

NEF: A PLEIOTROPIC MODULATOR OF PRIMATE LENTIVIRUS INFECTIVITY AND PATHOGENESIS*

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The lentiviral protein Nef recruits cellular signalling proteins to lipid rafts at the cell membrane and acts thereby as a master regulator affecting the transcription of a series of cellular genes. By activating resting T cells, Nef creates an optimal environment for lentivirus replication. In human immunodeficiency virus (HIV) infected macrophages and microglial cells Nef activates the production of T-cell attracting chemokines and contributes to the development HIV infection associated brain damage. Nef also functions as an adaptor or connector protein downregulating CD4 and CCR5, the key receptor and one of the coreceptors for HIV. It also downregulates cell surface expression of a subset of class I MHC molecules which contributes to viral immune evasion. Extracellular, soluble Nef may facilitate the spread of T-cell-tropic HIV variants and mediate a switch in dominant replicating HIV strains (from macrophage-tropic to T-cell-tropic viruses) in AIDS (acquired immunodeficiency syndrome) patients. Virion-bound Nef enhances infectivity. Nef is a potential target of antiretroviral therapy and *nef*-deleted (attenuated) retroviruses have been considered as candidate vaccines against HIV. We suggest that *nef*-deleted or highly mutated defective HIV (dHIV) genomes interfere with replication of “wild type” HIV in certain long-term non-progressor individuals. This implies that introduction of artificially constructed dHIV genomes (by infusion of leukocytes carrying dHIV proviruses) into HIV infected individuals [115, 116] could slow disease progression and could be considered as a therapeutic possibility.

Keywords: Nef, long-term non-progressor, HIV, defective HIV (dHIV), SIV, viral interference, master regulator, AIDS therapy, attenuated retrovirus

* Dedicated to Professor Ilona Béládi on the occasion of her 80th birthday.

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Introduction

The genomes of primate lentiviruses (human immunodeficiency viruses 1 and 2, simian immunodeficiency viruses 1 and 2) encode a unique *accessory protein*, Nef, which is dispensable for *in vitro* virus replication but necessary for the maintenance of high virus loads and the development of acquired immunodeficiency syndrome (AIDS) *in vivo*. Nef has no enzymic activity and coined its name from early *in vitro* experiments which suggested that it is a negative factor repressing virus replication in T-cell lines. Such an effect could not be confirmed, however, in later studies (reviewed by Harris, 1996) [1]. Nef contributes to efficient HIV replication in primary lymphocytes and macrophages infected *in vitro* [2, 3].

A role for Nef in lentivirus infectivity and pathogenesis was first demonstrated by infection of *rhesus monkeys* with *nef*-mutant and *nef*-deleted variants of simian immunodeficiency virus (SIV). A premature stop signal within the *nef* gene quickly reverted in the animals infected with the mutant virus, suggesting that Nef performs a critical function in the life cycle of SIV *in vivo* [4]. In contrast, the rate or extent of replication in cultured cells was similar for the wild type, *nef*-mutant or *nef*-deleted viruses. Replication of wild type or revertant viruses resulted in the development of AIDS (decreasing CD4⁺ lymphocyte number, lymphadenopathy, diarrhea, weight loss), while monkeys infected with the *nef*-deleted virus appeared to be healthy for over 1 year and their lymph node biopsies were normal. Thus, deletion of *nef* decreased the pathogenic potential of SIV [4].

Nef is a very important determinant of disease progression in HIV (human immunodeficiency virus) infected individuals as well. This is supported by the observation that *long-term non-progressors* (LTNPs, i.e. asymptomatic subjects with a low HIV-1 load in spite of 10–15 years of persistent HIV-1 infection) carried viruses with deletions in the *nef* gene. In contrast, wild type HIV-1 (coding for an intact Nef protein) results in a faster disease progression in the absence of treatment. [5–10].

Transgenic mice carrying HIV-1 genomes also develop severe AIDS-like pathologies [11]. Mutation of the *nef* gene, but not other HIV genes, prevented the development of AIDS-like symptoms suggesting a primary role for *nef* in pathogenicity. As a matter of fact, targeting the expression of a single HIV-1 gene, *nef*, to CD4⁺ T cells and macrophages was sufficient to the induction of an AIDS-like phenotype in transgenic mice [12, 13].

Nef: basic facts

As mentioned above, the *nef* gene has been initially described as a regulator gene of HIV-1 encoding a negative factor of virus replication. In primate lentivirus proviral genomes *nef* overlaps the 3' end of the long-terminal repeat (LTR). It is transcribed as a 2-kb doubly spliced mRNA. Nef mRNA is expressed early after HIV-1 infection but the Nef protein may play a role in the establishment of chronic infection as well [14]. Nef has a variable, strain-dependent amino acid sequence and length. The translated protein product undergoes modifications resulting typically in a 27-kDa phosphoprotein that associates with membranes through the myristoylated N-terminus [14]. Phosphorylation occurs predominantly at threonine-15 and affects cellular distribution, stability and association with cellular or viral proteins [15].

