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HIV Immune Evasion: Disruption of antigen presentation by the HIV Nef protein

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Abstract

The Human Immunodeficiency Virus (HIV) Nef protein is necessary for high viral loads and for timely progression to AIDS. Nef plays a number of roles but its effect on antigen presentation and immune evasion are among the best characterized. Cytotoxic T Lymphocytes (CTLs) recognize and lyse virally infected cells by detecting viral antigens in complex with host major histocompatibility complex class I molecules (MHC-I) on the infected cell surface. The HIV Nef protein disrupts antigen presentation at the cell surface by interfering with the normal trafficking pathway of MHC-I and thus reduces CTL recognition and lysis of infected cells. The molecular mechanism by which Nef causes MHC-I downmodulation is becoming more clear, but some questions remain. A better understanding of how Nef disrupts antigen presentation may lead to the development of drugs that enhance the ability of the anti-HIV CTLs to control HIV disease.

I. INTRODUCTION TO HIV-1 PATHOGENESIS

A. Summary of the HIV pandemic

Despite major advances in research and treatment, the human immunodeficiency virus (HIV) continues to persist as a pandemic. 33.4 million people are currently living with HIV, including 2.1 million children. In 2009 there were 2.7 million new infections and 2 million people died of acquired immunodeficiency syndrome (AIDS) (UNAIDS, 2009). 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.

B. Natural History of Untreated Disease

Following initial infection by HIV, there is evidence that a partially effective immune response reduces viral levels to a viral setpoint, the magnitude of which has prognostic significance with respect to how rapidly disease progression occurs (Mellors et al., 1996). HIV preferentially infects and destroys activated $CD4^+$ T lymphocytes, including those that are HIV-specific, which eventually leads to a defective anti-HIV immune response (Douek et al., 2002). 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.

C. The Virus

Like all retroviruses, HIV-1 reverse transcribes its single-stranded RNA (ssRNA) genome into a DNA intermediate prior to integration into the host cell genomic DNA. HIV-1 causes a chronic infection with a long incubation period, characteristic of viruses categorized into the genus lentivirus,. Also typical of retroviruses, HIV encodes group antigen (*gag*), polymerase (*pol*), and envelope (*env*) (Figure 1). (for review see (Ganser-Pornillos, Yeager and Sundquist, 2008), (Hill, Tachedjian and Mak, 2005)). HIV also encodes *tat* and *rev*, which promote transcription of the viral genome and nuclear export of viral RNA, respectively (for review see (Nekhai and Jeang, 2006)).

HIV is unique among retroviruses in that it has acquired accessory genes *vif, vpr, vpu,* and *nef,* which encode proteins that optimize viral fitness and spread in the host. The Vif protein counteracts intrinsic antiviral factor APOBEC3G by targeting it for degradation. Like Vif, Vpu also associates with 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. The role of Vpr is not entirely clear, but it is known that Vpr induces a G_2 mitotic arrest in infected cells, a state which favors transcription from the HIV-1 LTR. Recent data indicates 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 (Douglas et al., 2010; Kirchhoff, 2010))

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 been reported to alter the intracellular trafficking of a variety of proteins, such as major histocompatibility complex proteins class I (MHC-I), CD4, CD28, and CD8 (Garcia and Miller, 1991; Schwartz et al., 1996; Stove et al., 2005; Swigut, Shohdy and Skowronski, 2001). This review will focus on the role of Nef in MHC-I downmodulation.

II. HIV-1 Nef

Non-human primate research has revealed that the simian immunodeficiency virus (SIV) Nef protein is required for eventual immune collapse. In fact, rhesus macaques infected with a Nef-deleted (Δ*nef*) strain of SIV do not progress to AIDS (Kestler et al., 1991).

In addition there is a cohort of blood transfusion recipients exposed to an HIV-1 variant (Learmont et al., 1992) that contained a significant deletion in the viral genome including part of the *nef* gene and the long terminal repeat (LTR) (Deacon et al., 1995). Decades after infection and without anti-retroviral treatment (Dyer et al., 1997) none of these patients have been reported to progress to AIDS, though some do have reduced $CD4^+$ T cell counts (Birch et al., 2001; Learmont et al., 1999). These patients are considered long term non-progressors (LTNPs). The combination of non-human primate research and longitudinal patient cohort studies has revealed the requirement of Nef for progression from HIV disease to AIDS.

A. Disruption of Antigen Presentation to Cytotoxic T Lymphocytes

$CD8^+$ cytotoxic T lymphocytes (CTLs) are important for the control of chronic viral infections. CTLs bear receptors capable of distinguishing “self” from “non-self” peptide antigens presented by MHC-I on the cell surface (Figure 2). 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). Once the T cell receptor (TCR) on $CD8^+$ CTLs recognize 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 (Berke, 1995)).

