Implications of Nef: Host Cell Interactions in Viral Persistence and Progression to AIDS

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Contents

Abstract The HIV and SIV Nef accessory proteins are potent enhancers of viral persistence and accelerate progression to AIDS in HIV-1-infected patients and non-human primate models. Although relatively small (27–35 kD), Nef can interact with a multitude of cellular factors and induce complex changes in trafficking, signal transduction, and gene expression that together converge to promote viral replication and immune evasion. In particular, Nef recruits several immunologically relevant cellular receptors to the endocytic machinery to reduce the recognition and elimination of virally infected cells by the host immune system, while simultaneously interacting with various kinases to promote T cell activation and viral replication. This review provides an overview on selected Nef interactions with host cell proteins, and discusses their possible relevance for viral spread and pathogenicity.

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1 Introduction

The year 2008 has witnessed a sober reassessment of the state-of-the-art in AIDS (acquired immune deficiency syndrome) research since the discovery of the human immunodeficiency virus (HIV) 25 years ago. Despite many seminal advances in the field, HIV remains an elusive target for eradicating treatment or effective vaccination. Key to the elusive nature of the virus is its ability to evade host or treatment pressures through genetic hypervariability, its integration into the host cell genome, and its persistence in latent reservoirs (Stevenson 2003). In addition, the virus benefits from its ability to interact with components of the infected cell and subvert the cell trafficking, signal transduction, and transcriptional machineries to its advantage: to facilitate virus infection, increase the production of fully infectious progeny viruses, avoid recognition by the immune system, and establish latency.

The HIV and SIV Nef accessory proteins are particularly adept at interacting with their host cell and inducing complex changes that promote efficient virus spread and persistence. Although originally named on the mistaken understanding that it negatively regulates virus transcription (Nef is an acronym for *ne*gative *f*actor), Nef acts as a potent viral enhancer of primate lentiviral persistence.

Particular interest in Nef lies in early observations that correlate its expression with progression to AIDS. Rhesus monkeys infected with simian immunodeficiency virus (SIV) carrying a large deletion in the *nef* gene showed low viral loads and did not progress to simian AIDS (Kestler et al. 1991). Similarly, defective *nef* genes have been detected in several long-term survivors of HIV-1 infection with normal CD4+ T cell counts and very low viral loads (Kirchhoff et al. 1995; Deacon et al. 1995; Mariani et al. 1996; Salvi et al. 1998). As a result, much effort has been undertaken to identify the mechanisms by which Nef promotes viral persistence and accelerates progression to AIDS and to define the molecular interactions with the host cell that are involved.

Nef is unique to primate lentiviruses and present in all HIV and SIV strains characterized to date. Nef proteins have a molecular weight ranging from 27 to about 35 kD and are encoded by sequences extending from the 3' end of the viral envelope (*env*) into part of the 3' long-terminal repeat (LTR). Although the amino acid sequence and length of HIV-1 Nef is variable, several distinct conserved functional domains have been identified (Geyer et al. 2001). HIV-1, HIV-2 and SIV Nef proteins frequently share only about 30% amino acid identity, but most structural properties and the majority of functions are well preserved (reviewed in Kirchhoff et al. 2008).

The best conserved feature of Nef is its N-terminal myristoylation signal, required for membrane binding and critical for most Nef activities. The flexible myristoylated N-terminal anchor is followed by a flexible loop containing a conserved acidic cluster involved in Nef effects on trafficking and a proline-rich region, conserved in HIV-1 but not in HIV-2 nor some SIVs. The latter is involved in SH3-domain binding of tyrosine kinases and Nef effects on signaling (Saksela et al. 1995; Renkema and Saksela 2000; Collette et al. 2000). Other important interfaces for interaction with

cellular proteins are a highly ordered and well-conserved globular core domain and a flexible loop near the C-terminus of Nef, which contains a dileucine-based sorting motif that functions as an endocytosis signal (Craig et al. 1998). The remarkable ability of Nef to interact efficiently with multiple cellular partners may be due to the high degree of flexibility within its folded structure (Geyer and Peterlin 2001).

According to the current literature, Nef may interact with as many as 60 cellular factors and affect the function of more than 180 proteins. These are listed in the web site of the National Library of Medicine: http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/nef.html. All these interactions and effects could potentially be advantageous for the virus. However, most of them remain to be confirmed in virally infected primary T cells or macrophages. Moreover, the underlying mechanisms and the role in viral persistence and progression to AIDS have only been examined for a small proportion of these Nef–host cell interactions.

The aim of this review is to expose some of the reported interactions of Nef with its host cell, and to discuss their possible implications for viral persistence and progression to AIDS. Although an attempt has been made to categorize Nef–host cell interactions into those that facilitate immune evasion and those that directly enhance viral spread, it should be noted that most Nef interactions with its host cell cooperate to ensure the production of progeny virions and to generate an environment that facilitates viral spread.

2 Interactions Facilitating Viral Immune Evasion

2.1 CD8+ T Cell Evasion: MHC-I Down-Modulation

Like many other invading pathogens, HIV and SIV have developed multiple strategies to avoid elimination by the host immune system. One mechanism to reduce immune recognition of infected cells involves down-modulation of the major histocompatibility complex I (MHC-I) (Kerkau et al. 1989; Scheppler et al. 1989). The Nef protein is critical for this ability of HIV and SIV (Schwartz et al. 1996) and sufficient to protect infected primary T cells from killing by CD8+ cytotoxic T lymphocytes (CTL) (Collins et al. 1998). Although the exact mechanisms are still under investigation, consensus evidence indicates that Nef expression in infected cells leads to accumulation of MHC-I in the trans-golgi network (TGN) where it is redirected to TGN-associated endosomal compartments and to lysosomes for degradation (Roeth and Collins 2006). The literature supports two pathways for Nef-mediated down-modulation of MHC-I involving accelerated endocytosis of MHC-I from the plasma membrane (Greenberg et al. 1998a; Le Gall et al. 2000; Piguet et al. 2000) and disruption of normal anterograde transport of MHC-I from the Golgi to the cell surface (Le Gall et al. 2000; Swann et al. 2001; Kasper and Collins 2003; Roeth et al. 2004) (Fig. 1). The prevalence of one mechanism over the other may differ between T cell and non-T cell lines (Kasper and Collins 2003).