Nef has several major *domains* which mediate unique functions as summarized in Table I (based on Doms and Trono, 2000) [16]. Motifs involved in Nef-mediated CD4 and MHC I downregulation (see below) are located in flexible regions. In contrast, interaction sites for signalling molecules containing SH3 do-

Table I

Nef domains

Domain name	Feature	Function
N-terminal anchor domain	Composed of N-myristoyl glycine and a cluster of positively charged amino acids	– Targets Nef to the inner plasma membrane; – needed for P53 binding; – required for Nef incorporation into the virions and infectivity; – fusogenic activity
Acidic cluster domain	Contains sorting information	– Binds to PACS1, a TGN (Trans-Golgi Network) sorting protein; – binds to the GagPol polyprotein
SH3-binding domain	Contains PxxP motifs (3 or 4); located within the conserved core domain	– Binds to Src-family domain proteins and regulates their tyrosine kinase activity; – needed for efficient replication in macrophages
Oligomerization domain	FPD motif; located within the conserved core domain	– Mediates Nef oligomerization
C-terminal cholesterol-binding domain	Contains a recognition motif for cholesterol	– Transports newly synthesized cholesterol to sites of virion budding and release

mains or the p21-activated kinases are associated with the well-folded core domain which includes a motif (FPD) mediating oligomerization of Nef as well [17].

In infected cells Nef localizes at the plasma membrane and preferentially associates with the cytoskeleton, but it is also found in the cytoplasm, at the nuclear membrane, and in the nucleus [18]. Nef is also contained in the *virion* (see Table II). Each virion contains approximately 10–70 Nef molecules. The majority of virion-associated HIV-1 Nef is present as a 18–20 kDa isoform derived from the full-length, 27-kDa protein by proteolytic cleavage that is mediated by the viral protease [19–22]. The 20 kDa isoform retained in the virion enters into newly infected cells. It corresponds to the C-terminal portion of Nef containing conserved domains that are required for infectivity enhancement. A diarginine motif required for the association of Nef with a cellular serine kinase is also included in the 20 kDa fragment [22]. Nef is present in HIV-2 virions as well [23].

Table II

Localization of Nef

Location	Note
<i>1. Intracellular</i>	
Cytoplasm	Associates with the inner plasma membrane through its N-terminal anchor domain
Paranuclear area	In the TGN; correct localization requires PACS1
Nucleus	Binds to P53, blocks apoptosis in HIV infected cells
<i>2. Extracellular, virion-bound</i>	Nef is incorporated into the virions where it is cleaved; there are about 10–70 Nef molecules/virion
<i>3. Extracellular, free</i> (soluble, not virion-bound)	There is 1–10 ng soluble Nef in sera of HIV infected individuals; induces apoptosis in bystander (uninfected) T cells; renders macrophages resistant to HIV infection; probably facilitates the emergence of T-cell-tropic HIV variants in AIDS patients; reactivates latent HIV-1 genomes

Nef: intracellular activities

In vitro, Nef has several distinct activities that could affect, in principle, HIV and SIV pathogenicity (see Table III). These effects are attributed to interactions of Nef with cellular proteins and *Nef* can be depicted as a *connector* between targets and effectors [24–26, 18]. Although Nef was initially described as a GTP-binding protein resembling an oncogene product [27], its enzymic (GTPase) activity could not be confirmed (reviewed by Harris, 1996) [1].

Table III

Intracellular activities of Nef

Function	Mechanism
Downregulation of cell surface CD4 molecules	Interaction with the Src family kinase LCK Increasing clathrin-dependent endocytosis of CD4 to target it to lysosomal degradation
Downmodulation of cell surface MHC-I molecules	Internalization and sequestration to the trans-Golgi network (TGN)
Activation of T-cell signalling pathways	Interaction with the TCR-zeta chain Association with PAK2 (p21-associated kinase 2) Stimulation of FasL expression
Protection of infected cells from apoptosis	Inhibition of Fas and TNFR-mediated death signals by blocking MAP3K5 Interaction with p53 Inhibition of apoptotic signals regulated by the Bcl-2 family proteins through the formation of a Nef/PI3-kinase/PAK complex

Nef as a connector molecule: downregulation of HIV receptor CD4 and coreceptor CCR5 and establishment of superinfection immunity

CD4, the *primary receptor* for HIV, is part of the T-cell receptor complex. It is a transmembrane protein with a 38-amino acid cytoplasmic domain [28]. Nef (initially called the F protein, encoded by the *F-gene/3' orf* of HIV) downregulates the CD4 antigen [27] and reduces the formation of complexes between CD4 and newly synthesized HIV envelope proteins on the infected cell surface facilitating thereby the release of HIV virions [29].

