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HIV-1 Nef and T-cell activation: a history of contradictions

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Abstract

HIV-1 Nef is a multifunctional viral protein that contributes to higher plasma viremia and more rapid disease progression. Nef appears to accomplish this, in part, through modulation of T-cell activation; however, the results of these studies over the past 25 years have been inconsistent. Here, the history of contradictory observations related to HIV-1 Nef and its ability to modulate T-cell activation is reviewed, and recent reports that may help to explain Net's apparent ability to both inhibit and activate T cells are highlighted.

Keywords

HIV-1; Nef; pathogenesis; replication; signal transduction; T-cell activation; T-cell receptor

HIV-1 is a major global health challenge. In 2011, approximately 34 million individuals were living with HIV-1, resulting in 2.5 million new infections and 1.7 million deaths [1]. HIV-1 replicates predominantly in $CD4^+$ T cells, which serve a critical role as 'helper' cells for the immune system [2]. Loss of $CD4^+$ cells as a result of infection impairs host immunity and increases morbidity and mortality associated with normally benign infections and cancers, resulting in AIDS [3,4]. The HIV-1 Nef protein contributes to high plasma viremia and disease progression [5–8], in part, through modulation of T-cell activation, including cytokine expression, transcription factor function and other signal transduction events [9]; however, the results of these studies have been inconsistent [10]. Here, the authors review the history of contradictory observations related to Nef and T-cell activation, and highlight new reports that may help to explain Nef's apparent ability to both inhibit and activate T cells. A better understanding of the role of Nef in HIV-1 pathogenesis may enhance treatment strategies and assist in the development of novel approaches to curing HIV-1.

HIV-1 Nef: a 'negative factor'?

Nef is expressed early following infection of the host cell [11]. This approximately 206-amino acid, 24–32-kDa protein can manipulate host cell processes, including receptor

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endocytosis, protein degradation and intracellular signaling, which enhances *in vivo* replication, progeny production and immune evasion [5,12,13]. Many Nef functions have been identified, of which downregulation of the viral entry receptor CD4 and host immune molecules HLA-I are most well characterized [5,12,13]. Nef is modified by myristoylation and it associates with intracellular membranes. Localization of Nef to the plasma membrane is required to downregulate CD4 and HLA-I. Cytoplasmic- and cytoskeleton-bound fractions of Nef may modulate other cellular activities through interactions with intracellular kinases, protein-sorting complexes and actin network components [14].

Early studies of HIV-1 'open reading frame b' suggested that it inhibited virion production and viral replication [15,16], resulting in it being called 'negative factor' or Nef. Subsequent reports indicated that Nef silenced gene expression, supporting the notion that Nef dampens T-cell stimulation to prolong viral progeny production [17,18]. However, a series of later studies observed that Nef enhanced HIV-1 replication in primary cells. Spina *et al.* demonstrated that Nef-deleted viruses replicated poorly in quiescent $CD4^+$ T cells [19] and Miller *et al.* highlighted the ability of Nef to enhance virion infectivity [20]. Therefore, contrary to its name, a consensus has emerged that Nef enhances infection. This model of Nef as a 'positive factor' is consistent with *in vivo* observations that Nef-deleted strains of HIV and SIV are less pathogenic [7,8,21].

Relevance of T-cell activation for HIV-1 pathogenesis

Antigen-stimulated ('activated') $CD4^+$ T cells are highly permissive to HIV-1 infection [22,23], due, in part, to increased metabolic activity and reduced expression of intrinsic antiviral host restriction factors [24–26]. Maintenance of a 'semi-activated' state may be required for efficient production of viral progeny [27,28], since over-stimulation results in activation-induced cell death [29] that would shorten the lifespan of infected cells.

In addition to a productive replication cycle, HIV-1 establishes latent infection in resting $CD4^+$ T cells, allowing persistence of proviral genomes, despite immune responses or initiation of drug therapy [30]. Recent studies have begun to elucidate the viral and cellular mechanisms involved in the establishment and maintenance of latency [30,31], and differences in T-cell activation state are likely to play a role. An improved understanding of the factors involved in regulating HIV-1 latency could provide new avenues for treatment or lead to strategies capable of eradicating the virus from an infected individual [30].

$CD4^+$ T-cell activation occurs when its T-cell receptor (TCR)/CD3 complex engages a foreign peptide presented by HLA class II on an APC (Figure 1). TCR recognition initiates a series of events, including rearrangement of the actin cytoskeleton, stabilization of adhesion molecule binding and recruitment of new proteins to the plasma membrane [32]. This results in the formation of a TCR–APC contact zone, referred to as an 'immune synapse', which further enhances TCR signaling. A cascade of phosphorylation events ensues, involving p56-Lck and ZAP-70, adapter protein SLP-76, transmembrane protein LAT and others. These proximal events initiate distal signaling pathways, including MAPKs, PKC-θ and Ca^{2+}/ Calcineurin, resulting in translocation of three critical transcription factors into the nucleus: nuclear factor of activated T cells (NFAT), AP-1 and NF-κB.

