HIV-1 Nef control of cell signalling molecules: multiple strategies to promote virus replication

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HIV-1 has at its disposal numerous proteins encoded by its genome which provide the required arsenal to establish and maintain infection in its host for a considerable number of years. One of the most important and enigmatic of these proteins is Nef. The Nef protein of HIV-1 plays a fundamental role in the virus life cycle. This small protein of approximately 27 kDa is required for maximal virus replication and disease progression. The mechanisms by which it is able to act as a positive factor during virus replication is an area of intense research and although some controversy surrounds Nef much has been gauged as to how it functions. Its ability to modulate the expression of key cellular receptors important for cell activation and control signal transduction elements and events by interacting with numerous cellular kinases and signalling molecules, including members of the Src family kinases, leading to an effect on host cell function is likely to explain at least in part its role during infection and represents a finely tuned mechanism where this protein assists HIV-1 to control its host.

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1. Introduction

Human immunodeficiency virus (HIV) infection of humans is characterized by a progressive depletion of CD4+ve lymphocytes in peripheral blood and lymphoid organs culminating in severe immunodeficiency (Fauci 1988). While the precise mechanism is unknown by which infection with this virus causes CD4 T-cell loss and associated immune dysfunction, what is clear is that the *nef* gene of HIV-1 plays a fundamental role in determining viral pathogenicity.

The pivotal role for Nef in HIV-1 infection has been demonstrated in animal models and HIV-1-infected humans (Deacon *et al* 1995; Kirchhoff *et al* 1995; Hanna *et al* 1998, 2001; Simard *et al* 2002). The mechanisms by which Nef enhances virus replication and associated pathogenicity, whilst not well understood, are under intensive investigation. Evidence suggests that Nef, a myristoylated protein of 206 amino acids, is expressed abundantly during HIV-1 infection and localizes itself at the plasma membrane, cytosol, nucleus and within the virion, and can down-regulate cell surface signalling molecules, including CD4, CD28 and MHC class I and affects internal signalling pathways. These effects are likely to promote evasion of the virus from the immune system, extend the life span of the HIV-1-infected cell through control of apoptosis, regulate the expression of key cellular factors necessary for virus replication and increase virion infec-

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Abbreviations used: Ap, Adapter protein; CTLs, cytotoxic T-lymphocytes; IL-2R, IL-2 receptor; PAK2, p21-activated kinase 2.

tivity. Each of these activities undoubtedly assists virus replication.

2. Modulation of cell surface expression of receptors involved in signalling by Nef

2.1 *Down-regulation of CD4*

The best understood phenotypic effect of Nef is its ability to down-regulate cell surface CD4 (Garcia and Miller 1991; Garcia *et al* 1993; Mariani and Skowronski 1993; Aiken *et al* 1994; Salghetti *et al* 1995; Gratton *et al* 1996; Craig *et al* 1998; Piguet *et al* 1998; Mangasarian *et al* 1999; Piguet *et al* 1999a,b; Piguet and Trono 1999). A major benefit of Nef-induced CD4 down-modulation may be to enhance HIV replication by preventing detrimental superinfection events, which may lead to premature death of the infected cell (Benson *et al* 1993). Furthermore, as high levels of surface CD4 have been shown to interfere with the budding of progeny, virus down-regulation of surface CD4 by Nef would also be expected to enhance virus replication through augmented release of virus particles (Benson *et al* 1993). Equally plausible however is that this function of Nef represents a strategy to control signalling events in the infected cell. CD4 can enhance antigen-driven TCR signalling events through interactions between its extracellular domain and the MHC class II molecule, with the association of Lck tyrosine kinase with the CD4 cytoplasmic domain also being important (Weiss *et al* 1984; Weiss 1993; Weiss and Littman 1994). The effect of Nef on CD4 may interfere with TcR signalling to the advantage of the virus, possibly by decreasing activation induced apoptosis as will be discussed below.

Nef-induced CD4 down-regulation occurs early after viral infection (Chen *et al* 1996; Crise and Rose 1992; Willey *et al* 1992). Initially described as an undefined post-translational event, further studies demonstrated that Nef induces CD4 endocytosis in clathrin-coated pits followed by degradation of CD4 in lysosomes (Aiken *et al* 1994). Endocytosis of the CD4 receptor is a normal physiological response to T-cell activation by antigen presenting cells. However, Nef appears to by-pass the normal routes of CD4 down-regulation. Rather, it appears that Nef acts as a connector protein between CD4 and the endocytic machinery causing CD4 internalization. Specifically, data suggests that rather than interacting directly with endocytic machinery via its dileucine motif, CD4 (via the same motif) interacts with Nef, which in turn, possesses its own dileucine motif which acts as a connector with the endocytic machinery (Mangasarian *et al* 1997; Craig *et al* 1998; Mangasarian *et al* 1999). Indeed, the binding of Nef to CD4 has been demonstrated in a number of experimental systems (Harris and Neil 1994; Grzesiek *et al* 1996; Greenway *et al* 1995). The membrane proximal region of CD4 which contains a predicted α-helix, is important for the Nef-induced effect, as mutations significantly affecting this predicted structure, at least partially, disrupt Nef-induced down-regulation of CD4 (Gratton *et al* 1996). Included in this region is a dileucine motif that is critical for binding to Nef, and for the Nef-induced down-regulation (Grzesiek *et al* 1996; Aiken *et al* 1994; Salghetti *et al* 1995).

The main link between Nef and the endocytic machinery appears to occur through a direct interaction with the μ chain of adaptor complexes (Greenberg *et al* 1997; Greenberg *et al* 1998; Piguet *et al* 1999a,b). Adaptor protein (AP) complexes interact with cytosolic clathrin allowing the formation of clathrin-coated pits, mediating sorting of proteins at the plasma membrane and the *trans*-Golgi (reviewed in Holstein *et al* 1994). AP complexes recruit proteins for internalization in clathrin-coated pits through internalization signals, usually containing tyrosine or dileucine motifs (Letourneur and Klausner 1992). In the yeast two-hybrid system Nef binds to the μ chain of AP-2, which is specifically involved in clathrin-coated pit formation at the plasma membrane (Piguet *et al* 1998). Nef also co-localizes with a component of AP complexes at the cell margin (Greenberg *et al* 1998). The dileucine motif at position 164–165 and residues 174–179 in Nef are required for these activities (Greenberg *et al* 1997; Mangasarian *et al* 1999). Once Nef has internalized CD4, it has been suggested that it then interacts with endosomal COPI complexes which directs CD4 from early or recycling endosomes to late degradation compartments (Piguet *et al* 1999a,b).

2.2 *Down-regulation of CD28*

Recently, Nef has been reported to down-regulate the surface expression of CD28, which is a major co-stimulatory receptor that is necessary for maximal T-cell activation, by accelerating CD28 endocytosis (Bell *et al* 2001; Swigut *et al* 2001). This biological activity of Nef appears separable from its effects on CD4 and MHC class I molecule expression (discussed below). Studies by Swigut *et al* (2001), suggest Nef induces CD28 down regulation possibly via the AP-2 clathrin adaptor-dependent pathway, which involves a complex containing Nef, AP-2 and CD28.