Both HIV-1 and SIV have been shown, both *in vitro* and *in transgenic mice*, to induce an early state of *superinfection immunity* through a Nef-mediated downregulation of cell surface *CD4*. This effect does not involve changes in transcription or translation of CD4 [14, 30–33]. Nef downregulates cell surface *CCR5*, the *major HIV coreceptor* as well by enhancing its endocytosis and degradation [34]. Rendering cells refractory to subsequent infection by the same virus may facilitate high level replication and viral spread in the host.

In T-lymphoid cells, Nef does not affect the normal maturation and biosynthesis of CD4 or its transport to the cell surface. Rather, Nef triggers rapid *internalization of CD4*. While Nef membrane-association is essential for this effect, 20 membrane proximal residues of the CD4 cytoplasmic domain are sufficient to confer Nef-responsiveness to another integral membrane protein. There is a small

amino acid sequence in the CD4 cytoplasmic tail, including a dileucine motif at positions 413 and 414, that is required for CD4 internalization by Nef. Nef also induces the dileucine motif-dependent routing of CD4 from the Golgi to the lysosomal compartment, without transit through the cell surface [14, 35].

Nef binds to the cytoplasmic tail of CD4 directly and targets CD4 into *clathrin coated pits* (CCPs) followed by internalization and transport to lysosomes, where CD4 is degraded. An artificial CD4-Nef fusion protein is also internalized via CCPs and degraded. Thus, Nef indeed acts as a connector between CD4 and the cellular endocytic machinery.

Internalization of cell surface receptors *via* CCPs requires the action of *adaptor protein (AP) complexes* that bind to both clathrin and the receptor itself or clathrin and a connector protein, respectively. AP complexes exist in two major varieties termed AP-1, found in the trans-Golgi network (TGN), and AP-2, found at the plasma membrane. While both AP complexes are heterotetramers of similar size and structure, they do not share subunits. Nef can directly interact with both the $\mu 1$ component of AP-1 and the related $\mu 2$ component of AP-2. CD4 internalization primarily results from the recruitment of CD4 to CCPs due to the direct interaction of Nef with both CD4 and the $\mu 2$ subunit of AP-2 [29].

p56^{Lck}, a protein tyrosine kinase, stabilizes CD4 membrane expression and regulates the rate of CD4 internalization. It is possible, therefore, that CD4 endocytosis is a consequence of Nef-mediated dissociation of CD4 from p56^{Lck} [14, 35].

β -COP (human β -coat protein), a Nef-interacting cellular protein is a component of coatamers which form the coat of the non-clathrin-coated vesicles involved in the intracellular protein traffic between the endoplasmic reticulum and the Golgi apparatus. Tease (thioesterase II), another Nef-interacting protein affects the myristoylation status of Nef by regulating the intracellular steady-state level of myristoyl-CoA [36].

The major physiological role for CD4 is mediating activation of mature T lymphocytes. CD4 participates in the interaction between the T-cell receptor (TCR) and antigen presented in the context of MHC molecules on antigen-presenting cells by binding to the nonpolymorphic regions of MHC II. Interaction of CD4 with MHC II results in the activation and phosphorylation of Lck, followed by activation and phosphorylation of the ζ chain of the TcR, ultimately leading to expression of interleukin 2 (IL-2) and T-cell proliferation. Thus, by modulating CD4 expression Nef may affect T-cell activation events.

Nef also affects T-cell activation by modulating the IL-2 receptor (IL2R, CD25). As both CD4 and the IL2R play crucial roles in antigen-driven helper

T-cell signalling and T-cell proliferation, one critical role of Nef in the viral life cycle may be to perturb signalling pathways emanating from these receptors [37].

Downregulation of cell surface class I MHC molecules

Class I MHC proteins consist of a highly polymorphic, membrane-anchored heavy chain and a constant β_2 -microglobulin (β_2m) chain. The noncovalent assembly of the heavy chain with β_2m takes place in the endoplasmic reticulum (ER) or cis-Golgi apparatus, where the formation of stable complexes depends on the presence of antigenic peptides. The resulting trimolecular complexes follow the constitutive secretory pathway to reach the cell surface, where they are stably expressed. Spontaneous internalization of surface MHC-I molecules is either not detectable or involves only a minority of the complexes.

Nef downregulates surface expression of a subset of MHC class I (HLA-A and B) antigens in HIV infected cells [38]. Another accessory protein, Vif decreases cell surface expression of HLA-G1 [39]. The remaining HLA-C and -E molecules prevent recognition by natural killer (NK) cells, which lyse cells lacking MHC-I antigens. Nef-mediated MHC-I downregulation results from internalization of cell surface MHC-I receptors and sorting of MHC-I molecules from the TGN into AP-1-containing clathrin-coated vesicles. Downregulation requires specific sequences in the MHC-I cytoplasmic tail including a key tyrosine residue, and the activity of PI 3-kinase, a regulator of TGN-to-endosome/lysosome trafficking [40]. Nef, acting as an *adaptor* molecule, connects MHC-I to the intracellular protein sorting machinery by binding to MHC-I on the one hand and either AP-1 or AP-2 on the other [29].