The affinity and kinetics of TCR binding determines the effectiveness of signaling [33]. Induction of IL-2 expression requires stimulation of NFAT, AP-1 and NF-κB transcription factors (Figure 2A), and this cytokine is therefore a hallmark of robust $CD4^+$ T-cell activation. Secreted IL-2 enhances T-cell proliferation and is crucial for immunity against infection [34]. Notably, the HIV-1 long terminal repeat (LTR) promoter sequence also encodes binding sites for these transcription factors (Figure 2B) and TCR-mediated signaling events, among others, can reactivate latent proviral genomes [35].

Effects of HIV-1 Nef on *IL-2* gene expression

To investigate the impact of Nef on T-cell activation, its ability to modulate IL-2 has been assessed. Most reports indicate that Nef alters *IL-2* expression; however, both positive and negative effects of Nef have been observed using similar experimental approaches.

Initial studies indicated that Nef reduced the ability of Jurkat T cells to induce IL-2 mRNA or to activate IL-2 promoter-driven gene expression following stimulation with phorbol 12-myristate 13-acetate (PMA)/phytohemagglutinin or CD3 antibody [36,37]. However, a series of reports observed the opposite effect, namely that Nef enhanced IL-2 expression. Rhee *et al.* found that Nef augmented CD3 -mediated activation of a murine T-cell hybridoma to produce IL-2 38]. Nef was subsequently shown to increase IL-2 secretion following CD3/CD28 stimulation using both Jurkat cells and peripheral blood mononuclear cell [39,40]. Other results using Jurkat cells indicated that Nef protein or HIV-1 infection enhanced IL-2 promoter-driven gene expression [41,42], and indicated that myristoylation, the proline-rich SH3 -binding motif, and the PAK2 binding motifs of Nef were required for this function [43].

Further complicating the story, Nef was shown to enhance IL-2 production in resting $CD4^+$ cells following stimulation with PMA-ionomycin, but not CD3/CD28 [44]. Arhel *et al.* observed that HIV-1 Nef did not affect IL-2 production in virus-infected primary cells [45], and Thoulouze *et al.* found that although HIV-infected Jurkat cells displayed increased IL-2 production compared with uninfected cells, Nef-deleted viruses resulted in higher levels of IL-2 compared with wild-type HIV-1 [46].

Regulation of key transcription factors & signaling molecules by Nef

In the context of Nef and T-cell activation, the transcription factors NFAT, AP-1, and NF-κB, the tyrosine kinase p56-Lck, and the cytoskeleton modulator PAK2 have received greatest attention. We briefly review key observations for each here.

NFAT

The NFAT family of transcription factors is activated by TCR-mediated events through the Ca^{2+}/calmodulin (Calm) pathway, and is critical for induction of IL-2 [47]. TCR binding initiates an inositol triphosphate (IP) signaling cascade, leading to an influx of calcium into the cytoplasm, which binds Calm and, in turn, activates the Calm-dependent serine/threonine phosphatase Calcineurin (CaN). Dephosphorylation of cytoplasmic NFAT family members NFATl and NFAT2 by CaN allows their translocation into the nucleus and their binding to promoter sequences 48]. Since NFAT binding sites are present in the HIV-1 LTR, over-expression of NFAT-induced viral replication in resting T cells [49,50].

Nef has most often been described to hyperactivate NFAT [51,52]. Wang *et al.* observed that Nef localized to membrane lipid rafts and that increased IL-2 secretion following CD3/CD28 stimulation was associated with enhanced NFAT signaling [40]. NFAT was also determined to contribute to increased Nef-mediated responsiveness of infected $CD4^+$ T cells [53]. Other groups have observed similar results using NFAT promoter-driven luciferase constructs [42]. Nef may also activate NFAT in the absence of TCR signaling through synergistic interactions with the Ras/MAPK pathway [52] or through direct binding to IP_3 receptors (IP_3R), causing release of intracellular calcium stores [54]. TCR-independent activation of NFAT appears to be conserved among *Nef* alleles and is dependent on Nef motifs involved in membrane localization and SH3 binding [51]. Recent studies suggest that Nef's ability to modulate NFAT following TCR activation may be dependent on the status

of the cell, since Nef hyperinduces NFAT and IL-2 in quiescent T cells, but impairs NFAT and IL-2 in suboptimally activated T cells [55].

In contrast with these results, inhibition of NFAT-driven luciferase was observed when Jurkat T cells were transfected with a Nef-CD8 fusion protein and stimulated with peptide-pulsed APC [56]. Of interest, this function was associated with Nef-mediated modulation of Vav, possibly using PAK2 as an intermediary.

AP-1

The AP-1 transcription factor is a heterodimer of cFos and cJun proteins activated by MAPK. There are three major members of the MAPK family: ERK, JNK and p38. cFos is activated by ERK, while cJun is activated by JNK. MAPK cascades are essential for the regulation of cell differentiation, proliferation and death [57], and AP-1 binding contributes to the expression of numerous cellular genes, including *IL-2* [58]. The HIV-1 genome encodes three adjacent AP-1 binding sites located within the R5 region of the LTR (Figure 2B) and three intragenic AP-1 sites within the *pol* gene, which contribute to viral replication [59]. Other studies have demonstrated that ERK activity is required for HIV-1 infectivity [60] and reverse transcription [61], as well as virion assembly and release [62].

