HIV Nef: Role in Pathogenesis and Viral Fitness

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Abstract: Conserved in all primate lentivirus genomes, Nef promotes viral replication and infectivity, influences the trafficking of a large number of surface receptors and interferes with TCR signalling, consequently modulating T-cell activation. *In vivo* observations with Long Term Non-Progressors harbouring a Nef-defective HIV and vaccination studies with Nef-deleted SIV in Rhesus macaques have shown a prominent role for Nef in lentiviral pathogenesis. Here we review the functions of Nef involved in viral replication and infectivity and speculate on a possible role for Nef in HIV fitness.

Keywords: HIV, nef, fitness, pathogenesis, replication.

Since its discovery in the 1980's, the Human Immunodeficiency Virus type 1 (HIV-1) has infected millions of people worldwide, with current estimates totalling around 33.2 million and more than 2 million deaths during 2007 alone [1]. In the developed world, the use of antiretrovirals has significantly contributed to the increased survival of seropositive individuals. In stark contrast is the situation in developing countries, especially sub-Saharan Africa, where the vast majority of HIV-infected people live. Although current drug regimens (HAART) can suppress viral replication for extended periods of time (multiple years), no combination therapy is yet able to eradicate HIV from human cells.

HIV-1 was introduced into the human population through cross-species transmission from Chimpanzees (*Pan troglodytes troglodytes*) [2]. Recent field work has pointed to wild chimpanzee communities in southeastern and southcentral Cameroon as the sources of HIV-1 groups M (major) and N (new) [3], while the origin of the outlier group (O) may be related to a jump from *Gorilla gorilla* [4]. Since its introduction in humans, HIV-1 group M has diversified into nine subtypes, three sub-subtypes and an ever-expanding number of circulating recombinant forms (CRFs; currently 37 identified). Subtypes share 70-90% nucleotide sequence identity, groups share <70%, and HIV-1 and HIV-2 differ by as much as 50% [5]. In contrast to HIV-1 group M, HIV-2 viruses are geographically restricted to Western Africa and generally cause less aggressive infections. HIV-2 is also transmitted less frequently than HIV-1 in human populations with similar demographics and opportunities for sexual contact [6-8]. The genome of these primate lentiviruses consists of the prototypic structural *gag*, *pol* and *env* genes and six additional genes; *tat*, *rev*, *vif*, *vpr*, *vpu* (only in HIV-1), *vpx* (only in HIV-2), and *nef*. In this review we will focus on the role of Nef in HIV pathogenesis and more specifically on how Nef stimulates viral infectivity and replication. Finally, we will elaborate on the possible impact of Nef on viral fitness.

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NEF IN HIV PATHOGENESIS

In addition to CD4, HIV also requires a co-receptor of the chemokine receptor family (most commonly CCR5 or CXCR4) to successfully establish infection in humans. HIV preferentially infects CD4+ cells such as T-lymphocytes, macrophages, monocytes and dendritic cells and causes a complex and dynamic disease. Because CD4+ T-lymphocytes have a central regulatory function in the immune response, progressive depletion results in a weakened immune system that is less vigilant in controlling endogenous and environmental pathogens, eventually resulting in the acquired immunodeficiency syndrome (AIDS). Recent studies in SIVmac infected Rhesus macaques have shown massive death of gut-associated CD4+ T-cells in the first days to weeks post infection [9, 10]. These findings were confirmed in acutely infected HIV-1 patients, suggesting that the deterioration of lymphocytes hits hard from the onset of infection [11-13]. Infections of cells from the monocyte/macrophage lineage are particularly important in chronic HIV infection, as these cells are likely the major reservoir implicated in viral persistence. Furthermore, infected macrophages are key players in the neurological dysfunction associated with HIV pathogenesis, and may contribute to HIV-associated pathology of non-lymphoid organs such as the heart and kidney [14]. The period for progression from primary HIV-1 infection to terminal-stage AIDS can range from 1 year (fast progressors) to more than 20 years (slow progressors or long-term nonprogressors; LTNP), with a median time to onset of AIDS of 10 years (typical progressors). The variable time to onset of AIDS may depend upon host genetics (such as a 32 base pair deletion in the human *CCR5* gene (CCR5Δ32) or increased expression levels of CCL3L1) [15-17], humoral and cellular immune responses, endogenous and exogenous co-pathogens (such as Human Papiloma Virus, HPV; Tuberculosis Bacil, TB; Herpes Simplex Virus type 8, HSV8), and intrinsic viral factors such as viral genetic elements and virulence.

