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HIV-1 Nef Transfer and Intracellular Signalling in Uninfected Cells

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1. Introduction

Several lymphotropic viruses manipulate host innate immune response to escape immune recognition and improve viral replication and spreading. From this point of view HIV (Human Immunodeficiency Virus-1) represents a paradigmatic example (for review see Peterlin & Trono, 2003). HIV-1 encodes the classical structural and enzymatic factors of all retroviruses codified from the *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) genes. In addition it codes two regulatory proteins that are essential for viral replication (i.e., the transcriptional transactivator Tat and the regulator of virion gene expression Rev) and four accessory proteins (i.e., the ill-named 'negative effector' Nef, the viral infectivity factor Vif, the viral protein r Vpr and the viral protein u Vpu). With time has become increasingly clear that the so-called accessory proteins carry out several critical functions for both viral replication and pathogenesis (Malim & Emerman, 2008). In particular, the Nef protein was demonstrated to be an important virulence factor of primate lentiviruses. In fact Nef-defective HIV leads to an attenuated clinical phenotype with reduced viral loads in mouse models, monkeys, and in human disease (Daniel et al., 1992; Deacon et al., 1995; Gulizia et al., 1997; Kestler et al., 1991; Kirchhoff et al., 1995) and *nef* transgenic mice develop an AIDS-like disease (Hanna et al., 1998) confirming that this viral protein is a major determinant of pathogenicity.

Studies on structure and mechanism of action of the protein highlighted its multifunctional properties at cellular and molecular level. The open reading frame encoding Nef is located 3' of the *env* gene, overlaps the untranslated sequences of the 3' viral long terminal repeat and is translated from multiply spliced transcripts. Nef is expressed early and most abundantly during the infection cycle together with Tat and Rev and evidences have been reported of possible expression also before integration of the proviral genome (Wu & Marsh, 2001). It acts as a molecular adaptor inside the cell inducing genetically distinguishable, yet highly

conserved, effects via specific protein-protein interaction motifs (Arold & Baur, 2001; Doms & Trono, 2000; Geyer et al., 2001, Foster et al., 2011). In general, Nefs from all primate lentiviruses (*i.e.* HIV-1, HIV-2, and SIV) share multiple activities *in vitro*, but differences between the ability of human and simian immunodeficiency viruses to induce Nef-mediated internalization of the CD3 component of the T-cell receptor complex have been observed and have been correlated with the greater pathogenicity of HIV and the reduction of T-cell activation in simian immunodeficiency virus (SIV) (Schindler et al., 2006). It has been also observed that HIV-1 Nef treatment of uninfected cells in culture causes internalization of the protein in some cell types and/or activates specific intracellular signalling pathways. Interestingly, Nef has been found inside uninfected B cells of lymphoid follicles from infected individuals (Qiao et al., 2006) and recently it has been provided experimental evidencies that it can be transferred to uninfected cells from the infected ones via cellular protrusions and/or exosomes (Lenassi et al., 2010; Muratori et al., 2009; Xu et al., 2009) opening a new road to deepen our insight on the roles of this multifunctional protein. This review will focus on those recent observations trying to provide a unifying reading.

2. Nef: A multifunctional viral adaptor

Nef is a protein of about 200 aminoacids and the different alleles may vary slightly in length. Sequence analysis has identified a number of conserved motifs that are responsible for protein–protein interactions and for specific biological functions. The protein is co-translationally modified by N-terminal myristoylation and is phosphorylated on specific aminoacid residues. Its membrane binding is critical for Nef function on cell signalling and membrane trafficking and requires both the covalently attached myristic acid moiety and a cluster of N-terminal basic residues (Bentham et al., 2006; Gerlach et al., 2010; Szilluweit et al., 2009). Myristoylation appears only a weak membrane-targeting signal, but the N-terminal basic residues, especially the arginine-rich cluster (R^{17} to R^{22}), are needed for the stable association of the viral protein with cellular membranes. Nef appears to be a cytoplasmic protein partially associated with cell membrane and often accumulated in perinuclear regions. Cellular-fractionation assays from transient transfection experiments showed that less than 60% of the protein is localized at membranes, while the remaining portion was found to be cytosolic (Kaminchik et al., 1994). Structurally the 24-29 kDa HIV-1 Nef protein adopts a two-domain structure encompassing a flexible membrane anchor domain (residues 2-61 in Nef_{SF2}) and a folded core domain (residues 62-210). The core domain, again, contains a C-terminal flexible loop of 33 residues (152-184) that is thought to mediate trafficking interactions (Geyer et al., 2001). Arold and Baur speculated that after translation the protein adopts a close conformation where the myristoyl moiety interacts with a hydrophobic region on the core domain, which could explain why the majority of the protein is localized in the cytosol and not attached to membranes (Arold & Baur, 2001). Biochemical indication for myristoylation-dependent conformational changes in HIV-1 Nef has been obtained (Breuer et al., 2006; Dennis et al., 2005)**.** Indeed, Nef is readily soluble in aqueous solution, suggesting the shielding of the lipid moiety within the protein. Protein structures of Nef have been determined for the core domain by NMR spectroscopy and X-ray crystallography (Arold et al., 1997; Grzesiek et al., 1997; Lee et al., 1996) and for the flexible anchor domain by NMR (Geyer et al., 1999), but not yet for the full-length protein due to the low stability and solubility and the high degree of intrinsic flexibility. The protein mediates a multitude of functions, increasing the production and infectivity of viral particles

