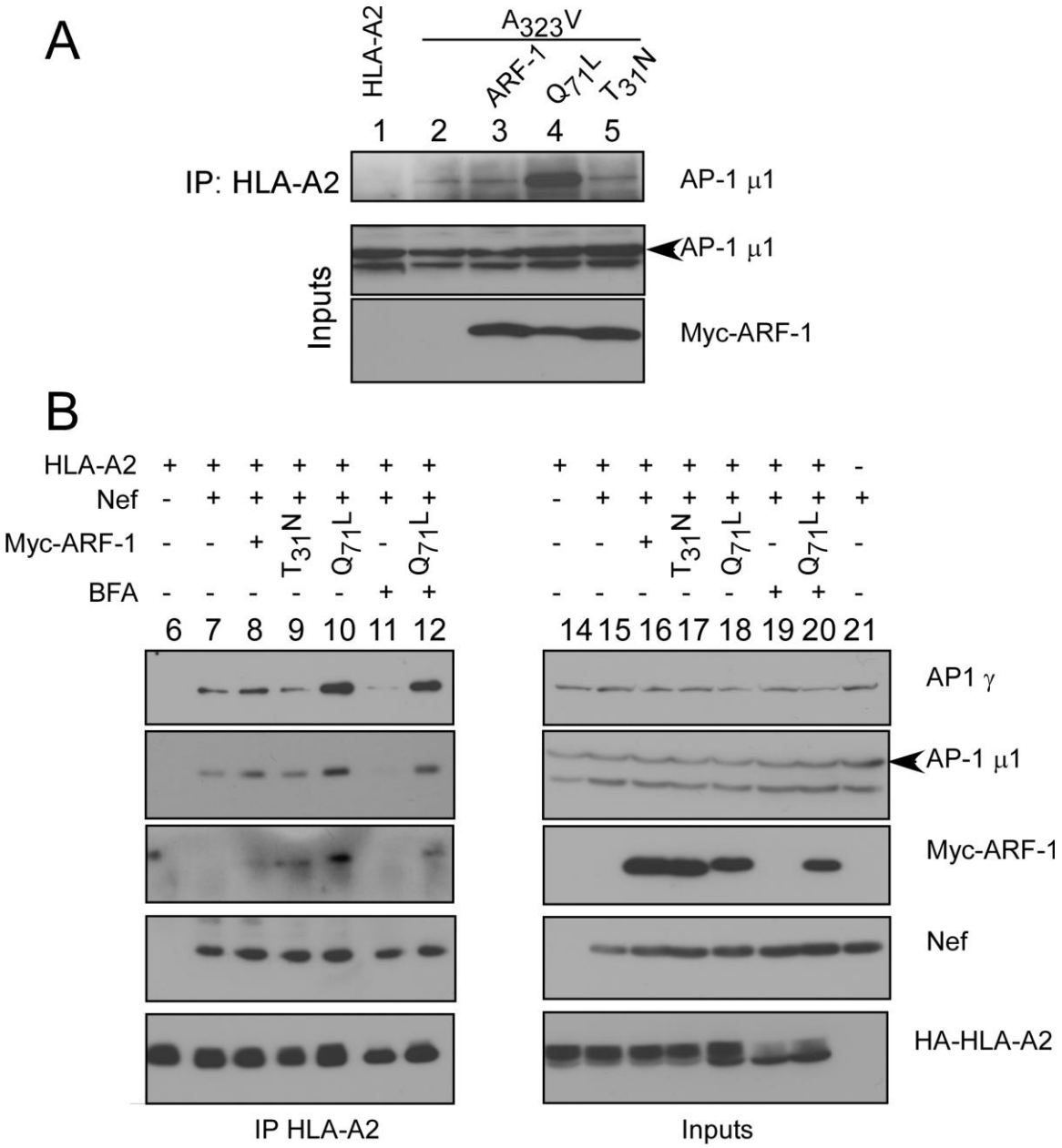


Figure 3.9. Dominant active ARF-1 stabilizes AP-1 binding to the Nef-MHC-I complex.

confirming previous data (58, 59) that Nef can bind to the cytoplasmic tail of HLA-A2 in the absence of AP-1.

We also performed the reverse experiment in which we asked whether we could observe co-precipitation of AP-1, Nef and HLA-A2 when we immunoprecipitated ARF-1. For this study we examined complexes formed both in the absence and presence of Nef. Indeed, we were able to detect AP-1 co-precipitating with ARF-1 $Q_{71}L$ (Figure 3.10). In Nef-expressing cells we were also able to detect Nef co-precipitating with ARF-1 independent of the GTP-bound state of the ARF-1 molecule (Figure 3.10 lanes 4, 5, and 6), as previously reported (36). Additionally, in Nef expressing cells, there was an enhancement in the amount of AP-1 that co-precipitated with ARF-1 $Q_{71}L$ (Figure 3.10, lane 6). Finally, we also detected HLA-A2 co-precipitating with ARF-1 $Q_{71}L$ in Nef expressing cells. These data suggest that ARF-1 $Q_{71}L$ potently stabilizes interactions amongst AP-1, Nef, and MHC-I HLA-A2 and that its mode of inhibition of MHC-I downmodulation is not disruption of complex formation but rather the formation of a static complex that sequesters necessary trafficking components. Therefore we propose a model in which ARF-1 is necessary to form the trafficking vesicles at the *trans*-Golgi network that contain MHC-I, Nef, and AP-1 (Figure 3.11).

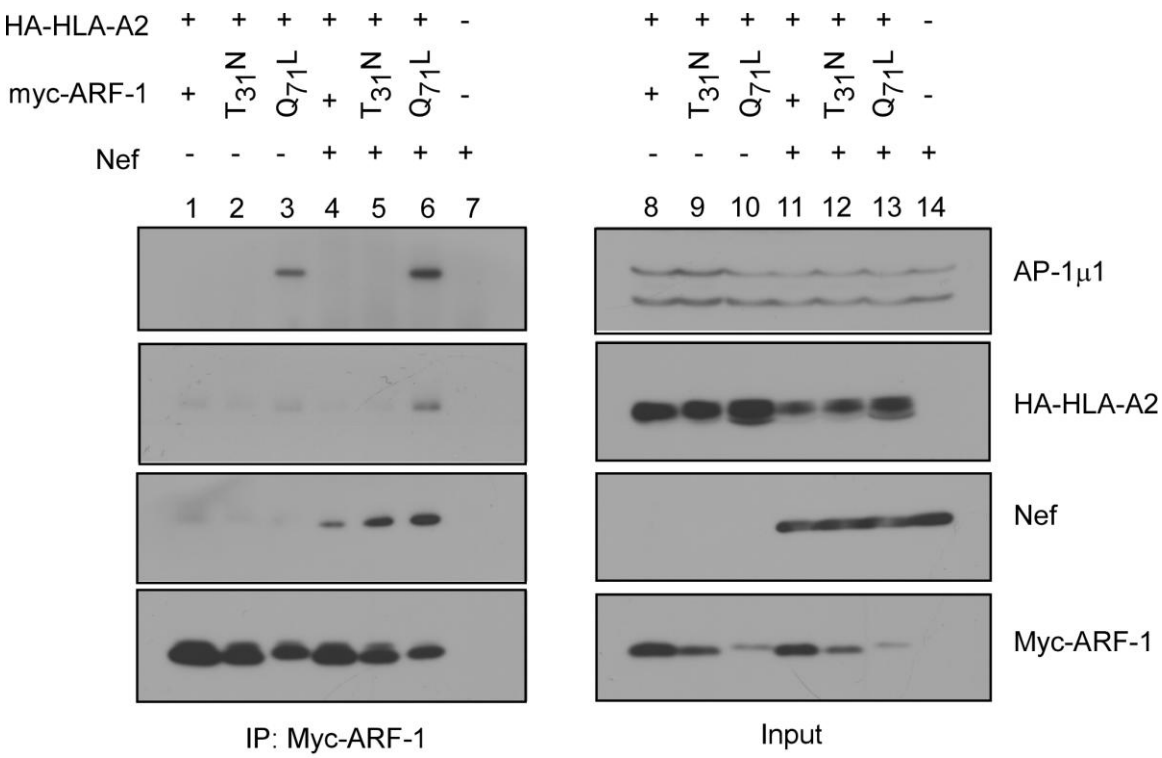


Figure 3.10. MHC-I, Nef and AP-1 co-precipitate with ARF-1 $Q_{71}L$. (A) Immunoprecipitation of myc-tagged ARF-1 with western blot analysis of associated proteins. ARF-1 proteins were immunoprecipated from cells treated as in figure 3 part B, Based on GFP expression 50-70% of the cells were transduced. (*n*=2)

3.4 Discussion

HIV causes a persistent infection that evades eradication by anti-HIV CTLs. Viral persistence is mediated in part by the activity of the HIV Nef protein, which disrupts antigen presentation by MHC-I to CTLs. A number of cellular factors have been implicated in this pathway and thus it is important to clarify the relative contribution of each to help focus the development of inhibitors.

Here we confirm a requirement for AP-1 and we provide new evidence that the small GTPase ARF-1 is also needed for Nef's effects on MHC-I trafficking. The ARF-1 dominant active mutant, ARF-1-Q_{71}L and the ARF-1 inhibitor, BFA both had dramatic effects on the formation of Nef-MHC-I–AP-1 complex and on surface MHC-I expression in Nef-expressing cells. Our new data, that ARF-1 is needed for Nef to recruit AP-1 to the MHC-I cytoplasmic tail, provide insights into what is required for complex formation and further elucidates how this process occurs (Figure 3.11).

The effect of dominant active ARF-1 Q_{71}L on MHC-I downmodulation may be explained by other studies examining COP-1 coats, which have demonstrated that when ARF-1 Q_{71}L is expressed, ARF-1-GTP cannot be hydrolyzed into ARF-1-GDP, resulting in a static coat (43, 50). This effect compromises the cycles of recruitment and dissociation from membranes.

Prior studies have shown that AP-1 is required for Nef-dependent trafficking and can be found in complexes with MHC-I and Nef in transformed T cell lines and in HIV-infected

