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Review

Role of Nef in primate lentiviral immunopathogenesis

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Abstract. More than a decade ago it was established that intact *nef* genes are critical for efficient viral persistence and greatly accelerate disease progression in SIVmac-infected rhesus macaques and in HIV-1-infected humans. Subsequent studies established a striking number of Nef functions that evidently contribute to the maintenance of high viral loads associated with the development of immunodeficiency in the 'evolutionary-recent' human and the experimental macaque hosts. Recent data show that many Nef activities are conserved across different lineages of HIV and SIV. However, some differences also exist. For example, Nef alleles from most SIVs that do not cause disease in their natural monkey hosts, but not those of HIV-1 and its simian precursors, down-modulate TCR-CD3 to suppress T cell activation and programmed death. This evolutionary loss of a specific Nef function may contribute to the high virulence of HIV-1 in humans.

Keywords. AIDS, HIV, SIV, pathogenesis, Nef, primate lentiviruses, immunological synapse, viral persistence.

Introduction

The accessory *nef* gene is unique to human and simian immunodeficiency viruses (HIV and SIV, respectively). It encodes a protein of 27–35-kDa that is abundantly expressed early during the viral life cycle. Based on NMR structure analyses Nef can be dissected into four major regions: a flexible myristoylated N-terminal anchor domain, a loop containing a proline-rich region, a conserved well-ordered globular core structure and a C-terminal flexible loop [1]. *N*-terminal myristoylation of Nef is critical for membrane association and essentially for all of its functions. The high amount of flexible surface might contribute to the ability of Nef to interact with a large number of cellular partners [1]. Early studies with an SIVmac molecular clone showed that a large deletion in *nef* greatly attenuates viral replication and pathogenicity in infected macaques [2]. Subsequently, grossly defective *nef* genes were detected in several long-term slow/non-progressors (LTNPs) of HIV-1 infection [3–5]. All these individuals showed low viral loads and usually maintained stable $CD4^+$ T cell counts for more than 10 years after infection. However, some of them developed signs of immunodeficiency after long asymptomatic periods [6, 7]. Moreover, a minority of adult and the majority of infant macaques also progressed to simian AIDS after infection with *nef*-defective SIV mutants [8]. Thus, Nef is not absolutely required for the development of disease but strongly accelerates progression to immunodeficiency.

The evidence that Nef is a major determinant of disease progression, at least in the evolutionary-recent human and experimental macaque hosts of HIV-1 and SIV, respectively, has stimulated intensive research on

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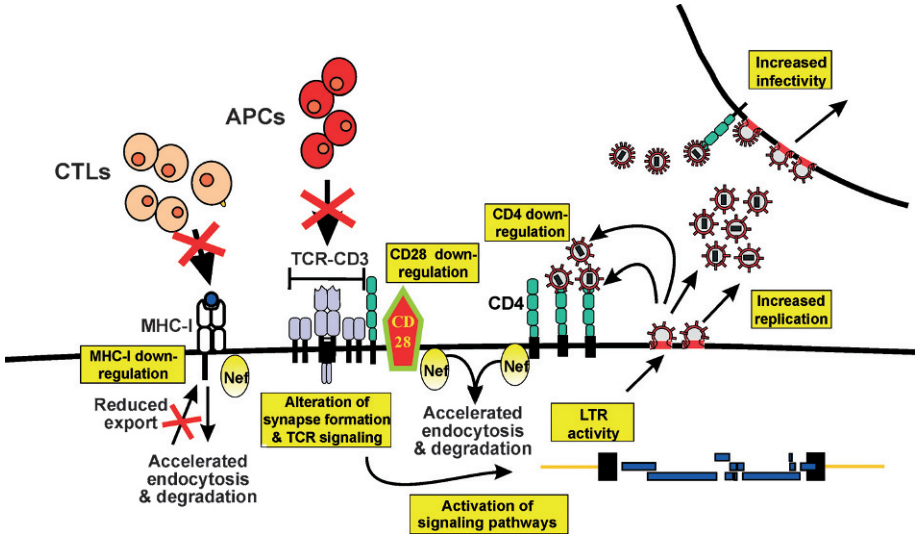

Figure 1. Overview on selected Nef functions in infected T cells. As outlined in the text, Nef performs a variety of functions in virally infected $CD4^+$ T cells, *e.g.*, it impairs MHC class I (MHC-I) antigen presentation to reduce cytotoxic T lymphocyte (CTL) lysis; affects the formation of the immunological synapse and TCR signaling by down-regulating CD28 and often also CD3 from the cell surface; induces downstream signaling events most likely by interacting with cellular kinases; down-modulates CD4 to promote virus release and to prevent superinfection; and enhances virus replication and virion infectivity to directly promote virus spread. Note that not all primate lentiviral Nefs perform all indicated functions.

Nef function. The results revealed that the HIV-1 Nef performs a striking variety of activities, including down-modulation of CD4, MHC class I and MHC class II, up-regulation of Ii, and enhancement of viral infectivity and replication (partly summarized in Fig. 1) [9–14]. Nevertheless, recent data demonstrate that HIV-1 Nef function is "crippled" in comparison to HIV-2 and most SIV Nefs, which in addition to these activities also efficiently down-modulate CD3 and CD28 cell surface expression [15–19]. The aim of this review is to summarize some of our knowledge on the role of specific Nef functions for viral persistence and pathogenesis in the SIV/macaque model, in HIV-1-infected humans and in monkeys naturally infected with SIV. Moreover, we recapitulate recent findings showing that various lineages of primate lentiviruses show major differences in Nef function and discuss the possible consequences for the outcome of viral infection. Other interesting model systems and further aspects of Nef function as well as detailed presentations of the underlying mechanisms and interactions have been summarized in recent reviews [1, 20–25].