One of the best characterized viral factors associated with HIV pathogenesis is 'Negative Factor' or Nef. Nef was initially identified as a 3'ORF element which negatively influenced viral replication [18] but more extensive research has revealed that Nef actually stimulates HIV replication and infectivity [19-21].

Nef is a small accessory protein (25-34 kDa), encoded early in the life cycle of HIV-1, HIV-2 and SIV. Two forms of Nef have been described, a membrane-bound myristoylated form and a non-myristoylated cytoplasmic form [22, 23], but since all Nef functions require an intact myristoylation signal, the biological relevance of non-myristoylated Nef is not clear. It was shown that Nef consists of a globular core domain (amino acids 54-205; NL4-3) and disordered N- and C-terminal structures using X-ray crystallography [24-26], nuclear magnetic resonance (NMR) [25, 27] and NMR spectroscopy [28] (Fig. **1**). It appears that the addition of a myristyl group to the N-terminus, which anchors Nef in the lipid membrane, partially orders this domain [28]. Based on models that predict the structure of Nef, it is suggested that Nef is a highly flexible protein that adapts its structural organization to its binding partners [29]. The core domains of HIV and SIV Nef are relatively well conserved, while the N- and C- terminal ends are more variable. The SIVsmm and HIV-2 Nef protein is slightly larger and contains more interaction sites in its termini compared to HIV-1 Nef. Interestingly, the HIV-2 *nef* sequence is more closely related to *nef* from SIVsmm than to that of HIV-1 *nef* (Fig. **1**). One striking example of this difference translates into differential properties in down regulating TCR-CD3 by *nef* alleles from HIV-1, HIV-2 and their simian relatives [30, 31].