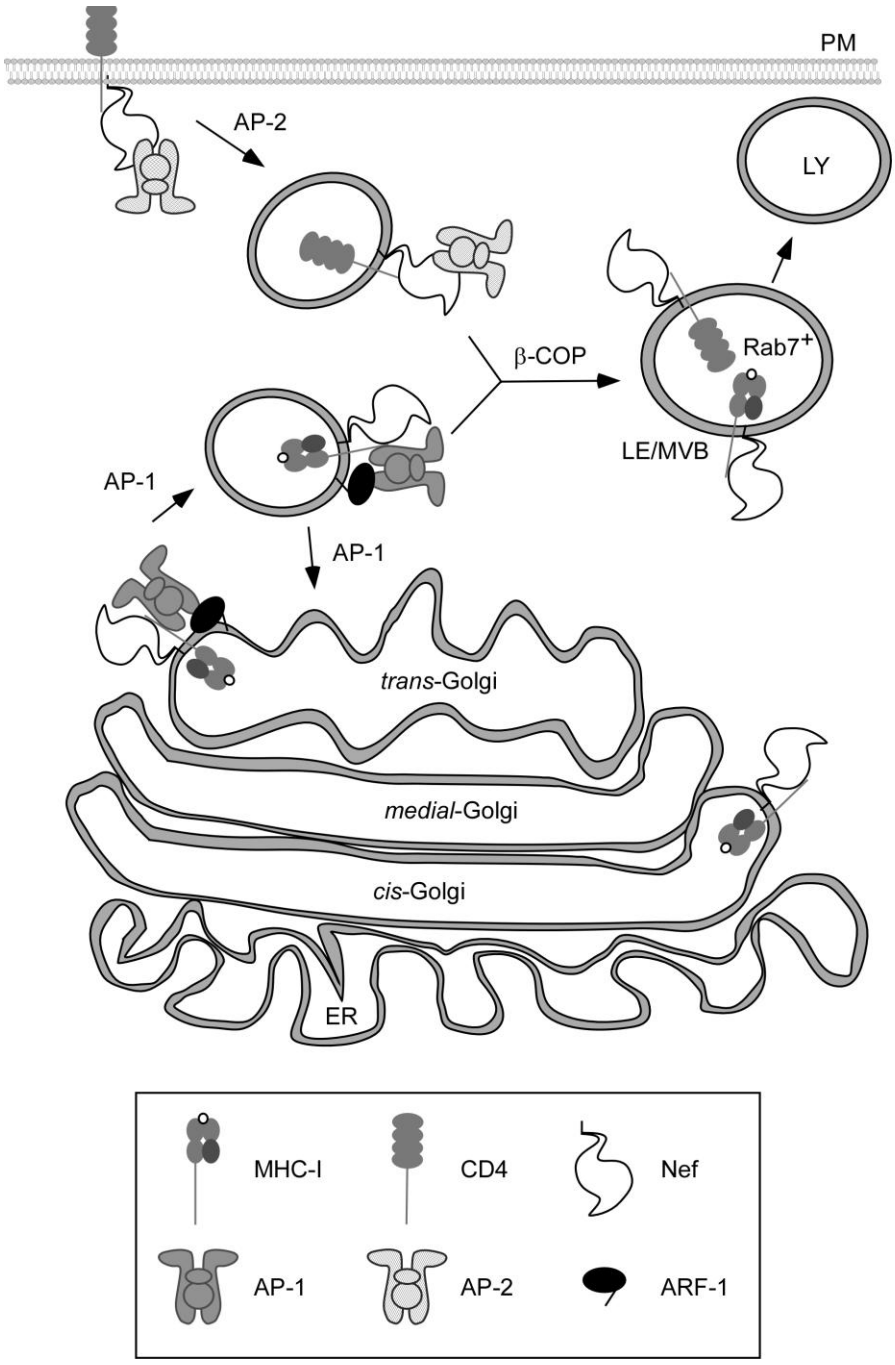


Figure 3.11. Model of Nef-dependent CD4 and MHC-I trafficking. LY, lysosome; LE, late endosome; MVB, multi-vesicular body; ER, endoplasmic reticulum.

primary T lymphocytes (41). Work with purified proteins has revealed that Nef directly contacts the MHC-I cytoplasmic tail (58) and that a Nef-MHC-I fusion protein directly interacts with the AP-1 μ subunit (32, 47). A tyrosine in the MHC-I cytoplasmic tail and the tyrosine binding pocket of AP-1 μ1 are both required for AP-1 to coprecipitate with HLA-A2 in Nef-expressing cells (59) and with purified proteins (32, 47). These data have led to a model in which Nef disrupts MHC-I trafficking from the TGN through an interaction with AP-1 that re-directs MHC-I into an endolysosomal pathway instead of the cell surface (Figure 3.11). In cells that demonstrate a greater degree of Nef-dependent MHC-I internalization (e.g. HeLa), AP-1 is also required for Nef-dependent internalization of MHC-I (23).

Nef also downmodulates the HIV receptor, CD4 to prevent viral superinfection (2) and to promote viral assembly and release (25, 42). Downmodulation of CD4 and MHC-I by Nef occurs by distinct mechanisms and requires different Nef domains (29, 44). Work from a number of laboratories has supported a model in which the separate Nef-dependent trafficking pathways of MHC-I and CD4 ultimately converge into a common β-COP-dependent pathway necessary for lysosomal targeting (15, 36, 44). In this model (Figure 3.11), Nef promotes accelerated internalization of CD4 in an AP-2 dependent manner (7, 17, 21, 49) and internalized CD4 is targeted into acidic compartments (36) and MVBs (12, 44) for accelerated degradation. Previous research has shown that ARF-1 is involved in COP-I coatomer recruitment to internalized Nef-CD4 complexes and is necessary for localization of these complexes to acidic compartments (15). However, GTP binding and hydrolysis is not needed for this process (15).

In contrast to our results with ARF-1, we did not detect a significant requirement for ARF-6 activity for Nef to reduce MHC-I surface expression. The difference in our results compared with prior publications from another laboratory may be related to a number of technical factors. The effect of ARF-6-Q_{67}L was reported in HeLa cells (4), which have been shown to traffic MHC-I differently than T cells (23). In addition, the prior study (4), did not examine the effects of these mutants on steady state surface expression as was done here.

In sum, we have further defined the mechanism by which Nef allows HIV-1 infected cells to evade the host immune response. Implicating ARF-1 in downmodulation of MHC-I by Nef provides further support for the role of the AP-1 dependent pathway shown in Figure 3.11. These data help to elucidate the mechanism by which Nef downmodulates MHC-I and reveal further targets for pharmaceuticals that may inhibit immune evasion.

3.5 Materials and methods

3.5.1 Cell lines and primary cell isolation

CEM T cells and SupT1 cells expressing HA-tagged HLA-A2 were created and maintained as previously published (59). Primary T cells were purified from buffy coats obtained from the New York Blood Center. Mononuclear cells were purified by ficoll-hypaque centrifugation, and primary T lymphocytes were isolated through adherence, CD8 and CD56 depletion of mononuclear cells. Following isolation, T cells were

stimulated with PHA at 10μg/mL (Sigma-Aldrich). After 24 hours, 50U/mL interleukin-2 [IL-2] was added to the culture medium. 48 hours post-IL-2 stimulation, the T lymphocytes were used for HIV transduction. Donor MHC-I allotype (HLA-A2, HLA-BW4, or HLA-BW6) was determined at the time of $CD4^+$ T cell isolation using flow cytometric techniques.

3.5.2 DNA constructs

3.5.2.1 Construction of pMSCV IRES GFP vectors expressing ARF-1 and ARF-6

pCB6 expressing Myc-tagged wild type or $T_{31}N$ ARF-1 were obtained from Didier Trono (Ecole Polytechnique Fédérale de Lausanne). The *arf-1* ORF was amplified by PCR using either wild type or $T_{31}N$ pCB6 Myc-ARF-1 as a template with the primers listed in Supplementary Table 1. MSCV Myc-ARF-1 $Q_{71}L$ IRES GFP was created through standard two-step PCR mutagenesis using wild type MSCV ARF-1 IRES GFP as a template with the primers listed in Supplementary Table 1. The PCR products were cloned into the BamHI site of MSCV IRES GFP (pMIG) (55).

pXS expressing HA-tagged wild type, $T_{27}N$, or $Q_{67}L$ ARF-6 was obtained from Julie Donaldson (National Institutes of Health). ARF-6 constructs were amplified using pXS HA-ARF-6 as a template with the primers listed in Supplementary Table 1. The PCR product was cloned into Bgl II and EcoRI sites of pMIG.

3.5.2.2 Construction of HIV vectors expressing ARF-1 and ARF-6

To construct HIVs that also contained both GFP and ARF ORFs, we first made a version of HIV (pNL-GI) in which a portion of the *env* ORF was replaced by a GFP IRES multiple cloning site cassette. PCR was used to amplify the IRES from pNL-PI (8) and to add additional restriction enzyme sites downstream of the IRES. The PCR product was ligated into the NheI and BglII sites in the *env* ORF of pNL4-3-deltaE-EGFP (63) just downstream of GFP. pNL-GI*nef*$^{-}$ was generated by creating a frame shift within the *nef* ORF of pNL-GI by digesting with XhoI, filling-in and re-ligating the ends.

pNL-GI was then used to create HIV constructs expressing ARF-1 and ARF-6. To create pNL-GI*arf* constructs, linker primers were designed to create a XbaI site in the 5' end and a MluI site in the 3' end of the amplicon during PCR amplification of the ARF-1 and ARF-1 $Q_{71}L$ from MSCV ARF-1 IRES GFP and MSCV Myc-ARF-1 $Q_{71}L$ IRES GFP respectively. Primers for this step are listed in Supplementary Table 1. Digested PCR products were ligated into the XbaI/MluI sites downstream of the IRES element in pNL-GI.

Due to an internal XbaI site present in ARF-6, pNL-GI ARF-6 +/- *nef* and pNL-GI ARF-6 $Q_{67}L$ +/- *nef* were engineered by designing linker primers to create a SpeI site in the 5' end, which is compatible with XbaI overhang ligation, and a MluI site in the 3' end of the amplicon during PCR amplification of the ARF-6 and ARF-6 $Q_{67}L$ from MSCV ARF-6 IRES GFP and MSCV ARF-6 $Q_{67}L$ IRES GFP respectively. PCR primers used are listed

in Supplementary Table 1. Digested PCR products were ligated into the XbaI/MluI digested parental vector. All constructs were confirmed by sequencing.

3.5.2.3 shRNA constructs

FG12 shRNA lentiviral vectors were constructed as previously described (37, 44). The negative control shNC construct was previously described (44). The target sequence for shARF-6 began at position 247:

GATCCCCGGTCTCATCTTCGTAGTGGTTCAAGAGACCACTACGAAGATGAGA
CCTTTTTGGAAA.

3.5.3 Virus preparation and transductions.

3.5.3.1 Retrovirus

Retroviral supernatants were prepared as described previously (34, 55). Bosc cells (34) were transfected with MSCV constructs described above, the retrovirus packaging vector pCL-Eco (31), and pHCMV-G (34). Briefly, $5x10^5$ CEM or SupT1 cells were spin-transduced with 1 ml of retroviral supernatants plus 8 μg/ml polybrene at 2500 RPM for two hours in a tabletop centrifuge at room temperature.

3.5.3.2 Adenovirus

Replication defective adenovirus was produced by the University of Michigan Gene Vector Core facility. Adenoviral transductions were performed as previously described (57). Transductions were performed using $1x10^6$ cells in 1 mL of RPMI 1640 containing 2% fetal bovine serum; 10mM HEPES; and 2mM penicillin, streptomycin, and glutamine. Multiplicity of infection was 200 for CEM and 100 for SupT1 (based on 293

cell infectivity, which is greater than CEM infectivity). Low serum transductions ranged from 4-6 hours.

3.5.3.3 HIV

HIV constructs were produced by transfecting 293 cells with each construct and harvesting the supernatant. $5x10^5$ PHA and IL-2 stimulated CD8-CD56-depleted T lymphocytes were transduced using 1 mL of supernatant with 8μg/mL polybrene using a spin-transduction protocol. Primary T lymphocytes were harvested for analysis of MHC-I downmodulation at 36 hours post transduction.

3.5.4 Flow cytometry and antibodies

Cells were stained for 20 minutes on ice in FACS buffer (PBS, 1% Human Serum, 1%FBS, 1% HEPES, and 1% NaN3), washed, and then stained for 20 additional minutes in secondary antibody. Mouse antibody to HLA-A2 antibody, BB7.2 (33) and mouse antibody to CD4, OKT4 (39) were purified from ascites (22) provided by the University of Michigan Hybridoma core facility. Mouse antibody to PLAP antibody was obtained from Serotec. The secondary antibodies used were goat anti-mouse IgG_{2B}-phycoerythrin (Invitrogen, 1:250) and goat anti-mouse IgG_1-Alexa Fluor 647 (Invitrogen, 1:250). Isotype controls were obtained from BD Biosciences. Stained cells were analyzed on a Becton Dickinson FACSCanto cytometer. Analysis was performed using FlowJo software (Tree Star Inc.).

3.5.5 Immunofluorescence microscopy and antibodies

ARF-6-transduced CEM cells were adhered to glass slides with Celltak (BD Biosciences), fixed, permeabilized, and stained for indirect immunofluorescence as previously described (Roeth JBC 2004). Images were collected using a Zeiss LSM 510 confocal microscope and processed using Deneba Canvas software. The following antibodies were utilized to localize proteins via microscopy: anti-HLA-A2 (BB7.2) and anti-GFP (3E6, Invitrogen). Secondary antibodies were obtained from Molecular Probes and were used at a dilution of 1:250: goat anti-mouse IgG_{2B}-Alexa Fluor 546 and goat anti-mouse IgG_{2A}-Alexa Fluor 488.