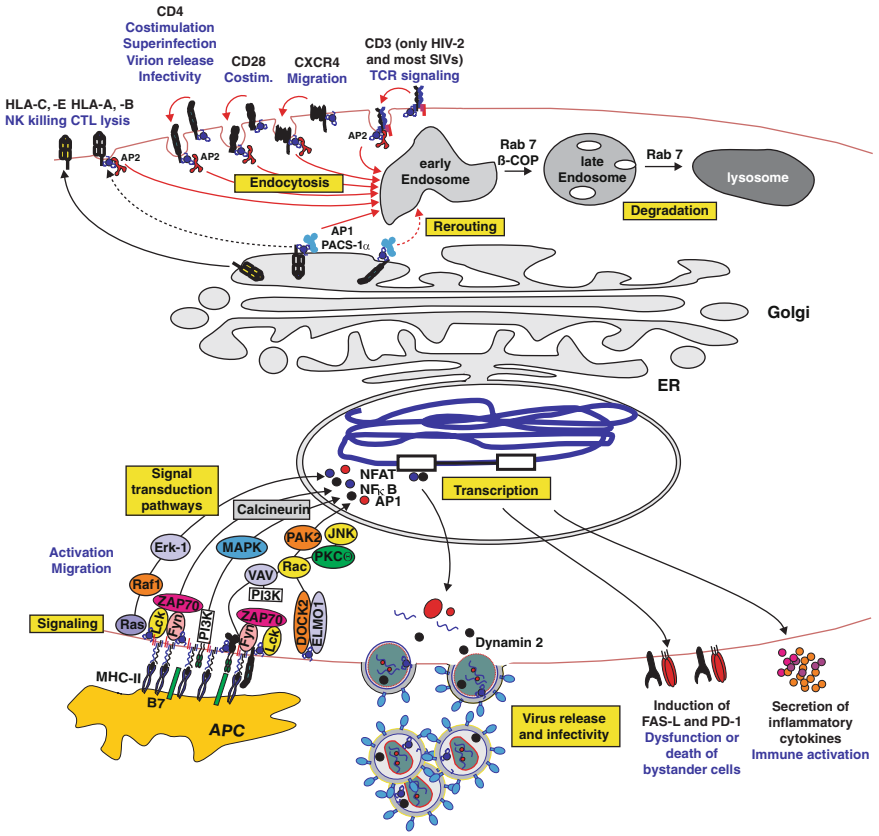

Fig. 1 Schematic presentation of selected Nef functions and interactions in infected T cells. Nef interacts with HLA molecules, CD4, CD28, CXCR4, and CD3 to reduce their surface expression on infected CD4+ T cells, thereby reducing CTL lysis, suppressing cell migration, facilitating virus release and modulating signal transduction by the immunological synapse. Furthermore, Nef interacts with a variety of cellular kinases and other factors to modulate downstream signaling events. HIV-1 Nef promotes the activation of transcription factors, such as NF-AT, NF-κB, and AP-1, to induce the efficient transcription of the viral LTR promoter and of various cellular genes, e.g., those encoding for inflammatory cytokines, activation markers and death receptors. Furthermore, Nef down-modulates CD4 to promote virus release and to prevent superinfection and enhances virus replication and virion infectivity to directly promote virus spread. Please note

Nef interacts directly with the cytoplasmic tail of MHC-I, although this interaction is weak and may be transient or stabilized by other factors (Williams et al. 2002). An N-terminal α helical region, the polyproline repeat, and the acidic domain in HIV-1 Nef are involved in MHC-I down-modulation (Greenberg et al. 1998a; Mangasarian et al. 1999). Notably, however, some HIV-1 and SIV *nef* alleles lead to efficient MHC-I down-modulation although they contain alterations in these residues (Specht et al. 2008).

For rerouting of MHC-I to the lysosomes for degradation, Nef recruits the clathrin adaptor complex AP1 via its μ1 subunit and subsequently ß-COP, which are both implicated in endosomal trafficking and transport through the early secretory pathway, to the cytoplasmic tail of MHC-I (Roeth et al. 2004; Noviello et al. 2008; Schaefer et al. 2008). It is a matter of debate whether accelerated endocytosis of MHC-I from the cell surface requires an interaction between Nef and a coat protein called PACS-1 (phosphofurin acidic cluster sorting protein-1) (Piguet et al. 2000; Lubben et al. 2007; Blagoveshchenskaya et al. 2002). It has also been suggested that Nef-mediated endocytosis of MHC-I involves the ARF-6 (ADP-ribosylation factor 6) endocytic pathway (Blagoveshchenskaya et al. 2002), which is normally involved in the clathrin-independent trafficking of MHC-I between the plasma membrane and endosomal compartments, and PI3K (phosphoinositide 3-kinase) (Swann et al. 2001; Hung et al. 2007). However, the significance of these mechanisms requires further study (Larsen et al. 2004), and the exact mechanism of MHC-I down-modulation is still under investigation (Atkins et al. 2008; Schaefer et al. 2008).

It has been clearly shown that down-modulation of MHC-I by Nef contributes to the ability of SIV to avoid CTL responses in vivo. A point mutation of Y223F near the C-terminus of SIVmac Nef that selectively disrupts the effect on MHC-I reverted within 4 weeks after infection, shortly after the peak of the CTL response (Münch et al. 2001). Furthermore, macaques infected with SIVmac mutants containing difficult-to-revert Nef mutations specifically eliminating MHC-I down-modulation exhibited higher levels of CD8+ T cell responses and showed compensatory mutations in Nef that restored MHC-I down-modulation (Swigut et al. 2004). In support of a relevant role of Nef-mediated down-modulation of MHC-I for viral immune evasion and effective persistence in HIV-1-infected individuals, it has been shown that *nef* alleles from non-progressor perinatally infected children were less efficient in MHC-I down-modulation than those from rapid progressors (Casartelli et al. 2003). The efficiency of CTL responses in infected patients seems to exert a selective pressure on the ability of Nef to down-modulate MHC-I (Carl et al. 2001), and the ability of Nef to down-modulate MHC-I correlated positively with the breadth of the HIV-1-specific CTL response (Lewis et al. 2008). Finally, unusually strong CTL responses have been detected in individuals infected with Nef defective HIV-1 strains (Dyer et al. 1999). Thus, together with the high variability of HIV and SIV leading to the emergence of CTL escape variants and other factors, the ability of Nef to down-modulate MHC-I provides an explanation why CTL responses are usually unable to effectively control viral replication.