and inducing alteration of specific cellular signalling and trafficking pathways. It has been also demonstrated that the protein is able to induce the transient translocation to the cell membrane of the Polycomb Group protein Eed, a nuclear transcriptional repressor, leading to a potent stimulation of Tat-dependent HIV transcription (Witte et al., 2004). The cytoplasmic translocation of Eed seems to result in the removal of a block on Tat-mediated HIV transcription essential to promote viral transcription at low concentrations of the transactivator protein found at the very early phase of the infection or reactivation from latency. It has been proposed that Nef adopts different structural conformations inside the cell that allows different localizations and interaction with different partners, realizing the so called "Nef interaction cycle" (Arold & Baur, 2001). In particular, Nef, after translation, could adopt the closed conformation in which its binding sites are mainly hidden (closed conformation). Contact with the cell membrane could then trigger a conformational change via the interaction of the negative charges of the membrane lipid heads with the positive charges in the N-terminus of Nef, thus relieving the interaction between the N-terminus and the core. This conformational change could also expose several motifs capable of binding signalling molecules (signalling conformation), many of which are present in lipid rafts. The association of Nef molecules with the membrane might persist for only a short period of time because of the exposure of the core loop, which would then bind molecules of the cellular endocytotic machinery mediating internalization of Nef together with specific interaction partners.

The best characterized Nef functions are: (a) acceleration of endocytosis and lysosomal degradation of CD4, thereby avoiding both super-infection of infected cells and the interaction of budding virions with CD4 of the infected cells; (b) down-regulation of HLA-A and -B MHC-I molecules thereby protecting infected cells from recognition and lysis by cytotoxic T lymphocytes (CTL); (c) induction of a pre-activation state in CD4+ T cells favouring viral gene expression (Simmons et al., 2001); (d) regulation of apoptosis, promoting apoptosis in bystander uninfected cells meanwhile protecting the infected cells by apoptotic stimuli through more than one mechanism (Das & Jameel, 2005). Nef perturbs the trafficking of many different plasma membrane-associated proteins but how Nef traffics within the endosomal system to reach the perinuclear endosomal region where it is concentrated at steady state is not yet understood (Burtey et al., 2007; Roeth & Collins, 2006). The list of cellular proteins whose transport is affected has continued to grow over the past several years. To date it has been reported to decrease the cell surface expression of MHC-I, MHC-II, CD4, CD28, transferrin and mannose receptors, CD80, CD86, CD8, and CCR5 and to increase the expression of TNF, LIGHT, DC-SIGN, and the invariant chain (Anderson et al., 1993; Chaudhry et al., 2005; Lama & Ware, 2000; Madrid et al., 2005; Schindler et al., 2003; Schwartz et al., 1996; Sol-Foulon et al., 2002; Stove et al., 2005; Stumptner-Cuvelette et al., 2001). The acceleration of endocytosis and lysosomal degradation of the transmembrane glycoprotein CD4 was one of the first discovered actions. To recruit CD4 into the endocytotic pathway, Nef acts as an adaptor between CD4 and components of the clathrin coated pits. In T cells this involves disruption of the CD4-Lck tyrosine kinase complex by Nef and its interaction with adaptor protein (AP) complexes and the regulatory subunit of the vacuolar proton pump v-ATPase. Nef binds also to the beta subunit of COPI coatomers (β-COP) to direct CD4 to a degradation pathway. Surprisingly, Nef uses different domains and mechanism to downregulate MHC-I molecules (Blagoveshchenskaya et al., 2002; Doms & Trono, 2000). Regarding the Nef ability to induce a state of pre-activation in T cells several publications have reported its direct interaction with T Cell Receptor (TCR) ζ chain,