There is a great deal of evidence that CTLs play an important role in the control of HIV infection (for review see (Collins, 2004)) and recent evidence indicates that individuals mounting a Gag-specific CTL response have improved parameters with regard to controlling disease (Geldmacher et al., 2007; Kiepiela et al., 2007). Despite the efficacy with which CTLs control viral load early in infection, 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 review will focus on the effect of the HIV Nef protein on antigen presentation by the infected cell.

Studies performed *in vitro* have detected different degrees of susceptibility of HIV-infected T cells to anti-HIV CTL killing (Collins et al., 1998; Shankar, Xu and Lieberman, 1999; Yang et al., 1996; Yang et al., 1997). These differences are probably due to the use of viral strains that have variable expression of the HIV Nef protein. Under conditions where killing of HIV-infected cells was directly compared plus or minus Nef expression, it was clear that Nef protected infected cells from CTL-mediated lysis (Collins et al., 1998; Lewinsohn et al., 2002; Tomiyama et al., 2002; Yang et al., 2002). Nef has been shown to protect HIV-infected primary T cells from CTL lysis using flow cytometric killing assays (Collins et al., 1998; Lewinsohn et al., 2002), CTL co-culture assays (Yang et al., 2002) and chromium release assays (Tomiyama et al., 2002). 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 (Tomiyama et al., 2002). 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 (Wong et al., 2010).

B. In vivo Evidence for Nef-induced MHC-I Downmodulation

Based on *in vivo* studies, it is known that an intact *nef* gene is necessary for the timely development of AIDS in most humans and monkeys (Deacon et al., 1995; Kestler et al., 1991; Kirchoff et al., 1995). However, Nef has multiple functions, therefore these studies do not prove an important role for Nef-mediated MHC-I downmodulation *in vivo*. Several studies have used SIV systems to demonstrate that the capacity to downmodulate MHC-I is selected for *in vivo* (Carl et al., 2001; Munch et al., 2001; Swigut et al., 2004). 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* (Lewis et al., 2008). 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.

C. Natural killer cells

To counteract the effects of viral pathogens on MHC-I expression, 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. There are three classical MHC-I genes expressed by all nucleated cells; 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 (Natarajan et al., 2002)).

Nef has been shown to directly interact with an amino acid sequence (Y_{320}SQAASS$_{326}$) present in the cytoplasmic tail of HLA-A and HLA-B molecules (Williams et al., 2002). This region of the MHC-I cytoplasmic tail is also necessary for Nef-dependent

downmodulation of MHC-I molecules (Cohen et al., 1999; Le Gall et al., 1998; Williams et al., 2002). In contrast, HLA-C and HLA-E have amino acid variations within this domain (Cohen et al., 1999; Le Gall et al., 1998; Williams et al., 2002) and thus remain unaffected by Nef. Therefore it has 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 (Schaefer, 2007).

D. Functional Domains Required for Nef to Downmodulate MHC-I

Nef can be divided into the N-terminal anchor domain, the core domain, and the C-terminal flexible loop (Figure 1). Two sites in Nef are required for most of Nef's functions. First, Nef is myristoylated at the glycine residue at position 2, which allows Nef to bind the inner leaflet of the plasma membrane (Fackler et al., 1999). In addition, an aspartic acid at position 123 (D_{123}) is required to form homodimers of Nef (Liu et al., 2000). If either of these sites is mutated (G_2A or $D_{123}G$), Nef is inactive for almost all of its functions.

Three regions of Nef, an N-terminal α-helix ($R_{17}ERM_{20}RRAEPA_{26}$ and specifically M_{20}) (Akari et al., 2000; Mangasarian et al., 1999), an acidic cluster ($E_{62–65}$), and a polyproline repeat ($P_{72/75/78}$) are required for Nef to bind to the cytoplasmic tail of MHC-I (Williams et al., 2005) and for Nef to downmodulate MHC-I (Greenberg, Iafrate and Skowronski, 1998; Mangasarian et al., 1999) (Figure 1). 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 (Roeth and Collins, 2006)). However, for unclear reasons, the C-terminal loop of Nef is only active against other Nef targets, such as CD4 (Mangasarian et al., 1999) unless Nef is directly fused to the MHC-I cytoplasmic tail (Wonderlich, Williams and Collins, 2008). These observations suggest 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.

III. Candidate host factors that partner with Nef

A. Adaptor protein complexes

Clathrin-coated vesicles transport cargo from the *trans*-Golgi network, 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) (Robinson, 2004; Robinson and Bonifacino, 2001; Traub, 2003). 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 (Doray et al., 2002; Klumperman et al., 1993; Waguri et al., 2003). AP-2 localizes to the plasma membrane and is necessary for internalization of some types of cargo into endosomes (Traub, 2003). AP-3 localizes to endosomes and is thought to transport proteins into acidic, degradative compartments (Peden et al., 2004).