2.2 NK Cell Evasion: Selective Down-Modulation of HLA-A and –B

Regulation of cell surface expression of MHC-I is a mechanism used by a number of viruses to evade recognition by CTL (for example herpes viruses, papillomaviruses, HIV). As a counteractive measure, natural killer (NK) cells can gage the

level of MHC-I expressed on cell surfaces and preferentially lyse cells that lack MHC-I. Under normal conditions, NK cell cytotoxicity is blocked by specific recognition of MHC-I molecules by inhibitory NK cells receptors (iNKRs). Reduced expression of MHC-I molecules on the surface of infected cells results in reduced engagement of iNKRs and triggers NK-cell-mediated cytolysis. Thus, down-modulation of MHC-I by HIV-1 Nef (among other viral proteins) should expose infected cells to lysis by NK cells. However, evidence shows that HIV-1 Nef down-modulates class I MHC proteins selectively, reducing surface expression of HLA-A and -B, which are recognized by the majority of CTL, but not of HLA-C and -E, which are recognized by iNKRs (LeGall et al. 1998; Cohen et al. 1999). Selective down-modulation of class I proteins by Nef is accounted for by differences in the cytoplasmic tail of MHC-I molecules, and residues Y320, A323, and/or D327 important for Nef-dependent down-modulation of HLA-A and -B are mutated in HLA-C and -E (LeGall et al. 1998; Cohen et al. 1999). The ability of Nef to selectively down-modulate HLA-A and -B reduces the ability of NK cells to kill HIV-infected cells despite reduced MHC-I surface molecules (Bonaparte and Barker 2003) and is a well conserved property among primate lentiviruses (Specht et al. 2008).

Nevertheless, studies have demonstrated that 30% or more of NK cells present in the peripheral blood do not express any receptors able to bind to HLA-C or -E (Bonaparte and Barker 2004; Mavilio et al. 2003), and that these NK cells have a greater ability to kill CD4+ T cells infected with HIV in which HLA-A and -B are decreased (Bonaparte and Barker 2004). It is therefore very likely that HIV-mediated perturbation of NK cell function (through aberrant expression and function of inhibitory receptors, defective cytokine production, and preferential expansion of NK cell subsets) also plays a role in the decreased ability of NK cells to kill HIV-infected cells. A recent work showed that Nef-pulsed dendritic cells (DCs) modulate NK cell effector function, inhibiting cytotoxic NK cell function while stimulating the pro-inflammatory cytokine-producing NK fraction (Quaranta et al. 2007).

Interestingly, although HLA-C presents self-peptides to NK cells to inhibit cell killing, it also has the ability to present viral peptides to CTL and thus restrict HIV-1 infection (Goulder et al. 1997; Adnan et al. 2006). Moreover, a single nucleotide polymorphism (SNP) upstream of the HLA-C locus associates with increased HLA-C expression and lower viral load at set-point (Fellay et al. 2007). Therefore, the resistance of HLA-C to Nef-mediated down-modulation could offer a promising opportunity for vaccine developments targeting HLA-C-restricted CTL responses.

2.3 Restricting MHC-II Antigen Presentation: Ii up-Modulation

MHC class II molecules, expressed chiefly on B cells, macrophages, and DCs, are specialized in exogenous antigen presentation to CD4+ T cells and are synthesized in the endoplasmic reticulum (ER) together with the invariant chain (Ii or CD74). Ii caps the MHC-II peptide binding site during its transport to endosomal compartments, where acidic pH leads to proteolytic cleavage of Ii thus allowing loading of

appropriate peptides on the MHC-II groove and subsequent transport of mature MHC-II-antigen complexes to the cell surface (reviewed by Rocha and Neefjes 2008). Interestingly, a fraction of MHC II-Ii complexes reaches endosomes not directly from the ER but rather by rapid internalization from the cell surface (Roche et al. 1993). It seems that this MHC-II-Ii fraction shuttles between the endosomes and plasma membrane before peptide loading in endosomes, undergoing repeated cycles of surface delivery and rapid internalization (Lindner 2002) via adaptor protein 2 (AP-2)-dependent endocytosis (Dugast et al. 2005).

Expression of HIV-1 Nef may perturb MHC-II-restricted antigen presentation by up-modulation of Ii cell surface expression (Stumptner et al. 2001; Stumptner et al. 2003). Indeed, stable expression of Ii hampers peptide presentation (Roche et al. 1992; Bertolino and Rabourdin 1996), and Nef-mediated up-modulation of surface Ii might therefore contribute to impaired helper T cell responses observed in AIDS patients (Norris and Rosenberg 2002). Nef interacts directly with both AP-2 and Ii in a dileucine-dependent manner (Toussaint et al. 2008), and it has been suggested that Nef may up-modulate Ii because both compete for AP-2 binding (Mitchell et al. 2008). More recently, however, it has been suggested that Ii up-modulation by Nef is due to impaired AP-2-mediated endocytosis rather than direct competition for AP-2 (Toussaint et al. 2008). Efficient Nef-mediated up-modulation of surface Ii is a well conserved property of primate lentiviruses (Schindler et al. 2003) and can be observed at very low levels of Nef expression (Stumptner et al. 2001). Significant Nef-mediated up-regulation of Ii was also observed in HIV-1-infected macrophages (Schindler et al. 2007a). Further studies are required to obtain definitive proof but our current knowledge suggests that the effect of Nef on Ii represents an important viral immune evasion mechanism in vivo in HIV-1-infected individuals.

2.4 Modulation of Signaling from the Cell Surface

2.4.1 Down-Modulation of TCR-CD3

The T cell receptor (TCR) is a heterodimeric protein consisting of α and β chains. Its function as a receptor is entirely dependent on its association with the CD3 complex, comprised of four transmembrane protein chains: γ, δ, ε, and ζ (zeta), which mediates signal transduction after antigen recognition. All four CD3 chains have intracellular immunoreceptor tyrosine-based activation motifs (ITAM) that become phosphorylated upon receptor ligation, enabling their interaction with cytoplasmic signaling proteins.

Early work showed that the central region of SIVmac239, SIVsmm, and HIV-2 Nef can directly associate with the TCR zeta chain and that this interaction correlates with their ability to down-modulate CD3 from the cell surface (Bell et al. 1998; Howe et al. 1998; Schaefer et al. 2002). Although Nef's endocytic motifs do not appear to be involved, TCR-CD3 endocytosis occurs via the AP-2 clathrin adaptor pathway (Schaefer et al. 2002; Swigut et al. 2003). HIV-1 Nef, on

the other hand, is unable to induce CD3 down-modulation. One study reported that HIV-1 Nef maintains some ability to interact with TCRζ (Xu et al. 1999), whereas several others found that only SIV and HIV-2, but not HIV-1, Nef proteins show this association (Bell et al. 1998; Howe et al. 1998; Schaefer et al. 2002).