glycolipid enriched membrane microdomains (i.e., GEMs also called lipid "rafts") and proteins of the submembrane TCR environment including the adaptor proteins Vav and LAT, and the kinases Lck, PAK and PKC (Renkema & Saksela, 2000). Mutational analysis suggests that most of the signaling molecules that bind to Nef interact with its core domain, often via the Pro-rich sequence that binds the SH3 domain of Src kinases. Nef displays high (*i.e.* nanomolar) affinity for the SH3 domain of Hck and Lyn, and rather modest (*i.e.* micromolar) affinity for those of Lck, Fyn and Src. *In vivo,* the individual tissue distribution of Src kinases become relevant because Hck is restricted to macrophages, therefore in T cells Nef might interact with lower-affinity targets such as Fyn or Lck. As far as regulation of apoptosis concerns, it appears that Nef causes bystander cell apoptosis in uninfected cells through its induction of FasL expression on the infected CD4+ T cell (Xu et al., 1999; Xu et al., 1997). Conversely, it inhibits apoptosis in virally infected host T cells both through its concomitant suppressive effects on ASK1, a key intermediate in the Fas and TNFα death signaling cascade, and via Akt-indipendent phosphorylation of Bad (Geleziunas et al., 1996; Geleziunas et al., 2001; Wolf et al., 2001). In addition it has been described to inhibit p53-mediated apoptosis binding the tumor suppressor (Greenway et al., 2002).

As a consequence of Nef ability to bind multiple targets it is plausible that depending on Nef subcellular localization and the availability of particular subset of targets (for example Hck versus Lck tyrosine kinase), certain Nef effects dominate over others in a time- and cell type-dependent manner thus differing in infected monocyte/macrophages versus T lymphocytes, DC, astrocytes or microglial cells (see Quaranta et al., 2009 for the different regulatory effects of Nef in immune cells). In macrophages, that are important target of HIV infection and reservoir of the virus, Nef expression induces the release of a set of paracrine factors including a marked increase of CCL2/MIP-1α and CCL4/MIP-1β chemokines. These factors are able to recruit T cells and make them susceptible to HIV replication (Swingler et al., 2003; Swingler et al., 1999). In particular, Nef expression in macrophages induces also the production of the soluble forms of the intercellular adhesion molecule ICAM-1 (sICAM) and of the coactivation molecule CD23 (sCD23). sICAM and sCD23 act on B cells that cooperate with macrophages in inducing T cell recruitment and their permissivity to productive infection *in vitro*. The effects of Nef expression in macrophages mimics those of CD40 Ligand (CD40L) in activating the CD40 signalling cascades suggesting that Nef intersects a macrophages pathway that is regulated by the CD40 receptor and requires nuclear factor kappa B (NF-κB) activation (Swingler et al., 2003; Swingler et al., 1999).

3. Effects induced by extracellular Nef in uninfected cells

Evidences that Nef produced by recombinant DNA technology can induce cell signalling effects when added to cell cultures has been provided (Alessandrini et al., 2000; Brigino et al., 1997; Fujii et al., 1996c; Fujinaga et al., 1995; Huang et al., 2004; James et al., 2004; Lehmann et al., 2006; Okada et al., 1998; Okada et al., 1997; Qiao et al., 2006; Quaranta et al., 1999; Quaranta et al., 2003; Tobiume et al., 2002; Varin et al., 2003). Different Nef alleles have been expressed in *E. coli* and both myristoylated (myr+) or not myristoylated (myr-) proteins have been produced (Breuer et al., 2006; Dennis et al., 2005). Myr+ Nef was obtained via co-trasformation of *E. coli* with expression vectors coding the viral protein and the human N-myristoyl-transferase and supplementing the culture medium with myristic acid. Purified myr+ and myr- wild type (wt) Nef proteins showed different oligomerisation properties *in vitro*. Indeed, myristoylated Nef prevails in a monomeric state in solution whereas the