The interaction between Nef and MHC-I appears to be weak. It may be transient or stabilized by other factors. Nef could link MHC-I to other cellular trafficking proteins as well, like PACS-1, a molecule that recycles proteins containing acidic cluster motifs to the TGN. The ability of Nef to downregulate MHC-I surface expression may influence the *pathogenesis* of the virus in its host. Despite a strong immune response, HIV infected individuals are unable to eradicate the virus and become chronically infected. This is due to HIV's ability to evade the immune system. One important mechanism of viral *immune evasion* is downmodulation of cell surface MHC-I molecules by Nef, which results in protection of infected cells from cytotoxic T-lymphocyte (CTL) killing *in vitro*.

One has to note that in organ cultures HIV replication *induces* MHC-I expression in immature thymocytes [41]. This is due to the fact that – like other vi-

ruses (see Béládi, 1999) [42] – HIV-1 induces interferon (IFN) synthesis. In fetal thymic organ cultures IFN- α (interferon- α) produced by type 2 predendritic cells acts on IFN-receptor expressing thymocytes to induce MHC-I expression. This effect is *not mediated by Nef* and apparently overrules the MHC-I downregulating effect of Nef in HIV-infected thymocytes. Thus, there is an additional level of complexity regarding HIV-host cell interactions. IFN- α secretion, as part of an innate immune response to HIV-infection, is insufficient to inhibit HIV-associated pathogenesis. HIV-1 also induces interferon gene expression and upregulates a set of interferon-stimulated genes, as assessed using oligonucleotide arrays, in monocyte-derived macrophages infected *in vitro* [43].

Infectivity enhancement

Nef facilitates virion infectivity only when present during viral particle assembly in the virus producer host cells. The enhanced infectivity is manifested during infection of new target cells. *In the absence of CD4 molecules* HIV-1 virion infectivity enhancement could occur via facilitation of proviral DNA synthesis [44; for review see 45]. It was also suggested that Nef enhances virion entry into the cytosol, perhaps reflecting changes in the lipid composition of the virion membrane. A polyproline-rich region of Nef (representing a high-affinity binding site for the SH3 domain of the Src family protein tyrosine kinase Hck) is necessary to confer enhanced, CD4-independent infectious potential upon HIV-1 particles. *In CD4+ HIV producer cells* Nef may enhance virion release via CD4 downregulation. It could ensure a more effective envelope engagement of CD4 receptors present on the subsequent target cells as well [46]. In CD4+ cells viral particles produced in the absence of Nef incorporate fewer envelope proteins and more CD4 molecules. The resulting viral particles are less infectious. In addition, high levels of CD4 proteins apparently sequester HIV-1 structural proteins in the host cells, reducing virion release [47].

An emerging research area connects enhancement of virion infectivity by Nef and the influence of Nef on the *synthesis and transport of cholesterol to lipid rafts* [48]. Cholesterol is essential for the egress of HIV virions from cells. It also facilitates HIV entry into the cells. Nef increases the transcription of cholesterologenic enzymes and the biosynthesis of cholesterol and its incorporation into DRMs (detergent-resistant membranes or lipid rafts) and progeny virions. Lipid rafts are microdomains in the plasma membrane that are enriched in sphingolipids, cholesterol, and a subset of cellular proteins. Two major pathways contribute to

cholesterol homeostasis in mammalian cells. Most exogenous cholesterol, which originates from low-density lipoproteins, is internalized *via* coated pits and distributed to intracellular pools. In addition, cells can synthesize cholesterol in their endoplasmic reticulum when the uptake of exogenous cholesterol is blocked. The newly synthesized cholesterol is then transported into the Golgi apparatus and distributed to various intracellular pools. Because cellular cholesterol is *compartmentalized*, some sites (including DRMs) are enriched in this newly synthesized lipid. *Nef binds cholesterol* via its C terminus *in vitro* and *in vivo*. The C terminus of Nef is conserved in all clades of HIV and shows similarity to a cholesterol recognition motif (CRM) identified in several other cholesterol-binding proteins. Mutation of this domain blocks cholesterol binding and decreases viral infectivity. An intact CRM in Nef is indispensable for increasing the level of newly synthesized cholesterol in lipid rafts and viral particles. In addition, N-terminal myristoylation and binding of cholesterol are both required for tight association of Nef with progeny virions. *Nef acts as a transporter of newly synthesized cholesterol* to sites of virion budding and release, increasing thereby HIV infectivity. Cholesterol may enhance the fusion of the virion membrane with the cell membrane by facilitating the formation of a complex between gp120, CD4, and CXCR4. One could speculate that certain rapidly replicating enveloped viruses harbour genes encoding cholesterol transporters to provide building blocks (i.e. cholesterol) for their membranes [48]. Assembly and budding of HIV virions at lipid rafts could be mediated by an interaction of Nef with the GagPol polyprotein. The C-terminal flexible loop in Nef (involved in binding of cellular sorting proteins) binds to the transframe p6* protein in GagPol. Nef increases the level of Gag in lipid rafts at the plasma membrane. The same interaction ensures incorporation of Nef into the virions [49].