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 (Owen and Evans, 1998). 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 (Kelly et al., 2008).

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 (Bresnahan et al., 1998; Craig, Pandori and Guatelli, 1998; Craig et al., 2000; Erdtmann et al., 2000; Greenberg et al., 1998; Janvier et al., 2003a; Janvier et al., 2003b; Le Gall et al., 1998; Piguet et al., 1998). 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 or δ subunits (Janvier et al., 2003b). 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 (Noviello, Benichou and Guatelli, 2008; Roeth et al., 2004; Singh et al., 2009; Wonderlich, Williams and Collins, 2008).

B. β-COP

COP-I and COP-II coatomers are needed for normal protein trafficking within the Golgi and ER (Rothman, 1994; Schmid, 1997). More recently, COP-I coatomers have been found associated with low pH endosomes in an ARF-1-dependent manner (Aniento et al., 1996; Gu and Gruenberg, 2000). These COP-I coatomers are implicated in recycling endosome function (Daro et al., 1997) and in transport from the endolysosomal network into multivesicular bodies (Gu et al., 1997).

The COP-I subunit β-COP was first identified as a binding partner for Nef in a yeast two-hybrid screen (Benichou et al., 1994). A diacidic motif ($EE_{155, 156}$) in the C-terminal loop domain of Nef mediates this interaction and targets internalized CD4 to degradative compartments. (Faure et al., 2004; Piguet et al., 1999; Schaefer et al., 2008). The interaction between Nef and βCOP requires ARF-1 but interestingly ARF-1 does not need to be in the activated, GTP bound state (Faure et al., 2004). β-COP binding to a separate region in the N-terminal alpha helical domain of Nef has also been implicated in targeting MHC-I for degradation (Schaefer et al., 2008).

C. PACS proteins

There is evidence that Nef interacts with phosphofurin acidic cluster sorting proteins (PACS-1 and PACS-2) through its acidic cluster $E_{62–65}$ (Piguet et al., 2000). PACS 1 and 2 were originally discovered by studying proteins that bind to the phosphorylated cytoplasmic tail of furin (Wan et al., 1998). Models for PACS-1 and PACS-2 function propose that these proteins help recruit AP-1 or AP-3 to cargo with acidic clusters (Crump et al., 2001). While it has yet to be found in coated vesicles (cited as data not shown in (Youker et al., 2009)), PACS has been shown to recruit AP-1 to a protein important for vesicle-membrane fusion, the SNARE vesicle-associated membrane protein (VAMP)-4 (Hinners et al., 2003). 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 and a CD4-Nef fusion protein in A7 astrocytic cells. 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 (Piguet et al., 2000). This model was later modified to indicate that PACS-2 was more important for localizing Nef to the TGN and that PACS-1 played a greater role in recycling in mouse embryonic fibroblasts and HeLa cells (Atkins et al., 2008). Another group confirmed that knockdown of PACS-1 inhibited Nef-induced MHC-I downmodulation in HeLa cells but not Jurkat T cells (Yi et al., 2010). Arguing against an important role for PACS proteins is data from other 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 (Lubben et al., 2007). Additionally, Baugh *et al* was unable to demonstrate a significant interaction between the acidic cluster in

Nef and the PACS-1 furin binding region (Baugh, Garcia and Foster, 2008). Moreover, mutating three of the four glutamates in the acidic cluster only decreased Nef's effects on MHC-I by about 50% (Baugh, Garcia and Foster, 2008).

D. 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 (Balch, Kahn and Schwaninger, 1992; Kahn et al., 1992). ARFs are important for clathrin dependent (Donaldson et al., 1992; Orcl et al., 1993; Palmer et al., 1993; Robinson and Kreis, 1992) and clathrin independent (Taylor, Kahn and Melancon, 1992; Waters, Serafini and Rothman, 1991) trafficking pathways. ARF activation and recruitment to cellular membranes is cyclical and regulated by its GTP binding state. Guanine nucleotide exchange factors (GEFs) are required for the recruitment of GTP to ARF and are necessary for the maintenance of overall Golgi structure ((Helms and Rothman, 1992; Jackson and Casanova, 2000) and reviewed in (Donaldson and Klausner, 1994)). GTPase-activation proteins (GAPs) promote GTP hydrolysis, thus inactivating ARFs (Turner, West and Brown, 2001). ARF-6 localizes to the plasma membrane and is involved in clathrin-independent endocytosis and recycling (Radhakrishna and Donaldson, 1997).