Importance of specific Nef functions for viral pathogenicity

It has been established that a variety of Nef functions, such as down-modulation of CD4, CD28 and MHC class I (MHC-I) and enhancement of viral replication and infectivity, are conserved between HIV-1 and SIVmac239 [26–30]. SIVmac239 is a well-characterized molecular clone [31] that has frequently been used to study determinants of AIDS because it is highly pathogenic in experimentally infected Asian rhesus macaques [32], although it does not cause disease in its original host, the African sooty mangabey (SM) [33]. More recently, it has been shown that HIV-1 and SIVmac Nefs also down-modulate mature MHC class II (MHC-II) and up-regulate surface expression of Ii (CD74) associated with immature MHC-II molecules [11, 34]. Structure-function analyses revealed that these Nef functions usually require distinct elements and are often genetically separable [17, 28, 35–39]. This knowledge allowed the generation of SIVmac Nef mutants selectively impaired in some functions. As outlined below, the results obtained in the SIV/macaque model (see Table 1) and data derived from HIV-1-infected individuals imply that together with host factors a combination of genetically separable Nef activities contributes to the maintenance of high viral loads and development of disease in experimental and evolutionary recent primate lentiviral infections.

Down-modulation of CD4

The importance of CD4 down-modulation is already evident from the fact that HIV-1 utilizes three of its gene products, Vpu, Env and Nef, to down-regulate its primary receptor (reviewed in [40]). Nef is expressed early during HIV-1 infection. It removes CD4 from the cell surface by enhancing its endocytosis *via* recruitment to AP-2 adapter complexes and directing the receptor to lysosomes for degradation [36, 41–45].

Table 1. Overview on selected SIVmac239 Nef mutants analyzed in rhesus macaques[a].

SIVmac239 Nef allele	Modulation of					Enhancement of		*In vivo*	References
	CD4	CD28	CD3	MHC-I	Ii	Infectivity	Replication		
Wild type	+++	+++	+++	+++	++	+++	+++	Virulent	[2]
nef defective	–	–	–	–	–	–	–	attenuated	[2]
R17Y,Q18E	+++	+++	+++	++	++	+++	+++	acute pathogenicity	[125]
R16Y	+++	+++	+++	++	++	+++	+++	rapid disease	[98, 160]
EDR	–	–	+++	+++	++	–	–	early att./rev./rest.	[98, 52]
Δ64–67	–	–	+++	+++	+++	++	(+)	moderately attenuated	[53]
Y223F	+++	+++	+++	(+)	++	+++	+++	rapid reversion	[98, 90]
G238*/fs/fs	+++	+++	+++	–	++	+++	+++	strong CTL rev./rest.	[91]
Δ239–240	+++	+++	+++	–	++	+++	+++	strong CTL rev./rest.	[91]
tNef	–	–	+++	–	++	–	–	attenuated	[98, 161]

[a] Functional activity was measured by FACS or in *in vitro* assays for viral infectivity and replication as described [11, 98, 122]. The *in vivo* phenotype was examined in rhesus macaques. +++, high; ++, moderate; +, weak; (+), marginal activity; –, no activity; att., attenuated; rev., reversion; rest., restoration of function.

In contrast, Vpu and Env are expressed late during the viral life cycle and interfere with the transport of newly synthesized CD4 to the cell surface [47–49]. Importantly, only Nef acts on CD4 molecules that were already at the cell surface prior to HIV-1 infection and plays the most prominent role in CD4 down-modulation from HIV-1-infected T cells [50, 51].

A number of findings support that Nef-mediated CD4 down-modulation plays a relevant role in the pathogenesis of AIDS (reviewed in [40]). Point mutations in SIVmac Nef disrupting CD4 down-modulation but not most of its other functions (Table 1) attenuate viral replication in acutely infected macaques and eventually revert [52]. An SIVmac239 Nef mutant containing a difficult-to-revert deletion of amino acids 64–67 disrupting the ability of Nef to down-regulate CD4 and CD28 and to stimulate viral replication, but not down-modulation of CD3 and MHC-I, up-regulation of Ii and enhancement of virion infectivity, showed a phenotype intermediate between grossly *nef* deleted and wild-type SIVmac239 [53]. Thus, both the disrupted Nef functions and those that were not impaired by this deletion contribute to the pathogenicity of SIVmac in infected rhesus macaques. Notably, *nef* alleles from some LTNPs of HIV-1 infection are unable to down-modulate CD4 but are fully capable of performing other functions [54–57]. In further support of a relevant role in the pathogenesis of AIDS, it has been shown that *nef* alleles derived from AIDS patients and from SIV-infected macaques after the development of disease show increased activity in CD4 down-modulation [58, 59].

While it is accepted that CD4 down-modulation is a key function of Nef, it remains largely elusive which consequences of diminished CD4 cell surface expression are critical for HIV-1 and SIVmac pathogenesis. For example, CD4 down-modulation might weaken the antiviral immune response because CD4 interacts with MHC-II on antigen-presenting cells (APCs) and is an important costimulatory factor of T cell receptor (TCR)-mediated T cell activation [60]. Furthermore, it has been reported that CD4 down-modulation enhances the release and infectivity of HIV-1 particles [61–66]. This might explain why the efficiency of Nef-mediated CD4 down-modulation correlates with its ability to enhance HIV-1 replication in primary T cells and in *ex vivo*-infected human lymphoid tissues [67, 68]. However, Nef also enhances viral infectivity of HIV-1 particles produced in $CD4^-$ cells [69, 70]. Some effects on viral particle production and infectivity were only observed under artificially high Nef expression levels. Another reason why it might be advantageous for HIV-1 to down-modulate its primary receptor is to avoid superinfection [26, 51, 71, 72]. Dissecting which consequences of CD4 down-modulation are critical for efficient viral spread and persistence will be a very challenging task and most likely several aspects contribute to the importance of this Nef function *in vivo*.

Down-regulation of MHC-I molecules

Besides the effect on CD4, down-modulation of MHC-I is one of the best-defined Nef activities [10, 35, 39, 73]. Multiple studies have analyzed the mechanism(s) of this Nef function (reviewed in [21]). Altogether, they show that Nef interacts with the cytoplasmic tail of MHC-I [74] and utilizes at least two pathways to reduce its expression on the cell surface: (i) recruitment of AP-1 to the MHC-I cytoplasmic tail to re-route MHC-I from the trans-Golgi network (TGN) to lysosomes, and (ii) endocy-

tosis of MHC-I from the plasma membrane to the TGN in a PACS-1, AP-1 and clathrin-dependent manner [75–83]. However, the exact mechanisms are controversial [21]. Furthermore, the magnitude of the effects may be cell-type dependent, possibly because Nef inhibits export and increases turnover of MHC-I in HIV-1-infected T cells but mainly affects endocytosis in other cell types commonly used to study Nef function, such as HeLa-derived cell lines [76, 77].