Effects on cellular signal transduction

Lipid rafts are crucial structures where transport processes occur and signal transduction processes are initiated. *Nef mimicks cellular processes by recruiting signalling intermediates to lipid rafts*, initiating thereby signal transduction processes. Nef interacts with a series of cellular proteins involved in immune functions (T-cell receptor; cellular tyrosine kinases: the haematopoietic cell kinase Hck and the lymphocyte-specific tyrosine kinase Lck and the T-cell specific isoform of Fyn; mitogen-activated protein kinase(s) or MAP kinase(s); serine/threonine kinases called p21-associated kinases 1 and 2 (Pak1, Pak2); a

CD4-associated thioesterase; proteasome subunit HsN3; the μ clathrin-associated adaptor protein; a vacuolar ATPase and CD4) [see 50, 51, and references therein].

Bell et al. demonstrated that Nef can bind directly to the ζ chain of TCR and reduce surface expression levels of TCR $\alpha\beta$, CD3 and CD4 [50]. They suggest that reducing the availability of the antigen-specific TCR-CD3 complex for stimulation by antigen on antigen-presenting cells could contribute to the pathogenic outcome of infection *in vivo*. Downmodulation of the TCR-CD3 complex, which initiates the T-cell signalling cascade could certainly affect T cell responses to subsequent antigen stimulation. The cytoplasmic domain of TCR ζ is sufficient for interaction with SIV Nef which also binds to the AP50 and AP47 subunits of the clathrin coat-associated adaptor protein complexes AP2 and AP1. In addition, the product of an extremely pathogenic SIV *nef* allele binds directly to the ZAP70 PTK and enhances its kinase activity [50].

The ability of Nef to directly associate with the T-cell signalling machinery might contribute to disease progression and provide a selective advantage for the virus by reducing the ability of the host immune system to mount a response against the virus.

Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) ζ chain leads to TCR downmodulation [50]. Nef activates the T-cell receptor (TCR) cascade by recruiting a 1 MDa multiprotein complex including the cellular serine/threonine *p21-activated kinases* Pak1 and Pak2 that mediate downstream effector functions. Two highly conserved interaction surfaces are required for the association of Nef with Pak, the SH3 binding domain (PxxP) and two arginine residues or one lysine and one arginine (R/KR). The PxxP motif also recruits and activates the guanine exchange factor Vav and *via* Vav, the GTPases Rac and/or Cdc42, whose activity is also required for the activation of Pak. Lck, which is a component of a Nef-associated kinase complex [51] and acts upstream of Pak, is also recruited to the lipid rafts. Thus, Nef in lipid rafts mimicks a physiological trigger of T-cell activation, creating thereby an optimal environment for HIV replication [52].

In *macrophages* Nef activates the Hck kinase as well as a Pak-related kinase and STAT1 resulting in the release of T-cell attracting soluble factors (chemokines) [53–55]. Macrophages represent a persistently-infected cellular reservoir involved in *virus dissemination* by transferring HIV not only into T cells but also to astrocytes and neurons [56]. Nef and another HIV-encoded accessory protein, Tat, stimulate the production of quinolinic acid, a neurotoxin, in macrophages which may contribute to the HIV infection associated *brain damage* (see also below) [57].

In astrocytes and neurons Nef (and gp41) can induce the synthesis of complement factor C3 which may contribute to HIV-induced pathogenetic processes in the brain [58]. Expression of transcripts for the HIV-1 coat protein gp120 in astroglial cells of transgenic mice can induce, however, central nervous system damage in the absence of Nef expression [59]. The possible interactions of Nef and other HIV proteins with neurons was reviewed by van de Bovenkamp et al. [60]. SIV Nef is required for optimal replication of neurovirulent SIV strains in primary rhesus macaque astrocytes *in vitro* [61]. This implies that Nef has an important function in SIV neuropathogenesis.

In microglial cells Nef (and Vpr) induces the beta-chemokine RANTES/CCL5 [62]. Intracisternal injection of soluble Nef induces elevated levels of IL-6, TNF- α and IFN- γ in rat cerebrospinal fluid. *In vitro* soluble Nef and Nef expressed by human astrocytoma cells induces chemotaxis of mononuclear cells. Thus *Nef may recruit leukocytes into the central nervous system* [63].

Nef as a master regulator: switching on the T-cell activation program

Analysis of Nef-induced gene expression in T cells revealed that 97% of the genes switched on by Nef are identical with those activated by plate-bound anti CD3-antibodies [64]. Transcripts upregulated by Nef but not by anti-CD3 activation include positive regulators of the HIV-1 long terminal repeat (LTR) promoter. Transcripts upregulated by anti-CD3 activation but not by Nef include the transcription factor YY1 (a repressor of HIV-1 LTR) and IL-16, a cytokine inhibiting HIV-1 replication *in vitro*. Thus, large-scale expression profiling resulted in an unifying picture integrating the observations (see above) on specific T-cell activation pathways. The exact mechanism of Nef mediated T-cell activation remains, however, to be elucidated (see Steffens and Hope, 2001 and Arendt and Littman, 2001 for review) [65, 66].