nonlipidated protein forms dimers, trimers, or even oligomers of greater magnitude. When cell signalling effects induced by myr+ and myr- wtNef were compared in cultures of human monocyte-derived macrophages (MDMs), myr+ wt Nef was found to be much more active than the myr- protein (Mangino et al., 2007; Olivetta et al., 2003). Table 1 briefly summarizes the effects induced by cell treatment with Nef. In particular, negative effects on CD4+ T cell survival has been described (Fujii et al., 1996a; b; Fujii et al., 1996c), *e.g.* Nef treatment of CD4+ T lymphocytes in culture induced apoptosis via the interaction with the CXCR4 receptor (Huang et al., 2004; James et al., 2004). On immature dendritic cells (iDCs) exogenous Nef enhances CXCR4 expression and up-regulates MHC-II molecules, possibly favouring their migration and nonspecific CD4+ T cell activation (Quaranta et al., 2002). Exogenous Nef enters iDCs, promoting their functional and morphological differentiation. Specifically, Nef promotes interleukin (IL)-12 release, which closely fits with nuclear factor NF-κB activation, and targets Vav promoting its tyrosine phosphorylation associated with its nucleus-to-cytoplasm redistribution. Nef induces also the rearrangement of actin microfilaments, leading to uropod and ruffle formation and increases the capacity of DCs to form clusters with allogeneic CD4+ T cells, improving immunological synapse formation (Quaranta et al., 2003). In addition to iDCs, Nef is internalized by primary human MDMs and IgD+ B cells in culture (Alessandrini et al., 2000; Qiao et al., 2006). Myr+ Nef treatment of primary human IgD+ B cells induced to differentiate in culture by the addition of CD40L, IL-4 and IL-10 inhibits switching to IgG, IgA, and IgE by inducing the negative regulators I-κB-α and SOCS proteins, which block CD40L and cytokine signalling rendering Nef-containing B cell less responsive to CD4+ T cell help (Qiao et al., 2006). Nef treatment of primary human MDMs down-regulated CD4 surface expression, thus reproducing an effect widely observed in cells endogenously expressing the viral protein. In addition, myr+ Nef treatment of MDMs induces the rapid (15-30') activation of IKK/NF-κB, MAPKs (i.e., ERK1/2, JNK and p38) and IRF-3, the main transcriptional regulator of the IFNβ gene expression, thereafter regulating the expression of many cellular transcripts (Federico et al., 2001; Mangino et al., 2007; Olivetta et al., 2003; Percario et al., 2003). The prompt transcriptional reprogramming leads in 2 hours to the synthesis and release of a set of proinflammatory cytokines/chemokines, including TNFα, IL-1β, IL-6, CCL2/MIP-1α and CCL4/MIP-1β, and of IFNβ that, in turn, immediately activate the signal transducers and activators of transcription STAT1, -2 and -3 in autocrine and paracrine manner. A transient STAT1, -2 and -3 tyrosine phosporylation was also observed early after (*i.e.* 8-16 hrs later) *in vitro* infection of 7-day old human MDMs with *nef*-expressing Δ*env*, but not Δ*nef*/Δ*env*, HIV-1 pseutotypes suggesting that intracellular signalling induced in Nef-treated MDMs might also be activated via Nef intracellular expression soon after MDMs infection with HIV-1 or viral reactivation from latency. In an attempt to identify the Nef structural motifs required for the activation of those signaling pathways, MDMs were treated with different myr+ recNef$_{SF2}$ proteins lacking specific conserved aminoacid residues (Mangino et al., 2007). In particular, the viral protein was modified in the consensus sequence required for myristoylation (mutant $G^2{\rightarrow}A$), in the polyproline-rich region (mutant $P^{76}XXP^{79}XR^{81}{\rightarrow}AXXAXA$), in the domain required for the interaction with CD4 (mutant $C^{59}AWL^{62}{\rightarrow}AAAA$) or with elements of the endocytic machinery, such as the V1H subunit of the vacuolar-membrane ATPase (mutant $E^{178}D^{179}{\rightarrow}AA$) or the adaptor protein complex (mutant $L^{168}L^{169}{\rightarrow}AA$) Finally a Nef protein with a deletion of the first 44 amino acids (ΔN-term) was also used. Overall the experimental results indicated that myristoylation of the protein was required for the activation of the signaling cascades, because $G^2{\rightarrow}A$ and ΔN-

term Nef were the only two Nef mutants unable to induce signalling. These data were in agreement with lack of induction of STAT1, STAT2 and STAT3 tyrosine phosphorylation in MDMs 8 to 16 h after infection with VSV-G Δ*env* HIV-1 pseudotypes expressing the $G^2{\rightarrow}A$ Nef mutant (Mangino et al., 2007; Percario et al., 2003). As previously mentioned myristoylation of Nef is a weak membrane-targeting signal and N-terminal basic residues, especially an arginine-rich cluster (R^{17} to R^{22}), are needed for the stable association of the viral protein with cellular membranes. Presently, it is not possible to exclude the hypothesis that also these residues are important together with myristoylation for the activation of NF-κB and IRF-3 in MDMs.