E. ARF-6 and PI3-kinase

ARF-6 is regulated by ARNO, an ARF-6 GEF that is activated and recruited to the plasma membrane by PI3-kinase(Venkateswarlu and Cullen, 2000). 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 (Blagoveshchenskaya et al., 2002). 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 (Yi et al., 2010). These antibodies recognize all MHC-I allotypes, including those that are unaffected by Nef and thus relatively small effects of Nef are usually detected. Based on these data, the prior model of how PACS proteins were involved in Nef-dependent MHC-I trafficking was modified. Instead of proposing that Nef physically recruits MHC-I and links it to a PACS-1 based TGN retrieval pathway, it was instead proposed that PACS proteins were needed to localize Nef to the TGN and that this localization of Nef was important for PI3-kinase and ARF-6 activation (Blagoveshchenskaya et al., 2002) (Figure 3). Subsequent studies provided evidence that localization of Nef to the TGN was important for Nef to recruit a SRC family tyrosine kinase needed for activation of a kinase cascade that culminated in PI3-kinase activation (Hung et al., 2007). Arguing against this possible model is evidence that additional, more specific ARF-6 mutants had no effect on MHC-I downmodulation in Jurkat T cells (Larsen et al., 2004). Furthermore, inhibition of PI3-kinase, had no effect on the internalization step in U373mg astrocytoma cells (Larsen et al., 2004). Instead, other investigators provided evidence that PI3-kinase inhibitors affected localization of intracellular MHC-I to the TGN in Nef-expressing U373mg astrocytoma cells (Larsen et al., 2004; Swann et al., 2001).

IV. Downmodulation of MHC-I: Endocytic Mechanism

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 (Schwartz et al., 1996). 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 (Roeth and Collins, 2006)). T lymphocytes and macrophages spontaneously internalize and recycle MHC-I back to the plasma membrane at high rates in an AP-2 dependent manner (Machy et al., 1987; Tse and Pernis, 1984). However, in Nef-expressing cell lines, over-expression of a dominant negative dynamin (Le Gall et al., 2000; Swann et al., 2001) or a dominant negative mutant subunit of AP-2 (Blagoveshchenskaya et al., 2002) did not affect Nef-induced MHC-I endocytosis suggesting this process could be clathrin and AP-2 independent. 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% (Yi et al., 2010). Furthermore, Greenberg *et al* determined that MHC-I co-localizes with AP-1, and not AP-2 in Nef-expressing cells (Greenberg, Iafrate and Skowronski, 1998) arguing against an AP-2-dependent internalization pathway. As discussed above, there is evidence for an ARF-6-dependent, clathrin-independent pathway by which Nef affects MHC-I, at least in some cell types (Figure 3).

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

A. Disruption of MHC-I transport

A fairly dramatic effect of Nef on MHC-I is required for HIV-infected primary T cells to effectively evade anti-HIV CTLs (up to 300-fold reduction (cited as data not shown (Collins et al., 1998)). The degree of downmodulation of MHC-I in HeLa cells (2–4 fold reduction (Blagoveshchenskaya et al., 2002) is small relative to the effect of Nef on an endogenous MHC-I allotype (HLA-A2) in HIV-infected primary T lymphocytes (Collins et al., 1998). 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 (Kasper and Collins, 2003).

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 Simpex 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(Hansen and Bouvier, 2009)). Alternatively, older MHC-I 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, previous 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 rather than only affecting MHC-I after it had reached the cell surface. The first experiment supporting this model examined the effect of Nef on an HLA-A2-GFP fusion protein in U373mg astrocytoma cells (Swann et al., 2001). In this series of experiments investigators utilized a temperature block (20°C) to prevent TGN exit and to allow accumulation of MHC-I in the TGN. When cells were subsequently shifted to 37°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 juxta-nuclear compartment (Swann et al., 2001). These investigators were the first to report an effect of PI-3 kinase inhibitors on this pathway. However, long incubation times (overnight) with inhibitors were required to

observe substantial re-accumulation of MHC-I at the cell surface in Nef-expressing cells. 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 (Kasper and Collins, 2003). An effect of Nef on intracellular transport of endogenous MHC-I HLA-A2 was confirmed in HIV-infected primary T cells (Kasper and Collins, 2003). PI3-kinase inhibitors did not reduce the ability of Nef to disrupt 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 (Kasper and Collins, 2003) as was subsequently proposed (Larsen et al., 2004).

The HLA-A2 cytoplasmic tail is phosphorylated at specific serines *in vivo* upon exiting the TGN (Eichholtz et al., 1992). Interestingly Nef preferentially binds immature, hypophosphorylated forms of HLA-A2 and inhibits phosphorylation of the MHC-I cytoplasmic tail (Kasper et al., 2005). Based on these data it was proposed that Nef binds MHC-I very early in the secretory pathway (Kasper et al., 2005). 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 (Yi et al., 2010). 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 (Kasper et al., 2005; Roeth et al., 2004)(Figure 4).