It is interesting to note that extracellular Nef activates Ras and *reactivates* thereby, *via* mitogen-activated protein kinase cascades, *latent HIV genomes* by switching on the viral promoter in LTR sequences [67, 68].

Nef as a superantigen

Nef resembles superantigens because it binds to MHC class II antigens and can activate T cells *causing cell proliferation* and production of IL-2 and IFN- γ (T helper

1 cytokines) even if presented in an unprocessed form. Induction of cell proliferation is important for HIV replication: the virus replicates in T-cell blasts but cannot replicate in quiescent cells (reviewed by Torres et al.) [69].

Protection of HIV infected cells from apoptosis and induction of apoptosis in uninfected bystander T lymphocytes

Localization of Nef in the cytoplasm and nucleus suggests that it may also control signal transduction events other than those which occur immediately at the plasma membrane. The ability of Nef to bind to p53 and the overlapping subcellular distribution of Nef and p53 within the cytoplasm and nucleus suggest that Nef may influence p53 function. p53 regulates HIV-1 gene expression by suppressing transcriptional activation of the long-terminal repeat (LTR). Nef may block this activity, enhancing thereby HIV-1 replication. Nef, *via* its N terminus binds to the N-terminal region of p53, blocking thereby the interaction of p53 with transcriptional coactivators [18]. Thus, Nef inhibits p53-dependent apoptotic events. It was suggested that in CD4⁺ T cells Nef may inhibit apoptosis by other mechanisms as well, i.e. by blocking TCR-mediated apoptosis *via* its interactions with *src* family kinases Lck and Fyn [18].

The apoptosis-inhibiting effect of Nef in HIV-infected cells seems to be in *contradiction* with the fact that HIV infection causes depletion of CD4⁺ T lymphocytes in AIDS patients [70]. T lymphocyte depletion may certainly be a result of programmed cell death. This contradiction could perhaps be resolved by the observation of Finkel *et al.*, who reported that apoptosis of CD4⁺ T lymphocytes in HIV infected individuals occurs predominantly in uninfected or bystander cells, with a distinct lack of cell killing in the productively infected cells themselves [71]. The “*bystander effect*” is either mediated directly by viral proteins or indirectly by virus induced cellular factors. A number of studies support the hypothesis that Nef contributes to the bystander effect. While Nef protects the HIV-1-infected host cells from apoptosis as described above and by blocking ASK1 (an apoptosis signal regulating serine/threonine kinase), it promotes, simultaneously, the killing of bystander cells through *the induction of FasL activation-induced cell death (AICD)*. Direct association of Nef with the ζ chain of the T-cell receptor complex is needed for HIV-mediated up-regulation of FasL. In addition, *cytokine dysregulation* (overproduction of type 2 cytokines like IL-4, IL-10 and decreased production of type 1 cytokines, IL-2 and IFN- γ) may also *increase susceptibility to AICD* in AIDS patients (reviewed by Alimonti et al., 2003) [72]. Thus, extra-

cellular Nef is capable to induce IL-10 production in peripheral blood mononuclear cells and T-cell and promonocytic cell lines [73]. Nef induces, however, a specific Th1 impairment by down-regulating IL-2 and IFN- γ production [74]. In CEM-GFP cells (a T-cell line) HIV-1 infection results in a decrease of overall host mRNA synthesis and suppression of selected mitochondrial and DNA repair gene transcripts. At the same time increased expression of proapoptotic genes results in apoptosis [75]. Thus, apoptosis induction by HIV is certainly not restricted to “by-stander” (uninfected) cells.

Extracellular Nef: a cytotoxic protein favouring replication of T-cell-tropic over macrophage-tropic HIV strains

The fusogenic activity of N-terminal region of Nef may be involved in its extracellular release [76]. Extracellular Nef switches on the apoptotic program in T-cell lines, PBMCs and other cell types through the CXCR4 receptor. Stable interaction between Nef and CXCR4 may either initiate apoptotic signals at the cell membrane or Nef could be taken up in coated pits and targeted to specific compartments of the cell, such as the mitochondria, by endocytic vesicles [77]. Exogenous Nef protein can also inhibit the proliferation of a T-cell line at doses lower than the dose necessary for apoptosis induction [78]. Human monocyte-macrophage cultures treated with recombinant Nef (rNef) protein downregulate the CD4 HIV receptor and develop thereby a resistance to HIV entry and replication [79]. In doubly-infected lymphocyte-macrophage cocultures rNef treatment facilitates the spread of a T-cell-tropic HIV strain over macrophage-tropic strains. This suggests that extracellular (soluble) Nef [80] may *mediate a switch in the dominant replicating HIV strains* (from macrophage tropic to T-cell-tropic HIV variants) in AIDS patients [79]. Such a switch is frequently observed during progression of acquired immunodeficiency syndrome and altered cell tropism can be correlated with altered coreceptor usage of HIV variants (reviewed by Fenyő 2000) [81].