To date, SIVs have been detected in about 40 African non-human primate species (reviewed in Pandrea et al. 2008), and phylogenetic studies indicate that SIVs from two of these species, chimpanzees and sooty mangabeys, have been transmitted to humans and gave rise to HIV-1 and HIV-2, respectively (Hahn et al. 2000). A recent study of a wide range of primate lentiviral Nef proteins revealed that the ability of SIVmac239, SIVsmm, and HIV-2 Nefs to down-modulate CD3 is shared by the great majority of primate lentiviruses, and blocks the responsiveness of infected T cells to activation (Schindler et al. 2006). Only Nef proteins from HIV-1 and its SIV counter parts failed to down-modulate TCR-CD3 and to suppress T cell activation and programmed cell death. The structural basis for this fundamental difference in the ability of primate lentiviral Nef proteins to modulate CD3 remains elusive since the core region of Nef that is critical for the interaction with the TCR zeta chain (Schaefer et al. 2002; Swigut et al. 2003) is generally well conserved.

While HIV-1 infection in humans is typically associated with high levels of immune activation and progressive CD4+ T cell depletion, natural SIV infections, such as by SIVsmm in sooty mangabeys and by SIVagm in African green monkeys, do not bring about generalized chronic immune activation or disease. The effect of HIV-1 Nef versus SIVsmm/SIVagm Nef on CD3 surface expression and T cell activation suggests a protective role for SIV Nef against immune activation (Schindler et al. 2006), and TCR-CD3 down-modulation may help the natural hosts of SIV to maintain stable numbers of CD4+ T cells (Schindler et al. 2008). It is interesting, however, that even profound depletion of CD4+ T cells is not associated with disease in naturally infected sooty mangabeys, and it has been proposed that attenuated immune activation following acute viral infection protects these animals from progressing to AIDS (Gordon et al. 2007; Milush et al. 2007).

2.4.2 Down-Modulation of the CD4 Receptor

CD4 assists signal transduction and T cell activation after TCR ligation. It is involved in clustering at the immunological synapse and binding to MHC-II on antigen presenting cells (APCs). CD4 is also the primary receptor for HIV and SIV entry. In support of a critical role for virus replication, three HIV-1 encoded proteins, Vpu, Env, and Nef, ensure that CD4 molecules are kept away from the cell surface upon HIV-1 infection.

It has long been known that Nef down-modulates CD4 (Garcia and Miller 1991) and the underlying mechanisms have been extensively investigated (reviewed by Lama 2003; Roeth and Collins 2006). Notably, Nef uses distinct surfaces to down-regulate MHC-I, CD3 and CD4 from the cell surface. Nef-mediated down-modulation of CD4 involves a dileucine motif in the membrane-proximal cytoplasmic domain of CD4 (Aiken et al. 1994) and an intact dileucine motif, two diacidic

motifs, and a hydrophobic pocket in Nef (Mangasarian et al. 1999; Bresnahan et al. 1998; Craig et al. 1998; Piguet et al. 1998; Lindwasser et al. 2008).

Expression of Nef leads to the endocytosis of surface CD4 by recruitment of the AP-2 clathrin adaptor complex, which directs the receptor to lysosomes for degradation (Aiken et al. 1994; Piguet et al. 1998; Greenberg et al. 1998b; Bresnahan et al. 1998; Craig et al. 1998). Recent findings suggest that ubiquitination of Nef may play a role in CD4 down-regulation (Jin et al. 2008). In contrast to Nef, Vpu and Env are expressed during the late stage of the viral life cycle and interfere with the transport of newly synthesized CD4 to the cell membrane rather than down-modulating CD4 molecules already present at the cell surface (Malim and Emerman 2008). However, intracellular retention mechanisms may also contribute to Nef-mediated down-modulation of CD4 (Rose et al. 2005). In either case, down-modulation of CD4 by Nef is highly effective and most likely important for efficient viral persistence in vivo (Brenner et al. 2006).

The role of CD4 in mediating T cell activation following receptor ligation suggests that down-modulation of CD4 by Nef may be advantageous for the virus because it limits T cell activation. However, since essentially all primate lentiviral Nef proteins down-modulate CD4 with high efficiency, while having very different effects on T cell responsiveness to activation (Schindler et al. 2006), it is likely that other consequences of low CD4 cell surface levels discussed Sect. 3.2.3 are of higher physiological relevance.

2.4.3 Down-Modulation of Co-Stimulatory Molecules

The complete activation of a T cell following antigen-MHC recognition requires a second co-stimulatory signal, provided for predominantly by the CD28 receptor on T cells and the B7 (CD80/CD86) ligand on APCs. Absence of this co-stimulatory signal results in the suppression of the immune response, and induces antigen-specific tolerance and T cell anergy.

In T cells, the Nef proteins of SIVmac239, SIVsmm and HIV-2, and to a lesser extent of HIV-1, down-modulate surface CD28 expression by binding to the cytoplasmic domain of CD28 and accelerating its endocytosis via the AP2 clathrin adaptor (Swigut et al. 2001; Bell et al. 2001; Münch et al. 2005). Thus, the mechanism is similar to CD4 and CD3 down-modulation, although the ability of Nef to down-regulate CD28 can be genetically separated from these functions (Swigut et al. 2001).

In APCs, the HIV-1 Nef protein has been shown to redirect the co-stimulatory molecules CD80 and CD86 away from the surface by binding to their cytoplasmic tails and rerouting them to the Golgi apparatus by a clathrin- and dynamin-independent actin-based endocytic pathway that seems to involve the activation of c-src and Rac (Chaudhry et al. 2005, 2007, 2008). By down-modulating co-stimulatory molecules, as well as other surface receptors, HIV-1 Nef manipulates the functional interaction between T cells and APCs (discussed further in Sect. 2.4.5) to impede the mounting of an effective immune response against the virus.

2.4.4 CXCR4 Down-Modulation

In addition to CD4, chemokine receptors are also essential as co-receptors for HIV-1 entry into target cells (Deng et al. 1996; Feng et al. 1996). Transmitted HIV-1 strains, and those that persist during chronic infection, generally use the CCR5 co-receptor, while HIV-1 variants that utilize CXCR4 or are dual tropic are observed in about 50% of all AIDS patients. HIV-2, on the other hand, is more promiscuous in its use of chemokine co-receptors, and most SIVs use only CCR5, but not CXCR4, as entry co-factors (Marx and Chen 1998).

Some HIV-2 and SIV Nef proteins effectively down-modulate CXCR4 to inhibit T cell migration to the CXCR4 natural ligand, the chemokine stromal-derived factor 1 (SDF-1), whereas HIV-1 Nefs display only weak activity (Choe et al. 2002; Hrecka et al. 2005; Venzke et al. 2006; Wildum et al. 2006). Similar to its effects on CD4, CD28, and CD3, Nef seems to down-modulate CXCR4 by recruiting it to sites of the AP-2 clathrin-adaptor-dependent endocytosis (Hrecka et al. 2005). Notably, HIV-1 and SIVmac239 Nefs also inhibit chemotaxis by binding to the guanine exchange factor DOCK2-ELMO1, a key activator of the Rho GTPase Rac in antigen- and chemokine-initiated signaling pathways (Janardhan et al. 2004; Hrecka et al. 2005).