Regarding the production of IFNβ we propose that Nef, abundantly expressed early after HIV infection together with Tat and Rev, induces IFNβ at a very early stage of viral infection or soon after reactivation of viral gene expression in latently infected cells, before the appearance of other viral proteins that can inhibit type I IFN production or mechanisms of action to allow the viral release from infected cells. In fact, even if type I IFNs inhibit the release of retroviral particles and their infectivity in cells chronically infected by the simplest retroviruses without inducing relevant reduction of viral proteins synthesis (Peng et al., 2006; Pitha-Rowe & Pitha, 2007), the lentivirus HIV has evolved specific strategies to inhibit IFN-mediated antiretroviral effects at least via Vif and Vpu accessory protein functions. Both these viral proteins are produced later than the early HIV regulatory protein Nef and inhibit the function of APOBEC3 and tetherin, two cellular antiretroviral proteins specifically increased by type I IFN via positive transcriptional regulation (Malim & Emerman, 2008). In addition a recent analysis of antiviral defences in CD4$^+$ cells during HIV-1 infection indicates that viral expression can also direct a global disruption of innate immune signalling through suppression of IRF-3 (Doehle et al., 2009). In particular, a marked depletion of IRF-3, but not IRF-7, was observed in HIV-1-infected cells which supported robust viral replication. Indeed, IRF-3 depletion was dependent on a productive HIV-1 replication cycle and caused the specific disruption of Toll-like and RIG-I-like receptors innate immune signalling. In agreement with these *in vitro* results, IRF-3 levels were found reduced *in vivo* within CD4$^+$ T cells from patients with acute HIV-1 infection, but not from long-term non-progressors (Doehle et al., 2009). A decrease of IRF-3 expression after HIV-1 infection was observed also in THP-1 cells, a human monocyte/macrophage-like cell line, as HIV-1 infection progressed over a 48-h period. The ability of Nef to induce transient IFNβ production in HIV-1 infected cells *in vivo* needs to be investigated. Nef might induce IFNβ production in cells neighbouring the infected ones if the protein is secreted or released after cell lysis or when transferred via cell-to-cell contact to uninfected cells (Peterlin, 2006). This cytokine could exert its effects on bystander cells, such as the plasmacytoid dendritic cells, priming them to IFNα production. Other HIV products (*i.e.*, gp120) induce the production of type I IFNs in MDMs, as well as in PBMCs (Ankel et al., 1994; Capobianchi et al., 1993; Capobianchi et al., 1992; Gessani et al., 1994). Induction of both IFNα and -β as a consequence of *in vitro* infection of MDMs cultures has been reported (Gessani et al., 1994; Szebeni et al., 1991) and a rapid and transient elevations in IFNα has been observed during the cytokine storms that accompany the increase in plasma viremia in

acute HIV-1 infection (Stacey et al., 2009). It is interesting to note that in early 1980's one of the first clear-cut HIV isolate was obtained using cultured T lymphocytes, derived from a lymphonode biopsy specimen of a patient with lymphadenopathy, with the help of IL-2 and anti-IFNα serum (Barre-Sinoussi et al., 1983).

Ref.	recNef Alleles	Cell type	Induced Effects
Alessandrini et al., 2000	NL4-3	primary hu MDMs	- CD4 downmodulation - Inhibition of M- and Dual-tropic strains replication
Brigino et al., 1997	LAV	PBMC H9 U937	- IL-10 induction in a calcium/calmodulin dependent manner
Creery et al., 2002		PBMC primary hu monocytes	- CD14 upregulation
Federico et al., 2001 Mangino et al., 2007 Olivetta et al., 2003 Percario et al., 2003	NL4-3 BH10 SF2	primary hu MDMs	-- Activation of NF-kB, MAPKs and IRF-3 signalling pathways - Synthesis of pro-inflammatory cyto- and chemokines including CCL2/MIP-1α, CCL4/MIP-1β, TNFα, IL-1β, IL-6 and IFNβ that induce STAT1, STAT2 and STAT3 phosphorylation in autocrine and paracrine manner - Induction of IRF-1
Fujii et al., 1996 a,b,c	NL4-3	CD4+ T lymphocytes	- Cytotoxic effects on T cells - Inhibition of proliferaton
Fujinaga et al., 1995		MOLT-20-2	- HIV reactivation from latency
Huang et al., 2004	NL4-3	PBMC Jurkat	- Apoptosis induction through Nef-CXCR4 interaction
James et al., 2004	NL4-3	Jurkat H9	- Apoptosis induction
Okada et al., 1997 Okada et al., 1998	ELI III B	JA-4, Balb3T3 EL-4, WEHI	- Fas-independent apoptosis induction
Qiao et al., 2006	BaL	primary B cells	- Suppression of CD40-dependent Ig class switching
Quaranta et al., 1999	ELI	hu monocyte /macrophages	IL-15 synthesis induction
Quaranta et al., 2002 Quaranta et al., 2003	ELI	primary hu Dendritic Cells	- DC maturation - Cytoskeleton rearrangements through Vav/Rac-1 dependent signaling
Tobiume et al., 2002			- HIV activation from latency through Ras/MAPKs signaling
Varin et al., 2003	SIVmac SF2 BH10	U937 U1	- Stimulation of HIV transcription through NF-κB, AP-1 and c-JNK signaling