Elegant *in vitro* experiments demonstrated that Nef-mediated removal of MHC-I from the cell surface protects HIV-1-infected cells against killing by cytotoxic T lymphocytes (CTLs) [84, 85], and is highly selective, *i.e.*, Nef specifically down-modulates HLA-A and –B but not HLA-C or -E alleles from the cell surface [86]. This selectivity is conserved between different groups of primate lentiviruses [87, 88]. The reason is most likely that reduced cell surface levels of MHC-I may increase the susceptibility of virally infected cells to lysis by natural killer (NK) cells. Thus, selective down-modulation of specific MHC types most likely allows HIV and SIV to balance escape from CTL lysis with protection from NK attack. The inability of HIV-1 to down-modulate HLA-C may explain why overexpression of HLA-C is protective in infected humans [89].

A major obstacle for conclusive studies on the relevance of specific Nef functions in the SIV/macaque model is that many mutations have pleiotropic effects. Mutations in the C-terminal domain of SIVmac239 Nef, however, selectively disrupt MHC-I down-regulation but no other known Nef function [28]. Experiments with such highly specific SIV Nef mutants clearly demonstrated that efficient MHC-I down-modulation is associated with a strong selective advantage and reduces $CD8^+$ T cell responses in infected rhesus macaques [90, 91]. MHC-I down-modulation by Nef seems to be of similar importance for viral immune evasion in HIV-1-infected individuals. For example, it has been shown that *nef* alleles obtained during chronic HIV-1 infection are frequently more active in down-modulating MHC-I than those from late stage AIDS patients [58], suggesting a strong selective pressure for this specific Nef function in immunocompetent hosts. In further support of this assumption it has been shown that a 36-bp deletion in *nef* alleles that impaired overall Nef function in an LTNP of HIV-1 was partially "repaired" by an adjacent duplication that restored the ability of Nef to down-modulate MHC-I and to enhance virus infectivity but not to down-regulate CD4 [54]. Finally, particularly strong HIV-specific CTL activity has been detected in individuals infected with *nef* defective HIV-1 strains [92]. Altogether, these studies clearly show that down-modulation of specific MHC alleles by Nef is an important immune evasion mechanism of primate lentiviruses.

Modulation of other receptors on T cells

Besides CD4 and MHC-I some Nef alleles are capable to modulate the surface expression of a substantial number of additional receptors on T cells, such as CD28 [93, 94], CXCR4 [95] and perhaps other chemokine receptors [72, 96]. Notably, many HIV-2 and SIVmac Nef alleles down-modulate CD28 and CXCR4 more efficiently than those of HIV-1 [51, 95] and also remove CD3 from the cell surface [19]. The possible relevance of these differences in Nef function for primate lentiviral pathogenesis is discussed below.

CD28 is a major costimulatory factor of T cell activation and critical for normal antigen-specific T cell responses. Thus, its removal from the surface of infected T cells may suppress the immune response and cause anergy. Studies of the Skowronski lab have shown that both HIV-1 and SIVmac Nef proteins interact directly with CD28 and use a similar mechanism to down-regulate this receptor as that established for CD4, which involves accelerated endocytosis *via* the AP-2 clathrin adaptor pathway [93]. In support of a selective advantage *in vivo*, it has been shown that an H196Q substitution in SIVmac Nef, which selectively disrupts its effect on CD28 [94], reverts in infected rhesus monkeys [97, 98]. However, these reversions occurred more slowly than those of other inactivating point mutations in *nef*. Thus, the selective advantage of CD28 modulation is only moderate, possibly because other Nef functions also affect TCR signaling.

Some SIV Nef alleles are highly effective in down-modulating the chemokine receptor CXCR4 from the cell surface and strongly inhibit lymphocyte migration to the CXCR4 ligand, the chemokine stromal cell-derived factor 1 (SDF-1α) [95]. In comparison, HIV-1 Nef proteins do generally not efficiently down-regulate CXCR4 [51, 95]. Nonetheless, they also inhibit lymphocyte migration to SDF-1α, albeit less efficiently than those of SIV, by activation of Rac2 and/or Rac1 *via* the DOCK2-ELMO1 guanine exchange factor [95, 99]. Thus, primate lentiviruses use at least two different mechanisms to inhibit trafficking of infected leukocytes, possibly to facilitate their dissemination or to impair the antiviral immune response [95]. It has also been suggested that Nef down-modulates CXCR4 from the surface of target cells to enhance their resistance to superinfection [100]. HIV-1 strains that utilize CXCR4 as entry cofactor emerge in approximately 50% of late stage AIDS patients (reviewed in [101]). However, Nef alleles from primate lentiviruses using CCR5 but not CXCR4 for

entry into target cells down-modulate CXCR4 with much higher efficiency than those of HIV-1 [51, 95] arguing against a role of this Nef function in preventing superinfection. It has also been reported that Nef down-modulates CCR5 and other chemokine receptors in stably transfected Chinese hamster ovary cells overexpressing various chemokine receptors [72, 96]. However, only marginal effects were observed in HIV-1-infected human indicator cell lines or in PBMC [51, 72, 96, 100]. Thus, the significance and specificity of these findings remains elusive.

Manipulation of antigen presenting cells by Nef

Recent data show that Nef not only reduces MHC-I cell surface expression but also affects MHC-II antigen presentation by two distinct mechanisms: (i) down-regulation of surface expression of mature MHC-II and (ii) up-regulation of the MHC-II-associated invariant chain (Ii, CD74) [11, 34]. APCs such as dendritic cells and macrophages, but also activated $CD4^+$ T cells, express MHC-II and are permissive to HIV-1 infection. Antigen-specific activation of T helper cells orchestrates the humoral and cellular immune responses and is crucial for an efficient anti-HIV immune response [102, 103]. It is well established that stable surface expression of Ii prevents antigen peptide presentation [104, 105] and might contribute to the impaired helper T cell responses observed in AIDS patients [106]. In HeLa CIITA cells and in the human monocytic THP-1 cell line, Ii surface expression is already efficiently up-regulated at low levels of HIV-1 Nef expression [11, 34]. Notably, marked Nef-mediated up-regulation of Ii could also be demonstrated in HIV-1-infected macrophages, whereas the effects on mature MHC-II expression were marginal [107].