While direct evidence for the biological significance of Nef down-regulation of CD28 does not yet exist, it has been hypothesised that this function of Nef may promote disengagement of the HIV-1-infected or SIV-infected T-cell from antigen presenting cells. This effect may promote the subsequent movement of the infected T-cell into

circulation, hence facilitating the spread of virus (Swigut *et al* 2001; Bell *et al* 2001). Alternatively, it seems that the viruses have evolved a sophisticated mechanism to divert signalling cascades in the infected cell, providing protection from activation-induced apoptosis. For example, high intensity TcR signalling can induce apoptosis (Weiss 1993; Weiss and Littman 1994). Down-regulation of CD28 may be one way by which the infected cell can minimize signalling upon engagement of the infected T-cell with APC; or down-regulation of CD28 may precipitate the interference by Nef of normal TCR-initiated signalling (Luria *et al* 1991; Greenway *et al* 1995; Collette *et al* 1996; Greenway *et al* 1996; Iafrate *et al* 1997). This effect may be necessary to prevent activation-induced apoptosis of already activated infected cells by further stimulation through CD3. As Nef proteins have been reported by several researchers to activate certain down stream effectors of signalling pathways, it is plausible that the infected cell can still remain activated yet protected from improper signalling through CD3 and/or CD28 by CD28 down-regulation and disconnecting activation from antigen presentation.

2.3 *Down-regulation of MHC-class I*

Maintenance of persistent infection undoubtedly relies upon virus evasion of immune surveillance. Many pathogenic viruses down-regulate the cell surface expression of MHC-I to avoid cytotoxic T-lymphocytes (CTL's) from attacking infected cells including HIV-1 (Kamp *et al* 2000). Without the expression of MHC-I the CTL does not detect foreign peptide antigen, and as such MHC-I molecules can be considered a signalling complex for the purpose of engaging CTL's. HIV-1 Nef has also included a strategy in its armour to circumvent CTL attack by down-regulating MHC-I by targeting the surface expression of the complex (Collins *et al* 1998). Studies have identified that in the presence of Nef the synthesis of the MHC-I molecule and its transport through the endoplasmic reticulum and *cis*-Golgi apparatus occur normally, but that surface MHC-I are rapidly internalized and directed towards the endosomal pathway for protein degradation. Furthermore, upon budding from the *trans*-Golgi network, Nef misdirects MHC-I molecules to clathrin-coated vesicles (Schwartz *et al* 1996; Le Gall *et al* 1997, 1998; Chang *et al* 2001). Internalization of MHC class I molecules by Nef relies on determinants, particularly Tyr 320 within the cytoplasmic tail of MHC class I molecules. These determinants are a part of a cryptic tyrosine-based motif that mediates endocytosis and sorting of a number of surface molecules (Le Gall *et al* 1998).

Again, Nef appears to harness AP complexes to down-regulate MHC-I. For example, MHC-I co-localizes with clathrin and AP-1 in the *trans*-Golgi network in the presence of Nef, and Nef also partially localizes with these complexes suggesting an active role for Nef in targeting of MHC-I to these regions (Greenberg *et al* 1998). Although Nef binds μ1 and μ2 subunits of adaptor protein complexes in yeast, the dileucine motif in HIV-1 Nef which mediates this binding is dispensable for MHC-I down-regulation (Mangasarian *et al* 1999; Riggs *et al* 1999). This suggests Nef does not act as an adaptor between MHC-I and the endocytic machinery, but rather it may expose the tyrosine-containing motif in HLA-A and B molecules, which acts as a signal for binding to AP-1 and AP-2, and internalization.

3. Nef control of internal cell signalling: positive effect on virus replication?

3.1 *Control of TcR signalling by Nef*

In addition to its ability to down-regulate cell surface receptors important in T-cell activation and development of the immune response, Nef appears to play a significant role in manipulating multiple signalling pathways within both CD4+ T-cells and macrophages. Although specific aspects of Nef perturbation of T-cell signalling cascades are somewhat confusing because of conflicting reports, Nef does target at least the TcR signalling pathway (Bandres and Ratner 1994; Baur *et al* 1994; Rhee and Marsh 1994; Greenway *et al* 1995; Collette *et al* 1996; Arold *et al* 1997; Iafrate *et al* 1997; Schrager and Marsh 1999; Manninen *et al* 2000; Simmons *et al* 2001), IL-2 receptor (IL-2R) signalling (Greenway *et al* 1994), pathways in macrophages leading to chemokine/monokine production (De *et al* 1998; Swingler *et al* 1999) as well as multiple anti-apoptotic cascades (Geleziunas *et al* 2001; Greenway *et al* 2002).

As mentioned above, the down-modulation of CD4, CD28 and IL-2R is likely to modulate T-cell activation events. For example down-regulation of CD4 by Nef dissociates Lck from CD4. Normally this association is necessary for the proximal localization of Lck with the CD3 complex, once T-cells have been activated via the TcR or anti-CD3 cross-linking. This tyrosine kinase is responsible for the phosphorylation of the zeta chain of the TcR which facilitates recruitment and binding of ZAP-70 leading to subsequent up-regulation of sensitive genes, such as IL-2 (Weiss 1993; Weiss and Littman 1994). Without the proximal localization of Lck these events are unlikely to occur. Furthermore, down-regulation of IL-2R will impair the ability of the infected T-cell to respond to IL-2. Similarly down-regulation of CD28 by Nef is likely to impact on further stimulation of the infected cell by antigen presenting cells, as CD28 is necessary for approp-

riate co-stimulation with CD3. Down-regulation of these receptors may facilitate the development of a pool of quiescent latently infected T-cells.

Certainly, initial reports showed that Nef interfered with TcR signalling. Stable transformants of the Jurkat T-cell line, which express Nef, failed to up-regulate the expression of IL-2 mRNA in response to either PMA and PHA or anti-CD3 cross-linking stimuli that normally result in the up regulation of IL-2 (Luria *et al* 1991). This suggests that Nef interferes with a signal emanating from the TcR that induces IL-2 gene transcription. Negative regulation of TcR signalling by Nef is consistent with reports describing Nef-mediated down-regulation of transcription factors NF*k*B and AP-1 (Niederman *et al* 1993; Bandres and Ratner 1994) which was observed in Nef-expressing cells stimulated either by mitogens or by antibodies to the TcR-CD3 complex. As Nef was reported in these experiments not to affect the surface expression of the TcR-CD3 complex, it was concluded that Nef down-regulates these transcription factors through effects on TcR signalling.

Despite the large number of reports that describe the negative effect of Nef on TcR signalling, opposing effects have been reported regarding the same signalling events. For example, Nef expression in an antigen-specific murine T-cell hybridoma results in both the down-modulation of CD4 and a positive enhancement of the TcR response (Rhee and Marsh 1994). However, these results contrast with others (Brady *et al* 1993; Skowronski *et al* 1993). While Nef has also been shown to lower the threshold required for activation of T-cells via CD3 and CD28 stimulation, the same study showed no effect of Nef on CD3 signalling alone (Schrager and Marsh 1999).

3.2 *Nef regulation of cell signalling can induce or protect cells from apoptosis*

The confusion surrounding the effects of Nef on the TcR pathway may reflect differences in cell lines, cell culturing conditions and the types of Nef alleles used in the plethora of reports. Also differences may be due to either variation in the levels of or duration of Nef expression in the experiments. Of course, both the positive and negative effects of Nef on the TcR signalling appear valid strategies that could be adopted to augment virus replication. Indeed, because of the positive correlation between HIV replication and cellular activation, it would seem logical that the virus would encode proteins, such as Nef to facilitate this activation event and replication in turn. Furthermore, up-regulation of TcR signalling may also help explain, in part, the demise of uninfected cells of the immune system. Concomitant with TcR activation is the up-regulation of the death factor, Fas-ligand (Van Parijs *et al* 1998). Up-regulation of Fas-ligand on the infected T-cell allows this cell to engage other cells expressing Fas and induce apoptosis (Oyaizu *et al* 1995; Van Parijs *et al* 1998). As immune surveillance cells express Fas, it is easily envisioned how the infected cell employs this strategy as a means, further to MHC class I down-regulation, to escape immune attack.