Reversion of a SIV strain carrying a deletion in *nef* delineates Nef functions essential for pathogenicity

Connor et al. observed that SIVmac239 Δ nef, a strain carrying a 182-bp deletion in *nef* and used as a live, attenuated vaccine in adult rhesus macaques, induced simian AIDS in one of 16 vaccinated animals [82]. The virus recovered from the diseased

animal proved to be virulent causing T-cell depletion in 3 out of 4 inoculated animals and carried a *nef* gene coding for a truncated Nef protein [83]. This was due to a second deletion in *nef* which reversed the frameshift introduced by the original 182 bp deletion and resulted in a longer *nef* open reading frame. The predicted Nef- $\Delta 2$ protein lacked the acidic domain of Nef (see above) and most of the core (conserved) region of wild-type Nef, and lost the capacity for downregulation of CD4, CD3 and MHC class I molecules. Thus, the latter properties of Nef are dispensable for pathogenicity of SIV. These data are comparable to an analysis of HIV-1 Nef mutants using *in vitro* fetal thymic organ cultures [84] which demonstrated that Nef-mediated downregulation of CD4, CD8 β , or MHC class I molecules (due to altered trafficking) or oligomerization of Nef is dispensable for Nef-induced defects in human intrathymic T-cell development. It is interesting, however, that mutations in the myristoylation site, the acidic cluster, the PxxP motifs and mutations affecting PAK2 binding abolished Nef-induced disturbance of thymopoiesis. One may add that HIV-1 Nef proteins from long-term non-progressors are highly variable at the core domain where epitopes recognized by cytotoxic T cells are located [10]. These changes could impair critical Nef activities necessary for HIV pathogenesis.

Nef as a target of antiretroviral therapy

Nef is a unique viral protein with no cellular counterpart. Its functions critical for HIV and SIV infectivity and pathogenesis render Nef an attractive therapeutic target and make the inhibition of Nef-induced enhancement of viral infectivity a logical therapeutic goal [85]. Choi and Smithgall proposed that conserved residues forming a hydrophobic pocket contacting the Hck SH3 domain could be targeted with a *small molecule inhibitor* [86]. The hydrophobic pocket of the Nef core region is critical for Hck recruitment and activation [8], which occurs by SH3 domain displacement [87]. Thus, disruption of this signaling process could, in principle, slow down disease progression in HIV infected individuals. As a matter of fact, based on the crystal structure of the conserved core of Nef with another Src family SH3 domain (representing a mutant Fyn tyrosine kinase) Lee et al. also pinpointed that compounds binding to the hydrophobic cavity would prevent the interaction of the specificity-determining isoleucine side chain of the SH3 domain with Nef, and recognized the therapeutic implications of this possibility [88]. Based on a similar cocrystallization study using the wild type Fyn SH3 domain, Arold et al. reached a similar conclusion as well [89]. One has to consider, how-

ever, that the truncated Nef protein encoded by the pathogenic SIVmac239 Δ nef strain lacks most of the Nef core region [83] and mutations in the SH3 ligand domain of Nef do not affect SIV Nef function *in vivo* [90]. These observations imply that targeting other domains of Nef could perhaps be more beneficial for therapeutic purposes. In addition, the side chains of the residues of the HIV-1 protease cleavage site in Nef insert themselves into the hydrophobic crevice [91] which may prevent, in principle, the access of small molecule inhibitors to the hydrophobic cavity.

Olszewski et al. used guanidine alkaloid analogs to inhibit HIV-1 Nef interactions with the cellular proteins p53, actin and p56^{Lck} [92]. These synthetic analogs of batzellidine and crambescidin natural products proved to be, however, cytotoxic and cannot be used, therefore, for therapeutic purposes in their present form.

In addition to small molecule inhibitors, a *nef* mutant coding for a *dominant negative* form of Nef (called F12Nef) was also suggested as a potential tool to counteract HIV-1 replication [93]. F12Nef differs from wild type Nef in 3 amino acid positions. It interferes with viral assembly or release and blocks HIV-1 release. Schiavoni et al. constructed an inducible lentivirus vector coding for a transmembrane fusion protein of nerve growth factor receptor and F12 Nef which successfully protected the transduced cells from HIV-1 spread [93].