In support of a relevant role *in vivo* it has been suggested that Ii up-regulation provides an advantage for viral replication in SIVmac-infected macaques [98]. Moreover, *nef* genes derived from some adult HIV-1-infected LTNPs do not up-regulate Ii [34]. However, Nef alleles derived from HIV-1-infected children with nonprogressive infection were significantly more active in up-regulation of Ii than those derived from rapid progressors [107]. It will be necessary to analyze larger sample numbers to challenge the possibility that effective Ii up-modulation may have a different impact on the clinical course of adults and perinatal HIV-1 infection. For example, strongly impaired MHC-II function might primarily contribute to lower levels of immune activation and decelerated loss of $CD4^+$ T cells in the context of an immature host immune system. Such studies seem highly warranted because of the fact that Ii up-modulation is conserved between different groups of primate lentiviruses and already observed at very low levels of Nef expression, suggesting an important role *in vivo* in HIV-1-infected individuals.

Notably, Nef also affects the function of macrophages, *i.e.*, it induces the production of two CC-chemokines, macrophage inflammatory proteins 1α and 1β, possibly to recruit and activate $CD4^+$ T cells at sites of virus replication [108]. Furthermore, it has been reported that Nef induces the release of soluble factors (sICAM-1 and sCD23) from macrophages that stimulate B cells to render resting T lymphocytes more permissive to HIV-1 infection [109] (Fig. 2). These findings suggest that Nef not only affects the activation status of the infected cells to generate a suitable environment for virus production [110] but also increases the susceptibility of the surrounding cellular reservoir to infection.

Nef-mediated enhancement of viral infectivity and replication

In addition of being a master manipulator of the function of T cells and APCs, Nef also directly enhances virion infectivity and stimulates viral replication [12–14, 29, 69, 70, 111]. The exact mechanisms of both effects are still not well understood and the overall effect of Nef on virus spread *in vivo* may depend on a large number of cellular properties and interactions (some of which are shown in Fig. 2). Enhancement of virion infectivity requires expression of Nef in the virus-producing cell [112] and involves an early step of the viral replication cycle [12, 70]. Furthermore, it seems to be dependent on the route of virus entry because Nef does not enhance the infectivity of HIV virions pseudotyped with envelopes mediating virus entry through an endocytic compartment rather than fusion at the plasma membrane [113, 114]. Nef is associated with cellular membranes and small quantities of Nef are present in virions [112, 115]. However, particle association of Nef seems dispensable for efficient HIV-1 infectivity [116]. Enhancement of cytoplasmic delivery by increased HIV-1 entry [117] (perhaps due to enhanced cholesterol content of progeny virions [118]), reduced susceptibility of virions to proteasomal degradation in the target cells [119], as well as facilitated transport of the viral genome through the cortical actin network [120], were all proposed to play a role in Nef-mediated HIV-1 infectivity enhancement. Recent data suggest that dynamin 2, a regulator of vesicular trafficking, is a binding partner of Nef that is required for its ability to increase viral infectivity [121]. Further studies are required to elucidate how Nef modifies progeny virions to enhance their infectivity. In agreement with a relevant role *in vivo* Nef-mediated infectivity enhancement is conserved between different groups