Nef has no enzymatic activity, but rather functions as an adaptor protein linking several cellular proteins in functional clusters. The involvement of Nef in HIV pathogenesis became apparent in a cohort of individuals that was accidentally infected during blood transfusion with an HIV variant containing a large deletion in *nef* [32, 33]. Although these individuals have remained asymptomatic for extended periods of time (>15 years), some have now shown clinical signs of disease progression (drop in CD4+ cell count) [34-36]. The role of Nef in HIV pathogenesis has also been examined in animal models. The expression of Nef in natural HIV target cell populations in a transgenic mouse model recapitulates several important AIDS related abnormalities, such as preferential loss of CD4+ T-cells, T-cell activation, lymphadenopathy, and immunodeficiency [37]. Using SCID-hu and hu-PBL-SCID mice, it was shown that Nef is of significant importance for viral replication and CD4+ T-cell depletion [38-40]. Interestingly, although neonatal macaques develop disease, adult rhesus macaques experimentally infected with a variant of SIV that lacks a functional *nef* gene, also appear to resist disease progression [41]. Moreover, Rhesus macaques vaccinated with live SIV Δ*nef* were completely protected against challenge by intravenous inoculation of live, pathogenic SIV [42]. Furthermore, when macaques were intravenously infected with SIV carrying a *nef* gene with a premature stopcodon, the *nef* open reading frame was swiftly restored. These observations clearly suggest a role for Nef in HIV pathogenesis and the development of AIDS.
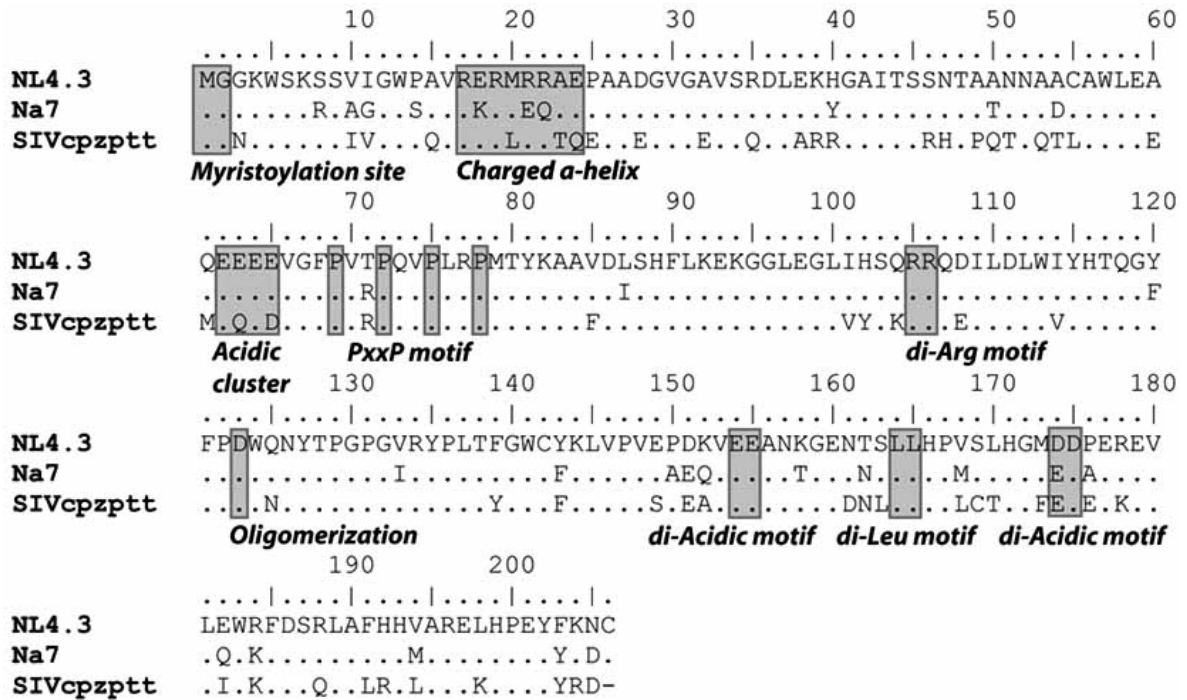
Nef contributes to HIV pathology by modulating protein trafficking, interfering with signalling pathways and apoptosis, and stimulating viral replication/virulence. Nef also affects normal human T-cell development [43]. Although the exact molecular mechanism underlying Nef-induced T-cell depletion is yet to be elucidated, we have provided evidence suggesting that the signalling functions of Nef, particularly