3.5.6 Immunoprecipitations and western blotting

Immunoprecipitations were performed as previously described (59). $15x10^6$ CEM cells were transduced with either control or Nef-expressing Adenovirus. At 48 hours post-adenoviral transduction, the cells were spin-transduced with either DMEM (mock) or MSCV ARF-1 IRES GFP viral supernatants. At 72 hours post-adenoviral transduction, all cells were incubated in 20mM NH_4Cl for 16 hours. Where indicated, samples were also incubated in 50μM BFA for 16 hours. Cells were then harvested and lysed in 1% digitonin (Wako) lysis buffer described previously (44, 59). Lysates were normalized for total protein and GFP transduction rates, when appropriate, prior to immunoprecipitation. Input controls were 1% of the immunoprecipitated protein. After an overnight pre-clear at 4^oC, lysates were immunoprecipitated for either HLA-A2 (BB7.2 crosslinked beads) (59) or Myc-tagged ARF-1 (9e10 (14) crosslinked beads). Immunoprecipitates were eluted

and analyzed by western blot as previously described (57). Western blot antibodies used were anti-Nef (AG11 (6)) and anti-Myc (9e10 (14)), which were produced by the University of Michigan Hybridoma Core Facility and purified as previously described (22). Antibodies also used for western blotting were anti-AP-1 γ (BD Biosciences), HA (HA.11, Covance), anti-PTEN (Cell Signaling Technology), anti-GAPDH (Santa Cruz Biotechnology), and anti-AP-1 μ1 (RY/1 (53)). The secondary antibody for anti-Nef, anti-HA, and anti-Myc was goat anti-mouse IgG1-HRP (Zymed Laboratories Inc). The secondary antibody for anti-AP-1 μ1 and anti-PTEN was goat anti-rabbit-HRP (Zymed Laboratories Inc). The secondary antibody for AP-1 γ was rabbit anti-mouse-HRP (Zymed Laboratories Inc).

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Notes

This work is the result of a collaborative effort. This project was the primary effort of Elizabeth Wonderlich, who performed the experiments shown in Figures 3.1, 3.2, 3.3, 3.9, and 3.10. Jolie Leonard contributed the work depicted in Figures 3.3, 3.5, 3.6, and 3.8. Deanna Kulpa performed the experiments in Figure 3.7, and cloned NL-GI*arf* from constructs previously created by Jason Normam.

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Chapter 4

Cell-based high throughput screening methodology to identify small molecule inhibitors of HIV-1 Nef

4.1 Introduction

HIV is a worldwide epidemic, with approximately 2.6 million new infections occurring annually (UNAIDS, 2009). Moreover, while treatments do exist, there is no cure for HIV. Instead, HIV is a chronic infection, persisting over the lifetime of an infected individual. Therefore, treatment must be also delivered over the lifetime of the patient. Many of the drug therapies currently available have significant toxic side effects, making long term adherence difficult. Furthermore, drug resistance can arise, making additional viral targets important.

The existing therapies for HIV target viral enzymes, including reverse transcriptase, protease, and integrase. Another class of inhibitors prevents viral entry by blocking envelope fusion or attachment to the CCR5 coreceptor. These drugs all target viral particles and inhibit viral replication. None of these inhibitors target HIV-infected cells

or enable the host immune system to better recognize and respond to HIV. There are currently no drugs on the market or in clinical studies which target the HIV-1 Nef protein, despite ample evidence that Nef is critically important for viral pathogenesis and disease progression in both animal models and human patients. Macaques infected with a Nef-deleted strain of SIV do not progress to AIDS (22). Furthermore, a cohort of blood transfusion recipients who received an HIV strain with a deletion in Nef and the LTR demonstrate delayed disease progression or elite control of viral loads (10, 19, 24). These observations highlight the potential efficacy of a Nef inhibitor in HIV treatment.

Traditional drug development strategies often rely on enzyme substrate modification and active site targeting. These methods are not suitable for identifying a Nef inhibitor, as Nef has no enzymatic activity and therefore requires a different approach. Nef is a structurally flexible, multifunctional adaptor protein with multiple cellular binding partners (13, 15-17, 21, 25). Nef downmodulates MHC-I, allowing infected cells to evade detection by cytotoxic T lymphocytes (CTLs) (9, 27, 38, 41, 44), as well as the viral receptor CD4, preventing superinfection and increasing virion release (14, 23, 37). Nef also modulates the activity of kinases, such as Pak2 and Hck, and promotes T cell activation, which may in turn promote viral replication (1, 4, 5, 18, 31, 39, 45). Additionally, Nef enhances viral infectivity *in vitro* by a poorly understood mechanism that is independent of CD4 downmodulation (6, 29, 34-36). It is likely that all of these Nef functions make contributions to HIV pathogenesis *in vivo.* However, the ability of Nef to downmodulate MHC-I and evade CTL detection is a particularly tempting target for therapeutic intervention. Several studies have used SIV systems to demonstrate that

the capacity to downmodulate MHC-I is selected for *in vivo (7, 32, 40)* . In addition, it has recently been demonstrated that the ability of *in vivo*-derived Nef to down-regulate MHC-I predicted the resistance of HIV-1 to suppression by CTL *in vitro (28)* . Taken together, these data demonstrate that the ability of Nef to down-regulate MHC-I *in vivo* is maintained by the need of HIV-1 to cope with the antiviral CTL response. Therefore, inhibition of this Nef function early in infection may enable the host immune system to better counteract, or even clear, HIV-1. While recent efforts have been applied to identifying Nef inhibitors, these studies have not specifically targeted the ability of Nef to downmodulate MHC-I.

The first report to identify chemical inhibitors of Nef function used a competition ELISA assay to identify guanidine alkaloid analogs capable of disrupting Nef's interactions with $p56^{lck}$, p53, and actin (33). These proteins all bind to the N-terminus of Nef, although the functional significance of these interactions is not clear. Furthermore, all of the putative Nef inhibitors were highly cytotoxic even at submicromolar concentrations and therefore could not be tested for their impact on viral replication or Nef function within cells. These compounds have limited therapeutic value.

A few studies have focused on inhibiting interactions with the polyproline, or SH3-binding, motif in Nef. Mutation of this domain reduces Nef's ability to downmodulate MHC-I and also inhibits association of Nef with cellular Src family kinases such as Hck (5, 20, 21, 30, 31, 42, 45). The importance of Hck activation by Nef in HIV pathogenesis

is unclear, as mutation of the SH3-binding domain of Nef results in a Nef protein which is defective for both Hck activation and MHC-I downmodulation. An *in silico* screen resulted in the identification of ten candidate molecules (2). Follow-up *in vitro* screening of those compounds, as well as structural analogs, narrowed the candidates to two molecules capable of inhibiting Nef-SH3 interactions in COS7 African green monkey kidney cells. Neither compound demonstrated significant cytotoxicity in the COS7 cell line. Inhibitor treatment did not affect CD4 downmodulation by Nef, which is independent of the SH3-binding domain of Nef, indicating that the inhibition was specific to this Nef domain. Disappointingly, treatment of resting peripheral blood mononuclear cells (PBMC) with up to 25μM inhibitor, the highest concentration tested, inhibited Nef-induced downmodulation of MHC-I A,B, and C by only 25%.

Subsequent studies also focused on the SH3-binding domain of Nef, specifically looking for inhibitors of Nef-interactions with Src family kinases (SFK) such as Hck. Screening of a 10,000 compound library enriched for kinase and protease inhibitors identified a compound capable of inhibiting Hck in the presence of Nef (12). The compound, termed DFP-4AP, was found to inhibit Hck activation even in the absence of Nef, and therefore is likely not specific enough for therapeutic use. However, analysis of DFP-4AP analogs lead to the discovery of DFP-AB, a molecule capable of specifically inhibited Hck activity in a Nef-dependant manner. Interestingly, both DFP-4AP and DFP-AB inhibited HIV replication in U87MG astrocytoma cells with an IC50 in the low μM range. Another study also identified a chemical inhibitor of Nef-induced activation of Hck which also partially reduced MHC-I HLA-A, B, and C downmodulation by Nef in

primary T lymphocytes at 8-days post HIV infection, a late time point (11). While mutation of multiple prolines within the SH3-binding domain of Nef inhibit both SFK interactions with Nef and downmodulation of MHC-I, point mutations disrupt kinase activation but do not affect MHC-I downmodulation (8, 43). It is therefore perhaps unsurprising that compounds which target this region of Nef have relatively small impact on MHC-I surface expression in the presence of Nef. While inhibition of kinase activity is a tractable readout for high throughput methodology, the correlation between this Nef function and HIV disease progression is unclear.

While targeting the polyproline motif of Nef has had limited success, another recent study took a different approach by simultaneously targeting multiple Nef functional domains in conjunction with the SH3-binding activity. The investigators designed a series of soluble peptides that wrap around Nef, interacting with several domains (3). The SH3 domain of Hck containing six amino acid modifications that enhance affinity for Nef was fused to the CD4 cytoplasmic tail with a GGGG linker in the presence of an N-terminal lipidation signal. This molecule was found to bind to purified Nef with a dissociation constant of 26 nM, the strongest interaction of a molecule with Nef ever reported. Additionally, this inhibitor (termed NI3-9) inhibited CD4 and MHC-I downmodulation by Nef, and blocked Nef-dependant enhancement of virion infectivity in HeLa and Chinese hamster ovary cells, which may not recapitulate Nef activity in primary T lymphocytes. The therapeutic potential of an inhibitory protein, however, is complicated by technical challenges in delivery, stability, and immunogenicity.

To our knowledge, none of the reported Nef inhibitors is being developed for therapeutic use. Targeting of the SH3-binding domain of Nef makes high throughput assay development simpler, as kinase activation through this Nef domain is technically straightforward to measure. However, the contribution of kinase activation in HIV pathogenesis is unclear, and for this reason is not a highly desirable target. Inhibitors that target one or more Nef functions of known pathogenic importance are highly desirable. However, protein-based inhibitors are difficult and expensive to manufacture and deliver. Furthermore, compounds that inhibit Nef *in vitro* may not accurately predict inhibition within live T cells, or may simply have chemical properties that make them toxic or otherwise unfit for use as therapeutic agents.

To this end, we have developed a high throughput assay system to screen for small molecule inhibitors of Nef-induced MHC-I downmodulation in T cells. This system is of particular value as we assay for inhibition of an important Nef function, not a Nef domain. Furthermore, we only identify molecules that inhibit Nef function within physiologically relevant cells. Any chemical compounds which are cytotoxic by nature will be screened out in our protocol. Here we describe the technical considerations in developing the assay, as well as the methods used to implement screening. Specifically, we designed an assay to measure increases in MHC-I surface expression in Nef-expressing T cells. We adapted flow cytometric analysis for minimal sample manipulation in order to be logistically amenable to high throughput handling. We also

optimized the assay for maximal statistical power. Preliminary findings suggest that molecules identified under the stringent conditions of this screen will be valid and effective Nef inhibitors.

4.2 Methods and Results:

4.2.1 Assay design

In order to screen for molecules which inhibit downmodulation of MHC-I by Nef in T lymphocytes, we first wanted to develop a cell-based flow cytometric screening protocol to measure MHC-I surface expression. A typical FACS staining protocol requires multiple antibody incubations and a number of rigorous washes. These steps are not amenable to high throughput methodology, as minimal manipulation of sample is technically desirable in these systems. Therefore, we needed to establish a FACS staining protocol in which we could reliably differentiate between high and low MHC-I surface expression, in as few steps as possible. We tested two strategies for reducing sample manipulation: condensing antibody incubations, and using dilution of the staining reaction to reduce background.

We examined three conditions for condensing antibody incubations. First, we tested a primary antibody against HLA-A2 (BB7.2) directly conjugated to Alexa Fluor-488 to eliminate separate primary and secondary antibody incubation steps. As two-step antibody stains can amplify signal, we also tried co-incubating primary and secondary

antibodies, as well as sequential primary and secondary antibody incubations in the absence of intervening wash steps. We used these staining protocols to compare CEM SS cells stably expressing exogenous HLA-A2, a common MHC-I allele, with the same cell line (CEM A2) transduced with an adenoviral vector expressing NL4-3 Nef. We found that the directly-conjugated antibody (BB7.2-488) was most amenable to a staining protocol without washes, resulting in the highest signal over background (Figure 4.1A-B).