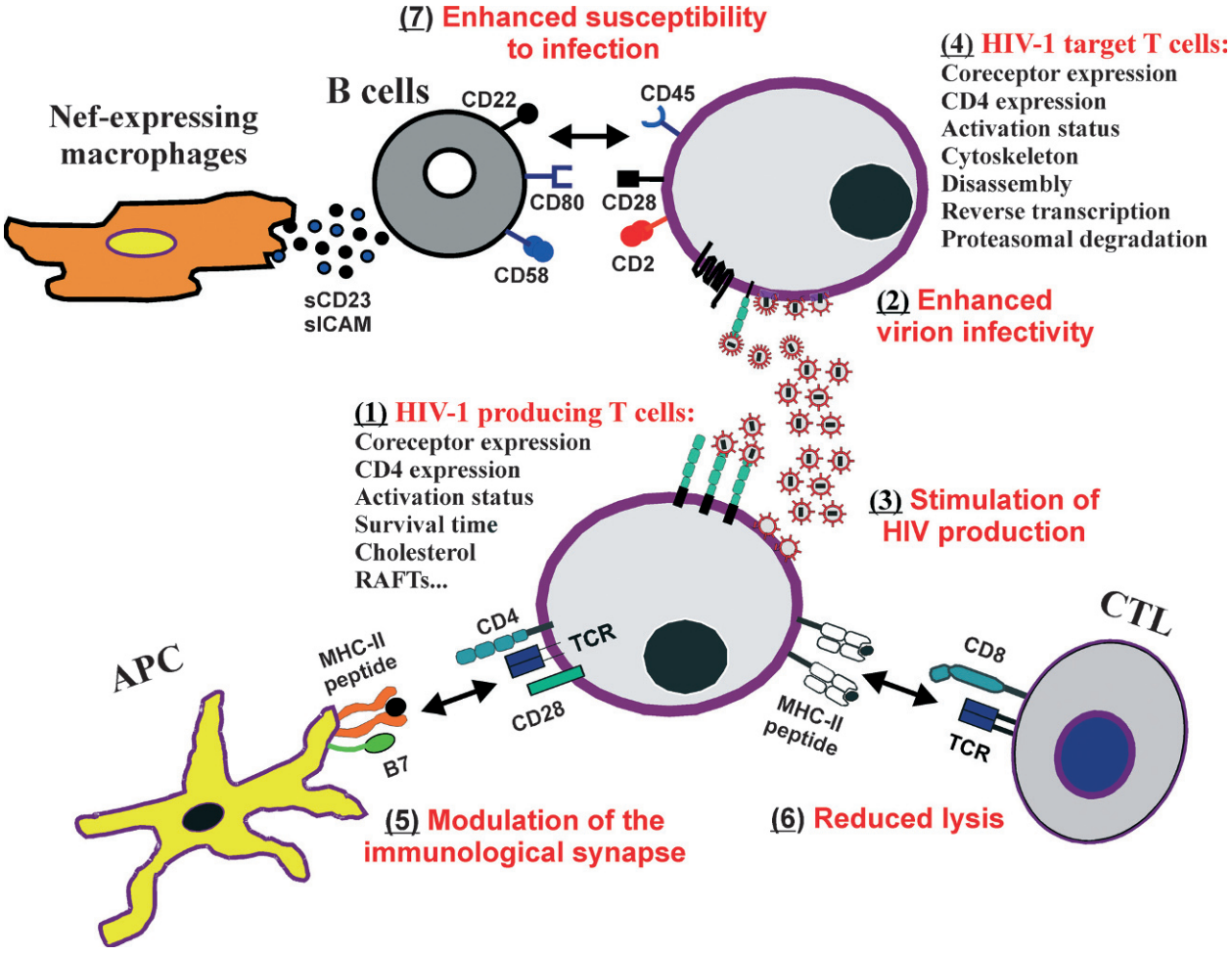

Figure 2. Complex role of Nef in HIV-1 replication. Nef manipulates various features of HIV-1-infected cells (1) to increase virus production (2) and enhance virion infectivity (3). The magnitude of the effects of Nef on virus infectivity and replication also depends on the properties of the target cells (4). Furthermore, Nef affects the activation status and life span of virally infected cells by modulating the interaction with antigen-presenting cells (APCs) (5) and reducing CTL lysis (6). Finally, it has been proposed that Nef induces soluble CD23 and ICAM in macrophages that up-regulate expression of costimulatory molecules on B cells, which interact with resting T cells to render them susceptible to HIV-1 infection (7) [109].

of primate lentiviruses [12, 18, 122] and apparently contributes to efficient spread of SIVmac in infected rhesus macaques [53]. However, usage of HeLa-derived cell indicator lines is a caveat of most studies on Nef-mediated infectivity enhancement and recent data suggest that the effects may be less pronounced in primary $CD4^+$ T cells [123]. To avoid possible artifacts it will be important to further define the effects of Nef on virion infectivity in producer and target cells relevant for viral spread *in vivo* in the infected host. Nef efficiently enhances HIV-1 replication in primary T cell cultures, particularly if these are exposed to HIV-1 prior to stimulation [13, 14], and in *ex vivo*-infected human lymphoid tissue (HLT) [111], but hardly in transformed T cell lines [14]. As mentioned above, the potency of CD4 down-modulation and not of infectivity enhancement by Nef correlates with the efficiency of viral replication in primary lymphocyte culture and *ex vivo*-infected HLT [67, 68]. In support of a relevant role in viral pathogenesis is has been shown that Nef alleles from AIDS patients are particularly active in promoting HIV-1 replication [58, 124]. Studies in the SIV/macaque model showed that effects of Nef on T cell activation also affect the clinical course of infection. For example, an SIVmac Nef variant containing an additional SH2 domain (YE-Nef) is highly active in causing T cell activation, replicates in unstimulated PBMC cultures and is acutely pathogenic in rhesus macaques [125]. Notably, introduction of the ITAM motif in Nefs also enhanced the virulence but not the levels of viral replication of SIVagm from African green monkeys and SIVsmm from SMs in experimentally infected pigtail macaques [126]. These results are evidence that enhanced activity of Nef in causing T cell activation is associated with increased virulence in

the SIV/macaque model. However, no HIV-1 Nef allele with a YE-Nef-like phenotype has been described and usually Nef does not directly activate T cells but rather sensitizes them for activation to allow effective viral spread. It is beyond the scope of the present review to discuss the complex effects of Nef on the transcriptional responses of host T cells but it is important to note that HIV-1 Nef may even be transcribed and modulate the transcriptional activity of resting T cells prior to integration [127]. Further studies are required to unravel the underlying mechanisms but it seems that at least three HIV-1 Nef activities, *i.e.*, CD4 down-modulation, alteration of T cell activation and enhancement of virion infection, contribute to efficient viral replication. Obviously, the relative importance of these Nef functions may depend on the initial cellular activation status and CD4 expression levels. *In vivo* in the SIV/macaque model the effect of Nef on virus replication is more pronounced during the chronic phase of infection [2]. Possible reasons are that Nef may be less important for virus replication in an inflammatory environment or that its immune evasion mechanisms are not critical for virus spread during acute infection prior to the onset of the adaptive immune response.

Lineage-specific differences of primate lentiviral Nef functions

Since it is the major causative agent of AIDS, most studies on Nef function have focused on HIV-1 and to a much lesser extent on HIV-2 that also causes disease in humans, as well as on SIVmac because infection of macaques is commonly used as an animal model for AIDS in humans. However, these viruses represent only a small fraction of primate lentiviruses. To date SIVs have been detected in about 40 African non-human primate species [128, 129]. Although all of them contain *nef* genes our current knowledge suggests that they do not usually induce disease in their natural monkey hosts [130, 131]. The recent analysis of HIV and SIV strains from 14 different primate species showed that several Nef activities, *i.e.*, the ability to down-modulate CD4, CD28 and MHC-I [19] but also to enhance virion infectivity and to stimulate virus replication [122], are conserved across most or all primate lentiviral lineages (summarized in Table 2). This was unexpected since many SIV Nef alleles show only about 30% amino acid identity to those of HIV-1 [132]. Thus, although primate lentiviral Nef proteins are highly variable some functional interactions are obviously well conserved. It is noteworthy, however, that in some cases the same activities are mediated by different domains in Nef proteins from different groups of HIV and SIV [28, 133–135], suggesting that they may have evolved independently during primate lentiviral evolution.