Alternatively, but equally plausible is that Nef down-regulation of TcR/CD3 signalling also promotes virus replication. Down-regulation of cell surface CD28 may leave the activated infected cell exposed to potential re-stimulation, only through CD3. Re-stimulation of already activated cells via CD3 can induce apoptotic cell death (Mountz *et al* 1995; Oyaizu *et al* 1995). Inhibition of signalling, emanating from TcR/CD3 receptor engagement, may represent one strategy whereby the infected cell avoids apoptosis, promoting virus replication. This idea is congruent with reports of Nef triggering a transcriptional programme in T-cells imitating CD3 T-cell activation (Simmons *et al* 2001). This may mean that Nef substitutes at least partially for CD3 signalling so that required activation for virus replication is achieved without the deleterious effect of re-stimulation of CD3 in the absence of CD28 co-stimulation.

Indeed, the balancing effect of Nef in maintaining sufficient activation of the infected T-cell for virus production without causing its own death via apoptosis may be further achieved by disrupting the process of apoptosis directly. This opposing approach by Nef has the advantage of protecting the infected cell and killing surrounding cells that may seek to destroy it. Multiple strategies whereby Nef may do this have now been reported. Geleziunas *et al* (2001) show that Nef protects the infected cells against CD95 (Fas) and TNF-alpha receptor-mediated death via inhibition of the apoptosis signal regulating kinase (ASK-1), one of the signalling molecules in one of three known death pathways. These studies, supported by others, show that Nef affects the Fas signalling pathway through inhibition of caspase-3 and caspase-8 activation (Yoon *et al* 2001).

Further examples of Nef inhibition of apoptosis signalling pathways have been described. Wolf *et al* (2001) demonstrated that Nef also represses death signalling by Bad which is a proapoptotic member of the BcL-2 family. As HIV-1 infection results in the induction of Bad, Nef serves to balance the apoptosis inducing effects of HIV-1. The mechanism of Bad inhibition involves Nef-mediated activation of phosphatidylinositol-3-kinase (PI3-K) and the p21-activated kinase 2 (PAK2), which results in the phosphorylation and subsequent inactivation of Bad. PI3K and PAK modulation by Nef will be discussed below.

We have also reported an anti-apoptotic effect of Nef that involves the tumour suppressor protein p53 (Green-

way *et al* 2002). In our studies we showed that Nef binds to p53, and that an N-terminal, 57-residue fragment of Nef (Nef 1–57) contains the p53-binding domain. Nef was also shown to interact with p53 during HIV-1 infection *in vitro*. As p53 plays a critical role in the regulation of apoptosis, we investigated whether Nef could affect this activity of p53. Indeed, Nef inhibited p53-dependent apoptosis is most likely due to its observed ability to decrease p53 protein half-life and, consequently, p53-DNA-binding activity and transcriptional activation. These effects correlated with the ability of Nef to bind to p53. These data show that HIV-1 Nef may augment HIV replication by prolonging the viability of infected cells by blocking p53-mediated apoptosis.

The protective effect of Nef against apoptosis seen in T-cells may also extend to monocyte-derived macrophages. Recent data from our own laboratory shows that HIV-1 infected monocyte-derived macrophages are also protected from apoptosis by the presence of Nef during HIV-1 infection (Cornall A and Greenway A, unpublished data). Specifically, while infection of monocyte-derived macrophages with the molecular clone AD8 showed little indication of apoptosis, cells infected with AD8 lacking the *nef* gene or encoding Nef containing a mutation within the central proline repeat motif showed evidence of apoptosis as detected by TUNEL. This data shows that Nef may assist the survival of the HIV-1 infected macrophage thereby augmenting virus replication by this population. This effect may be dependent upon Nef interaction with signalling proteins, such as members of the Src kinases, through its proline repeat motif and shall be further discussed below. Similarly, Nef has been shown to promote survival of myeloid cells by a Stat3-dependent pathway. This effect is also dependent on the proline repeat motif of Nef (Briggs *et al* 2001).

3.3 *Effect of Nef on further signalling pathways: regulation of factors necessary for HIV-1 replication*

The signalling pathways discussed above are not the only cascades modulated by Nef. Introduction of highly purified Nef protein into peripheral blood mononuclear cells caused reduced proliferative responsiveness to IL-2 in this cell population. This may be a direct effect on signalling by the TcR but may also be a consequence of the effect of Nef on the IL-2R (Greenway *et al* 1994). Further still, Nef expression in murine NIH-3T3 cells resulted in significantly decreased proliferative responsiveness to bombesin and platelet-derived growth factor (PDGF), suggesting that multiple pathways may be affected at common intersection points by Nef (De and Marsh 1994).

In monocyte-derived macrophages, Nef expression caused a dramatic increase in the expression of IL-6, TNF-*a* and IL-1*b* (Greenway A and Allen A, unpublished data). The up-regulation of these cell factors, may be as a result of Nef modulation of several Src family tyrosine kinases which will be discussed in more detail below (Saksela *et al* 1995; Greenway *et al* 1995, 1996, 1999; Collette *et al* 1996; Dutartre *et al* 1998; Collette and Olive 1999; Collette *et al* 2000; Moarefi 1997; Biggs *et al* 1997). These studies complement others investigating the effect of Nef on monocyte-derived signalling. For example, adenovirus-mediated expression of Nef in primary macrophages induces the production of two CC-chemokines, macrophage inflammatory proteins 1alpha and 1beta (Swingler *et al* 1999). As supernatants from Nef-expressing macrophages induced both the chemotaxis and activation of resting T-lymphocytes, thereby permitting productive HIV-1 infection a role for Nef in lymphocyte recruitment and activation at sites of virus replication may exist.

4. Nef binds host cell protein kinases: mechanism for control of signalling and implications for virus replication

The molecular mechanisms underlying the effect of Nef on signal transduction pathways may be explained, at least in part, by the ever increasing number of cellular protein kinases with which Nef interacts. Extensive investigation of the cellular proteins which interact with Nef has been undertaken and many of the proteins identified are protein tyrosine kinase or serine/threonine kinases which play key roles in the generation of signals in multiple signalling cascades (Bodeus *et al* 1995; Greenway *et al* 1995, 1996, 1999; Lee *et al* 1995, 1996; Smith *et al* 1996; Wiskerchen and Cheng-Mayer 1996; Benichou *et al* 1997; Dutartre *et al* 1998; Manninen *et al* 1998; Fackler *et al* 1999; Lock *et al* 1999; Xu *et al* 1999).

Table 1 represents a list of the signalling proteins reported to interact with Nef and the domains of each or either protein involved in the interaction. A number of these interactions will be discussed in light of their potential impact on signalling in the infected cell and ultimately the ability of the virus to replicate efficiently. References describing these interactions are found in throughout the text.

4.1 *Nef interaction with Src kinases*

HIV-1 Nef has been shown by numerous investigators to bind multiple members of the Src family kinases (Greenway *et al* 1995, 1996, 1999; Lee *et al* 1995, 1996; Saksela *et al* 1995; Collette *et al* 1996, 2000; Arold *et al* 1997; Baur *et al* 1997; Dutartre *et al* 1998; Cheng *et al* 1999). These include Lck, Fyn, Hck and Lyn.

Table 1. Cellular proteins reported to bind to HIV-1 Nef.