Table 1. Effects induced by cell treatment with recombinant Nef

Regarding the response of astrocytes to extracellular Nef, treatment of uninfected cells (*i.e.* human glioblastoma/astrocytoma cell line U373MG) with the purified viral proteins Nef induces the synthesis of C3 (Speth et al., 2002). A significant difference in the C3 synthesis in Nef-treated cells was visible after 3 days and maximal effect was reached after 9 days. Besides Nef, also whole HIV virions and gp41 were biologically active in upregulating C3, whereas Tat, gp120, and gp160 were not (Speth et al., 2002). The complement system is of special importance in the brain because the elements of adaptive immunity have only

limited access due to the blood-brain barrier. The complement factor C3 is a central protein of the cascade, and its fragments (C3b, iC3b, C3d, and C3a) affect many cellular processes in the brain, such as activation of signalling pathways and modulation of cytokine synthesis. In general, all complement proteins can be synthesized by various brain cells, including astrocytes, neurons, microglia, and oligodendrocytes, with astrocytes being the most potent complement producers. Although normal synthesis in the brain is low, inflammatory cytokines such as IFNγ and TNFα considerably increase complement production, especially of complement factor C3. Increased levels of C3 and C4 were found in the cerebrospinal fluid of HIV-infected patients with neurological symptoms and signs of central nervous system dysfunction, supporting the hypothesis of an association between complement and HIV-induced neurodegeneration. HIV-induced upregulation of C3 expression in astrocytes may be an important reason for increased complement levels in the cerebrospinal fluid of HIV-infected patients. HIV-1 increased the C3 levels in astrocyte culture supernatants from 30 to up to 400 ng/ml. Signal transduction studies revealed that adenylate cyclase activation with upregulation of cyclic AMP is the central signalling pathway to mediate that increase. Furthermore, activity of protein kinase C was necessary for HIV induction of C3, since inhibition of protein kinase C by prolonged exposure to the phorbol ester tetradecanoyl phorbol acetate partly abolished the HIV effect.

Confocal microscopy analysis of human MDMs treated with myr- and myr+ Nef–FITC indicated that both proteins are internalized and localize in an intracytoplasmic punctate pattern and at the cell margin (Alessandrini et al., 2000; Olivetta et al., 2003) as described for cells that endogenously express the protein (Greenberg et al., 1997). The protein lacking the acceptor signal for myristoylation (G2→A Nef) is internalized, but loose at least in part the co-localization signal with the cell membrane (Alessandrini et al., 2000). The wt protein does not bind MDMs at 4 °C suggesting that it does not recognize specific receptor on the cell membrane, but it might be internalized exploiting the endocytic/pinocytotic MDMs machinery. Indeed Nef was shown to perturb model membranes opening an avenue for its translocation through cell membranes (Gerlach et al., 2010; Szilluweit et al., 2009).

All these experimental evidences suggest that extracellular Nef if present *in vivo* could induce specific cellular response in uninfected cells that might contribute directly and indirectly to the onset of AIDS. Indeed the presence of extracellular Nef in infected individuals has not been extensively evaluated. The viral protein has been found in the serum of some HIV-1 infected patients at concentration ranging from 1 to 10 ng/ml (Fujii et al., 1996c). This concentration might be even higher in the lymphonodal germinal centers, where virion-trapping dendritic cells, virion-infected CD4+ T cells and macrophages are densely packed (Pantaleo et al., 1991). In addition antibodies directed against this viral protein have been found in HIV-1 infected seronegative individuals supporting the possible detection of extracellular molecule (Ameisen et al., 1989). Nef can be released in culture supernatant of 293, Jurkat and THP-1 cells transfected with HIV-1$_{NL4-3}$-, HIV-2- or SIV-Nef expressing vectors indicating that it can be secreted (James et al., 2004). The genetic characterization of Nef-induced secretion has identified that the N-terminal 70 amino acids were sufficient for induction of secretion of Nef- containing vescicles in several cell lines and identified critical aminoacid residues: (1) a basic cluster of four arginine residues (aa 17, 19, 21, 22), (2) the phosphofurin acidic cluster sequence (PACS; Glu62–65), and (3) a previously uncharacterized domain spanning amino acid residues 66–70 (VGFPV), which has been named the secretion modification region (SMR). Interestingly, *in vivo* analysis performed on infected follicles of lymphoid tissue showed the presence of Nef positive IgD+ B cells at the

edge of the germinal center and in the interfollicular area even if these cells were unlikely to be infected by HIV-1 (Qiao et al., 2006). This observation suggested that Nef, produced inside the HIV infected cells via the transcription of the proviral genome, can be transferred *in vivo* to bystander non infected cells.