Analyses of Nef function from a wide variety of primate lentiviruses revealed that many activities are conserved but also identified lineage-dependent differences in the ability to modulate receptors involved in the interaction and communication between T cells and APCs (Table 2; Fig. 3). The most striking finding was that Nef alleles from the great majority of SIVs and HIV-2 down-modulate TCR-CD3 with high efficiency, whereas those of HIV-1 and its closest simian relatives from chimpanzees and some *Cercopithecus* monkeys generally failed to perform this function [19]. Interestingly, phylogenetic analyses revealed that Nef-mediated down-modulation of TCR-CD3 was lost twice during primate lentiviral evolution. Firstly, after a *vpu* gene was acquired by an ancestor of SIVgsn/mus/mon now found in *Cercopithecus* monkeys (Table 2) and secondly, when SIVrcm recombined with a *vpu* containing precursor of SIVgsn/mus/mon in chimpanzees to become SIVcpz [19, 136]. It will be interesting to clarify why Vpu reduces the selective pressure for Nef-mediated down-modulation of CD3. Recently, it has been shown that Vpu suppresses an IFN-α-induced host restriction factor, named tetherin (also called BST2 or HM1.24), to facilitate virion release [137]. Thus, while many alternative explanations exist, it is tempting to speculate that viruses carrying a *vpu* gene could afford to loose the ability to down-modulate CD3 and hence to cause higher levels of immune activation because they are able to counteract the host restriction induced by high levels of inflammatory IFN-α.

Efficient T cell activation by APCs requires the interaction of the antigen/MHC-II complex and a costimulatory signal mediated by the interaction of CD28 with B7 [138, 139] (Fig. 3A). HIV-1 Nefs interfere with this process by modulating CD4 surface expression and impairing MHC-II antigen presentation (Table 2, Fig. 3B). In comparison, *nef* alleles from most SIVs and HIV-2 Nefs impair the function of T cells and their interaction with APCs more severely because they also efficiently down-regulate CD3, CD28 and CXCR4 (Table 2, Fig. 3C), most likely to suppress T cell activation, migration and apoptosis. In agreement with its role as the key ligand of the TCR, Nef-mediated CD3 down-modulation was required and sufficient to prevent activation and programmed death in virally infected T cells [19]. Notably, *nef* alleles from different groups of primate lentiviruses differed fundamentally in their effect on the responsiveness of infected T cells to activation: those from HIV-2 and most SIVs blocked cellular activation and suppressed activation-induced cell death (AICD),

Table 2. Preliminary overview on the activity of primate lentiviral Nef proteins in selected assays[a].

Clone(s)	Species/subspecies	CD4	MHC-I	CD3	CD28	Replication	Infectivity
HIV-1 M	Human (*Homo sapiens*)	+++	++	−	(+)	++	++
HIV-1 O	Human (*Homo sapiens*)	+++	++	−	(+)	++	++
HIV-1 N	Human (*Homo sapiens*)	+++	++	−	(+)	++	++
SIVcpz	Centrl West. Chimp. (*Pan t. troglodytes*)	+++	++	−	+	++	++
SIVcpz	Eastern Chimp. (*Pan t. schweinfurthii*)	+++	++	−	+	++	++
SIVgsn	Greater spot-nosed monkey (*C. nictitans*)	+++	++	−	+	++	+++
SIVmus	Mustached monkey (*C. cephus*)	+++	++	−	(+)	++	++
SIVmon	Mona monkey (*Cercopithecus mona*)	+++	++	−	+	++	+++
SIVrcm	Red-capped mangabey (*C. torquatus*)	+++	(+)	+	−	++	++
HIV-2	Human (*Homo sapiens*)	+++	++	++	++	+	++
SIVsmm	Sooty mangabey (*Cercocebus atys*)	+++	++	++	++	++	+++
SIVmac	Rhesus macaque (*Maccaca mulatta*)	+++	++	++	++	++	+++
SIVdeb	De Brazza monkey (*C. neglectus*)	++	+	+++	++	+++	+++
SIVsyk	Sykes' monkey (*C. albogularis*)	+++	+	++	++	++	++
SIVblu	Blue monkey (*Cercopithecus mitis*)	+++	++	+++	+++	++	++
SIVsun	Sun-tailed monkey (*C. solatus*)	++	+	+++	+	++	++
SIVagm	Tantalus monkey (*Chlorocebus tantalus*)	+++	++	+++	+++	++	++
SIVagm	Green monkey (*Chlorocebus sabaeus*)	++	++	+++	+++	++	++

[a] The properties of most primate lentiviral Nef proteins shown have recently been described [19, 122]. Note that the data are preliminary because for some species only a very limited number of *nef* alleles has been analyzed and most data were generated in human-derived cells. Abbreviations and symbols: see Table 1.

whereas *nef* alleles derived from HIV-1 and its simian counterparts usually increased the responsiveness of virally infected T cells to stimulation and did not prevent cell death [19]. The latter result is consistent with previous studies suggesting that the HIV-1 Nef contributes to the high levels of immune activation and apoptosis associated with progression to AIDS in infected humans by enhancing the responsiveness of virally infected T cells to activation [19, 140–146]. Based on the observation that Nef affects several aspects of the functional interaction between T cells and APCs, it has been suggested that Nef may uncouple T cell activation from the antigen-specific interactions of T cells with APCs to facilitate virus replication [17]. In agreement with this hypothesis it has been shown that the HIV-1 Nef protein impairs the formation of the immunological synapse [147] and triggers a transcriptional program in Jurkat T cells that is highly similar to anti-CD3 T cell activation [110]. Whether or not HIV-2 and SIV Nefs trigger similar signaling pathways in T cells derived from the respective host species remains to be determined. However, the result that primary T cells infected with viruses expressing *nef* alleles that down-modulate CD3 show substantially lower expression levels of activation markers and apoptosis compared to those infected with otherwise isogenic viruses containing *nef* alleles unable to perform this function suggests that overall HIV-2 and most SIV Nefs suppress rather than enhance T cell activation [19].