The chemotaxis of T cells mediated by SDF-1 through CXCR4 is essential for trafficking of T cells during development and the initiation of immune responses, with recruitment of lymphocytes to lymphoid tissues. CXCR4 down-modulation also reduces superinfection of infected cells (Venzke et al. 2006; Wildum et al. 2006). However, this effect is obviously of limited relevance since the primate lentiviruses that down-modulate CXCR4 with the highest efficiency do not utilize it as an entry cofactor. Nef-mediated impairment of T cell chemotaxis, with or without affecting CXCR4 surface expression levels, likely reduces contact with APCs and contributes to immune evasion.

2.4.5 Effects on the Immunological Synapse

As outlined above, Nef most likely forms trimeric complexes with AP-2 and various receptors, i.e., CD3, CD4, CD28, and CXCR4, to target them for endocytosis via AP-2 clathrin adaptors and subsequent degradation in lysosomes. Notably, Nef can use distinct surfaces to interact with AP-2 and to recruit different receptors. As a consequence, all these Nef activities are genetically separable (Akari et al. 2000; Craig et al. 1998; Iafrate et al. 1997; Swigut et al. 2000, 2001), and can (at least in part) be independently adapted to the specific host environment to optimize viral spread (Carl et al. 2001; Patel et al. 2002). Another interesting aspect is that some conserved functions are mediated by different domains in Nef proteins derived from various lineages of HIV and SIV (Swigut et al. 2000; Bresnahan et al. 1999; Hua and Cullen 1997; Lock et al. 1999) suggesting that they evolved independently during primate lentiviral evolution.

Many of the receptors modulated by Nef are involved in the formation of the immunological synapse (Fig. 1), which requires the interaction of the TCR-CD3 complex on T cells with the Ag/MHC-II complex expressed by APCs and of co-stimulatory and adhesion molecules on both cells. As described above, HIV-1 Nefs interfere with this process by modulating MHC-II, Ii, CD4, and, to some extent, CD28. It has been suggested that the ability of HIV-1 Nef to deregulate the function of the immunological synapse may reduce T cell activation and help to prevent damaging high levels of immune activation (Fackler et al. 2007). It is obvious, however, that HIV-1 Nef does a poor job in protecting the infected host because HIV-1 infection is almost invariably associated with high levels of chronic immune activation and progression to AIDS in infected humans. In comparison, HIV-2 and most SIV Nefs impair synapse formation more severely because they also down-regulate TCR-CD3 and are usually more effective in down-regulating CD28 and CXCR4 (Hrecka et al. 2005; Schindler et al. 2006). Cell cultures infected with viruses expressing HIV-1 Nefs are characterized by high levels of activation and apoptosis, whereas PBMCs expressing HIV-2, SIVsmm, or SIVagm Nefs show low levels of activation and cell death. The distinct characteristics of these in vitro cultures are similar to the documented different characteristics of HIV-1, HIV-2, and SIVsmm or SIVagm infection in vivo (Pandrea et al. 2008). Altogether, these findings suggest that HIV-1 Nefs dysregulate the functional interaction of infected T cells with APCs, whereas those of most other primate lentiviruses may disrupt it entirely. The possible importance of this differential ability of primate lentiviruses to disrupt the immunological synapse has been addressed in a recent review (Kirchhoff et al. 2008).

3 Interactions Supporting SIV and HIV Replication

Besides performing multiple functions that facilitate viral immune evasion, Nef also modulates the activation status of the infected T cells and enhances the infectivity of progeny virions to promote viral replication (Fig. 1). As described in the previous chapters, Nef down-modulates several receptors to dysregulate or disrupt antigen-specific signaling by the TCR-CD3 complex. However, HIV-1 Nef proteins also interact with numerous cellular factors to increase the responsiveness of virally-infected T cells to stimulation. The induction of downstream signaling pathways leads to the activation of transcription factors that increase the expression of the HIV-1 provirus as well as of many cellular genes. In addition, HIV and SIV Nefs also act at the latest stage of the virus life cycle by enhancing the infectivity of progeny virions. The effect of Nef on viral replication is most pronounced in primary T cell cultures, particularly if these are infected prior to stimulation (Miller et al. 1994; Chowers et al. 1994; Schwartz et al. 1995) and is most likely dependent on a variety of Nef effects, such as modulation of signal transduction pathway, induction of transcription factors and cellular activation, CD4 down-modulation, and enhancement of virion infectivity.

3.1 Enhancement of Virus Production

3.1.1 Subversion of T Cell Signaling Pathways

Although HIV-1 Nef down-modulates CD4 and (to some extent) CD28, it enhances the responsiveness of T cells to stimulation (Skowronski et al. 1993; Schrager and Marsh 1999; Fenard et al. 2005; Fortin et al. 2004; Wang et al. 2000) and interacts with a large number of cellular signaling proteins (reviewed in Greenway et al. 2003; Renkema and Saksela 2000). While the physiological relevance of these interactions is often poorly understood, they may lead to the induction of various signaling pathways and the activation of transcription factors (Maninnen et al. 2000; Wang et al. 2000; Fortin et al. 2004) that enhance viral and cellular gene expression (Arendt and Littman 2001; Simmons et al. 2001). Thus, HIV-1 Nef may partly uncouple T cell activation from the "normal" antigen-dependent interaction with APCs to increase virus production (Fig. 1). Notably, Nef can manipulate the infected cells very rapidly, as it is abundantly produced early during the viral life cycle. Moreover, it has been shown that selective transcription of *nef* and *tat* in quiescent cells can increase T cell activation and viral replication even prior to viral integration into the host cell genome (Wu and Marsh 2001).

It is well established that the PxxP motif in HIV-1 Nef interacts with the SH3 domains of a number of tyrosine kinases, including Lck, Fyn, Hck, Lyn, and c-Src itself (Saksela et al. 1995; Greenway et al. 1996; Collette et al. 1996; Baur et al. 1997). Lck and Fyn are the first recruited kinases upon T cell receptor ligation and Lck also mediates signaling from CD4, CD8, and IL-2 receptors. In yeast, Nef activated the kinase activities of Hck, Lyn, and c-Src but not of Fyn, Lck, or Yes (Trible et al. 2006). It is conceivable that the well-conserved albeit frequently low-affinity interactions of HIV-1 Nef with various Src kinases contribute to its effect on signal transduction and T cell activation (Renkema and Saksela 2000). It has also been reported that Nef interacts with the SH3 domain of VAV, a Rac1 guanine nucleotide exchange factor (GEF), possibly to trigger the JNK/SAPK signaling cascade and to induce rearrangements of the cytoskeleton (Fackler et al. 1999). However, another study using a comprehensive proteomics approach to directly identify Nef interacting proteins did not detect several of the proteins mentioned above including VAV, but suggests that binding of Nef to the DOCK2–ELMO1 complex, a key activator of Rac, plays a major role in its ability to inhibit chemotaxis and to promote T cell activation (Janardhan et al. 2004).