Early studies reported that HIV-1 Nef inhibited AP-1 activity through an effect on TCR-dependent signaling [18,63]. Greenway *et al.* also demonstrated that Nef's proline-rich domain bound to MAPK and inhibited its kinase activity [64]. These findings contrast with subsequent studies, which have described either little effect of Nef on AP-1 [65] or increased activation of MAPK by Nef. Fortin *et al.* reported higher AP-1 activity due to Tat and Nef in virus-infected T cells 53]. Schrager *et al.* indicated that Nef increased ERK activity following TCR stimulation [66]. These results are consistent with Witte *et al*, who reported that Nef enhanced Tat-mediated HIV transcription through an Lck-mediated process that required stimulation of ERK by PKC-8 [67]. Activation of cJun by JNK is also required for AP-1 function, and HIV-1 Nef has been reported to induce the JNK pathway through interaction with the guanine nucleotide exchange factor, Vav [68]. However, in other studies, Nef failed to enhance JNK activity in the presence or absence of TCR stimulation [66].

NF-κB

The NF-κB transcription factor plays a key role in cellular activation and proliferation. It is a heterodimer comprised of one ankyrin repeat-containing subunit (p50 or p52) and one trans-activation domain-containing subunit (p65/ RelA, RelB or c-Rel). Cytoplasmic NF-κB is sequestered by I-κB and activated by various stimuli, including TCR activation coupled with a costimulatory signal typically provided by CD28 [69]. Binding of CD28 to CD80 (or CD86) on the APC activates PI3K which triggers Akt and then I-κB kinase to phosphorylate I-κB, resulting in its ubiquitinylation and degradation, thereby releasing NF-κB for translocation to the nucleus [69]. NF-κB binding sites located in the HIV-1 LTR are critical for viral replication [70–73].

Consistent with early evidence that Nef inhibited IL-2, initial studies indicated that Nef suppressed NF-κB by interfering with a TCR-derived signal [63,74]. In contrast, other reports suggested that Nef enhanced NF-κB activity. Wang *et al.* observed that Nef recruitment to membrane lipid rafts promoted NF-κB [40]. Fortin *et al.* reported that Nef increased NF-κB nuclear translocation in Jurkat cells after stimulation with PMA-ionomycin or CD3/CD28 [53]. Yet another series of studies indicated that Nef had no effect on NF-κB: Collette *et al.* observed that CD28 signaling did not contribute to Nef-mediated modulation of IL-2 [75], and Yoon *et al.* reported that there was no influence of Nef on NF-κB or AP-1

[65]. Furthermore, Schrager *et al.* found that Nef did not alter I-kB phosphorylation in $CD4^+$ T cells [66]. Echoing these results, a report by Witte *et al.* observed that induction of HIV-1 transcription by Nef involved Lck and PKC-θ recruitment to membrane lipid rafts, but resulted to activation of ERK rather than NF-κB 67]. In addition, Neri *et al.* demonstrated that Nef had no effect on early substrates in the NF-κB pathway (including I-κBα) or phosphorylation of NF-κB following stimulation with CD3/CD28 [55].

p56-Lck

NeF's proline-rich motif binds to the SH3 domain of Lck, a member of the Src family of protein tyrosine kinases [76–78]. Lck is a 'master regulator' of TCR signaling that is recruited to the plasma membrane by CD4. Upon TCR engagement, Lck phosphorylates immunoreceptor tyrosine-based activation motifs on the TCR/CD3C chain. Phosphorylated TCR recruits ZAP-70 kinase, which is also phosphorylated by Lck. Activated ZAP-70 phosphorylates downstream signaling molecules, including transmembrane protein LAT and adapter protein SLP-76. Of note, Nef is reported to interact with other Src family kinases, including Hck and Fyn [79–81], which may alter intracellular signaling.

CD4-Lck binding & altered Lck localization—Bandres *et al.* observed that Nef-mediated downregulation of CD4 was more efficient in the presence of Lck [82]; however, other studies indicated that Nef and Lck interact with the same hydrophobic motif on CD4's tail 83]. Furthermore, Nef's proline-rich motif is dispensable for CD4 downregulation [78] and Nef's ability to disrupt the CD4-Lck complex and to target CD4 to lysosomes for degradation are genetically separable [84,85]. Nef inhibits Lck clustering at the TCR, and Lck is instead retained in endosomal compartments [46,86,87]. Haller *et al.* further demonstrated that Nef uses distinct mechanisms to retain Lck within these compartments and to block Lck recruitment to the TCR [86], and Rudolph *et al.* have indicated that this function is conserved among HIV and SIV Nef proteins [88]. These results suggest that Nef displaces Lck from CD4 and redistributes it to an intracellular location.

Modulation of Lck phosphorylation—Several studies have indicated that Nef inhibits Lck phosphorylation and blocks its ability to activate downstream targets [64,76,77,89,90]. Reduced Lck phosphorylation was associated with Nef's ability to disturb immune synapse formation [46,86], for which Nef's proline-rich motif appeared to be essential [87]. However, Abraham *et al.* reported that Nef did not reduce Lck's ability to phosphorylate its immediate downstream target, ZAP-70 [91]. Rather, they observed that Nef blocked subsequent steps required for TCR signaling: namely LAT recruitment to TCR microclusters, LAT phosphorylation, and the interaction between LAT and SLP-76 [91]. This is consistent with previous work by Haller *et al*, which also found that Nef inhibited the recruitment of phosphorylated LAT to the immune synapse [87]. In contrast, other studies have concluded that Nef activates Lck, which, in turn, stimulates PKC-8 and upregulates ERK signaling [67,92].