4. Nef transfer from infected to uninfected cells

The transfer of HIV viral proteins by nanotubes (Sowinski et al., 2008) and the transfer of Nef by vesicles (Campbell et al., 2008) has been recently documented. Interestingly in September 2009, Xu and collaborators (Xu et al., 2009) reported that HIV-1 infected macrophages form B cell–targeting conduits in response to Nef expression. These conduits translocate membrane-bound Nef and Nef-containing endosomes from macrophages to follicular B cells via a mechanism propelled by actin, mediated by Vav and dependent on GTPases. These results provided the evidence that infected cells can transfer the viral protein via cell to cell contact to uninfected cells. It appears that Nef-mediated activation of membrane trafficking is bidirectional, connecting endocytosis with exocytosis as occurs in activated T cells. Interestingly Nef expression induced an extensive secretory activity also in infected and, surprisingly, in non infected T cells, leading to the massive release of microvesicle clusters, a phenotype observed in vitro and in 36%–87% of primary CD4+ T cells from HIV-infected individuals. Consistent with exocytosis in noninfected cells, Nef is transferred to bystander cells upon cell-to-cell contact and subsequently induces secretion in an Erk1/2-dependent manner. Thus, Nef alters membrane dynamics mimicking those of activated T cells and causing a transfer of infected cell signalling to bystander cells (Muratori et al., 2009). Based on these findings, it was hypothesized that Nef transfer could potentially explain the effects observed in bystander cells *in vivo*. In agreement with this hypothesis conduits-mediated shuttling of Nef from infected macrophages to B cells attenuated IgG2 and IgA class switching in systemic and intestinal lymphoid follicles suggesting that HIV-1 exploits intercellular 'highways' as a 'Trojan horse' to deliver Nef to B cells and evade specific humoral immunity systemically and at mucosal sites of entry. The stronger virus-specific IgG2 and IgA responses observed in Long Term Non Progressor infected by Nef-deficient HIV-1 further supports this possibility (Xu et al., 2009). In addition Lenassi et al. (Lenassi et al., 2010) demonstrated that the expression of Nef not only augmented the production of the exosomes from T cells (Jurkat, SupT1 and primary T cells) but also resulted in the packaging of Nef into these vesicles which, upon contact, leads to an activation-induced cell death in the resting CD4+ T lymphocytes (bystander cells). It is known that subsets of cellular proteins are specifically targeted to exosomes reflecting the site of their formation. Proteomic profiling of Nef exosomes originating from Jurkat and SupT1 cells indicates that Nef exosomes form at the plasma membrane in Jurkat and in Multivescicular Bodies in SupT cells. Instead Nef exosomes from PBLs represent a mixture of secreted vesicles of plasma membrane and late endosomal origin. Therefore, Nef increases the production of exosomes from several distinct cellular compartments. Nef release through exosomes was conserved also during HIV-1 infection of peripheral blood lymphocytes (PBLs). Thus, HIV-infected cells export Nef in bioactive vesicles that might facilitate the depletion of CD4+ T cells *in vivo*.
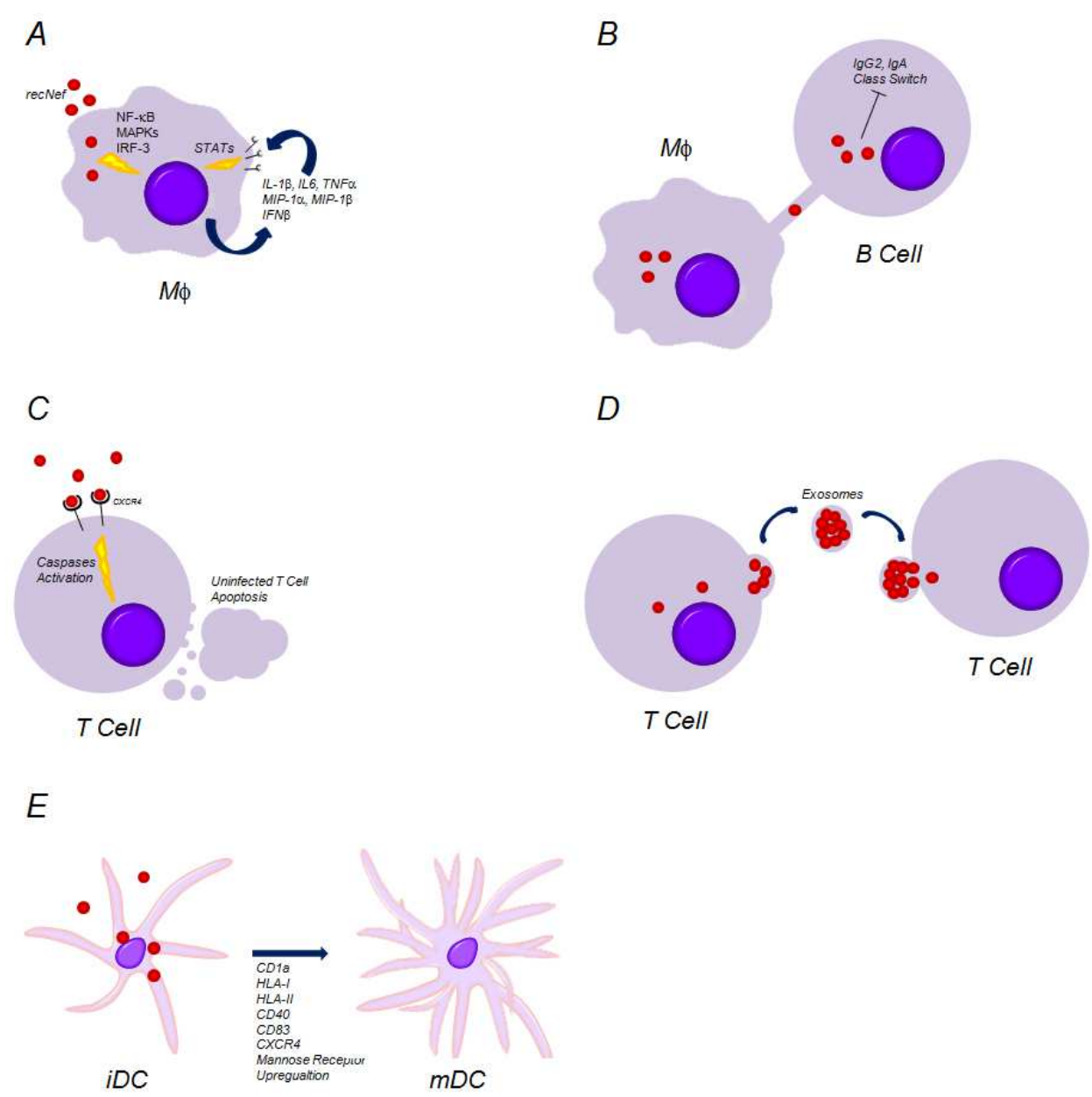Figure 1 schematically summarizes Nef-induced effects and transfer in uninfected cells.