	Domain of Nef important for interaction	Domain of associating protein important for interaction
Hck	PxxP	SH3 domain
Lck	PxxP, N-term	SH3 domain
Fyn	PxxP	SH3 domain
Lyn	PxxP	SH3 domain
PAK	Arg 107 of the DiArg motif, PxxP Leu 112, F121	
PKC theta	Unidentified	Unidentified
Vav	PxxP	SH3 domain
MAPK	PxxP	Unidentified
p53	N-term (aa 1–57)	Unidentified
CD4	Unidentified	Unidentified
Raf 1	Core sequence Asp-Asp-X-X-X-Glu (aa 174–179)	Unidentified
PI3K	C-terminus predominantly N-terminus	P85 subunit
TcR zeta chain	PxxP	Unidentified

The minimal consensus region of Nef involved in the interaction with each of the Src kinases is the proline repeat motif (PxxP, where x is any amino acid) present between amino acid residues 69–78. This region is highly conserved among Nef proteins of different HIV-1 strains and bears strong resemblance to SH3 (Src homology 3) domain binding sites (Greenway *et al* 1995, 1996, 1999; Lee *et al* 1995, 1996; Saksela *et al* 1995; Collette *et al* 1996, 2000; Arold *et al* 1997; Dutartre 1998; Cheng *et al* 1999). SH3 domains are modular protein units of approximately 60 amino acids which have been shown to mediate protein : protein interactions with ligands such as Nef that contain proline repeat motifs. An additional mechanism of Nef interaction with Lck has also been proposed whereby the first N-terminal 22 amino acid residues of Nef can support indirect binding of Lck (Baur *et al* 1997). Further, work is necessary to fully elucidate the relationship between each binding domain to the Nef-Lck interaction event. Whether the two domains of Nef co-operate to bind to Lck or whether they operate independently under different circumstances is being investigated.

Nef association with the Src kinases modulates their catalytic activities. Several reports describe the dramatic up-regulation of Hck catalytic activity when it is SH3 domain is bound by Nef (Briggs *et al* 1997; Moarefi *et al* 1997; Greenway *et al* 1999). The addition of the HIV-1 Nef protein to, either the down-regulated or activated form of, Hck causes a large increase in Hck activity. The intact central proline-rich motif in Nef is crucial for this effect which is considered a consequence of Nef displacement of the SH3 domain of the kinase from a polyproline type II helix chain linking the SH2 and the catalytic domains in an inactive form, causing a conformational change in the amino terminal lobe of the catalytic domain which enhances phosphotransfer (Moarefi *et al* 1997). Lyn kinase activity is also up-regulated by Nef-SH3 binding, presumably in a similar manner (Greenway *et al*, unpublished data).

In contrast to these findings, however, direct binding of Nef to either Lck or Fyn has been shown to result in the inhibition of Lck and Fyn catalytic activities (Greenway 1996, 1999; Collette *et al* 1996). The inhibition of Lck and Fyn kinase activities is dependent upon the central proline repeat motif within Nef and correlates with binding directly to each of the kinases.

The differential regulation of Src kinase activity by Nef suggests it has adopted different strategies in CD4+ T-cells and monocyte-derived macrophages to augment virus replication. Both Lck and Fyn are expressed in T-lymphocytes and are intimately involved in mediating signals derived from the co-receptors CD4 and CD8 (Lck), IL-2R (Lck) and the TcR (Lck and Fyn) (Rudd *et al* 1988; Veillette *et al* 1988; Frank *et al* 1990; Horak *et al* 1991; Bolen *et al* 1992; Minami *et al* 1993). The modulation of Lck and Fyn activities by Nef could explain the inhibition of CD3-mediated signalling which has been reported. The inhibition of Lck and Fyn kinases by Nef may also result in the observed inhibition of IL-2 mRNA and protein production in Nef expressing/containing cells (Luria *et al* 1991; Greenway *et al* 1995; Collette *et al* 1996). As Lck signalling plays an essential role in the induction of apoptosis in Jurkat cells following TcR stimulation (Oyaizu *et al* 1995), it is also likely that the inhibition by Nef of Lck and Fyn will prevent apoptosis occurring in response to re-stimulation through CD3. This hypothesis is supported by the findings that Nef protein containing mutations within its proline-repeat motif

to abrogate binding to Lck and Fyn did not protect Jurkat cells from activation-induced death when simulated through the TcR (Greenway *et al*, unpublished data). Interception of the TcR pathway at an early point by Nef may prolong the life-span of an HIV-1 infected cell allowing increased virus production. As mentioned above, prevention of premature killing of HIV-1 infected cells by Nef may represent one mechanism, in addition to others proposed, by which Nef augments virus production.

Interaction of HIV-1 Nef with Hck, which is expressed in cells of the macrophage/monocyte lineage, may be related to Nef perturbation of macrophage signalling. Indeed, Nef activation of Hck has been linked to transformation of fibroblasts. Certainly this interaction, like that of Nef and Lyn, may impact on virus infection as the pathogenicity of Nef in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. This may be related to the ability of Nef to induce high-level constitutive expression of IL-6, TNF-a and IL-1b in monocyte-derived macrophages, which are potent activators of HIV-1 replication (De *et al* 1998). As Hck and Lyn are involved in the transduction of signals leading to the production of monokines such as IL-6 and TNF-a, it appears likely that Nef interaction with the Src kinases up-regulates the expression of these factors (Beaty 1994; Ernst *et al* 1994; Gupta *et al* 1995). The interaction of Nef with Src kinases may also be involved in MHC class I down-modulation by Nef as a dominant-negative mutant derived from the Hck protein-tyrosine kinase, composed of the Hck N-terminal region, as well as the SH3 and SH2 domains, was able to inhibit Nef-induced MHC class I molecule down-regulation (Chang *et al* 2001). These results suggest that this Nef-mediated effect requires an interaction between the Nef polyproline site and an SH3-containing cellular protein that is involved in MHC class I molecule turnover. Obviously such interactions impact positively on the ability of the infected cell to evade the host response to infection.

4.2 *TcR zeta chain interaction with Nef: Effects on T-cell activation and Fas-L*

Xu *et al* (1999) have reported that a plasma membrane localized form of Nef can interact with the zeta chain of the TcR. This interaction is again dependent on the proline repeat motif of Nef. The zeta chain of the TcR performs a critical function during T-cell activation, being phosphorylated by Lck after TcR stimulation. This event allows docking of ZAP-70 to the zeta chain and eventual IL-2 production. By interacting with the zeta chain, Nef may bypass the necessity for activation of Lck and activation of downstream events in the absence of CD3 stimulation. This hypothesis is complementary to the idea that inhibition of Lck and Fyn by Nef may prevent CD3-mediated apoptosis of already activated HIV-1 infected cells, while binding of the zeta chain of the TcR still promotes downstream activation and subsequently virus replication. The interaction of Nef with the zeta chain of the TcR has also been shown to be important for the induction of Fas-L on Nef expressing cells, thus this protein : protein interaction also facilitates the depletion of the immune system.

4.3 *Nef interaction with PAK2*

The interaction with a member of the PAK family, thought to be PAK2, appears to be a conserved function of Nef (Sawai *et al* 1994, 1995, 1996; Nunn and Marsh 1996; Baur *et al* 1997; Manninen *et al* 1998; Brown *et al* 1999; Arora *et al* 2000; Renkema *et al* 2001; Wolf *et al* 2001). Several regions within Nef have been shown to be important for the interaction. Firstly, the second arginine residue of the diarginine motif present within Nef is crucial for this interaction (Sawai *et al* 1994, 1995, 1996; Baur *et al* 1997). Furthermore, an intact PxxP motif is necessary for co-precipitation of Nef with PAK2 (Manninen *et al* 1998). As PAK2 is not known to contain an SH3, binding domain, it is likely that the requirement of the proline repeat motif within is indicative that an intermediary binding protein facilitates the Nef/PAK2 interaction. Members of the PAK family play important roles in organization of the cytoskeleton and apoptosis (Holly and Blumer 1999; Jakobi *et al* 2001; Zang *et al* 2001). As Nef is thought to interact with an activated from of PAK2, this interaction may be important in Nef's diversion of signalling pathways (Renkema *et al* 2001). Additionally or alternatively, Nef interaction with PAK2 may facilitate positive effect of Nef on lipid raft formation via the effect of PAK2 on cytoskeletal rearrangement. As HIV-1 has been shown to bud from cells through lipid rafts, the ability of Nef to faciliate their arrangement obviously impacts positively on virus replication (Rousso *et al* 2000; Campbell *et al* 2001; Ono and Freed 2001).