PAK2

P21-associated kinases (PAKs) are serine/threonine kinases that regulate the small GTPases Cdc42 and Rac. PAKs are involved in early signaling events that lead to MAPK [93], and they function in cytoskeleton remodelling and apoptosis [94]. PAK2 mutants that lack the kinase domain block TCR-induced CD69 upregulation, Ca^{2+} flux, NFAT activation and IL-2 production in T cells [95]. Sawai *et al.* first reported the presence of a Nef-associated kinase (NAK) [96], and Nef binding to NAK was found to depend on Cdc42 and Racl [97]. NAK was identified as a PAK family member [98–100], and Renkema *et al.* determined it to be PAK2 101]. Mutational analysis studies have identified amino acids within HIV-1 Nef that are critical in the interaction of Nef with PAK2. The Nef myristoylation residue G2,

SH3-binding domain and the highly conserved arginine R105/R106 were shown to be required for Nef interaction with PAK2 [102,103]. Agopian *et al.* further demonstrated that Nef amino acids at positions 85, 89, 187, 188 and 191 (L, H, S, R and F in the clade B consensus, respectively) are critical for PAK2 association [104]. Using primary Nef isolates Foster *et al.* found that Nef mutants S189R and F193I were defective for Nef-FAK2 association [105]. Mutational analysis of PAK2 revealed that an intact Cdc42–Racl interactive binding motif was required for Nef–PAK2 interaction [106]. While the majority of Nef research has been conducted on PAK2, other studies have indicated that PAK1 contributes to NAK activity [107], and that PAK1 may be important for Nef-mediated enhancement of virion production [108].

Nef association with PAK2 is a conserved of property of HIV-1, HIV-2 and SIV. This interaction occurs in membrane lipid rafts and forms a multiprotein complex comprised of Nef, PAK2, PI3K, GTPases Rac and Cdc42, and Vavl [109,110], referred to as the Nef–PAK2 signalosome. Through interactions with PI3K, Nef–PAK2 may increase virion production [109] and prevent apoptosis [111]. More recently, it was observed that PAK2 binding is important for Nef-mediated enhancement of cellular activation and viral replication in primary T cells [43]. TCR-mediated remodeling of the actin cytoskeleton is necessary for cellular activation [112,113]. Nef associates with actin [114] and Nef-PAK2 can inhibit actin rearrangement [86,87]. Nef has been shown to modulate the key actin organizer WASp [87] and to deregulate the actinsevering factor cofilin [115,116], which may alter the immune synapse formation required for early events in TCR activation.

Effects on surface receptors that may contribute to T-cell activation CD3/TCR

HIV-2 and most SIV Nef alleles efficiently downregulate CD3/TCR [117,118]. This Nef activity, which has been shown to potently suppress T-cell activation *in vitro*, was lost in the lentiviral lineage that gave rise to HIV-1 [41]. The resulting lack of CD3 downregulation function by HIV-1 Nef has been suggested to contribute to the high pathogenicity observed with human infection compared with that of natural hosts [45]. A recent study demonstrated that HIV-2 Nef-mediated downregulation of CD3 and CD28 was associated with higher CD4 counts [119], but symptomatic HIV-2 patients also displayed Nef clones able to downregulate CD3 [120], and CD3 down-regulation alone was insufficient to explain SIV virulence [121].

CD4

Nef's ability to downregulate CD4 is well characterized [122–124]. In addition to its role in viral entry, CD4 enhances TCR signaling by recruiting Lck to the immune synapse [125]. CD4 endocytosis is a normal response to TCR stimulation [10]; however, internalization of CD4 by Nef is independent of T-cell activation and has been suggested to modulate early events requiring Lck. Indeed several studies indicated that Nef-mediated downregulation of CD4 may alter TCR signaling [126,127], while other reports have determined that Nef-mediated inhibition of TCR signaling is independent of its CD4 downregulation function [46,51,128].

CD28

HIV and SIV Nef proteins downregulate cell-surface CD28 expression [119,129,130]. Costimulation provided by CD28 is necessary to elicit optimal antigen-specific T-cell activation, and absence of costimulation often results in T-cell anergy [131]. This Nef activity may contribute to pathogenesis, since *in vivo* reversion of H196Q, a codon

important for CD28 downregulation [129], was associated with progression in SIV-infected rhesus macaques [132].

Nef-mediated activation of uninfected bystander T cells

HIV-1 Nef can also alter the activation of bystander uninfected T cells by modifying protein expression in APCs. In HIV-infected macrophages, a cell type targeted for HIV infection [133,134], HIV-1 Nef induces the production and release of two CC-chemokines, macrophage inflammatory proteins lα (CCL2) and 1β (CCL4), resulting in chemotaxis and activation of resting T lymphocytes, and thereby facilitating productive viral infection [135]. Furthermore HIV-1 Nef intersects the CD40 signaling pathway in macrophages, inducing the release of the soluble forms of intercellular adhesion molecule ICAM-1 and the coactivation molecule CD23, which, in turn, promotes interactions between B cells and T cells that render the T cell permissive to HIV-1 [136]. However, HIV-1-infected dendritic cells (DCs) cannot significantly increase resting $CD4^+$ T-cell proliferation in DC–T-cell cocultures [137].