Fig. 1. Schematic representation of Nef-induced signalling and transfer in uninfected cells. A: Nef treatment of MDMs induces signal transduction events followed by production of inflammatory chemo/cytokines and IFNβ; B: infected MDMs transfer Nef via cellular protusions; C: exogenous Nef induces apoptosis in T cells via interaction with CXCR4; D: T cells expressing Nef release Nef-containing – exosomes that are transferred to bystander cells; E: extracellular Nef induces up regulation of specific maturation markers in immature DC.

5. Perspectives and future directions

In recent years, it has become apparent that many viruses induce signal transduction events in concert with virus entry and/or as a consequence of the intracellular expression and release of viral proteins. These events can trigger a variety of changes in the cell, including induction of apoptosis, cytoskeleton rearrangements and global reprogramming of cellular transcription to favour viral replication or persistence. At the same time the innate sensing

of some viral components by cells triggers signal transduction pathways to induce antiviral responses. At present, we are only beginning to decipher the mechanism and the consequences of these events to viral replication and pathogenesis in the host. As far as Nef concerns, its ability to induce exosome formation and cell to cell connection mediating its transfer and the transfer of signalling molecules between cells open new road to understand its complex role in HIV-1-host interaction and highlights the complexity of functions of biological molecules. How Nef is able to switch on signalling events in uninfected cells and the patho-physiological relevance of this phenomenon requires further investigations and might disclosure new therapeutic targets for treatment of seropositive patients.

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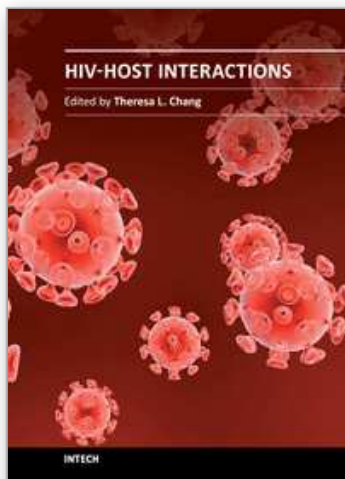
HIV-Host Interactions

Edited by Dr. Theresa Li-Yun Chang

HIV remains the major global health threat, and neither vaccine nor cure is available. Increasing our knowledge on HIV infection will help overcome the challenge of HIV/AIDS. This book covers several aspects of HIV-host interactions in vitro and in vivo. The first section covers the interaction between cellular components and HIV proteins, Integrase, Tat, and Nef. It also discusses the clinical relevance of HIV superinfection. The next two chapters focus on the role of innate immunity including dendritic cells and defensins in HIV infection followed by the section on the impact of host factors on HIV pathogenesis. The section of co-infection includes the impact of Human herpesvirus 6 and Trichomonas vaginalis on HIV infection. The final section focuses on generation of HIV molecular clones that can be used in macaques and the potential use of cotton rats for HIV studies.

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