We then optimized dilution conditions in order to achieve thorough background reduction while keeping sample volume low. Additionally, we further condensed the protocol by combining the dilution and fixation steps. This was accomplished by including paraformaldehyde (PFA) in the dilution buffer. To determine signal over background, we compared CEM A2 cells to parental CEM SS, incubated with BB7.2-488 and then subjected to a variety of dilution volumes. We found that dilution of two- to three-fold resulted in high specific signal and low background, while greater dilution factors reduced signal without further reducing background (Figure 4.1C-D). Thus we determined optimal conditions for a high throughput FACS stain, condensing a multi-step protocol into two simple steps which produce robust, specific signal.

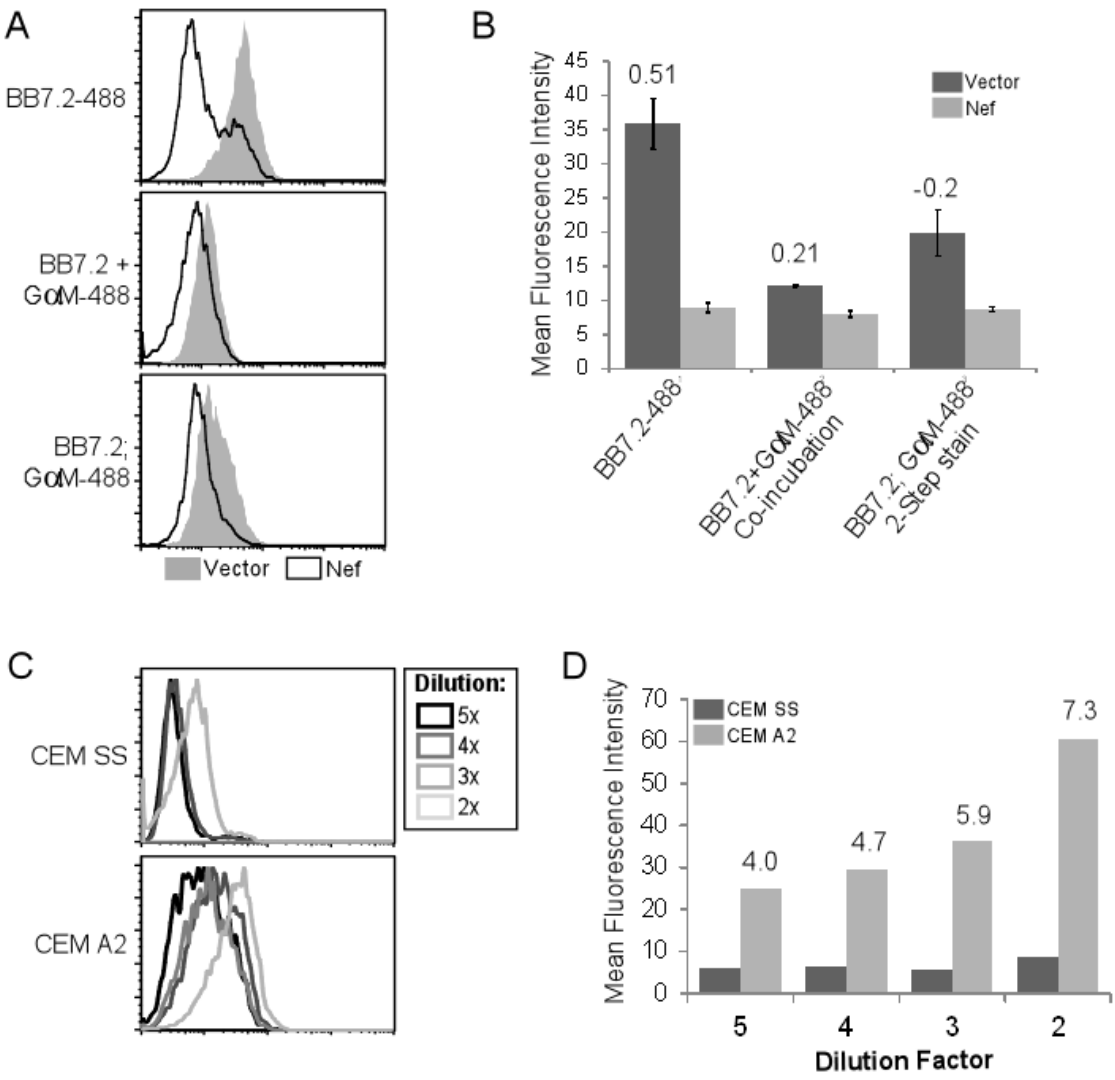

Figure 4.1 FACS stain protocol development. **(**A-B) CEM A2 cells infected with the indicated adenovirus were stained with the indicated antibodies. (A) Flow cytometric analysis of HLA-A2 expression. (B) Mean fluorescence intensity (MFI) plus standard deviation is shown, and the Z'-factor for each +/- Nef pair is displayed above the bars. (C-D) CEM SS and CEM A2 cells were stained with fluorophore-conjugated antibody and dilution with PFA was carried out at the indicated dilution factor. (C) Flow cytometric analysis of background and specific staining. (D) The MFI is shown and the

signal:noise ratio, calculated as CEM A2 MFI divided by CEM SS MFI, is displayed above the bars.

Next, we adapted this protocol to the Intellicyt high throughput sampling platform. To reduce overall assay costs, we used low volume, u-bottom, 384-well plates. Keeping sample volume low was desirable as this required nearly three-fold less of each reagent as compared to high-volume plates. This represents a significant cost savings when screening more than 100,000 samples. The u-bottom well shape was important for reliable sampling, as any cells settling to the bottom of the well were condensed into a small area for easy uptake. The Intellicyt sampling platform consists of a sample sip, run by a peristaltic pump, connected by tubing to an Accuri flow cytometer. The sip constantly samples so that the material from each well is separated within the tubing by a bubble of air which is introduced when the sample sip lifts to move to the next well. Therefore, samples are identified within the software as spikes in live events over time, separated by times with zero or low events, and thus can be assigned to specific wells. A limitation of the system is that the software is only able to record 1 million events within each plate. Therefore, we adjusted the density of cells that we seeded into each well such that we could sample sufficient cells per well without exceeding the number of data points we could record. As a result, we established a powerful and cost-effective FACS staining protocol that could be reliably sampled with high throughput robotic equipment.

4.2.2 Assay optimization

Once we had established a FACS staining protocol that was amenable to high throughput handling, it was important to assess the potential statistical power of this assay for screening. An important measurement when piloting high throughput assays is the Z'-factor. The Z'-factor is calculated as follows:

$$Z' = 1 - \frac{3(s_p + s_n)}{m_p - m_n}$$

Where "s" is the standard deviation of the positive (p) and negative (n) controls, and "m" is the mean value of the controls. Therefore this value relies on the magnitude of the difference between positive and negative controls, as well as the variability within these samples. This provides a measure of the reliability with which the assay may detect samples with a difference of more than three standard deviations from the control samples. Z'-factors fall between 0 and 1, with 1 being the ideal Z'. Assays with Z'-factors greater than 0.5 are considered excellent assays, while lower Z' values indicate that additional considerations are warranted. In this screen, CEM A2 cells expressing Nef, and therefore displaying low HLA-A2 surface expression, were the negative controls. CEM A2 cells transduced with control adenovirus were the positive controls, as a Nef inhibitor would restore high levels of HLA-A2 surface expression (Figure 4.2A-C). Hence our technical goals were to minimize standard deviation and maximize the fold-dowmodulation of HLA-A2 by Nef.

In order to improve downmodulation of HLA-A2 by Nef, we optimized our adenovirus transductions to achieve efficient delivery of Nef. The most obvious strategy for enhancing infection rate is to use a higher multiplicity of infection (MOI). However,

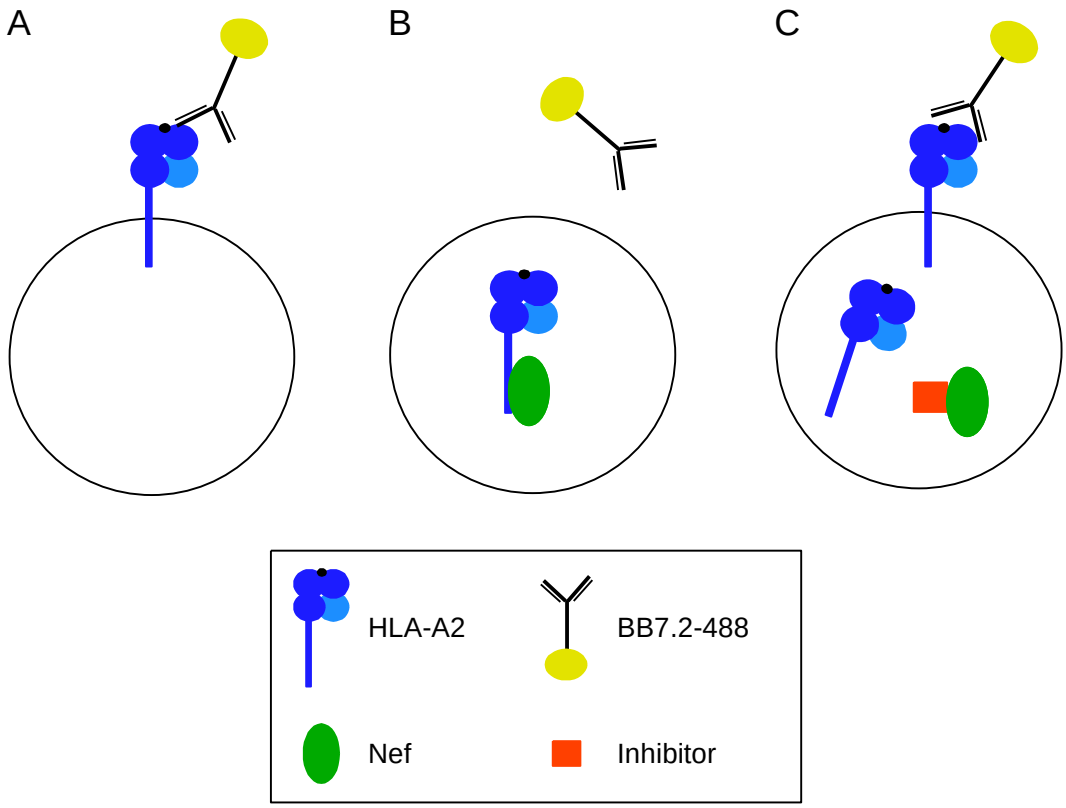

Figure 4.2. Schematic representation of experimental controls and potential inhibitor results. (A) Positive control. CEM A2 cells display high surface-expression of MHC-I in the absence of Nef. (B) Negative control. Nef reduces MHC-I surface expression, which in turn reduces antibody binding and fluorescent signal. (C) A chemical inhibitor prevents Nef from disrupting MHC-I surface expression, resulting in elevated fluorescent signal.

adenovirus preparations, which we obtained from the University of Michigan vector core, are a costly reagent when required in large amounts over the course of a screen.

Therefore, it was important to balance optimal infection rates with expense. An additional strategy was to improve infection efficiency without using more virus. Adenovirus is inhibited by serum, so we typically perform these infections in low serum media, RPMI 1640 supplemented with 2% fetal bovine serum (FBS). We tested whether eliminating serum from the infection medium altogether would enhance transduction over a range of MOIs. Indeed, we found that the use of serum-free infection media resulted in a greater fold-reduction in HLA-A2 surface expression as compared to the 2% serum-containing media (Figure 4.3A-B). We also determined that an MOI of 600 resulted in uniform infection of the cells within the population and nearly twice as much Nef-induced downmodulation of HLA-A2 as the lower MOIs (Figure 4.3A-B).