Nef can be phosphorylated on serine residues and associates with a number of serine and threonine kinases (Renkema and Saksela 2000). Particularly, the interaction of Nef with the p21-activated serine–threonine kinase 2 (PAK-2) has been extensively investigated (Sawai et al. 1994; Lu et al. 1996). PAK-2 is usually activated by Rac1 and Cdc42 and involved in the regulation of several cellular processes, such as cytoskeleton rearrangement, cell morphology, motility, apoptosis, and gene transcription (Daniels and Bokoch 1999; Chu et al. 2004).

It has been proposed that the interaction of PAK-2 with Nef plays a role in T cell activation, viral replication, apoptosis, and progression to AIDS (Lu et al. 1996; Wiskerchen and Cheng-Mayer 1996; Fackler et al. 2000; Linneman et al. 2002; Chu et al. 2004). However, data obtained using Nef mutants selectively impaired in this interaction (Agopian et al. 2006) failed to detect definitive effects on T cell activation, viral replication, and apoptosis (Schindler et al. 2007b). Further effects of HIV-1 Nef involve the modulation of additional effectors and signaling pathways such as protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) families (Lu et al. 1996; Smith et al. 1996; Greenway et al. 1996; Yang and Gabuzda 1999; Wolf et al. 2008), interactions with the Ras-Raf-MAP kinase pathway (Maninnen et al. 2000; Hodge et al. 1998), and modulation of calcium signaling (Skowronski et al. 1993; Baur et al. 1994; Maninnen and Saksela 2002).

The biological consequences of most of these Nef interactions are still poorly understood. Taken together, however, our current knowledge suggests that HIV-1 Nef recruits various signaling proteins to the inner cell membrane and lipid rafts and, by modulating their catalytic activity and bringing them into close proximity, primes T cells for activation. In fact, it has been shown Nef alone can trigger T cell activation signaling pathways, inducing a transcriptional program that is highly similar to that of anti-CD3 T cell activation at least in Jurkat T cells (Simmons et al. 2001). In primary HIV-1 infected T cells, however, expression of Nef is usually not sufficient to induce T cell activation and viral replication but rather increases their responsiveness to stimulation (Djordjevic et al. 2004; Fenard et al. 2005; Fortin et al. 2004; Wang et al. 2000). The exceptions are SIV *nef* alleles that contain an additional SH2 domain and are highly active in enhancing T cell activation and associated with an acute lethal disease in infected rhesus monkeys (Du et al. 1995). Typically, Nef expression in HIV-1-infected T cells increases the induction of various transcription factors, such as NFAT, NFκB, and AP1 Activation Protein 1 upon stimulation with various agents (Wang et al. 2000; Manninen et al. 2000; Manninen et al. 2001; Fortin et al. 2004). Since HIV-1 requires T cell activation for efficient replication, Nef's subversion of T cell signaling pathways increases the transcriptional activity of the viral LTR promoter to promote efficient viral replication (Fig. 1).

Although the polyproline motif mediating interactions between Nef and Src kinases is highly conserved between HIV-1 isolates, three amino acid substitutions in the HIV-2 and SIVmac Nef proteins result in the targeting of different Src kinases (Collette et al. 2000; Karn et al. 1998). To date, relatively little is known about the interaction of SIV and HIV-2 Nefs with cellular kinases and their effects on signal transduction pathways, T cell activation, and virus replication. Further studies seem warranted since differences in the ability of Nef to modulate cellular signaling pathways may affect the levels of immune activation and hence the development of immunodeficiency. The observation that an additional SH2 domain in Nef is associated with acute disease in SIV-infected rhesus macaques (Du et al. 1995) represents a particular striking example for the importance of cellular activation in the clinical outcome of primate lentiviral infections.

3.1.2 Activation of Viral and Cellular Transcription

By modulating the signal transduction machinery Nef augments the expression of its own genome and of a large number of cellular genes (reviewed by Arendt and Littman 2001). Gene expression profiling studies showed that Nef induces a transcriptional program in Jurkat T cells that is highly similar (but not identical) to that of anti-CD3 T cell activation and partly dependent on ZAP-70 and the zeta chain of the TCR (Simmons et al. 2001). In particular, Nef induces transcription factors that transactivate the HIV-1 LTR promoter [NFAT, NFκB, IRF-1/2 (interferon regulatory factor), c-fos, Jun-D] and several cellular co-factors of viral replication, such as the Tat co-factor CDK9, the transcription elongation factor Tat-SF1, the transactivator NFIB-2, and the spliceosome component U1 snRNP (small nuclear ribonucleic protein) (Simmons et al. 2001). Nef also induces the expression of cytokines and chemokines which are thought to favor viral replication (IL-2 IL-4, TGFβ, MIP1α, MIP1β) (Arendt and Littman 2001). At least in HeLa cells, many effects on cellular gene expression were dependent on an intact PxxP motif in HIV-1 Nef (Shaheduzzaman et al. 2002). Relatively little is known about the effect of other primate lentiviral Nef proteins on the transcription of cellular genes, but one study reported that SIVmac Nef down-modulates genes associated with antigen presentation and induces the transcription of genes involved in cell survival and in the synthesis of membrane glycolipids and phospholipids (Ndolo et al. 2006). In contrast to the HIV-1 Nef (Simmons et al. 2001), that of SIVmac did not significantly modulate genes involved in T cell activation (Ndolo et al. 2006). Although all these findings need to be confirmed in virally infected primary T cells, they indicate that Nef exerts profound effects on the transcriptional response of infected T cells to favor viral replication. Notably, HIV-1 Nef also induces the transcription of the genes encoding T cell activation markers and of death receptors, such as the programmed death receptor 1 (PD-1) and the Fas ligand (Xu et al. 1999; Muthumani et al. 2008). These effects of Nef may play a role in the pathogenesis of AIDS since they may cause the death or dysfunction of uninfected bystander CD8+ T cells.