B. AP-1 is a host factor that is required for disruption of antigen presentation by HIV Nef

Because AP-1 was a clathrin adaptor protein that acted 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 AP-1 μ1 inhibited downmodulation of endogenous HLA-A2 in U373mg astrocytoma cells and exogenous HLA-A2 expressed in CEM-SS T cells (Roeth et al., 2004). Recently, siRNA directed against AP-1 μ1 also abolished Nef-induced downmodulation of MHC-I in HeLa and Jurkat cell lines, as well as in primary T Lymphocytes (Yi et al., 2010).

In addition, AP-1 co-precipitated with Nef and endogenous HLA-A2 from lysates made from HIV infected primary T cells (Roeth et al., 2004). 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 (Kasper et al., 2005). These data help explain why investigators that focused on non-T cell lines did not detect this pathway.

C. Nef stabilizes an interaction between the AP-1 tyrosine binding pocket and an extended domain on the MHC-I cytoplasmic tail that includes a tyrosine residue

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 (Bresnahan et al., 1998; Craig, Pandori and Guatelli, 1998; Craig et al., 2000; Erdtmann et al., 2000; Greenberg et al., 1998; Janvie ret al., 2003a; Janvier et al., 2003b; Piguet et al., 1998). In contrast, MHC-I downmodulation and AP-1 recruitment in T cell systems did not require these amino acids (Greenberg, Iafrate and Skowronski, 1998; Roeth

et al., 2004; Williams et al., 2005). 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 mediated a key interaction between the Nef-MHC-I complex and AP-1 (Roeth et al., 2004). Remarkably, 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. However, 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 (Roeth et al., 2004).

Additional mutational analysis of the MHC-I cytoplasmic tail revealed two other amino acids (A_{324} and D_{327}) that were needed for co-precipitation of AP-1 but not Nef. Interestingly, all three MHC-I cytoplasmic tail amino acids necessary for formation of the Nef-MHC-I-AP-1 complex are only found in HLA-A and HLA-B allotypes but not in HLA-C or HLA-E. The fact that these three amino acids (Y_{320}, A_{324}, and D_{327}) are important for co-precipitation of AP-1 suggests that this binding site may have a normal and as yet unidentified function in uninfected cells. Consistent with the fact that this site can be utilized by AP-1 in the absence of Nef, mutation of the cytoplasmic tail to create a somewhat more hydrophobic signal ($Y_{320}SQV_{323}$) allowed for AP-1 recruitment in the absence of Nef and significantly enhanced Nef's ability to downmodulate HLA-A2 and recruit AP-1 (Wonderlich, Williams and Collins, 2008). Providing further support for the model that Nef stabilized 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-1 μ1 that had two amino acid substitutions in the tyrosine binding pocket (TBPM) dramatically and specifically inhibited Nef-mediated MHC-I downmodulation (Wonderlich, Williams and Collins, 2008).

D. 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 (Williams et al., 2005). 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 (Roeth et al., 2004). 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 (Roeth et al., 2004; Wonderlich, Williams and Collins, 2008). 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 (Roeth et al., 2004). However, when a digitonin detergent based buffer that lacked crosslinker was substituted, a requirement for these domains to stabilize the interaction between AP-1 and MHC-I was noted (Wonderlich, Williams and Collins, 2008). In addition, the N-terminal α-helix and specifically M_{20}, were required for AP-1 recruitment under all conditions tested (Roeth et al., 2004; Wonderlich, Williams and Collins, 2008). Therefore, at least three Nef domains are required for AP-1 recruitment and subsequent downmodulation of MHC-I in Nef expressing cells (Roeth et al., 2004; Wonderlich, Williams and Collins, 2008).

E. Binding studies with purified proteins

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-1 μ-1 subunit. Moreover, these experiments using purified protein 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-1 μ1 subunit also required an intact tyrosine binding pocket in the AP-1 μ1 subunit. However, no role for Nef M_{20} was identified and thus this amino acid, which is required for Nef-induced MHC-I downmodulation may not be directly involved in protein-protein interactions but may serve another role in intact cells (Singh et al., 2009).

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

Although Nef recruits AP-1 to reroute MHC-I into the endosomal network (Roeth et al., 2004) (Figure 4), it remained unclear how Nef promoted accelerated degradation of MHC-I (Roeth et al., 2004; Schwartz et al., 1996). Prior reports had determined that Nef accelerated the degradation of internalized CD4 through an interaction between Nef and β-COP, a component of COP-I coats. Interestingly, MHC-I and internalized CD4 co-localize in $Rab7^+$ late endosomes (Schaefer et al., 2008; Yi et al., 2010) and RNAi against β-COP disrupts degradation of both MHC-I and CD4 (Schaefer et al., 2008). 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 (Faure et al., 2004; Janvier et al., 2001; Lindwasser et al., 2008; Piguet et al., 1999). An arginine rich domain in the N-terminal alpha helix of Nef ($R_{17}XR_{19}$) mediate 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. (Piguet et al., 1999; Schaefer et al., 2008). The inability of Nef to utilize sequences within the C-terminal loop to affect MHC-I downmodulation and the inability of Nef to utilize 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 versus Nef bound to the CD4 tail.