Once we had maximized the measurable difference between our positive and negative controls, we investigated possible methods to reduce variability between samples. We suspected that compound addition was a source of variation, as we observed high standard deviation in plates to which we added the DMSO solvent control versus plates that had no solvent or compound addition. DMSO is the solvent for the chemical compounds within the test libraries and can affect HLA-A2 surface expression. Compound addition was performed with a robotic arm that has 384 small metal pins that dip into each well of plates containing the chemical libraries and rely on surface tension

Figure 4.3 High throughput assay optimization. (A) Flow cytometric analysis of HLA-A2 expression in CEM A2 cells infected with a control adenovirus or an adenovirus expressing Nef at a range of MOIs in the indicated infection medium. (B) Quantitation of (A) showing fold downmodulation, calculated as the mean fluorescence intensity (MFI) of vector transduced cells divided by the MFI of the Nef-expressing cells. (C) Adenovirus-infected CEM A2 cells were either directly subjected to pintool addition of DMSO, or dispensed into plates containing PBS to which DMSO had been added by pintool. Graph summarizing the results of high throughput sampling of each plate is represented by the median MFI for control vector treated or Nef-expressing cells. Error bars indicate standard deviation. Z' values are displayed above bars. (D) CEM A2 cells were transduced with adenovirus or an adenovirus expressing Nef. At 2 dpi, the cells were incubated overnight at high density in low serum assay media plus DMSO solvent control. Cell viability, as determined by forward scatter and side scatter profile, is shown. Error bars represent standard deviation.

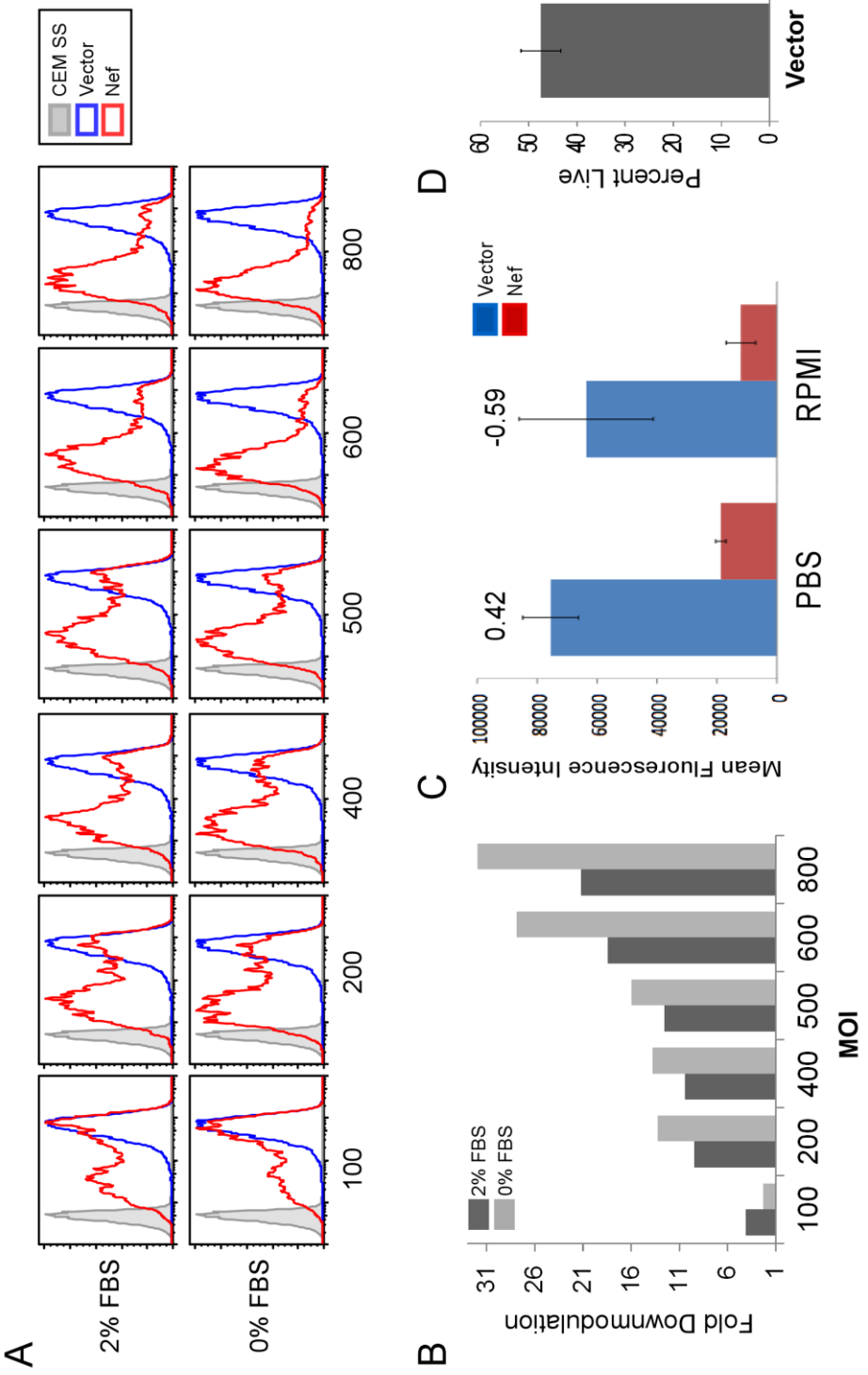

Figure 4.3 High throughput assay optimization

to pick up and deliver the contents of the library plates. Therefore, the depth (ie volume) within the wells of the experimental plate determines the amount of solvent or compound delivered. We found that pintooling solvent directly into cells and media in the experimental plates resulted in a high standard deviation in the concentration delivered to each well. Alternatively, delivery into 4 μL of PBS in each well of experimental plates resulted in lower standard deviations and a desirable concentration of 15-25 μM for the compounds within the library. A 2x cell suspension could then be delivered into plates already containing PBS and experimental compounds (Figure 4.3C). In this manner, we were able to reduce the variation between identical samples.

An additional factor to consider was potential cytotoxicity. It was important both that the cells survived the assay protocol and that we avoided registering toxic chemicals as potential hits for extensive follow up. With regard to the first, we examined viability of cells exposed to our assay protocol. A source of potential cytotoxicity was an overnight incubation in low-serum media in the presence of chemical compounds or DMSO solvent. The low-serum incubation was intended to avoid interference by undefined factors within serum. Therefore cells were incubated with the compounds in RPMI 1640 supplemented with 0.1% FBS. The cells were exposed to the putative inhibitors overnight, or approximately 16 hours, in hopes of achieving maximal delivery and activity of each experimental molecule. Cells subjected to this treatment in the presence of 1% DMSO tolerated this treatment well, as determined by cell viability (Figure 4.3D). Additionally, inclusion of a live-cell exclusion reagent, 7-Aminoactinomysin-D (7AAD), in the dilution buffer during the FACS stain allowed us to exclude dead or dying cells

from our analysis. Therefore, we were confident that we could identify compounds that inhibit Nef while excluding compounds with significant toxicity in T cells.Despite protocol optimization, pilot experiments of the assay yielded marginal Z' values of 0.2-0.4. The non-ideal Z' values were a reflection of relatively high variation in the positive controls, a variation that we found to be inherent to dipping the pintool into the wells. The negative controls, or Nef-expressing cells, displayed very little variation. Therefore, despite the low overall Z' values, we could quite reliably identify compounds which increased HLA-A2 expression greater than three standard deviations as compared to negative controls (Figure 4.1B and 4.3C). The high variation within the positive controls made elimination of false positive samples more difficult. Hence it was necessary to develop a method for eliminating false positives.

False positives could be the result of compounds that have intrinsic fluorescence within the detection channel (Figure 4.4B) or compounds that induce non-specific binding of the fluorophore-conjugated antibody to the cells (Figure 4.4C). To factor out such compounds, we developed a counter-screen. This involved testing the same compound set against CEM SS parental cells, which do not express HLA-A2, using the same screening protocol as that for the primary screen of CEM A2 cells. This counter-screened allowed us to detect any increase in fluorescence intensity that was not specific to increased HLA-A2 surface expression. Any compound which registered as a hit in the counter-screen could be eliminated as non-specific. Therefore, despite the non-ideal Z' value for the primary screen, the stringent counter-screen allowed us to confidently eliminate false positives.

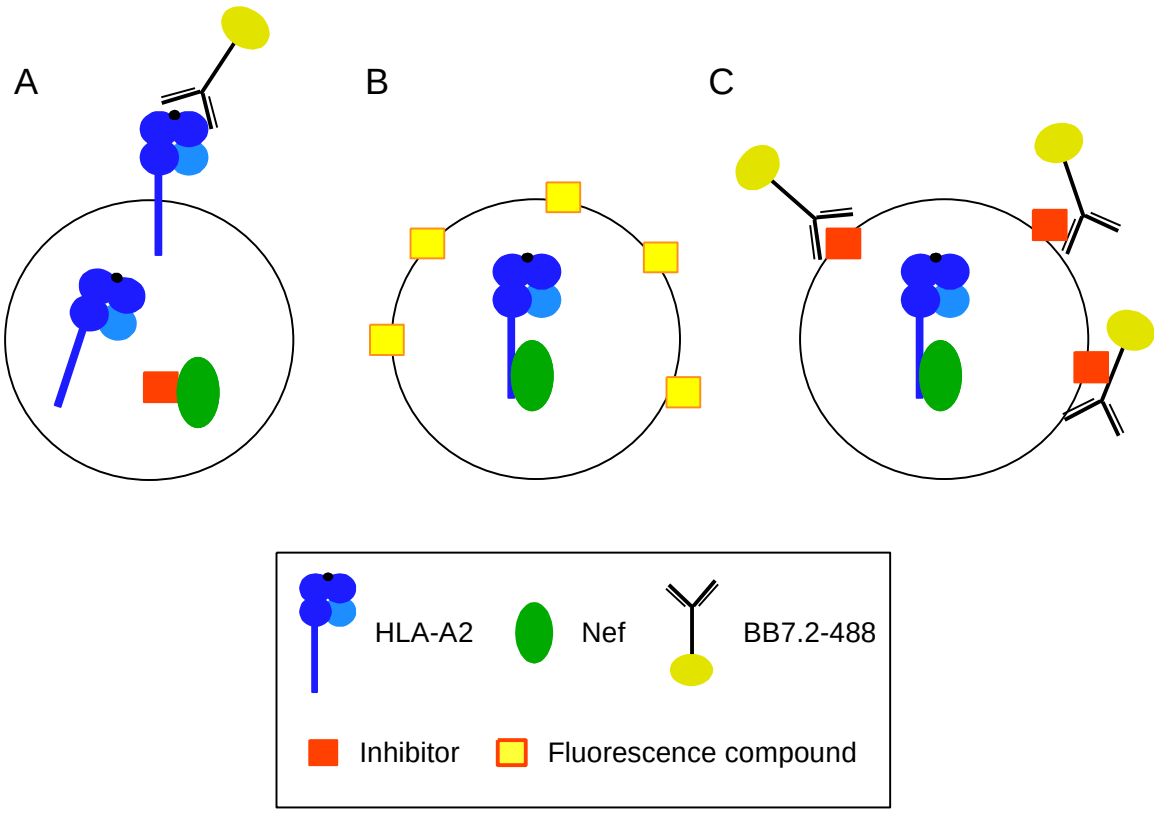

Figure 4.4. Schematic representation of possible positive sample outcomes. A) A chemical inhibitor prevents Nef from disrupting MHC-I surface expression, resulting in elevated fluorescent signal. B) A test molecule with intrinsic fluorescent properties coats the cell surface, artificially increasing the fluorescent signal. C) A sticky chemical compound induces non-specific binding of the BB7.2-AF488 antibody, resulting in spurious fluorescent signal.

4.2.3 Protocol

We have developed a high throughput screening system with sufficient statistical power to reliably identify small molecules that display potent Nef inhibition in T lymphocytes. The detailed protocol is as follows (Figure 4.5): First, CEM A2 cells were transduced with an empty adenoviral vector control or Nef-expressing adenovirus at an MOI of 600 for six hours in serum-free RPMI 1640. At two days post infection, the cells were counted and resuspended at a density of 2 million cells per mL in RPMI 1640 supplemented with 0.2% FBS. Experimental plates were then prepared by dispensing 4 μL PBS into each well, delivering experimental compounds, and subsequently dispensing 4 μL of cell suspension into each well. The cells were incubated with the compounds at 37°C for approximately 16 hours. On day three, 2 μL of a 5x antibody solution of BB7.2-488 (1:500) in 5x FACS buffer (10% FBS, 5% human A/B serum, 5% Hepes, and 0.02% sodium azide in PBS) was added directly to the cells and culture media within each well and incubated for 30 minutes at 4°C. After antibody incubation, each sample was diluted and fixed with 20 μL 1.5% PFA in 1x FACS buffer (2% FBS, 1% human A/B serum, 1% Hepes, and 0.02% sodium azide in PBS). After fixation, plates were stable at 4°C for up to one week and thus were sampled within this time frame.