Soluble Nef can be detected in HIV-1-infected patient sera [138], and extracellular Nef may also contribute to T-lymphocyte activation. Indeed, recombinant Nef can enter monocytes and macrophages, and induce IL-15 synthesis resulting in activation of peripheral blood mononuclear cell [139]. Furthermore, it has been shown that recombinant Nef triggers a series of phenotypic and functional changes that promote DC differentiation, enhancing the ability of these cells to activate bystander $CD4^+$ T cells [140].

Role of HIV-1 Nef in T-cell apoptosis

HIV-1 Nef is one of several viral proteins that have been described to modulate lymphocyte cell death. The role of Nef in apoptosis remains controversial, with both inhibitory and pro-apoptotic activities reported. The earliest study to assess the effects of HIV-1 Nef on apoptosis was by Fujii *et al.* [138]. This group observed that soluble Nef could bind to the surface of $CD4^+$ T cells, and that the cross-linking of these proteins using anti-Nef antibodies was cytotoxic to uninfected cells [138]. These findings were substantiated by other studies reporting that soluble Nef bound not only to $CD4^+$ T cells, but also $CD8^+$ T cells, B cells, macrophages and neutrophils, and that cross-linking of membrane-bound Nef led to an apoptosis pathway that was independent of Fas (CD95) [141] and that could be blocked by serine/ threonine protein kinase inhibitors [142]. Lenassi *et al.* later described that this soluble Nef exited infected cells via exosomes to cause the apoptosis of bystander $CD4^+$ T cells [143]. Various studies have reported enhancement of apoptosis by Nef through upregulation of cell surface Fas and Fas ligand [144,145], which could also serve as a mechanism to evade immune responses by initiating the death of antiviral $CD8^+$ T cells [144]. Another mechanism employed by Nef to modulate apoptosis is by upregulation of the programmed death-1 surface protein, which appears to be dependent on the activation of p38/MAPK [146]. Nef also reduces the expression of antiapoptotic proteins Bcl-2 and Bcl-XL as a mechanism to increase apoptosis [147]. Finally, soluble Nef is reported to induce CXCR4-mediated apoptosis [148,149].

HIV-1 Nef has also been reported to inhibit apoptosis. In association with PAK and PI3Ks, Nef phosphorylates the cellular protein Bad to enhance its antiapoptotic properties [111]. In addition, Nef inhibits the function of ASK1 [150]. This prevents cell death that could result from *cis*-ligation of Nef-induced Fas and FasL on the surface of virus-infected cells, and simultaneously protecting the virus-infected cells from Fas and TNF-α death signaling pathways. It has also been reported that the survival of productively infected $CD4^+$ T lymphocytes requires Nef expression by HIV-1, as well as activation by TNF-α expressed

on the surface of macrophages [151]. Finally, Nef has been reported to bind the tumor suppressor protein p53 and to inhibit its ability to mediate apoptosis [152].

Making sense of discrepancies in the literature

Research on Nef and T-cell activation has yielded divergent results. On the one hand, substantial data suggests that Nef enhances activation –including increased nuclear translocation of transcription factors and IL-2 expression. On the other hand, Nef appears to disrupt TCR signaling – including reduced Lck activity, LAT phosphorylation and cytoskeletal reorganization. These contradictions are not new to the field and several factors have been proposed to contribute to these diverse observations.

Intracellular localization

Baur *et al.* investigated the importance of intracellular localization by fusing Nef to CD8 glycoprotein [89]. They observed that plasma membrane-targeted Nef activated TCR signaling and induced the NF-κB pathway, whereas cytoplasmic Nef inhibited both of these events 89]. The notion that Nef causes differential effects on T-cell activity depending upon its intracellular location remains an attractive hypothesis to resolve discrepancies in experimental results. While early work by Kaminchik *et al.* indicated that a naturally occurring N-terminally truncated Nef protein failed to localize to the plasma membrane [153], to our knowledge, potential differences in the localization of patient-derived Nef alleles has not been examined systematically. In addition, the concentration of Nef or the method used for protein expression (transient or stable) might also affect the relative localization of Nef within a cell, thereby shifting the balance of activating and inhibitory mechanisms.

Alternative signaling pathways

Over the past year, the Fackler group has reported a series of observations showing that, although Nef disrupts classical TCR signaling, this protein also organizes an alternative pathway that can enhance cellular activation. Abraham *et al.* demonstrated that Nef inhibited early events in TCR signaling downstream of ZAP-70, including LAT recruitment to TCR microclusters, SLP-76 activation and actin reorganization [91]. Notably, Nef also redirects Lck from the plasma membrane to the trans-golgi network and stimulates Lck-mediated activation of Ras/MAPK signaling [154]. A recent review by Abraham and Fackler provides additional discussion of this topic [155].