3.1.3 CD4 Down-Modulation

As discussed in Sect. 2.4.2, down-modulation of CD4 is one of the hallmarks of primate lentiviral infections and the best characterized Nef function. However, it is still not well understood which of its multiple consequences is most critical for efficient viral spread in vitro and in vivo (reviewed by Lama 2003; Kirchhoff et al. 2008). It has been reported that cell surface CD4 interferes with viral particle release by interacting with the HIV-1 envelope protein present on budding virions, and that Nef-mediated down-modulation of surface CD4 relieves progeny virions from this block (Arganaraz et al. 2003; Cortes et al. 2002; Lama et al. 1999; Ross et al. 1999). Furthermore, Nef-mediated down-modulation of CD4 may increase the incorporation of functional Env glycoproteins into progeny virus (Lama et al. 1999; Argañaraz et al. 2003). In support of a relevant role in virus replication, it has been

shown that the potency of CD4 down-modulation correlates with the efficiency of viral replication in primary lymphocyte cultures in human lymphoid tissue ex vivo (Glushakova et al. 2001; Lundquist et al. 2002). Moreover, down-modulation by HIV-1 of its own entry receptor reduces superinfection (Benson et al. 1993; Wildum et al. 2006), which would otherwise likely lead to cell death before virus production is accomplished. However, effective down-modulation of CD4 is not sufficient for effective replication in primary T cells (Saksela et al. 1995). To our current knowledge, at least three HIV-1 Nef activities, i.e., CD4 down-modulation, T cell activation, and enhancement of virion infection, contribute to efficient viral replication. Some seeming discrepancies may be due to the fact that the relative importance of these Nef functions for viral replication varies depending on the activation status and CD4 expression level of the target cells. CD4 down-modulation is most likely particularly important for the efficient release of fully infectious progeny virions from primary T cells since they usually express high levels of this receptor (Pham et al. 2004).

3.1.4 Enhancement of Viral Transfer from DCs to T Cells

Although the main target cells for HIV and SIV are CD4+ T cells and macrophages, it has been proposed that immature DCs present in the mucosa can capture virus particles and, following maturation and migration towards secondary lymphoid organs, transmit the virus to T cells (Granelli-Piperno et al. 1999). Although DCs are poorly infected by HIV-1, they can capture HIV-1 virions using the lectin DC-SIGN and maintain them in an infectious state for several days before transmitting them to lymphocytes (Geijtenbeek et al. 2000).

HIV-1 Nef is required for optimal virus production in DC-T cell co-cultures (Petit et al. 2001) and is thought to be implicated in transmission of virus to T cells (Sol-Foulon et al. 2002). Moreover, increased CD4 surface expression in APCs impairs DC-SIGN-mediated transmission by increasing internalization of particles through productive infection, and Nef-mediated CD4 down-modulation in DCs correlates with enhanced viral transmission to T cells (Wang et al. 2007). Together, these studies provide possible mechanisms by which Nef expression converts DCs into more efficient HIV-1 transmitters to T cells.

3.2 Enhancement of Virion Infectivity

Nef also enhances virion infectivity in a CD4-independent manner. Early studies suggested that Nef acts at an early postentry step because viral particles produced in the absence of Nef have an impaired ability to undergo reverse transcription (Aiken and Trono 1995; Chowers et al. 1995; Miller et al. 1995; Schwartz et al. 1995). This Nef-mediated enhancement of infectivity may depend on the route of entry since it is lost with HIV-1 virions pseudotyped with the vesicular stomatits

virus glycoprotein (VSV-G) which targets virions for entry by endocytosis rather than surface fusion (Aiken 1997; Chazal et al. 2001). However, recent data suggest that entry via low pH-dependent Envs does not always bypass the requirement for Nef (Pizzato et al. 2008). Nef does not affect the efficiency of virion fusion with target cells (Tobiume et al. 2003; Cavrois et al. 2004). Instead, it may promote cytoplasmic delivery of HIV-1 virions (Schaeffer et al. 2001), to the detriment of endocytosis, which may lead to non-productive infection (Maréchal et al. 1998). Other reports suggested that Nef enables HIV-1 complexes to cross the cortical actin network underlying the plasma membrane (Campbell et al. 2004) and may reduce the susceptibility of incoming virions to proteasomal degradation in the target cells (Qi and Aiken 2007). Altogether, the results suggest that Nef may help the virus to penetrate the cortical actin barrier and that this function becomes dispensable if entry is mediated by endocytosis (Campbell et al. 2004).

The enhancement of virion infectivity is dependent on the expression of Nef in the virus producer cells, suggesting that Nef alters the molecular composition or properties of the progeny virions. Small quantities of Nef are incorporated into the viral particles and cleaved by the viral protease (Welker et al. 1996; Pandori et al. 1996). However, virion incorporation and cleavage of Nef does not correlate with its ability to enhance virion infectivity (Chen et al. 1998; Miller et al. 1997). Recently, it has been shown that Nef interacts with the GTPase Dynamin-2 (Dyn-2), an essential regulator of clathrin-mediated endocytosis, and that the infectivity enhancement of Nef is dependent on both Nef interaction with Dyn-2 and clathrin-coated pit formation (Pizzato et al. 2007). Nef itself has also been shown to increase clathrin-coated pit formation in an in vitro model (Foti et al. 1997), suggesting that Nef's ability to enhance virion infectivity may be linked to its ability to enhance clathrin-dependent endocytosis. In support of this possibility it has been shown that the dileucine motif in Nef, which acts as an endocytosis signal, is required for optimal viral infectivity (Craig et al. 1998; Madrid et al. 2005).

The capability of Nef to enhance virion infectivity is highly conserved between different primate lentiviral lineages (Münch et al. 2007), and some evidence suggests that it is relevant for viral spread in vivo (Brenner et al. 2007). However, it is noteworthy that the effect of Nef on virion infectivity does not correlate with its ability to promote viral replication in primary T cells or ex vivo infected human lymphoid tissues (Glushakova et al. 2001; Lundquist et al. 2002) and has mainly been examined using HeLa-derived indicator cell lines. Thus, it will be important to further investigate how Nef modifies progeny virions and enhances virion infectivity in primary producer and target cells.

3.3 Induction of Soluble Factors Facilitating Virus Spread

Nef not only manipulates the infected host cells but may also induce changes in the cellular environment to render it more conducive to viral spread. Specifically, it has been shown that Nef expression in HIV-1-infected macrophages induces the

secretion of the chemokines MIP-1alpha and MIP-1beta (Swingler et al. 1999). These chemokines can promote the chemotaxis of resting T-lymphocytes, thus recruiting them to sites of HIV-1 virion release from infected macrophages. Furthermore, it has been suggested that Nef expression in macrophages leads to the production of the soluble factors sCD23 and sICAM-1, which stimulate the production of accessory surface molecules on neighboring B cells (Swingler et al. 2003), which in turn can render resting T cells permissive for productive HIV-1 infection. These results indicate that HIV and SIV Nef proteins have evolved highly sophisticated ways to manipulate the cross-talk between different cell types, and that some effects that may be highly important for viral spread in vivo can be missed in standard cell cultures.