4.4 *Nef interaction with p53*

Our laboratory recently described the interaction of Nef with p53 (Greenway *et al* 2002). Using a panel of N-terminal and C-terminal truncation Nef mutants with purified recombinant p53 in binding studies, we have shown that Nef interacts directly with p53 and that this association is dependent upon the N-terminus of Nef, specifically amino acid residues 1 to 57. The region of p53 involved in the interaction is yet to be determined. The interaction of Nef with p53 can occur both in the cytoplasm

and the nucleus of the cell. The ability of Nef to interact with p53 resulted in decreased p53 half-life and consequently decreased p53 DNA binding and transcriptional activity and protection against p53-mediated apoptosis.

4.5 *Nef interaction with PI3 kinase*

As with p53, Nef interaction with PI3 kinase may also be responsible for Nef inhibition of apoptosis. Wolf *et al* (2001) reported that Nef co-precipitates PI3 kinase, specifically its p85 subunit, via sequences within the N- and C-termini of Nef. This interaction leads to the activation of PI3 kinase, which subsequently leads to the phosphorylation and hence inactivation of the pro-apoptotic protein Bad. Inactivation of Bad inhibits mitochondrial related apoptosis. PI3 kinase is, however, not the only protein required for this effect, as the inactivation of Bad by Nef is dependent on association of Nef with and activation of PAK2. These studies provide an example whereby Nef association with cellular kinases is a co-ordinated approach to controlling signalling and ultimately the life span of the infected cell for the benefit of virus replication. Undoubtedly, Nef association with the other described kinases will also be an intricately co-ordinated approach to reach the same outcome.

4.6 *PKC theta*

Finally, Nef has further been shown to associate with serine threonine kinases, including MAPK and PKC theta (Greenway *et al* 1995, 1996; Smith *et al* 1996). Smith *et al* (1996) identified the 80 kDa theta isoform of PKC to be amongst cellular proteins which co-precipitated with a GST-Nef fusion protein. As Nef can also serve as a substrate for PKC with a major phosphorylation site located in the very N-terminus of Nef, this interaction may have implications for PKC as well as for Nef (Coates *et al* 1997; Ellis P and Greenway A, unpublished data). Phosphorylation of Nef at its N-terminus may act as an electrostatic switch to alter association of Nef with plasma membrane by introducing a negative charge, thereby repelling Nef from the membrane. This may help explain why such a large proportion of myristoylated Nef exist in the cytosol (Coates *et al* 1997), and how Nef is able to interact with such a wide variety of cellular proteins located at the plasma membrane, in the cytosol and in the nucleus.

5. Virion infectivity: relevance to Nef regulation of signalling molecules?

In vivo studies show that Nef confers a positive advantage for virus replication (Kestler *et al* 1991; Deacon *et al* 1995; Kirchhoff *et al* 1995). Aside from the effects of Nef enabling the infected cell to escape immune surveillance, to up-regulate cellular factors important for virus replication and its anti-apoptotic function assisting the infected cell to survive, the positive influence of *nef* on viral growth rate is due, at least in part, to an infectivity advantage of virus produced with an intact *nef* gene.

Comparison of wild-type and *nef*-deleted virus production during single cycle replication initiated by infection with high-titer virus stocks or by transfection with viral DNA showed that wild type virus yielded a five-fold increase in p24 production relative to its *nef* deleted counterpart (Miller *et al* 1994). In contrast, single-cycle transfection yielded equal amounts of p24 production. These results imply that while Nef does not affect replication after the provirus is established supporting the findings of Schwartz *et al* (1995) – prior events are suggestive of being *nef*-sensitive. End-point titrations of isogenic wild-type and *nef*-deleted viruses have determined that virus containing an intact *nef* gene have a greater infectivity per picogram of HIV p24 antigen than *nef*-deleted virus (Pandori *et al* 1996, 1998).

The infectivity of HIV-1 deleted within *nef* can be restored to near wild-type levels by co-expression of Nef in *trans* in the virus-producer cell (Pandori *et al* 1996, 1998). This observation implies that the HIV-1 virions produced in the presence of Nef are intrinsically different from those produced in its absence. Certainly such virions would differ as Nef is incorporated, albeit in small quantities, into the virion. However, no structural or biochemical defects in Nef-defective HIV-1 have been demonstrated. The mechanism behind Nef enhancement of infectivity is not clear. However, analysis of reverse transcription within cells infected with HIV-1 or HIV-1 deleted within *nef* showed that the expression of Nef in virus-producing cells resulted in larger amounts of viral DNA accumulating in target cell (Schwartz *et al* 1995). This effect is not due to inhibition of reverse transcriptase activity *per se* but suggests that the expression of Nef in virus-producing cells is required for efficient processing of the early stages of virus replication in targe T-cells, which are concomitant with initiation of reverse transcription such as viral uncoating (Schwartz *et al* 1995; Zhou and Aiken 2001). Indeed, Zhou and Aiken (2001) report, using an intervirion fusion system, that Nef modifies the virion in a manner which can be transferred from donor to target virions during intervirion fusion. It is proposed by these authors that Nef may act by altering the function of the viral envelope, but in a fusion independent manner. Specifically, Nef may alter the lipid composition and structure of the envelope so as to enhance release of the core into the cytosol after fusion. This idea is congruent with association of Nef with lipid rafts, which is a proline repeat motif-dependent event. This association with lipid

rafts appears to increase HIV-1 infectivity (Rousso *et al* 2000; Campbell *et al* 2001; Ono and Freed 2001). As most Nef-associated kinases and particularly members of the Src family kinases, PAK2, TcR zeta, and Vav (Fackler *et al* 1999) will be present within the rafts and interact with Nef in a PxxP dependent manner, these kinases through Nef may be responsible for enhanced release of the core into the cytoplasm after fusion, in an as yet undefined manner. The ability of these Nef associated proteins to act in this way may be related to the fact that HIV-1 buds from the cell via lipid rafts or alternatively may be included along with Nef in the virion. This last idea is supported by the findings that the cellular kinase designated NAK (now Pak2) and MAPK, which associate with Nef may also be present in the virion (Wiskerchen and Cheng-Mayer 1996; Flaherty *et al* 1998; Jacque *et al* 1998). Thus Nef manipulation of signalling molecules may also be involved in augmenting virus replication though enhancement of viron infectivity.

6. Conclusion

Cellular receptors and signalling molecules are the tools used by cells to interact with and control their environment. Cell surface receptors on CD4 + T-cells are involved in regulating the way the cell responds to certain situations, transmitting signals for the activation of the cell to produce cytokines necessary for its survival or activating mechanisms whereby the cell can protect itself from immune invasion. Considering this, it makes perfect sense that a virus, such as HIV-1, encodes a protein, such as Nef, that can control these responses through the manipulation of key cell surface signalling molecules as well as their internal partners. The down-modulation of key cell surface receptors such as CD4, MHC-I and CD28 by Nef, represents a complex strategy whereby this enigmatic protein can control cell activation, and promote cell death of neighbouring uninfected cells yet protect itself from the same fate by controlling apoptosis and lysis by cytotoxic lymphocytes. The mechanisms that Nef employs to fulfil these activities rely heavily on association of Nef with multiple cellular proteins, including many cellular tyrosine, and serine/threonine kinases. These interactions are the likely factors which impact positively on virus infectivity and survival for the purpose of augmenting virus production. The manipulation by Nef of cell signalling receptors and molecules represents a finely tuned system for the virus to achieve its ultimate goal-survival.