Figure 4.5. Flow chart of the high throughput screening protocol. CEM A2 cells were transduced with an adenoviral vector control or Nef-expressing adenovirus. At two days post infection, the cells were seeded into 384-well plates. Experimental plates were prepared by delivering experimental compounds into PBS and subsequently dispensing a cell suspension into each well. The cells were incubated with the compounds for approximately 16 hours. On day three, the cells were stained, fixed and then sampled.

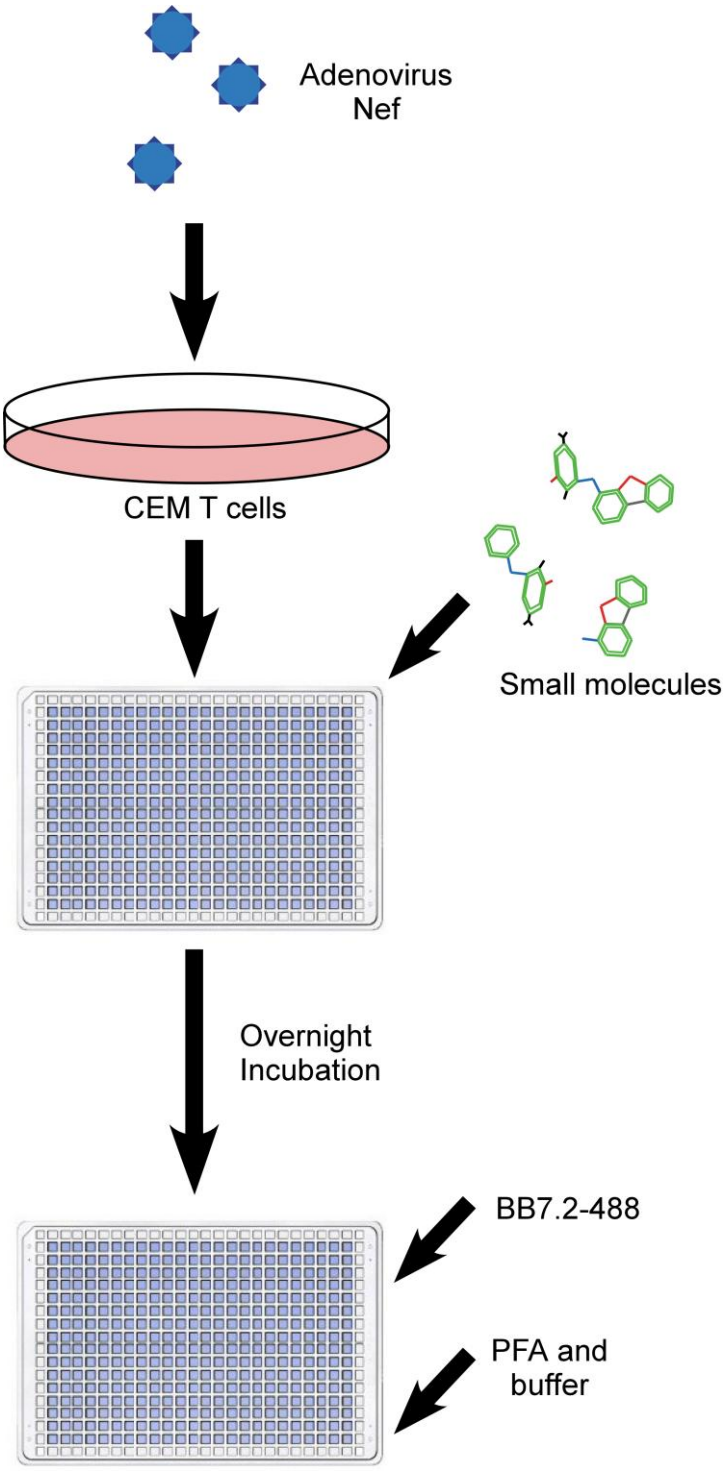

Figure 4.5. Flow chart of the high throughput screening protocol

4.2.4 Preliminary results

With an optimized protocol in place, we began screening experimental compounds. The initial phase consisted of screening 38,351 small molecules, including 2000 compounds within the Spectrum library of FDA-approved compounds, as well as Chembridge and Maybridge collections of drug-like compounds. The Z-factor for this portion of the screen averaged to 0.25, which is a marginal value. This screen identified 613 primary hits, as defined by activity greater than three standard deviations above the negative control samples within each plate, within the entire assay, or greater than 20% activity, where the average HLA-A2 staining intensity in adenovirus-treated cells is set to 100% activity, and that in Nef-expressing cells is set to 0%. This represents a hit rate of 1.3% within the primary screen. 110 of these hits occurred in wells where fewer than 50 live cell events were recorded. These compounds might be interpreted as cytotoxic, or, because of potential sampling error, may be considered as unsampled and thus worth including in a secondary screen. These 613 compounds were tested in triplicate in a confirmation screen with a protocol identical to that of the primary screen. Only compounds which tested as hits in at least 2 of 3 replicate wells were counted as confirmed hits. To factor out false positive hits, we counter-screened the same compound set against CEM SS parental cells. Any compound which registered as a hit in the counter-screen was eliminated as a false positive. Of the 613 primary hits, only 1 compound demonstrated Nef inhibition in replicate wells of the confirmation screen without non-specifically increasing fluorescence values in the parental cell counter-screen. Therefore, despite the non-ideal Z' value for the primary screen, we were able to

stringently eliminate false positive and identify a small number of functionally active small molecule Nef inhibitors.

The next test for putative Nef inhibitors is a dose response curve. This assay also consists of the same protocol as the primary screen, but tests each "hit" compound at a range of known concentrations. Unfortunately, our initial hit demonstrated negligible potency in the dose response assay. However, we felt there was value in continuing to screen a broader library of chemical compounds.

A phase 2 screen of 64,316 compounds within the Chemdiv 100,000 library resulted in 1084 primary hits, defined by the same standards as phase 1. This represents a hit rate of 1.7% within the primary screen. The Z' factor for this phase of the screen averaged to 0.45, an improvement that was likely the result of exclusion of DMSO from the positive control wells. This change was necessary due to the setup of the chemical library source plates, not a deliberate alteration in experimental design. In total, 113 of the hits occurred in low event wells. These 1084 compounds were subjected to confirmatory and counter-screening protocols. Of those, 506 compounds were confirmed as active in two of three replicate wells, a confirmation rate of 47%, which is quite typical for high throughput screens. However, all but 33 of the confirmed compounds also scored as active in the counter-screen, eliminating 95% of the hits as false positives. Thus, we had a final hit rate of 0.05% for phase 2 of the screen. The 33 compounds of interest will be subjected to dose response analysis in order to identify the most potent Nef inhibitors.

4.3 Discussion

Here we have described and verified a high throughput assay system to identify chemical compounds that potently inhibit a critical function of Nef in live T lymphocytes in the absence of significant cytotoxicity. To date we have screened 102,667 drug-like small molecules for their ability to inhibit downmodulation of MHC-I by HIV-1 Nef in T lymphocytes. Preliminarily, we observed 1,697 primary hits, a hit rate of 1.7%. While the Z'-factor measurement for the assay is non-ideal, this is a reflection of the relatively high variation in the positive control samples. Conversely, the Nef-expressing negative control samples have a very low standard deviation (Figure 4.1). Therefore, sample wells with a significant increase in HLA-A2 expression as compared to the negative controls are unambiguous. Hits are defined as compounds that have a signal greater than three standard deviations, or 20%, over the negative controls. In addition, we have implemented a stringent counter-screening protocol to eliminate false positive hits. Therefore, we have developed a powerful system for the discovery of chemical compounds that inhibit an important function of Nef in T cells.

We have not, as yet, identified a potent inhibitor of Nef. We have, however, identified a small pool of candidate molecules for further testing. We will also continue screening the chemical diversity libraries available to us through the University of Michigan Center for Chemical Genomics. This includes, among others, the remainder of the Chemdiv library, as well as a locally assembled natural compounds library, totaling approximately 50,000 additional chemical compounds. Should these libraries prove insufficient, NIH-

supported screening centers offer libraries of 300,000-500,000 small molecules for larger scale screening. We fully expect to identify compounds of interest for further characterization.

Once we have identified potent inhibitory molecules, we will perform cluster analysis in order to detect structural similarities. Using this information, we will obtain structurally related analogs and test these for improved activity. Our most promising inhibitory compounds will be subjected to rigorous follow up testing. We will first confirm that the inhibitor functions similarly in primary human T lymphocytes, as well as in other HIV targets, such as macrophages. Importantly, we will assess the functional impact of a Nef inhibitor in protecting HIV-infected PBMCs from anti-HIV CTLs, similar to previously published assays (9). Further work will involve elucidating the molecular mechanism of Nef inhibition, including mapping an interaction between the inhibitor and Nef through crystallography or NMR analysis. The ability of Nef to physically associate with cellular binding partners, such as MHC-I, SFKs, and clathrin-associated adaptor proteins, in the presence of the inhibitor will also be an important line of investigation.

While we are primarily interested in compounds that inhibit downmodulation of MHC-I by Nef, it is also interesting to determine whether other Nef functions are also inhibited. A compound that interferes with the effects of Nef on intracellular trafficking may also prevent downmodulation of other Nef targets such as CD4, CD28, and CD8. These molecules are affected by Nef through distinct mechanisms, but some common cellular

factors are involved (26). Furthermore, an inhibitor that had profound effects on Nef stability, subcellular localization, or conformation might also prevent Nef-induced kinase activation or enhanced HIV infectivity. A compound which inhibits multiple Nef functions might have the greatest impact on HIV pathogenesis.

While a Nef inhibitor would be a powerful laboratory tool for studying Nef function, our primary long term goals would be to develop such compounds for therapeutic use. Early steps would involve testing the efficacy of a Nef inhibitor in animal models such as humanized mice and macaques. It would be important to assess the effects of a Nef inhibitor on disease progression in animals with an established infection. It is also interesting to hypothesize that treatment with a Nef inhibitor as a prophylactic measure concurrent with exposure, or very early in HIV infection, might enhance the ability of CTLs to recognize and target HIV-infected cells before the virus can fully establish long-lived reservoirs. Therefore, it will be important to measure not only HIV replication and disease progression in animal models, but immune cell function as well. Depending upon the success of animal trials, we would then proceed to human clinical trials, with the end goal of bringing a Nef-targeting drug to market.

With this assay, we strive to identify compounds that potently inhibit a critical function of Nef in live T lymphocytes in belief that such a drug would have a significant impact on HIV-1 pathogenesis. Inhibition of MHC-I downmodulation by Nef during acute infection could fundamentally impact the ability of the human immune system to identify and eliminate HIV-1, and perhaps increase the efficacy of vaccine-induced memory

responses. This screen specifically targets this important Nef function without bias for a particular Nef domain and is performed in live T cells. Thus, the nature of the assay excludes cytotoxic molecules and identifies compounds that are active within a physiologically relevant environment. For these reasons, molecules identified in this screen will have significant therapeutic potential against HIV.

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Notes

The work presented here is the result of a long term collaborative effort. Jolie Leonard designed and performed the experiments presented above, developing and optimizing the primary and counter-screening assays, performed a pilot screen of 2,000 compounds, and wrote the text of this manuscript. Jolie Leonard supervised Kay Leopold in screening the 102,000 compounds, the results of which are detailed as preliminary data.