Cell type & intrinsic activation state

Numerous reports utilizing immortalized Jurkat T-cell lines have reported either Nef-mediated enhancement or inhibition of T-cell activation (Table 1), and it remains an important task to systematically evaluate and explain the contradictory results generated using presumably similar cells. Despite their utility as a well-established model for analysis of T-cell function, there are caveats to using Jurkat cells for Nef studies. Jurkat tumor cells display a constitutive partially active phenotype that manifests in an idiosyncratic expression profile of several signaling molecules, which has led to mischaracterization of PI3K–dependent signaling events [156] and of activation-dependent mechanisms of Nef-mediated HLA-I downregulation [157,158]. Therefore, Nef's effect on activation in Jurkat cell lines may not be fully representative of its effect in primary cells. A positive role for Nef in HIV-1 replication was demonstrated in primary $CD4^+$ T cells [19], and Nef-mediated enhancement of TCR signaling can contribute to this outcome [39,66]. Although these and other results using primary cells support an emerging consensus that Nef is a 'positive factor', Neri *et al.* recently reported that the intrinsic activation state of primary T cells is important for Nef function, since Nef was able to enhance IL-2 expression in quiescent

$CD4^+$ T cells, but not in preactivated cells [55]. Thus, it remains too simplistic to argue that Nef's negative effects on T-cell signaling are merely artefacts of using Jurkat cell lines, or that Nef always promotes activation in primary cells. Subtle differences in the phenotype of primary T cells, how they are prepared or propagated, or inherent differences between cell lines may affect signaling pathways or protein interactions and tip the balance of Nef function in favor of T-cell activation or suppression.

Stimulation conditions

Most early experiments that observed Nef-mediated inhibition used PHA/PMA [36,74] or anti-CD3 antibody alone [38,63,89] to stimulate cells. One related study found that stimulation with anti-CD28 did not affect Nef's inhibitory role [75]. By contrast, later studies have employed anti-CD3 and anti-CD28 that are either cross-linked [39,40,42,44,53], presented on beads [45,66,159] or bound to coverglass to mimic the immune synapse [86]; however, the stimulation dose in these assays may not reflect biological interactions with APC. This limitation may be overcome by activating T cells with peptide-pulsed APCs [45,46,56]. It would be of interest to systematically investigate whether reagents or dose can contribute to divergent findings.

Given that early and late signaling events require different kinetics, assay duration may be a complicating factor. Many studies noting a negative role for Nef in activation looked at transcription factor translocation or IL-2 transcription, requiring stimulation of 4 h [36,63,74] or less [89], while those assessing proximal TCR signaling events measured outcome after only minutes [45,46,55,86,89]. By contrast, many studies noting a positive role for Nef used IL-2 secretion or IL-2/NFAT-driven luciferase as activation markers, and often required longer assay times [39,40,55,159]. It would be useful to investigate whether longer stimulation conditions allow for compensatory processes or feedback loops.

Conclusion

Recent studies have attempted to reconcile a long history of divergent results by proposing more sophisticated models of HIV-1 Nef's ability to modulate T-cell activation state. Rather than describing Nef as a 'negative' or a 'positive' factor, new results indicate that this protein may act in both capacities in virus-infected cells. By inhibiting proximal TCR-mediated signaling events, Nef can prevent activation-induced cell death and, thereby, extend the lifespan of virus-infected cells. By redirecting Lck and other kinases to intracellular compartments, Nef can stimulate transcription factor activity that is required to maintain viral gene expression and to enhance progeny virion production. Different *in vitro* experimental approaches may favor one of these mechanisms over the other, leading to seemingly contradictory observations; however, it is likely that both Nef functions contribute to the ability of this protein to promote *in vivo* HIV-1 replication and pathogenesis. Additional details of these mechanisms may provide novel targets for antiviral therapy that can counteract Nef's impact on T-cell signaling.

Future perspective

Additional studies are necessary to characterize the two-pronged approach that Nef uses to modulate the activation state of virus-infected $CD4^+$ T cells. Systematic analyses of methods used to assess Nef function should be performed to assess the impact of cell type, stimulation conditions, and *Nef* allele on outcome.

As mechanisms of Nef function are further defined, novel therapeutic strategies should be tested and the role of Nef in determining the balance between productive viral replication and establishment of latency should be revisited.

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Nef & T-cell activation

- Antigen-stimulated $CD4^+$ T cells are highly permissive to HIV-1 infection and it is likely that Nef acts to maintain a 'semi-activated' state that is efficient for the production of viral progeny.
- Early studies observed that Nef inhibited virion production and replication; however, later reports found that Nef enhanced viral pathogenesis and progression to AIDS.
- Conflicting evidence of Nef's role in modulating T-cell activation status has continued to accumulate – suggesting that Nef can function as both a 'negative factor' and as a 'positive factor'.

Early T-cell receptor signaling events

- Nef appears to inhibit early signaling events following T-cell receptor activation, including recruitment and activation of LAT, as well as actin reorganization.
- Localization of Nef to the plasma membrane through myristoylation is likely to be required for this activity.
- By blocking antigen-specific signaling, Nef may prevent activation-induced cell death and prolong the lifespan of virus-producing cells.

Alternative pathways for cellular activation

- Nef also appears to increase stimulation of transcription factors and expression of host genes, such as *IL-2*.
- Localization of Nef to intracellular compartments (endosomes) or interactions with cellular kinases (Lck and PAK2) are likely to be required for this activity.
- Nef-mediated stimulation of gene expression may promote viral long terminal repeat function.