References

Aiken C, Konner J, Landau N R, Lenburg M E and Trono D 1994 Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain; *Cell* **76** 853–864

Arold S, Franken P, Strub M P, Hoh F, Benichou S, Benarous R and Dumas C 1997 The crystal structure of HIV-1 Nef protein bound to the Fyn kinase SH3 domain suggests a role for this complex in altered T cell receptor signaling; *Structure* **5** 1361–1372

Arora V K, Molina R P, Foster J L, Blakemore J L, Chernoff J, Fredericksen B L and Garcia J V 2000 Lentivirus Nef specifically activates Pak2; *J. Virol*. **74** 11081–11087

Bandres J C and Ratner L 1994 Human immunodeficiency virus type 1 Nef protein down-regulates transcription factors NF-kappa B and AP-1 in human T-cells in vitro after T-cell receptor stimulation; *J. Virol*. **68** 3243–3249

Baur A S, Sass G, Laffert B, Willbold D, Cheng-Mayer C and Peterlin B M 1997 The N-terminus of Nef from HIV-1/SIV associates with a protein complex containing Lck and a serine kinase; *Immunity* **6** 283–291

Baur A S, Sawai E T, Dazin P, Fantl W J, Cheng-Mayer C and Peterlin B M 1994 HIV-1 Nef leads to inhibition or activation of T-cells depending on its intracellular localization; *Immunity* **1** 373–384

Beaty C D, Franklin T L, Uehara Y and Wilson C B 1994 Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction; *Eur. J. Immunol.* **24** 1278–1284

Bell I, Schaefer T M, Trible R P, Amedee A and Reinhart T A 2001 Down-modulation of the costimulatory molecule, CD28, is a conserved activity of multiple SIV Nefs and is dependent on histidine 196 of Nef; *Virology* **283** 148–158

Benichou S, Liu L X, Erdtmann L, Selig L and Benarous R 1997 Use of the two-hybrid system to identify cellular partners of the HIV1 Nef protein; *Res. Virol*. **148** 71–73

Benson R E, Sanfridson A, Ottinger J S, Doyle C and Cullen B R 1993 Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection; *J. Exp. Med*. **177** 1561–1566

Bodeus M, Marie-Cardine A, Bougeret C, Ramos-Morales F and Benarous R 1995 In vitro binding and phosphorylation of human immunodeficiency virus type 1 Nef protein by serine/threonine protein kinase; *J. Gen. Virol*. **76** 1337–1344

Bolen J B, Rowley R B, Spana C and Tsygankov A Y 1992 The Src family of tyrosine protein kinases in hemopoietic signal transduction; *Faseb J*. **6** 3403–3409

Brady H J, Pennington D J, Miles C G and Dzierzak E A 1993 CD4 cell surface downregulation in HIV-1 Nef transgenic mice is a consequence of intracellular sequestration; *EMBO J*. **12** 4923–4932

Briggs S D, Scholtz B, Jacque J M, Swingler S, Stevenson M and Smithgall T E 2001 HIV-1 Nef promotes survival of myeloid cells by a Stat3-dependent pathway; *J. Biol. Chem*. **276** 25605–25611

Briggs S D, Sharkey M, Stevenson M and Smithgall T E 1997 SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1; *J. Biol. Chem*. **272** 17899–17902

Brown A, Wang X, Sawai E and Cheng-Mayer C 1999 Activation of the PAK-related kinase by human immunodeficiency virus type 1 Nef in primary human peripheral blood lymphocytes and macrophages leads to phosphorylation of a PIX-p95 complex; *J. Virol*. **73** 9899–9907

Campbell S M, Crowe S M and Mak J 2001 Lipid rafts and HIV-1: from viral entry to assembly of progeny virions; *J. Clin. Virol*. **22** 217–227

Chang A H, O'Shaughnessy M V and Jirik F R 2001 Hck SH3 domain-dependent abrogation of Nef-induced class 1 MHC down-regulation; *Eur. J. Immunol.* **31** 2382–2387

Chen B K, Gandhi R T and Baltimore D 1996 CD4 down-modulation during infection of human T cells with human immunodeficiency virus type 1 involves independent activities of vpu, env, and nef; *J. Virol.* **70** 6044–6053

Cheng H, Hoxie J P and Parks W P 1999 The conserved core of human immunodeficiency virus type 1 Nef is essential for association with Lck and for enhanced viral replication in T-lymphocytes; *Virology* **264** 5–15

Coates K, Cooke S J, Mann D A and Harris M P G 1997 Protein kinase C-mediated phosphorylation of HIV-I nef in human cell lines; *J. Biol. Chem.* **272** 12289–12294

Collette Y, Arold S, Picard C, Janvier K, Benichou S, Benarous R, Olive D and Dumas C 2000 HIV-2 and SIV nef proteins target different Src family SH3 domains than does HIV-1 Nef because of a triple amino acid substitution; *J. Biol. Chem.* **275** 4171–4176

Collette Y, Dutartre H, Benziane A, Ramos M, Benarous R, Harris M and Olive D 1996 Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects; *J. Biol. Chem.* **271** 6333–6341

Collette Y and Olive D 1999 The primate lentivirus-encoded Nef protein can regulate several steps of the viral replication cycle; *Virology* **265** 173–177

Collins K L, Chen B K, Kalams S A, Walker B D and Baltimore D 1998 HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes; *Nature* (*London*) **391** 397–401

Craig H M, Pandori M W and Guatelli J C 1998 Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity; *Proc. Natl. Acad. Sci. USA* **95** 11229–11234

Crise B and Rose J K 1992 Human immunodeficiency virus type 1 glycoprotein precursor retains a CD4-p56lck complex in the endoplasmic reticulum; *J. Virol.* **66** 2296–2301

De S K and Marsh J W 1994 HIV-1 Nef inhibits a common activation pathway in NIH-3T3 cells; *J. Biol. Chem.* **269** 6656–6660

De S K, Venkateshan C N, Seth P, Gajdusek D C and Gibbs C J 1998 Adenovirus-mediated human immunodeficiency virus-1 Nef expression in human monocytes/macrophages and effect of Nef on downmodulation of Fcgamma receptors and expression of monokines; *Blood* **91** 2108–2117

Deacon N J, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker D J, McPhee D A, Greenway A L, Ellett A, Chatfield C, Larison V A, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan J S, Cunningham A, Dwyer D, Dowton D and Mills J 1995 Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients [see comments]; *Science* **270** 988–991

Dutartre H, Harris M, Olive D and Collette Y 1998 The human immunodeficiency virus type 1 Nef protein binds the Src-related tyrosine kinase Lck SH2 domain through a novel phosphotyrosine independent mechanism; *Virology* **247** 200–211

Ernst C A, Zhang Y J, Hancock P R, Rutledge B J, Corless C L and Rollins B J 1994 Biochemical and biologic characterization of murine monocyte chemoattractant protein-1. Identification of two functional domains; *J. Immunol.* **152** 3541–3549

Fackler O T, Luo W, Geyer M, Alberts A S and Peterlin B M 1999 Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions; *Mol. Cell* **3** 729–739

Fauci A S 1988 The human immunodeficiency virus: infectivity and mechanisms of pathogenesis; *Science* **239** 617–622

Flaherty M T, Barber S A and Clements J E 1998 Neurovirulent simian immunodeficiency virus incorporates a Nef-associated kinase activity into virions; *AIDS Res. Hum. Retroviruses* **14** 163–170

Frank S J, Samelson L E and Klausner R D 1990 The structure and signalling functions of the invariant T cell receptor components; *Semin. Immunol.* **2** 89–97

Garcia J V, Alfano J and Miller A D 1993 The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4; *J. Virol.* **67** 1511–1516