Nef-disrupted and Nef-deleted retroviruses as candidate vaccines against AIDS

Although Nef is a preferential target for CD8⁺ T cells in primary HIV-1 infection [94] and CD4⁺ T cell responses (which may also contribute to the control of virus infections) are directed predominantly against Nef (and Gag) epitopes in established HIV infection [95], attenuated strains of SIV with deleted *nef* or *nef* coding for a truncated Nef protein have been evaluated as AIDS vaccine candidates in primate models (for review see Ruprecht, 1999) [96]. Although most of the immunodominant epitopes of Nef are absent from cells infected by such attenuated viruses, other viral proteins could elicit, in principle, an effective immune response in the infected hosts. The advantage of such strains is that they are usually non-pathogenic due to disruption of *nef*, although reversion of the vaccine strain to a pathogenic virus or residual virulence of the vaccine strain are important safety problems to cope with. It is important to note, that the coreceptor phenotype of a *nef*-deleted HIV strain evolved *in vivo* and this change (from macrophage tropic,

CCR5 coreceptor using to dualtropic, CCR5/CXCR4 coreceptor using phenotype) was associated with a higher cytopathic potential [97]. Comparison of chimeric simian and human immunodeficiency virus (SHIV) constructs with deleted *nef* or carrying a human IFN- γ gene showed that both could protect against a pathogenic virus challenge early after vaccination, in the absence of neutralizing antibodies to the attenuated or challenge viruses [98]. Nef deletion mutants of SIV, expressing IL-4 or IFN- γ , also induced protection against a homologous challenge virus and a subsequent SHIV challenge in the absence of neutralizing antibodies [99]. Similarly, although protection induced by attenuated SIV against superinfection [100] was associated with the presence of serum-neutralizing antibodies [101–103] or virus-specific cytotoxic T cells in certain studies [104, 105], these immunological mechanisms failed to explain the generation of superinfection resistance in other experiments [82, 106–112]. Thus, virus-enhanced innate immunity [108] or non-immunological phenomena, e.g. receptor modulation and/or occupancy of a critical niche by the attenuated strain have been suggested as putative mechanisms of superinfection resistance [107, 108]. Receptor downmodulation or receptor interference as a major mechanism of protection could be excluded, however, by using an SIV-murine leukemia virus (MLV) hybrid as a challenge virus carrying MLV *env* [110]. MLV and SIV belong to different interference groups and use different receptors. This experiments ruled out chemokine inhibition as a major mechanism of protection as well. Chemokines induced by the attenuated virus could block, in principle, coreceptor binding of the challenge virus coated by a related Env protein, but SIV-MLV entry is independent of HIV/SIV coreceptors [110]. Gundlach et al. speculate that Env-independent interference phenomena or cytotoxic T cells directed against viral proteins other than Env could contribute to protection [110].

Nef-deleted, defective HIV (dHIV) genomes as therapeutic tools to curb HIV infection

Khatissian et al. observed, that both the vaccine virus and the challenge virus could persist for long periods after challenge in rhesus macaques immunized with SIVmac251Deltanef and challenged with the pathogenic SIVmac251 isolate [111]. Similarly, Wei et al. observed replication competent HIV-1 (plasma viral load: 49,000 copies/ml) coexisting with highly mutated proviral genomes (with stop codons within *pol* and *vif*) in a long-term non-progressor [113]. These data raise the intriguing possibility that the presence of defective or crippled proviruses could interfere with replication of “wild type” retroviruses. This idea is supported

by the observation that Env-independent (postentry) interference could be established *in vitro* in the absence of CD4 downregulation in cells infected with either wild type HIV-1 or mutant HIV-1 (carrying inactive *vpu*, *vpr* and *nef* genes) [114]. We suggested that *defective HIV (dHIV)* genomes could induce superinfection resistance in their host cells [115, 116]. Their transcripts or proteins could either block the entry of “wild type” HIV or could interfere with the replication of “wild type”, nondefective HIV variants within the cell. In addition, “wild type” HIV could act as a helper virus permitting dHIV replication and facilitating dHIV spread in the form of pseudotypes. On this basis we suggested that introduction of leukocytes harbouring artificially constructed dHIV genomes (coding for a non-toxic Env protein and deficient in several viral genes including 3'-*orf1/nef*) into HIV infected individuals or AIDS patients could slow or arrest disease progression [115, 116]. Retroviral interference could certainly prevent disease progression in a mouse model. Czub et al. reported that coinfection of a nonneuropathogenic Friend murine leukemia with a highly neuropathogenic chimeric retrovirus prevented noninflammatory spongiform degeneration of the central nervous system [117]. Heat inactivated Friend murine leukemia virus was ineffective.

We suggest that defective HIV genomes and retroviral interference play a principal role in the establishment and maintenance of long-term non-progression state in certain HIV infected individuals. We propose that both *nef*-defective [5–10] or otherwise crippled [113] HIV genomes could compete with “wild type” HIV for receptors and viral and/or cellular enzymes and “wild type” HIV may act as a helper virus for such defective or seriously mutated HIV variants in these patients. Thus, based on the clinical and experimental data accumulated in the last 17 years we suggest that introduction of dHIV (by infusion of leukocytes carrying dHIV proviruses) [115, 116] into HIV infected individuals remains a viable therapeutic possibility.

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