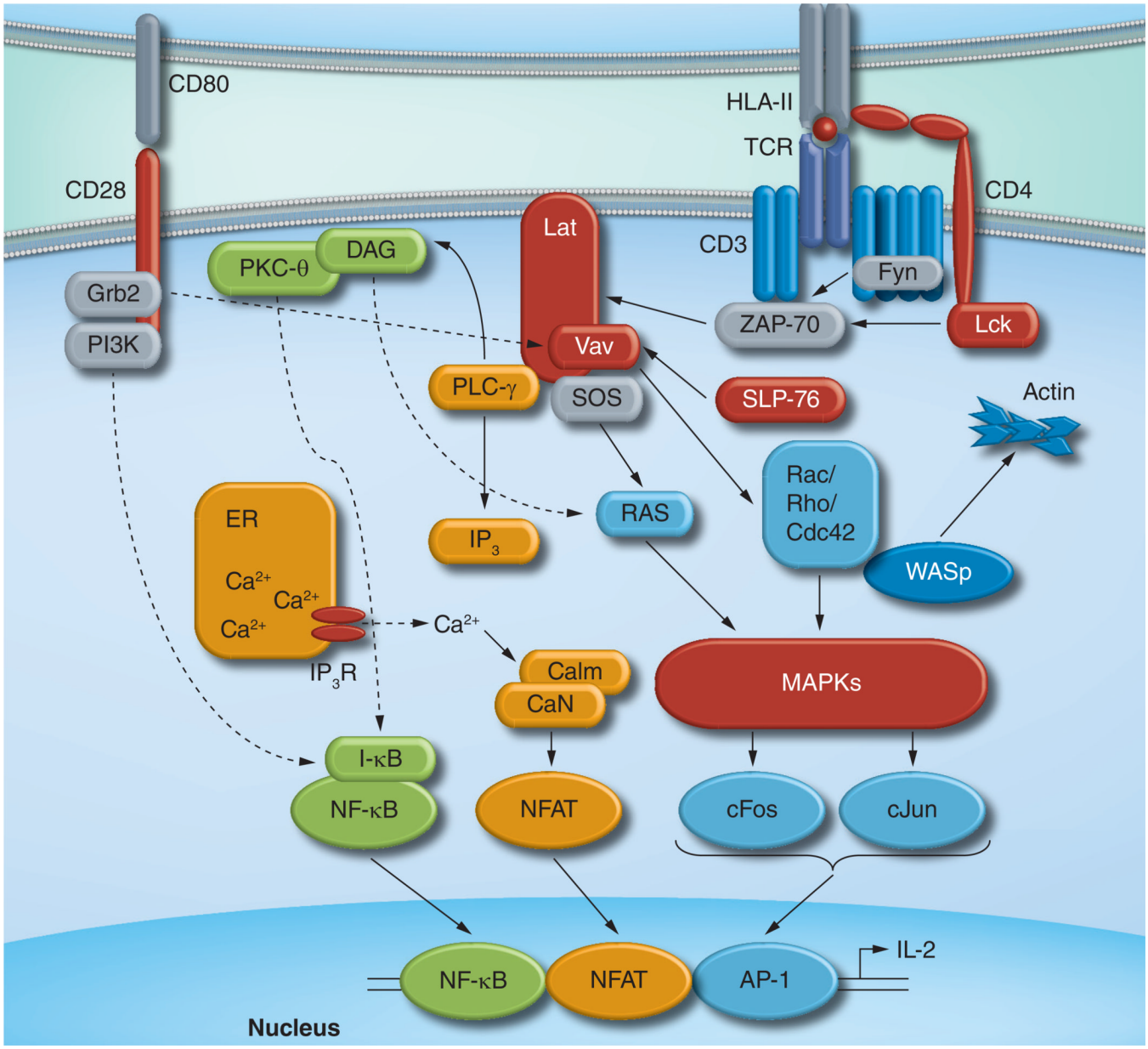

Figure 1. T-cell receptor signaling

Recognition of peptide/HLA ligand by TCR leads to cellular activation and IL-2 production. The TCR/CD3 complex mediates proximal signaling events through recruitment of cellular kinases (Lck, Fyn and ZAP-70), adapter molecules (SLP-76) and the scaffold protein LAT. Distal events result in the activation of three key transcription factors (NFAT, NF-κB and AP-1) and their translocation into the nucleus. NFAT is triggered by CaN through a process that requires PLC-γ, IP_3, release of Ca^{2+} from the ER and Calm (shown in orange). Activation of NF-κB requires degradation of I-κB, which is mediated by DAG and PKC-θ (shown in green) and enhanced by costimulatory signals provided by CD28. AP-1, consisting of cFos and cJun, is stimulated by MAPK cascades that are triggered by the Ras family GTPases, including Ras, Rac, Rho and Cdc42 (shown in blue). WASp initiates actin cytoskeletal rearrangement. Proteins whose activities are reported to be modulated by HIV-1 Nef are indicated in red.

Calm: Calmodulin; CaN: Calcineurin; DAG: Diacylglycerol; NFAT: Nuclear factor of activated T cells; TCR: T-cell receptor.