Garcia J V and Miller A D 1991 Serine phosphorylation-independent downregulation of cell-surface CD4 by nef; *Nature* (*London*) **350** 508–511

Geleziunas R, Xu W, Takeda K, Ichijo H and Greene W C 2001 HIV-1 Nef inhibits ASK1-dependent death signalling providing a potential mechanism for protecting the infected host cell; *Nature* (*London*) **410** 834–838

Gratton S, Yao X J, Venkatesan S, Cohen E A and Sekaly R P 1996 Molecular analysis of the cytoplasmic domain of CD4: overlapping but noncompetitive requirement for lck association and down-regulation by Nef; *J. Immunol.* **157** 3305–3311

Greenberg M, DeTulleo L, Rapoport I, Skowronski J and Kirchhausen T 1998 A dileucine motif in HIV-1 Nef is essential for sorting into clathrin-coated pits and for down-regulation of CD4; *Curr. Biol.* **8** 1239–1242

Greenberg M E, Bronson S, Lock M, Neumann M, Pavlakis G N and Skowronski J 1997 Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation; *EMBO J.* **16** 6964–6976

Greenway A, Azad A and McPhee D 1995 Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononuclear cells and T-cell lines; *J. Virol.* **69** 1842–1850

Greenway A, Azad A, Mills J and McPhee D 1996 Human immunodeficiency virus type 1 Nef binds directly to Lck and mitogen-activated protein kinase, inhibiting kinase activity; *J. Virol.* **70** 6701–6708

Greenway A L, Dutartre H, Allen K, McPhee D A, Olive D and Collette Y 1999 Simian immunodeficiency virus and human immunodeficiency virus type 1 nef proteins show distinct patterns and mechanisms of Src kinase activation; *J. Virol.* **73** 6152–6158

Greenway A L, McPhee D A, Allen K, Johnstone R, Holloway G, Mills J, Azad A, Sankovich S and Lambert P 2002 Human immunodeficiency virus type 1 Nef binds to tumour suppressor p53 and protects cells against p53-mediated apoptosis; *J. Virol.* **76** 2692–2702

Greenway A L, McPhee D A, Grgacic E, Hewish D, Lucantoni A, Macreadie I and Azad A 1994 Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4·3 down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells; *Virology* **198** 245–256

Grzesiek S, Stahl S J, Wingfield P T and Bax A 1996 The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR; *Biochemistry* **35** 10256–10261

Gupta D, Jin Y P and Dziarski R 1995 Peptidoglycan induces transcription and secretion of TNF-alpha and activation of lyn, extracellular signal-regulated kinase, and rsk signal

transduction proteins in mouse macrophages; *J. Immunol.* **155** 2620–2630

Hanna Z, Kay D G, Rebai N, Guimond A, Jothy S and Jolicoeur P 1998 Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice; *Cell* **95** 163–175

Hanna Z, Weng X, Kay D G, Poudrier J, Lowell C and Jolicoeur P 2001 The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is abolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck; *J. Virol.* **75** 9378–9392

Harris M P and Neil J C 1994 Myristoylation-dependent binding of HIV-1 Nef to CD4; *J. Mol. Biol.* **241** 136–142

Holly S P and Blumer K J 1999 PAK-family kinases regulate cell and actin polarization throughout the cell cycle of *Saccharomyces cerevisiae*; *J. Cell. Biol.* **147** 845–856

Holstein S E, Drucker M and Robinson D G 1994 Identification of a beta-type adaptin in plant clathrin-coated vesicles; *J. Cell. Sci.* **107** 945–953

Horak I D, Gress R E, Lucas P J, Horak E M, Waldmann T A and Bolen J B 1991 T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56lck; *Proc. Natl. Acad. Sci. USA* **88** 1996–2000

Iafrate A J, Bronson S and Skowronski J 1997 Separable functions of Nef disrupt two aspects of T cell receptor machinery: CD4 expression and CD3 signaling; *EMBO J.* **16** 673–684

Jacque J M, Mann A, Enslen H, Sharova N, Brichacek B, Davis R J and Stevenson M 1998 Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase; *EMBO J.* **17** 2607–2618

Jakobi R, Moertl E and Koeppel M A 2001 p21-activated protein kinase gamma-PAK suppresses programmed cell death of BALB3T3 fibroblasts; *J. Biol. Chem.* **276** 16624–16634

Kamp W, Berk M B, Visser C J and Nottet H S 2000 Mechanisms of HIV-1 to escape from the host immune surveillance; *Eur. J. Clin. Invest.* **30** 740–746

Kestler H W D, Ringler D J, Mori K, Panicali D L, Sehgal P K, Daniel M D and Desrosiers R C 1991 Importance of the nef gene for maintenance of high virus loads and for development of AIDS; *Cell* **65** 651–662

Kirchhoff F, Greenough T C, Brettler D B, Sullivan J L and Desrosiers R C 1995 Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection [see comments]; *N. Engl. J. Med.* **332** 228–232

Le Gall S, Erdtmann L, Benichou S, Berlioz-Torrent C, Liu L, Benarous R, Heard J M and Schwartz O 1998 Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules; *Immunity* **8** 483–495

Le Gall S, Heard J M and Schwartz O 1997 Analysis of Nef-induced MHC-I endocytosis; *Res. Virol.* **148** 43–47

Lee C H, Leung B, Lemmon M A, Zheng J, Cowburn D, Kuriyan J and Saksela K 1995 A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein; *EMBO J.* **14** 5006–5015

Lee C H, Saksela K, Mirza U A, Chait B T and Kuriyan J 1996 Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain; *Cell* **85** 931–942

Letourneur F and Klausner R D 1992 A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains; *Cell* **69** 1143–1157

Lock M, Greenberg M E, Iafrate A J, Swigut T, Muench J, Kirchhoff F, Shohdy N and Skowronski J 1999 Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis; *EMBO J.* **18** 2722–2733

Luria S, Chambers I and Berg P 1991 Expression of the type 1 human immunodeficiency virus Nef protein in T-cells prevents antigen receptor-mediated induction of interleukin 2 mRNA; *Proc. Natl. Acad. Sci. USA* **88** 5326–5330

Mangasarian A, Foti M, Aiken C, Chin D, Carpentier J L and Trono D 1997 The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane; *Immunity* **6** 67–77

Mangasarian A, Piguet V, Wang J K, Chen Y L and Trono D 1999 Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking; *J. Virol.* **73** 1964–1973

Manninen A, Hiipakka M, Vihinen M, Lu W, Mayer B J and Saksela K 1998 SH3-Domain binding function of HIV-1 Nef is required for association with a PAK-related kinase; *Virology* **250** 273–282

Manninen A, Renkema G H and Saksela K 2000 Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway; *J. Biol. Chem.* **275** 16513–16517

Mariani R and Skowronski J 1993 CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals; *Proc. Natl. Acad. Sci. USA* **90** 5549–5553

Miller M D, Warmerdam M T, Gaston I, Greene W C and Feinberg M B 1994 The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages; *J. Exp. Med.* **179** 101–113

Minami Y, Kono T, Yamada K, Kobayashi N, Kawahara A, Perlmutter R M and Taniguchi T 1993 Association of p56lck with IL-2 receptor beta chain is critical for the IL-2-induced activation of p56lck; *EMBO J.* **12** 759–768

Moarefi I, LaFevre-Bernt M, Sicheri F, Huse M, Lee C H, Kuriyan J and Miller W T 1997 Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement [see comments]; *Nature* (*London*) **385** 650–653

Mountz J D, Zhou T, Wu J, Wang W, Su X and Cheng J 1995 Regulation of apoptosis in immune cells; *J. Clin. Immunol.* **15** 1–16