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Chapter 5

Discussion of results and future directions

5.1 Overview

The data presented in the previous chapters advance our understanding of the primary cellular factors targeted by the HIV-1 Nef protein. Though a variety of reports existed previously, there was little agreement on which proteins were targeted by Nef, or the mechanisms involved. We developed tools that allowed us to directly compare the effects of Nef on putative target molecules. As a result, we found that HIV-1 Nef has three major cellular targets: MHC-I, CD4, and CD8, and, to a lesser extent, CD28. Moreover, we were able to demonstrate the ability of Nef to downmodulate endogenous CD8 in HIV infected primary $CD4^{+}CD8^{+}$ T cells. We also performed biochemical analyses to determine that the effect of Nef on MHC-I and CD8 depended at least in part on expression of cellular trafficking adaptors AP-1 and β-COP. In addition, we found that the AP-1 binding motif utilized by Nef to recruit AP-1 to cytoplasmic tails varied depending on which target protein Nef was bound to. Thus, one explanation for the capacity of Nef to affect multiple targets is that it has evolved redundant mechanisms to recruit the same adaptor proteins. Additionally, we provided evidence that ARF-1, but not ARF-6, is required for Nef-dependent MHC-I downmodulation, consistent with a Nef-induced block in MHC-I transport to the plasma membrane. Finally, we described our ongoing efforts to identify and develop a potent Nef inhibitor for clinical application.

5.2 Adaptor protein involvement in Nef-dependent trafficking

The data presented in chapter 2 is the first to assess the contribution of AP-1 and β-COP to the downmodulation of CD28 and CD8β. Another group has reported a partial requirement for AP-2 in the downmodulation of CD28 and CD8β that we were unable to reproduce (53). Knockdown of adaptor protein subunits is technically challenging, as highly efficient depletion of these proteins can be lethal to cells. Additionally, co-depletion of Eps15 and AP-2 may be required to observe an inhibition of Nef activity against CD4 (25). For these reasons we may have underestimated the contribution of AP-2 in CD28 and CD8β downmodulation by Nef in our system. AP-1- and AP-2-dependant mechanisms may represent parallel or redundant pathways, which Nef can utilize depending upon cellular localization and availability of cellular adaptors. Knocking down multiple adaptor proteins in combination to look for synergistic inhibitory effects on downmodulation of Nef targets would clarify whether multiple parallel pathways exist, or whether the adaptors function within the same downmodulation pathway. Our preliminary efforts to perform such experiments were unsuccessful due to technical issues. As mentioned, knockdown of even one cellular adaptor is cytotoxic, and knocking down two adaptors further reduces cell viability. Additionally, this requires introducing shRNA-encoding constructs singly on successive days, broadening the timeline during which we must maintain live cells. Furthermore, we have observed that successive viral transductions decrease in efficiency, and knockdown in our system requires highly efficient transduction and shRNA expression. Therefore, a recently developed tool for expressing up to three shRNAs from the same plasmid might allow us to target up to three adaptor proteins simultaneously, at equal efficiency, and

perhaps within a timeframe that would allow analysis of cells prior to massive death of the culture (54).

In chapter 2 we present direct evidence that Nef can recruit AP-1 by utilizing either the tyrosine motif recognition site or the dileucine recognition site of AP-1, and that this is determined by which cytoplasmic tail domain Nef is bound to. Previous studies found that the dileucine motif in Nef is required for glutathione-S-transferase (GST)-Nef pull-down of AP-1 complexes from mammalian cell lysates (5, 24) and for interactions detected in yeast two and three hybrid analyses (5, 9, 10, 12, 16, 23, 24, 32, 43) but the dileucine motif is dispensable for MHC-I downmodulation even though AP-1 is required (36). Conversely, CD4 downmodulation by Nef requires the dileucine motif, but does not involve AP-1 (16). Thus, the interaction between AP-1 and the dileucine motif has remained unexplained. Here we provide evidence that this interaction is utilized to target CD8, as well as CD28. Previously, we found that mutation of residues within the tyrosine binding pocket of the AP-1 μ subunit inhibited AP-1 binding to tyrosine-based trafficking motifs in a dominant negative manner (60). It is possible that mutating residues within the AP-1 and 2 σ subunits that mediate binding to dileucine motifs (A263 and L103 in σ2) may also result in a dominant negative phenotype (26). This may provide an alternative approach to assessing the involvement of AP-2 in CD8 and CD28 downmodulation by Nef. This approach may also be a less cytotoxic method of comparing the relative contribution of multiple adaptor proteins to dileucine-dependent downmodulation of proteins targeted by Nef.

5.3 β-COP is a common element in multiple Nef-induced pathways

We have also identified β-COP as a common factor required for maximal downregulation and degradation of all major targets of Nef: MHC-I, CD4, and CD8β and CD28. The evidence presented here is consistent with a model in which Nef utilizes AP-1 or AP-2 to direct cellular targets into endosomal compartments, where Nef subsequently recruits β-COP to route targets to the lysosomes for degradation as previously proposed for MHC-I and CD4. However, the fact that the degradation of CD8 was far more dependent on β-COP expression than on AP-1 expression indicates that β-COP may also directly target intracellular CD8 for degradation in Nef expressing cells without the need for additional adaptor proteins.

The requirement for β-COP in multiple Nef pathways may represent a good target for therapeutic inhibition of Nef. As presented in chapter 2, RNAi knockout of β-COP partially inhibits Nef-induced downmodulation of MHC-I, CD8, and CD28. Whether this level of inhibition is sufficient to restore antigen presentation by MHC-I could be tested by assaying for restored CTL activity against HIV-infected T cells when β-COP expression is knocked down. A fluorescence-resonance energy transfer (FRET) system could be used to screen for chemical compounds that disrupt Nef/β-COP interactions. However, the fact that Nef may use either of two domains to interact with β-COP may complicate the experimental setup, as the two types of interaction may not both orient

protein-conjugated fluorophores in sufficient proximity to induce FRET signal. Another system amenable to high throughput screening methodology would be a mammalian two-hybrid system similar to that described by Betzi et al (3). This system involves co-expressing a Nef-VP16 fusion protein and a β-COP-GAL4 fusion protein, where an interaction of Nef and β-COP would result in transcriptional activation of a GAL4-luciferase reporter construct (3, 47). However, this system may also be subject to the technical issues associated with the multiple conformations of the Nef-β-COP interaction. Therefore, using the inhibition of Nef function as an experimental readout, as described in chapter 4, may be a more reliable approach to identifying Nef inhibitors.

5.4 CD80 and CD86

We were unable to confirm an effect of Nef on CD80 and CD86 surface expression in chapter 2. This may be a result of differences in experimental systems. Previous mechanistic studies of CD80/86 downmodulation by Nef were largely conducted on endogenously expressed molecules in the monocytic cell line U937. Even when replicating the published culture conditions, we were able to observe only low levels of CD80 on the cell surface, and no CD86 at all, leading us to believe that our clone of the U937 line is inherently different from that used to report downmodulation of CD80 and CD86. Additionally, the method of Nef delivery may influence the outcome. Chaudhury et al. introduced Nef into U937 cells by transfection, while we used retroviral and adenoviral expression systems designed to express Nef at levels similar to that observed with HIV infection. Other investigators that used adenovirus to introduce Nef expression

also failed to observe CD80/86 downmodulation in primary dendritic cells (35). This discrepancy is the reason that we utilized primary human macrophages and DCs as a highly relevant system for investigating Nef activity against these potential targets. It is also possible that the culture conditions and activation state of primary cells might influence Nef activity. Studies in immature and mature DCs reported no effect of Nef on CD80/86 expression (35, 46, 50), while those performed in mouse DCs or human monocytes did observe Nef-induced CD80/86 downmodulation (6, 37). The differences in experimental systems are also reflected in the magnitude of Nef-induced MHC-I downmodulation observed. Chaudhury et al. report 5-10-fold reduction of MHC-I using a pan-MHC-I antibody which recognizes HLA-A and –B allotypes, but also allotypes that are not affected by Nef. In contrast, we consistently observed only a 2-3-fold Nef-induced reduction in HLA-A2 expression in both monocytic cell lines and primary antigen presenting cells. Because we were unable to detect an effect of Nef on CD80 or CD86 under established stimulation and culture conditions for primary macrophages and DCs in which Nef was active against other targets, we conclude that if Nef targets CD80 and CD86 it must do so under specialized circumstance that we were unable to replicate.

5.5 Nef has a small effect on CD1d

CD1d targeting by HIV-1 appears to be a special case. In chapter 2, we observed that not all Nef isolates retain the ability to downmodulated CD1d, yet one or more *nef* alleles are capable of doing so. Therefore, this function may be rare, evolutionarily driven by some infrequently occurring host factor, or may be a feature of certain HIV-1 clades.

Furthermore, the magnitude of the affect of Nef on CD1d expression is quite small, around 1.5-fold on average. This observation is consistent with published reports, yet previous work found that this minor reduction in CD1d surface expression had a functional impact on NKT cell activation *in vitro* (6, 7, 35). However, it is unclear what impact CD1d downmodulation might have on HIV-1 pathogenesis *in vivo*. While CD1d is able to present lipid antigens from bacterial and parasitic pathogens such as *Sphingomonas, Borrelia burgdorferi, Leishmania, and Mycobacteria (1, 14, 27, 28, 38, 52),* there is no evidence that an HIV-associated lipid antigen exists. It is also unknown whether CD1d-restricted NKT cells have any antiviral activity against HIV or whether CD1d expression influences HIV pathogenesis. $CD1d^{-/-}$ transgenic mice have been widely used to elucidate the impact of CD1d on a variety of pathogens (13, 51). While mice normally cannot be infected by HIV-1, the recent development of BLT (bone marrow, liver, thymus) mice with humanized immune systems allows mice to be used as a model organism for HIV studies (11, 39). Another useful technology is the use of homing endonucleases to specifically knock out genes of interest in hematopoietic progenitor cells, as recently developed for knock out of the HIV co-receptor, CCR5 (19). Combining these two technologies to create BLT mice transplanted with fetal bone marrow in which CD1d has been knocked out, followed by HIV infection, may shed light on the role CD1d plays in HIV-1 pathogenesis.

Reduced CD1d expression in HIV-infected cells has also been attributed to Vpu. Interestingly, it has been reported that deletion of either Nef or Vpu from HIV partially reduced CD1d downmodulation, with the greatest inhibition occurring when both HIV-1

accessory proteins were deleted. This data supports the conclusion that both Nef and Vpu remove CD1d from the plasma membrane through distinct mechanisms. There is precedence for such functional redundancy in HIV-1 proteins, as both Nef and Vpu also target CD4 for degradation. An alternative hypothesis is that either Nef or Vpu may retain the ability to affect CD1d expression depending upon the virus strain. This is supported by the observation in chapter 2 that some, but not all, HIV-1 Nef isolates have the ability to downmodulate CD1d. This scenario resembles that of the restriction factor BST-2/tetherin, which inhibits virus release from the plasma membrane (40, 58). Tetherin is antagonized by HIV-1 Vpu, while this function is performed by Nef in primate lentiviruses which lack Vpu, such as SIV strains that infect sooty mangabey, macaque, and African green monkey species (49). A comparison of the ability of Nef and Vpu proteins from a variety of human and primate lentiviruses, as well as additional HIV-1 isolates, to remove CD1d from the cell surface would be informative.

5.6 CD28 recycling may be regulated by Nef

We did confirm that HIV-1 Nef has a moderate effect on CD28 surface expression. CD28 appears to be a unique target of Nef, in that this function is conserved among a panel of Nef variants, but the effect is small relative to the other targets of Nef. CD28 is internalized at a rate similar to that of CD4 in the presence of Nef, yet displays a much smaller change in steady state expression (53). This discrepancy may be explained by the observation in chapter 2 that CD28 recycling is significantly increased in Nef-expressing cells. SIV Nef downmodulates CD28 more potently than HIV-1 Nef; therefore CD28

downmodulation may be more important for SIV pathogenesis. It would be interesting to assess the impact of SIV Nef on CD28 recycling; presumably CD28 would be recycled to a lesser extent in the presence of SIV Nef than HIV-1 Nef. It is also possible that a physiologically relevant set of conditions exists which would result in less recycling of CD28 and more downmodulation of CD28 by HIV-1 Nef.