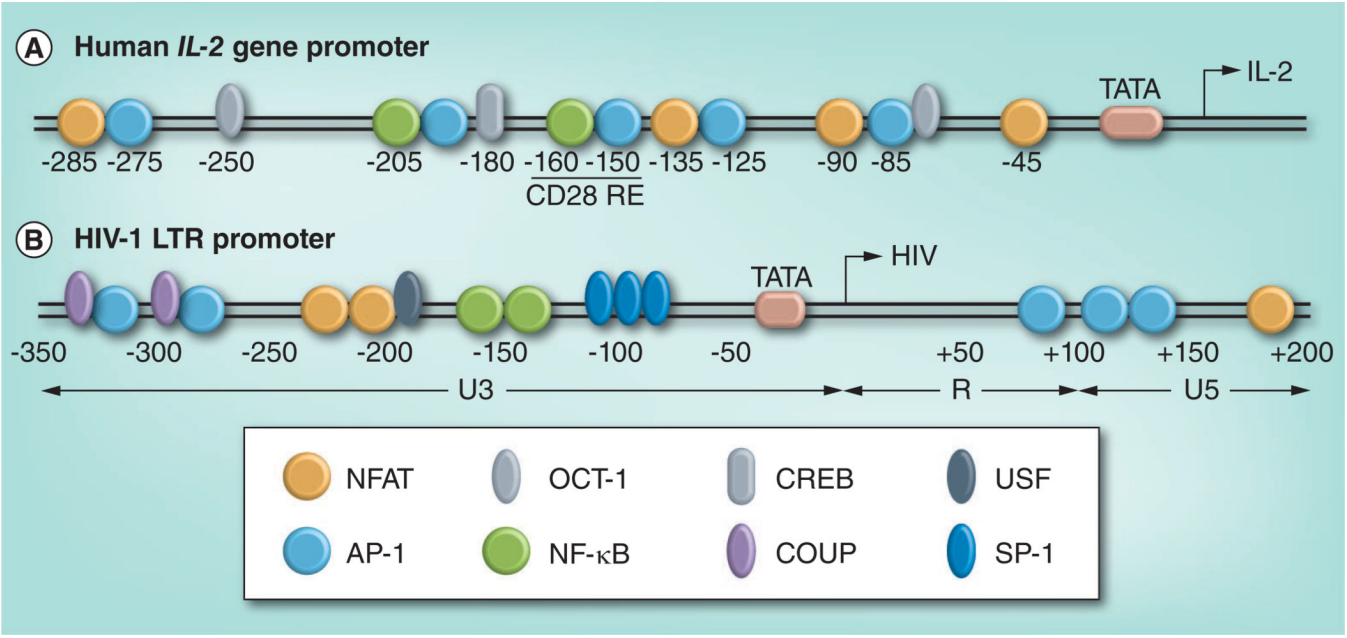

Figure 2. Human *IL-2* and HIV-1 long terminal repeat promoters

The location of critical transcription factor binding sites in (**A**) the human *IL-2* gene promoter and (**B**) the HIV-1 LTR sequence are indicated. In addition to NFAT, NF-κB and AP-1 (discussed in the text), constitutive transcription factors, OCT-1, COUP, USF-1 and SP-1, as well as CREB, contribute significantly to promoter activity.

COUP: Chicken ovalbumin upstream promoter; LTR: Long terminal repeat; NFAT: Nuclear factor of activated T cells; OCT-1: Octamer transcription factor 1; SP-1: Specificity protein 1; USF-1: Upstream stimulatory factor 1.

Table 1

Contradictory observations regarding HIV-1 Nef and T-cell activation[†].

Cell type	Expression system	Nef effect	Stimulation (outcome)	Ref.
Jurkat cells	Transient	Positive	PMA (NFAT); CD3/CD28 (ERK)	[51,52,56]
		No effect	PMA/PHA (NF-κB, AP-1); CD3/CD28 (NFAT); APC (NF-κB)	[52,56,65]
		Negative	PHA/PMA (NF-κB); CD3/CD28 (CD69, Actin, Lck); APC (NFAT)	[56,74,86,87,159]
	Stable	Positive	CD3 (NF-κB); CD3/CD28 (NFAT, NF-κB, IL-2)	[39,40,42,89,159]
		Negative	PMA/PHA (NF-κB, IL-2, HIV LTR); PMA/CD2 (IL-2); CD3 (Ca^{2+}, AP-1, NF-κB, TCR)	[36,63,74,89]
	Virus infection	Positive	CD3/28 (NFAT, AP-1, NF-κB, IL-2)	[41,53]
		Negative	APC (IL-2, Lck)	[46]
Primary $CD4^+$ T cells	Transient	Positive	CD3/CD28 (IL-2)	[44]
		Negative	PHA (Lck); IL-2 (proliferation)	[77]
	Stable	Positive	CD3 (ERK); CD3/CD28 (IL-2)	[39,66]
		No effect	CD3/CD28 (NF-κB)	[66]
	Virus infection	Positive	CD3/CD28 (IL-2, NFAT); APC (ERK)	[55,154]
		No effect	CD3/CD28 (Zap-70, NF-κB, Lck); APC (IL-2)	[45,55]
		Negative	APC (Lck)	[46]

[†]Selected publications describing the effect of HIV-1 Nef on T-cell activation. Stimulation reagent(s) and outcome(s) measured are indicated.

APC: Antigen-presenting cell; CD3: Anti-CD3 antibody; CD28: Anti-CD28 antibody; Lck: Lymphocyte-specific protein tyrosine kinase; NFAT: Nuclear factor of activated T cells; PHA: Phytohemagglutinin; PMA: Phorbol 12-myristate 13-acetate; TCR: T-cell receptor.