Niederman T M, Hastings W R, Luria S, Bandres J C and Ratner L 1993 HIV-1 Nef protein inhibits the recruitment of AP-1 DNA-binding activity in human T-cells; *Virology* **194** 338–344

Nunn M F and Marsh J W 1996 Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family; *J. Virol.* **70** 6157–6161

Ono A and Freed E O 2001 Plasma membrane rafts play a critical role in HIV-1 assembly and release; *Proc. Natl. Acad. Sci. USA* **98** 13925–13930

Oyaizu N, Than S, McCloskey T W and Pahwa S 1995 Requirement of p56lck in T-cell receptor/CD3-mediated apoptosis and Fas-ligand induction in Jurkat cells; *Biochem. Biophys. Res. Commun.* **213** 994–1001

Pandori M, Craig H, Moutouh L, Corbeil J and Guatelli J 1998 Virological importance of the protease-cleavage site in human immunodeficiency virus type 1: Nef is independent of both intravirion processing and CD4 down-regulation; *Virology* **251** 302–316

Pandori M W, Fitch N J, Craig H M, Richman D D, Spina C A and Guatelli J C 1996 Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein; *J. Virol*. **70** 4283–4290

Piguet V, Chen Y L, Mangasarian A, Foti M, Carpentier J L and Trono D 1998 Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes; *EMBO J*. **17** 2472–2481

Piguet V, Gu F, Foti M, Demaurex N, Gruenberg J, Carpentier J L and Trono D 1999a Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of beta-COP in endosomes; *Cell* **97** 63–73

Piguet V, Schwartz O, Le Gall S and Trono D 1999b The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors; *Immunol. Rev*. **168** 51–63

Piguet V and Trono D 1999 The Nef protein of primate lentiviruses; *Rev. Med. Virol*. **9** 111–120

Renkema G H, Manninen A and Saksela K 2001 Human immunodeficiency virus type 1 Nef selectively associates with a catalytically active subpopulation of p21-activated kinase 2 (PAK2) independently of PAK2 binding to Nck or beta-PIX; *J. Virol*. **75** 2154–2160

Rhee S S and Marsh J W 1994 HIV-1 Nef activity in murine T-cells. CD4 modulation and positive enhancement; *J. Immunol*. **152** 5128–5134

Riggs N L, Craig H M, Pandori M W and Guatelli J C 1999 The dileucine-based sorting motif in HIV-1 Nef is not required for down-regulation of class I MHC; *Virology* **258** 203–207

Rousso I, Mixon M B, Chen B K and Kim P S 2000 Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity; *Proc. Natl. Acad. Sci. USA* **97** 13523–13525

Rudd C E, Trevillyan J M, Dasgupta J D, Wong L L and Schlossman S F 1988 The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp 58) from human T lymphocytes; *Proc. Natl. Acad. Sci. USA* **85** 5190–5194

Saksela K, Cheng G and Baltimore D 1995 Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4; *EMBO J*. **14** 484–491

Salghetti S, Mariani R and Skowronski J 1995 Human immunodeficiency virus type 1 Nef and p56lck protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail; *Proc. Natl. Acad. Sci. USA* **92** 349–353

Sawai E T, Baur A S, Peterlin B M, Levy J A and Cheng-Mayer C 1995 A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity; *J. Biol. Chem*. **270** 15307–15314

Sawai E T, Baur A, Struble H, Peterlin B M, Levy J A and Cheng-Mayer C 1994 Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes; *Proc. Natl. Acad. Sci. USA* **91** 1539–1543

Sawai E T, Khan I H, Montbriand P M, Peterlin B M, Cheng-Mayer C and Luciw P A 1996 Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques; *Curr. Biol*. **6** 1519–1527

Schrager J A and Marsh J W 1999 HIV-1 Nef increases T-cell activation in a stimulus-dependent manner; *Proc. Natl. Acad. Sci. USA* **96** 8167–8172

Schwartz O, Marechal V, Danos O and Heard J M 1995 Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell; *J. Virol*. **69** 4053–4059

Schwartz O, Marechal V, Le Gall S, Lemonnier F and Heard J M 1996 Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein; *Nat. Med*. **2** 338–342

Simard M C, Chrobak P, Kay D G, Hanna Z, Jothy S and Jolicoeur P 2002 Expression of simian immunodeficiency virus nef in immune cells of transgenic mice leads to a severe AIDS-like disease; *J. Virol*. **76** 3981–3995

Simmons A, Aluvihare V and McMichael A 2001 Nef triggers a transcriptional program in T-cells imitating single- signal T cell activation and inducing HIV virulence mediators; *Immunity* **14** 763–777

Skowronski J, Parks D and Mariani R 1993 Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene; *EMBO J*. **12** 703–713

Smith B L, Krushelnycky B W, Mochly-Rosen D and Berg P 1996 The HIV nef protein associates with protein kinase C theta; *J. Biol. Chem*. **271** 16753–16757

Swigut T, Shohdy N and Skowronski J 2001 Mechanism for down-regulation of CD28 by Nef; *EMBO J*. **20** 1593–1604

Swingler S, Mann A, Jacque J, Brichacek B, Sasseville V G, Williams K, Lackner A A, Janoff E N, Wang R, Fisher D and Stevenson M 1999 HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages; *Nat. Med*. **5** 997–1103

Van Parijs L, Peterson D A and Abbas A K 1998 The Fas/Fas ligand pathway and Bcl-2 regulate T cell responses to model self and foreign antigens; *Immunity* **8** 265–274

Veillette A, Horak I D, Horak E M, Bookman M A and Bolen J B 1988 Alterations of the lymphocyte-specific protein tyrosine kinase (p56lck) during T-cell activation; *Mol. Cell. Biol*. **8** 4353–4361

Weiss A 1993 T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases; *Cell* **73** 209–212

Weiss A and Littman D R 1994 Signal transduction by lymphocyte antigen receptors; *Cell* **76** 263–274

Weiss A, Wiskocil R L and Stobo J D 1984 The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL 2 production reflects events occurring at a pre-translational level; *J. Immunol*. **133** 123–128

Willey R L, Maldarelli F, Martin M A and Strebel K 1992 Human immunodeficiency virus type 1 Vpu protein regulates the formation of intracellular gp160-CD4 complexes; *J. Virol*. **66** 226–234

Wiskerchen M and Cheng-Mayer C 1996 HIV-1 Nef association with cellular serine kinase correlates with enhanced virion infectivity and efficient proviral DNA synthesis; *Virology* **224** 292–301

Wolf D, Witte V, Laffert B, Blume K, Stromer E, Trapp S, d'Aloja P, Schurmann A and Baur A S 2001 HIV-1 Nef associated PAK and PI3-kinases stimulate Akt-independent Bad-phosphorylation to induce anti-apoptotic signals; *Nat. Med*. **7** 1217–1224

Xu X N, Laffert B, Screaton G R, Kraft M, Wolf D, Kolanus W, Mongkolsapay J, McMichael A J and Baur A S 1999 Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor zeta chain; *J. Exp. Med*. **189** 1489–1496

Yoon K, Jeong J G and Kim S 2001 Stable expression of human immunodeficiency virus type 1 Nef confers resistance against Fas-mediated apoptosis; *AIDS Res*. *Hum*. *Retroviruses* **17** 99–104

Zang M, Waelde C A, Xiang X, Rana A, Wen R and Luo Z 2001 Microtubule integrity regulates Pak leading to Ras-independent activation of Raf-1. Insights into mechanisms of Raf-1 activation; *J*. *Biol*. *Chem*. **276** 25157–25165

Zhou J and Aiken C 2001 Nef enhances human immunodeficiency virus type 1 infectivity resulting from intervirion fusion: evidence supporting a role for Nef at the virion envelope; *J*. *Virol*. **75** 5851–5859