An important remaining question is the functional importance of Nef-induced CD28 downmodulation. CD28 is an important co-stimulatory molecule for T lymphocyte activation; downmodulation of CD28 could conceivably regulate the activation state of HIV-infected T cells. Since all Nef domains known to be important for CD28 downmodulation are also required for either MHC-I or CD4 downmodulation, it is not possible to study the pathogenesis of HIV constructs in which Nef has been mutated in such a way that only CD28 downmodulation is affected. Until a Nef mutation is discovered which genetically separates CD28 downmodulation from other Nef functions, mutational analysis of CD28 may be more informative. It is not currently known what residues within CD28 participate in an association with Nef and/or cellular adaptor proteins. Identification of mutations that render CD28 insensitive to Nef would further illuminate the mechanisms by which Nef interacts with and affects cellular targets. Furthermore, it might be possible to "knock-in" a Nef-insensitive CD28 into an animal model in order to assess the importance of this Nef function in HIV replication and disease progression.

5.7 The role of Nef-induced CD8 downmodulation

We consistently observed that multiple Nef variants induced a robust decrease in CD8 surface expression in the presence of multiple Nef variants, yet it is not clear in what context Nef encounters CD8. Downmodulation of CD8, the T cell receptor co-receptor, would prevent activation of CTLs, perhaps providing a second mechanism by which HIV-infected cells avoid CTL lysis. However, CD8 is not expressed in cell types that are classically infected by HIV. Reports of CD8-tropic HIV strains do exist, which provide a potential scenario for Nef coming into contact with CD8 (17, 18, 33, 44, 48, 57). While this phenomenon may in fact occur, the relatively small body of evidence in support of this may suggest that this occurrence is rare, arguing against a strong contribution to HIV evolution.

Another plausible scenario is the transfer of Nef through cell-cell contacts such as virological synapses or nanotubules. This has already been reported to occur between APCs and B cells, resulting in disregulation of the humoral response without direct infection of B cells by HIV (45). It is conceivable that this could similarly occur when CTLs contact an infected target. Nef would be transmitted through cellular contacts and then downmodulate CD8 locally to prevent activation of an HIV-specific CTL. Preliminary attempts to demonstrate transfer of a Nef-GFP fusion protein from THP-1 monocytic cells to SupT1 T cells were inconclusive, as the GFP protein alone was observed to transfer to the bystander cells (data not shown). We also examined whether Nef protected primary human macrophages from lysis by non-autologous CTLs. Again,

initial studies were not promising (data not shown). However, the experiments were performed with an adenoviral system for expressing Nef and it is possible that other HIV proteins may mediate Nef transfer.

There is yet another possible scenario in which HIV Nef could encounter CD8. Usually, T lymphocytes are either CD4+ or CD8+, however a small population of CD4+CD8+ T lymphocytes does exist in peripheral blood (4, 15, 41, 42, 64). There is evidence that these cells may be a subset of CD8+ T cells that upregulate CD4 in response to activation stimuli (15, 28, 29, 54, 58. Importantly, these cells express the CD8α/β heterodimer, and thus a CD8 molecule which would be susceptible to Nef (41, 55). This subset of T cells is small, generally less than 5% of total circulating lymphocyte population. The frequency of these CD4+CD8+ double positive cells has been reported to increase in response to infection with a number of viruses, including HIV (22, 31, 34). Additionally, these cells are susceptible to infection by HIV (2, 7, 21, 22, 30, 61, 63). While the role of CD4+CD8+ double positive cells is not entirely clear, they are known to exhibit antigen-dependant IFN-γ and IL-2 secretion and cytolytic activity and are enriched for HIV-specific responses in chronically infected patients (20, 41, 56, 62). Therefore, CD4+CD8+ T lymphocytes are an infectable target cell, the inhibition of which would be beneficial to HIV (15, 29, 30, 55, 61). Indeed, when we infected peripheral blood mononuclear cells with HIV +/- Nef, we observed a marked reduction in the CD4+CD8- single positive cells and CD4+CD8+ double positive cells which expressed HIV-Gag, and a substantial population of CD4-CD8- cells which stained positive for Gag, indicating that both CD4 and CD8 were downmodulated by Nef.

Once again, CD8 downmodulation by Nef is not genetically separable from other functions of Nef. This means that we cannot create a mutated HIV construct lacking only the ability to downmodulate CD8 and assess this virus for pathogenic defects. However, studying the impact of Nef on CD4+CD8+ T lymphocyte function *in vitro* may shed light on the potential benefit of CD8 downmodulation to the virus. Such experiments are complicated by the fact that once these cells express Nef, they lose their identity as CD4+ or CD8+, as these molecules are removed from the cell surface. Therefore, sorting strategies will be important. Positive selection of CD8+ lymphocytes would result in a mixed population of CD8 single-positive and CD4, CD8 double-positive cells. We would then infect with population with HIV constructs, +/- Nef and pseudotyped with a natural HIV envelope, such that only the CD4-expressing cells could be infected. Thus, the CD4-CD8- HIV Gag-expressing cells would have originated from the double-positive T cells, and we could test these cells for functional responses. For example, if we obtained these cells from a blood donor with previous exposure to human cytomegalovirus (hCMV), we could examine whether Nef inhibits the ability of these cells to bind CMV tetramers, or four fluorophore-labeled peptide-loaded MHC-I complexes. We could also co-incubate the primary cells with target cells loaded with CMV peptide. We could then use multi-parameter flow cytometry assess the ability of the primary T cells to upregulate cytokine expression or display cytotoxic potential, as apparent by CD107a/Lamp1 expression at the plasma membrane. A cleaner way to do this would be to positively select for CD8+ cells, followed by CD4 positive selection in order to isolate only the CD4+CD8+ T lymphocytes. This may be fraught with technical

problems, as this population is quite small. If we were to succeed in sorting this population, we would perform the experiments described for the CD8+ mixed population. These experiments would help elucidate the impact of Nef on CD4+CD8+ T cell activity, but would not provide evidence that Nef gains access to these cells *in vivo*.

In order to explore the ability of Nef to downmodulate CD8 in vivo, we could obtain lymphocytes from SIV-infected primates. Again, CD4+CD8+ T lymphocytes lose their identity as such if they express Nef. Therefore, we could sort lymphocytes into CD4+, CD8+, and double-negative populations and use qPCR methods to detect Nef and CD8β mRNA in the absence of CD8 protein expression. These same methods could also be applied to lymphocytes acquired from HIV-infected human donors. This would provide direct evidence that Nef removes CD8 from the surface of lymphocytes *in vivo*.

5.8 Nef inhibits antigen presentation in an ARF-1-dependant manner

We have also provided additional detail of the mechanism by which Nef affects MHC-I expression and addressed a controversy within the field. In chapter 3, we observed a requirement for ARF-1 in Nef-induced MHC-I downmodulation, while we found ARF-6 to be dispensable for this function. We observed that a constitutively GTP-bound mutant of ARF-1 (ARF $Q_{71}L$) reduced the effects of Nef on MHC-I in human T cell lines and in primary human T lymphocytes. The analogous ARF-6 mutant (ARF-6 $Q_{67}L$) did not inhibit downmodulation of MHC-I HLA-A and –B allotypes by Nef in human T cell lines, regardless of PTEN expression levels, as well as in primary human T lymphocytes.

RNAi knockdown of ARF-6 expression also had no effect on MHC-I downmodulation by Nef. An ARF-1 inhibitor prevented Nef from recruiting AP-1 to the cytoplasmic tail of HLA-A2, while the dominant active ARF-1 mutant stabilized the Nef-AP-1-MHC-I complex. This data supports a model in which Nef recruits AP-1 to the MHC-I cytoplasmic domain in an ARF-1-dependent manner to prevent forward transport of MHC-I. Experiments using shRNA to knock down ARF-1 expression in T cells are ongoing in order to confirm a requirement for this molecule. However, Golgi-associated ARFs may be capable of substituting for one another, and ARF-1 knockdown has been shown to have no effect on membrane trafficking unless co-depleted with other ARF isoforms (59). Therefore, it would be unsurprising if we were unable to demonstrate a requirement for ARF-1 in Nef-induced MHC-I downmodulation with this experimental strategy.

5.9 Methods for the identification of a pharmacologic inhibitor of Nef

Understanding the specific mechanisms through which Nef impairs the host immune response may help direct future efforts to design anti-HIV drugs. An alternative approach to developing pharmacologic agents against Nef is to screen libraries of drug-like small molecules for Nef inhibitors. In chapter 4, we have presented preliminary results from a screen of over 100,000 chemical compounds from which we have identified a pool of 33 potential Nef inhibitors. The next step is to test the efficacy of each of these active compounds over a range of concentrations. Once we have identified potent inhibitory molecules, we will perform cluster analysis in order to detect structural

similarities. This structure-activity relationship analysis will allow us to determine key chemical moieties that likely participate in Nef inhibition. Using this information, we will obtain structurally related analogs and test these for improved activity.

Our most promising inhibitory compounds will be subjected to rigorous follow up testing. We will first confirm that the inhibitor similarly disrupts Nef-induced MHC-I downmodulation in primary human T lymphocytes, as well as in other HIV targets, such as macrophages and DCs, without significant cellular toxicity. Importantly, we will assess the functional impact of a Nef inhibitor in protecting HIV-infected PBMCs from anti-HIV CTLs in co-culture assays (8).

The functional impact of a Nef inhibitor on HIV pathogenesis is of primary importance. However, it will also be important to elucidate the molecular mechanism of Nef inhibition. First, we will use crystallography or NMR analysis to determine whether the inhibitor directly binds to Nef, and where, as well as how this affects the conformation of Nef. We will also assess the effect of the inhibitor on Nef protein stability and subcellular localization. The ability of Nef to physically associate with cellular binding partners, such as MHC-I and clathrin-associated adaptor proteins, in the presence of the inhibitor will also be an important line of investigation. These questions can be answered with immunoprecipitation experiments like those conducted in chapters 2 and 3.

While we are primarily interested in compounds that inhibit downmodulation of MHC-I by Nef, it is also interesting to determine whether other Nef functions are also inhibited. A compound that interferes with the effects of Nef on intracellular trafficking may also prevent downmodulation of other Nef targets such as CD4, CD28, and CD8. Furthermore, an inhibitor that had profound effects on Nef stability, subcellular localization, or conformation might also prevent Nef-induced kinase activation or enhanced HIV infectivity. Kinase activation can be detected with phospho-specific antibodies. Effects on viral infectivity can be measured by producing HIV +/- Nef in T cell lines or primary T lymphocytes, and determining the relative efficiency with which equal titers of the viruses, as determined by p24 ELISA, infect a second round of target cells. If a Nef inhibitor does impact this poorly understood Nef function, it would be interesting to determine whether the inhibitor is effective when delivered to producer cells or target cells. A compound which inhibits multiple Nef functions might have the greatest impact on HIV pathogenesis.

While a Nef inhibitor would be a powerful laboratory tool for studying Nef function, our long term goals are be to develop such compounds for therapeutic use. Early steps will involve testing the efficacy of a Nef inhibitor in animal models such as humanized mice and non-human primates. Important parameters to measure will include the time to AIDS progression, as well as virus titers and CD4+ T cell counts. It will be important to assess the effects of a Nef inhibitor on disease progression in animals with an established infection, as this would resemble the most common clinical scenario. It is also interesting to hypothesize that treatment with a Nef inhibitor as a prophylactic measure concurrent

with exposure, or very early in HIV infection, might enhance the ability of the immune system to recognize and target HIV-infected cells before the virus can fully establish long-lived reservoirs. Therefore, it will be essential to measure not only HIV replication and disease progression in inhibitor-treated animals, but immune cell function as well. Adaptive immune responses can be measured by HIV-tetramer staining, IFN-γ ELISpot, and intracellular cytokine staining. Contingent upon success in animal trials, we would then proceed to human clinical trials with the goal of bringing a Nef-targeting drug to market.

5.10 Concluding remarks

The work presented in this dissertation advances our understanding of Nef, a protein required for maximal HIV-1 pathogenesis. The identification of which cellular proteins are targeted by Nef will help direct future study of how Nef influences host immune responses to HIV. Additional work is needed to determine how Nef's functions contribute to HIV-1 persistence and disease progression. In addition to a greater mechanistic understanding of Nef, this work represents preliminary efforts in developing chemical inhibitors of Nef for pharmaceutical intervention in HIV-1 pathogenesis. Furthermore, understanding how modulation of host proteins by Nef affects HIV-specific immune responses will expand the current understanding of host immune responses to intracellular pathogens.

5.11 References

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