4 Effects of Nef on Programmed Cell Death

One can conceive how it might be advantageous for primate lentiviruses to inhibit apoptosis of the infected host cell to prolong the time of virus production. No less than five viral proteins, Tat, Nef, Vpr, Vpu, and Env, were reported to modulate the programmed cell death of virally infected T cells (Gougeon 2003; Fackler and Baur 2002). As discussed in Chap. 2, most SIV and HIV-2, but not HIV-1, Nefs inhibit apoptosis by blocking the responsiveness of infected T cells to activation (Schindler et al. 2006). Furthermore, Nef induces the expression of the Fas ligand (CD95L), possibly to induce the apoptosis of "attacking" bystander CD8+ cytotoxic T-cells (Xu et al. 1999; Muthumani et al. 2005). It has also been suggested that Nef directly represses pro-apoptotic signaling in infected cells by inhibiting apoptosis signal-regulating kinase 1 (ASK1) (Geleziunas et al. 2001) and by the inactivation of the pro-apoptotic Bad-2 protein through phosphorylation (Wolf et al. 2001). However, it is controversial whether Nef inhibits or enhances apoptosis. In support of a pro-apoptotic role, it has been described that Nef sensitizes CD4+ T cells to apoptosis by up-regulating CD95 and CD95L (Zauli et al. 1999) and by reducing the expression of the anti-apoptotic proteins Bcl-2 and Bcl-XL (Rasola et al. 2001). Experiments using HIV-1 constructs coexpressing Nef and eGFP from single bicistronic RNAs that allowed to directly correlate the levels of cell death and Nef expression failed to detect a significant effect of Nef on apoptosis in HIV-1-infected primary T cells (Schindler et al. 2005). This suggests that Nef affects the survival of the infected cells mainly indirectly, e.g., by reducing CTL lysis and suppressing T cell activation (in the case of HIV-2 and most SIVs), rather than by direct effects on apoptosis.

5 Interactions of Exogenous Nef with Host Cells

In addition to its expression in virally infected cells, Nef is also present in the extracellular environment and can reach concentrations of up to 10 ng/ml in the sera of HIV-infected individuals (Fujii et al. 1996). Extracellular Nef may activate

various transcription factors in monocytes and macrophages (Alessandrini et al. 2000; Mangino et al. 2007; Olivetta et al. 2003; Varin et al. 2003) and induce apoptosis (Fujii et al. 1996; Okada et al. 1997; Huang et al. 2004). It has been proposed that soluble Nef is internalized and blocks immunoglobulin class switch DNA recombination in B cells by perturbing CD40 ligand activation of B cells by T cells (Qiao et al. 2006). Recently, soluble Nef has also been shown to interact with CD34+ hematopoietic stem cells and to inhibit their clonogenic potential through induction of PPARγ and down-modulation of STAT5A and 5B expression (Prost et al. 2008), suggesting that it may contribute to the hematopoietic abnormalities observed in HIV-infected patients (Marandin et al. 1996; Sloand et al. 1997). These studies suggest that extracellular Nef may play a relevant role in the pathogenesis of AIDS. However, it is currently largely unknown how effectively Nef is released and how it can interact or be taken up by uninfected bystander cells. Furthermore, the significance of some of these findings is difficult to assess since they were obtained using rather artificial experimental conditions, such as high levels of Nef produced by bacteria or insect cells.

6 Conclusion

HIV and SIV Nef proteins perform a large number of interactions and functions that help the virus to persist in the infected host by facilitating immune evasion and by increasing virus spread. Although (or perhaps because) a myriad of Nef effects has been reported, we are still far away from a comprehensive understanding of the role of this "all-rounder" protein in viral persistence and pathogenesis. However, some principles of Nef function have become clear: (1) Nef interacts with the cytoplasmic domains of various cellular receptors to recruit them to the endocytic machinery, or to reroute them to endosomes, for ultimate degradation in lysosomes. The reduced surface expression of MHC-I prevents CTL lysis, that of CXCR4 impairs cellular migration, and that of several others, such as CD4, CD28, and CD3, interferes with TCR signaling. Together, these effects of Nef help the virus to evade the host immune system by making infected cells less "visible" to the immune system and prolonging their survival time and by preventing or deregulating the crosstalk between infected T cells and APCs. (2) Simultaneously, Nef interacts with various cellular factors involved in signaling, trafficking, cell activation and migration to alter the responsiveness of virally infected cells to stimulation and to facilitate the transcription of the viral genome and various cellular genes. The preliminary evidence suggests that HIV and SIV have evolved highly elaborate mechanisms to favor the expression of cellular genes that promote viral spread at the cost of antiviral cellular factors. (3) By reducing the surface levels of CD4 and by another mechanism that is currently incompletely understood, Nef facilitates viral release from primary T cells and enhances the infectivity of progeny virions. (4) Nef may also facilitate viral immune evasion by up-modulating several death receptors and inducing the secretion of cytokines that affect the survival and

function of uninfected bystander CD8 T cells. (5) Finally, by inducing the release of cellular factors, Nef may recruit T cells to the sites of infection and render them susceptible to infection. Thus, although Nef is commonly considered as an early viral gene product, it acts essentially at every stage of the virus life cycle and may even modify the microenvironment of the infected cells to facilitate viral spread. The combination of these Nef interactions and functions allows HIV and SIV to persist efficiently at high levels in their respective hosts. Most likely, the great majority of primate lentiviral Nefs also limits the detrimental effects associated with these high viral loads by efficiently suppressing the responsiveness of virally-infected T cells to activation. Exceptions are *nef* alleles from *vpu*-containing viruses, i.e., HIV-1 and its simian precursors, which increase rather than block the responsiveness of infected T cells to stimulation. These differences in Nef function could contribute to the differential levels of immune activation associated with pathogenic and nonpathogenic primate lentiviral infections and hence play a relevant role in the clinical outcome of infection (Kirchhoff, 2009). Understanding the sophisticated mechanisms that primate lentiviruses have evolved to evade the host immune response and to manipulate cells to their advantage may help us to develop novel preventive and therapeutic strategies.

Note Some SIV Nef Proteins Are Tetherin Antagonists: Very recent data demonstrate that the Nef proteins of some SIVs antagonize the recently identified interferon-inducible host-cell factor Tetherin, also known as BST2, CD317 or HM1.24 (Jia et al., 2009; Zhang et al., 2009). This further adds to the list of Nef functions that facilitate virus spread. Furthermore, these studies identify the second function (beside the suppression of CD4 cell surface expression) that Nef shares with the small accessory viral protein U (Vpu), which is used by HIV-1 to counteract tetherin (Neil et al., 2008).

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