VI. Summary

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 (Dikeakos et al., 2010; Lubben et al., 2007; Roeth et al., 2004; Schaefer et al., 2008; Wonderlich, Williams and Collins, 2008; Yi et al., 2010). Additionally, there is agreement that three Nef domains are clearly required (acidic, polyproline and N-terminal alpha helix, including $M_{20}A$) for Nef-induced MHC-I downmodulation (Blagoveshchenskaya et al., 2002; Greenberg, Iafrate and Skowronski, 1998; Mangasarian et al., 1999; Noviello, Benichou and Guatelli, 2008; Roeth et al., 2004; Singh et al., 2009; Williams et al., 2005; Wonderlich, Williams and Collins, 2008). 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 (Roeth et al., 2004; Singh et al., 2009). 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 (Noviello, Benichou and Guatelli, 2008; Roeth et al., 2004; Singh et al., 2009; Wonderlich, Williams and Collins, 2008). 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 (Singh et al., 2009; Wonderlich, Williams and Collins, 2008). 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 (Blagoveshchenskaya et al., 2002; Hung et al., 2007; Larsen et al., 2004; Swann et al., 2001).

In conclusion, data from a number of laboratories have contributed to our current understanding of the mechanism by which Nef downmodulates MHC-I and its role in immune evasion *in vitro* and *in vivo*. While questions about the detailed molecular

mechanism remain, much has been learned. A clearer understanding may promote the development of a compound designed to specifically inhibit the effect of Nef on MHC-I antigen presentation. The capacity to rescue viral antigen presentation to CTLs and allow the host's immune system to maintain low-level viremia may improve the treatments available for HIV infected people.

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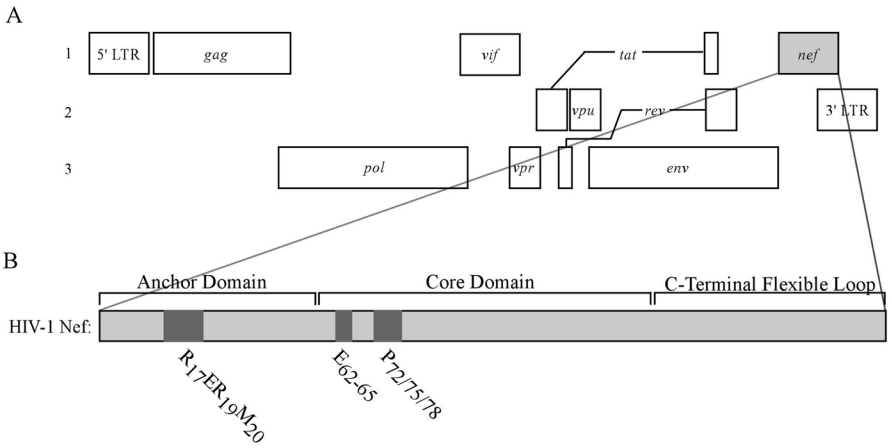

Domain Name		Endocytic Model	Disruption and degradation of newly synthesized MHC-I
Arginine Rich Region	($R_{17,19}$)		• β-COP Recruitment
M_{20}	(M_{20})	• Sorting internalized MHC-I	• Binding to MHC-I (in cells) • AP-1 Recruitment (in cells)
Acidic Cluster	(E_{62-65})	• PACS-1/2	• Binding to MHC-I • Stabilizing AP-1 in complex with Nef and MHC-I
Polyproline Domain	($P_{72/75/78}$)	• Src Family Kinase	• Binding to MHC-I • Stabilizing AP-1 in complex with Nef and MHC-I

Figure 1.

The genome of HIV-1 and a detailed view of domains in Nef.

(A) Three reading 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. (B) Domains in Nef that are pertinent to reducing the surface expression of MHC-I and their proposed functions in either of the two models of MHC-I downmodulation.