Possible implications of differences in Nef function for HIV and SIV pathogenesis

Nef is commonly considered a "virulence" factor because disrupted *nef* genes are associated with an attenuated clinical course in HIV-1 and SIVmac infection [2–4]. Studies in transgenic mice [148] and the finding that Nef may render HIV-1-infected T cells hyperresponsive to activation [142] support a direct role in the pathogenesis of AIDS. Other lines of evidence suggest, however, that Nef may only accelerate the development of disease in HIV-1-infected individuals and in SIVmac-infected macaques because it drastically enhances the viral loads and numbers of virally infected cells and not because it directly increases the virulence of these primate lentiviruses. Primary T cells infected with *nef* deleted HIV-1 constructs show high levels of activation and apoptosis upon stimulation [19]. Moreover, some humans and rhesus macaques infected with grossly *nef* deleted forms of HIV-1 and SIVmac developed signs of immunodeficiency in the absence of detectable virus loads [6, 7, 149]. Finally, *nef* deleted SIV is pathogenic in neonatal macaques [8], possibly because Nef is less critical for effective replication in the absence of a functional mature immune system. Thus,

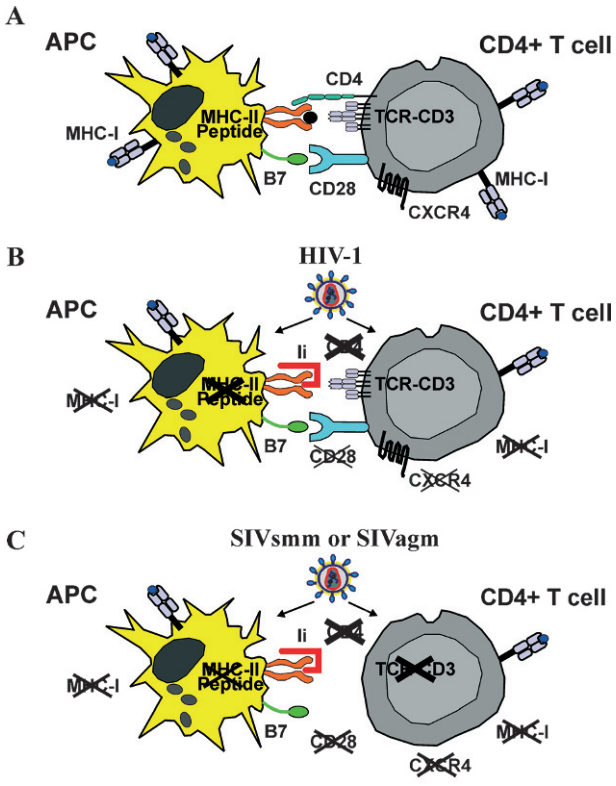

Figure 3. Manipulation of T cell/APC interaction by primate lentiviral Nef proteins. Schematic presentation of (*A*) the interaction between uninfected APCs and CD4$^+$ T cells and the effect of (*B*) HIV-1 and (*C*) SIVsmm or SIVagm infection on specific receptors expressed by these cell types. Receptors down-modulated by the *nef* alleles of the respective viruses are crossed out and erased if the effects are highly effective. Note that this outline is preliminary because some effects remain to be demonstrated in primary cells from the respective primate species.

even highly attenuated HIV-1 and SIV strains with *nef* deletions are ultimately pathogenic in humans and macaques, respectively, if they replicate to significant levels.

Importantly, the high viral loads associated with infection by primate lentiviruses expressing functional Nef only lead to the development of immunodeficiency in poorly adapted hosts, like humans, who acquired HIV-1 and HIV-2 very recently in the first half of the last century [128, 129, 150–152], or Asian macaques, which are not a natural host of SIV [153]. In contrast, SIVs seem to replicate to high levels and persist efficiently in their natural monkey hosts without causing disease [130–132]. Further work is required to fully elucidate the reasons for the different clinical outcome of natural and recent or experimental primate lentiviral infections. However, an increasing number of studies suggests that deregulation of T cell function and high levels of chronic immune activation and programmed cell death drive the development of AIDS [130, 131, 154–156]. Low levels of chronic T cell activation resulting in reduced proliferation and apoptosis might allow SIV-infected mangabeys or African green monkeys to maintain functional helper T cell responses [130–132]. As outlined above, SIVsmm and SIVagm Nefs affect the function of T cells to activation much more severely than those of HIV-1 because they efficiently down-regulate CD3 and CD28 (Table 2). These functions should reduce the stimulation of virally infected CD4$^+$ helper T cells by APCs and might be advantageous for both the virus and its host. Inefficient CD4$^+$ helper T cell activation would weaken the antiviral immune response and might allow the virus to persist at high levels. However, reduced T cell activation, proliferation and apoptosis might also allow the host to maintain a functional immune system. In agreement with this hypothesis, inefficient down-modulation of TCR-CD3 correlates with low CD4$^+$ T cell counts in SIVsmm-infected SMs [19]. In other words, a more "HIV-1-like" Nef phenotype of SIVsmm, correlates with declining CD4$^+$ T cell counts and hence a course of infection more reminiscent of pathogenic HIV-1 infection in natural SIV infection. At first, it may seem strange that down-modulation of CD3, which is critical for the function of T cells, protects against the loss of CD4$^+$ T cells. Thus, the functionality of the small fraction of CD4$^+$ T cells is presumably not critical for the overall immune competence of the infected host. However, the rate at which these virally infected T cells die and must be replaced, might exhaust the regenerative capacity of the host immune system. Furthermore, hyperactivated helper CD4$^+$ T cells likely contribute to high levels of immune activation by sequestering cytokines that induce the migration, inflammatory response and death of uninfected bystander cells. Thus, while Nef may act to uncouple T cell activation from interaction with APCs, it obviously also helps to prevent the escalation of immune activation to harmfully high levels at least in natural SIV infection. It is conceivable that Nef alleles that down-modulate the key ligand CD3 and the major costimulatory molecule CD28 of T cell activation are particularly well suited to exert protective effects.

Altogether, our current knowledge suggests that Nef limits the damaging effects of high levels of SIV replication in the majority of natural primate lentiviral infections by suppressing the activation and programmed death of infected CD4$^+$ T cells [19]. However, it is obvious that a large number of host factors also play an important role in AIDS progression (reviewed in [157]). For example, SIV from SMs is usually non-pathogenic in its natural host, moderately pathogenic in humans and highly virulent in macaques [131, 158]. Thus, even *nef* alleles that down-regulate TCR-CD3

and inhibit the responsiveness of infected T cells to activation are unable to prevent the escalation of immune activation to harmfully high levels in non-adapted hosts that are highly susceptible to disease. For example, the fact that the SIVmac239 molecular clone frequently causes fatal disease in rhesus macaque within 1 year after infection [32] seemingly argues against a protective role of Nef-mediated down-modulation of CD3 *in vivo*. However, SIVmac239 does not cause disease when reintroduced in its original host, the SM [33, 159]. Thus, the terms "pathogenic or virulent" and "non-pathogenic or apathogenic" should be used with the understanding that they represent relative and not absolute terms because the clinical and virological outcome of infection depends on a complex interplay between many viral and host factors. Notably, mutations in Nef that increase its ability to cause T cell activation result in acute pathogenicity in SIV-infected macaques [125, 126, 160], whereas a virus strain expressing a Nef allele that down-modulates CD3 but is otherwise defective was even more attenuated than an entirely *nef* defective SIVmac strain [161]. Thus, increased levels of T cell activation obviously accelerate disease progression also in the experimental macaque host. Furthermore, it is also well established that HIV-2, which is closely related to SIVmac and also originates from SIVsmm-infected mangabeys [151], is less virulent than HIV-1 in the human host [162, 163]. Thus, when compared in the same human host the SIVsmm/mac/HIV-2 group seems to be less virulent than the SIVcpz/HIV-1 group. Notably, SIVsmm usually expresses functional Nef proteins and the infection in its natural simian host is asymptomatic despite high levels of viral replication [19, 131, 164]. In contrast, high frequencies of defective *nef* alleles and low viral loads are frequently found in HIV-2-infected individuals with nonprogressive infection [165–169]. Thus, the reasons for asymptomatic infection may usually be different in the natural SM and the evolutionary recent human host.

It has been proposed that chimpanzees may not develop high levels of chronic immune activation in response to infection because their T cells are less responsive to TCR stimulation than those of humans [170]. This is an interesting hypothesis and differences in the responsiveness of different primate species may play a relevant role in the pathogenesis of AIDS [33, 159, 171]. It is also important to consider, however, that although it is commonly assumed that SIVs do not usually cause disease in their natural hosts, convincing experimental evidence has only been presented for SIVsmm and SIVagm, which both efficiently down-modulate TCR-CD3 as well as CD28 (Table 2). However, even natural SIVsmm infection is not always asymptomatic [172], and it is currently unclear whether SIVgsn, SIVmus and SIVmon that do not down-modulate CD3 nor inhibit cellular activation and AICD may cause disease in their respective hosts. Interestingly, SIVgsn and SIVmon are only found in up to 5% of animals of their respective primate host species [173]. This is very low compared to the prevalence of SIVsmm and SIVagm in SMs and AGMs, respectively, which frequently exceeds 50% [174]. Clearly, a lot more remains to be done to clarify the basis for this different distribution of the different groups of SIV. Nonetheless, the finding that SIVagm, SIVsm, SIVdeb and SIVsyk, all expressing *nef* alleles that down-regulate CD3, show a much higher seroprevalence in their host species than SIVgsn, SIVmon and SIVcpz, which are all unable to modulate this receptor, supports the hypothesis that this function might be beneficial for both the host and the virus. It will be of high interest to further challenge the dogma that SIV does not cause disease in its natural simian hosts.

A well-balanced virus-host relationship is obviously common in well-adapted natural but not in experimental or recent primate lentiviral infections. For example, infection of rhesus macaques with SIVmac239 constructs expressing HIV-1 *nef* alleles (called Nef-SHIVs) from a different genomic location and at markedly reduced levels resulted in rapid disease in about half of infected macaques, whereas the remaining usually controlled Nef-SHIV replication very efficiently and remained asymptomatic [175–177]. This "all or nothing" phenotype – elimination of either the virus or the host – is essentially the opposite from natural SIV infection where both parties coexist. Notably, these observations were made after infection of rhesus macaques with isogenic molecular Nef-SHIV clones. Thus, subtle differences in the properties of the non-adapted host can result in a totally different clinical and virological outcome of viral infection. The observation that Nef-SHIVs that are unable to down-modulate CD3 usually show an "all or nothing" phenotype is in agreement with a role of this Nef function in "balanced" virus-host relationships.

Summary and perspectives

Primate lentiviral Nef proteins generally perform a large number of functions, such as modulation of CD4, MHC-I and Ii surface expression as well as enhancement of virion infectivity and replication. The combination of these activities obviously helps the virus to persist efficiently in the infected host by facilitating evasion of the immune system and by increasing virus spread in a direct manner. The resulting high viral loads are associated with disease in poorly adapted hosts but not in natural SIV infection. Hence Nef

should be considered a "persistence" rather than a "virulence" factor. It is obvious that a large number of inherent host and viral properties determine the different clinical outcome of primate lentiviral infections [89, 157, 178, 179]. Nef performs a number of functions that should dampen the antiviral immune response and most likely prevent the escalation of immune activation to levels that are harmful to the host in natural SIV infection. The evolutionary loss of the most effective Nef function in suppressing the responsiveness of virally infected T cells to activation, *i.e.*, down-modulation of TCR-CD3, may contribute to the high levels of chronic immune activation and loss of $CD4^+$ T cells associated with HIV-1 infection [19]. Further studies in appropriate SIV/monkey models, such as SIV-infected SMs or AGMs, may teach us how the destruction of the host immune system can be prevented and hence offer new prospects for the therapy of AIDS. Moreover, it is conceivable that primate lentiviruses have "learned" to manipulate exactly those immune functions that are most relevant for the control of virus replication. Lack of Nef-dependent immune evasion functions may also explain why infection with attenuated *nef*-deleted strains of SIV exerts strong protective immune responses [180]. Further studies aiming to elucidate how HIV and SIV evade the host immune system should help to learn how better immune control can be achieved and may help to design vaccines with improved efficacy.

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