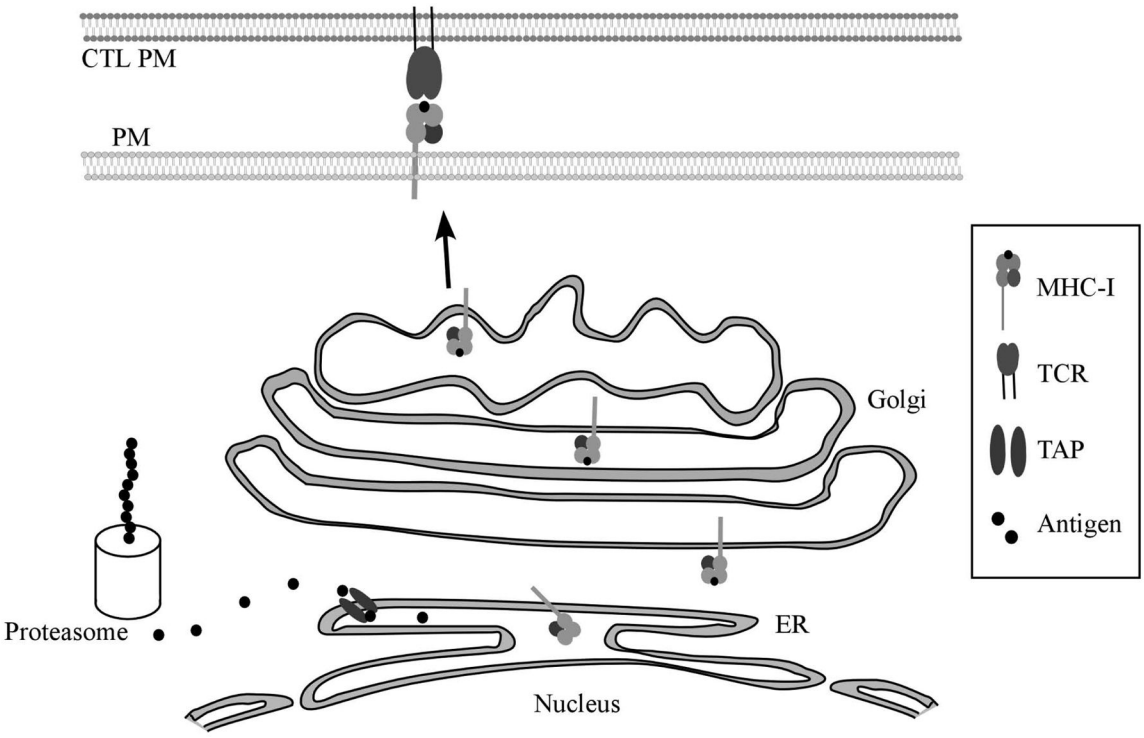

Figure 2.

Antigen presentation by class-I major histocompatibility complexes (MHC-I). Intracellular peptides (antigens) are produced through protein synthesis and subsequent breakdown by proteasomes. The peptides are then transported into the endoplasmic reticulum through the transporter associated with antigen processing (TAP) and loaded onto MHC-I molecules. Complete MHC-I molecules are transported through the Golgi Network out to the plasma membrane (PM) where the antigen is recognized as “self” or “non-self” by the T Cell Receptor (TCR) on a $CD8^+$ CTL. Cells expressing MHC-I in complex with “non-self” peptides are lysed to minimize spread of infection.

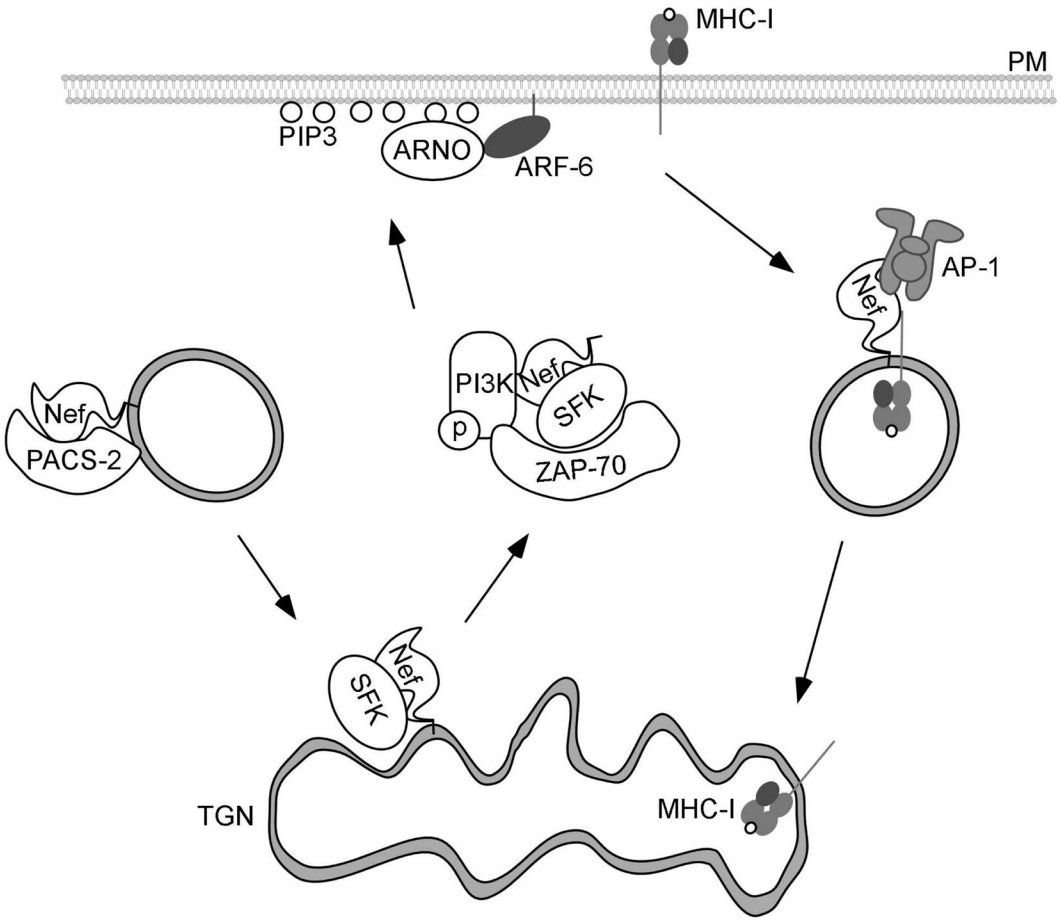

Figure 3.

Nef-induced endocytosis of MHC-I.

In this model, Nef is bound by the acidic cluster sorting protein, PACS-2, and localized to the *trans*-Golgi network (TGN). Nef binds to the Src Family kinase (SFK), Hck, which activates the tyrosine kinase ZAP-70. ZAP-70 then binds to and activates PI-3-Kinase. PI-3-Kinase creates PIP_3 on the inner leaflet of the plasma membrane (PM), which recruits the ARF-6 GEF, ARNO, subsequently recruiting and activating ARF-6. MHC-I is then internalized by an ARF-6 – dependent mechanism into endosomes. Nef then recruits AP-1, which transports MHC-I to the TGN.

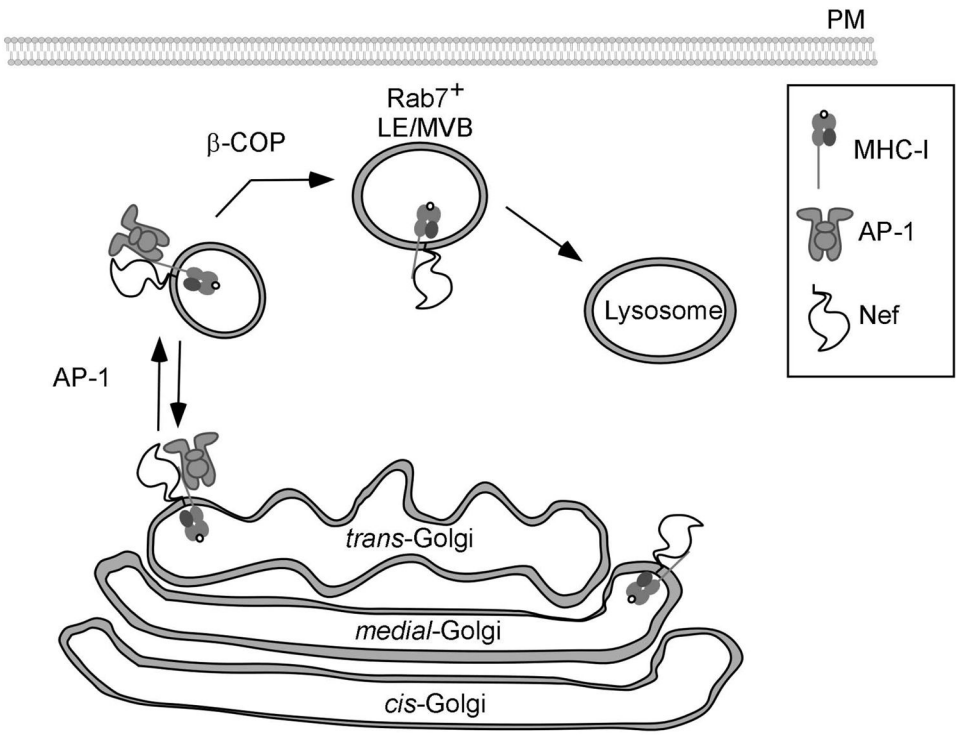

Figure 4.

Nef reroutes newly synthesized MHC-I into the endolysosomal network.

Nef binds to the cytoplasmic tail of MHC-I early in the secretory pathway. Nef blocks normal secretion of MHC-I to the plasma membrane by recruiting AP-1 to redirect MHC-I into the endosomal network. β-COP is then required to transport MHC-I bound to Nef from the endosomal network into late endosomal compartments for subsequent degradation in lysosomes.