the interaction with p21-activated kinase 2 (PAK2), are implicated [44; Ariën & Verhasselt, unpublished observations]. Nef is equipped with several conserved protein-interaction domains on its surface such as the well defined Src homology (SH3) binding domain (Fig. **1**). This domain is involved in interactions between Nef and Hck, Lck and PAK2 [reviewed in 45], while tyrosine-based motifs, dileucine motifs and diacidic motifs are essential in interactions with adaptor proteins (AP) from vesicular coats and induce the down regulation of certain surface receptors (CD4, MHC-I, CD8β, CD28) [reviewed in 46]. Nef also induces the up regulation of Fas ligand on the surface of HIV-infected cells, inducing programmed cell death of nearby HIV-specific cytotoxic T-lymphocytes (CTL). Another strategy exploited by HIV in order to subvert CTL killing is Nef-induced down regulation of major histocompatibility complex class I (MHC-I). Nef specifically affects the membrane expression of HLA-A and –B (CTL recognition) and not that of HLA-C and –E (NK cell recognition) [47, 48]. There is a vast amount of literature on Nef and its effects on receptor trafficking and signal transduction pathways but because this falls outside the scope of this article, we refer to excellent reviews that cover these topics [45, 46, 49]. In this review we will focus on the role of Nef in HIV infectivity, replication, and fitness.
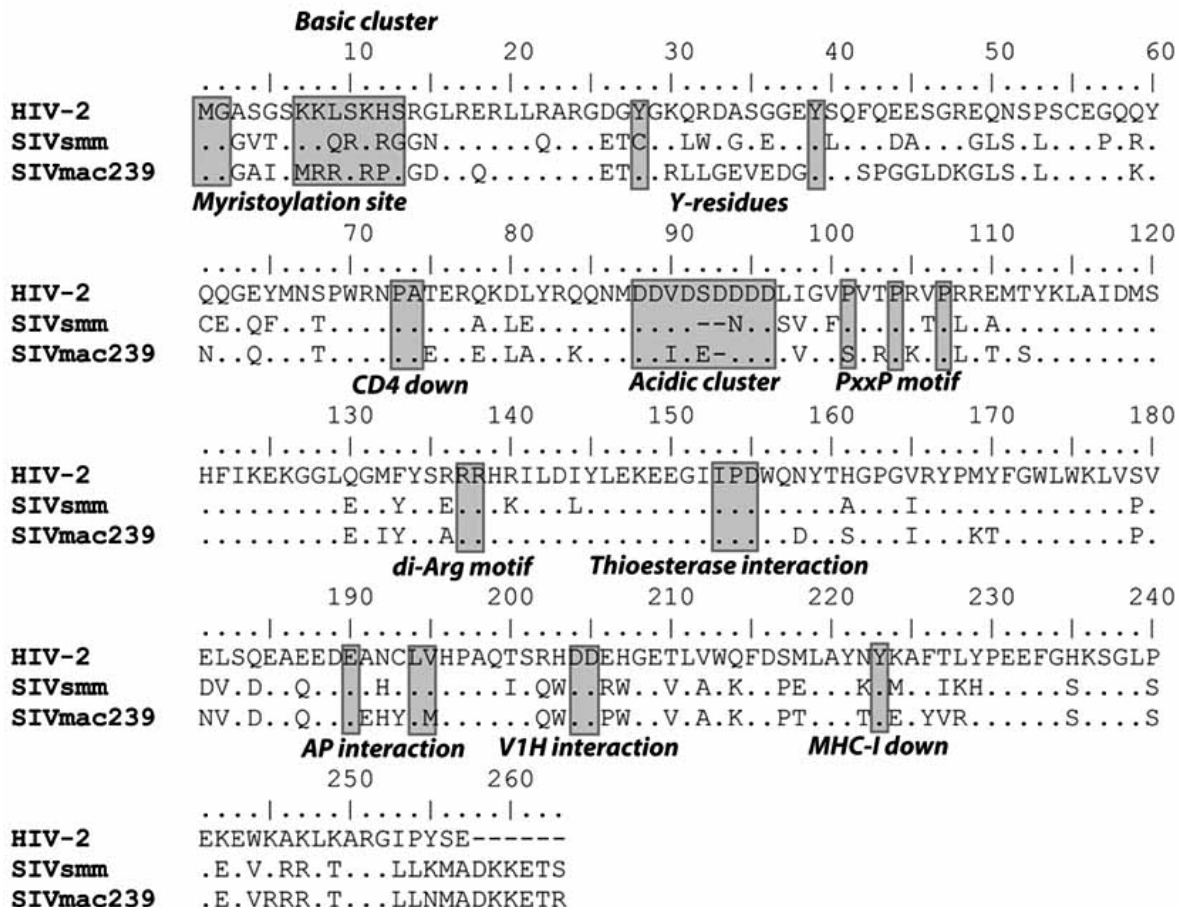
NEF ENHANCES HIV INFECTIVITY BY STIMULATING REPLICATION

In a seminal paper, Chowers and coworkers observed that, when using equivalent amounts of p24, wild type HIV particles infected at least five times more cells than Nef-deleted variants, suggesting a role for Nef in virus infectivity [50]. Schwartz *et al.* showed that Nef stimulates viral replication irrespective of the cell line and multiplicity of infection and that the enhancement of virion infectivity could be dissociated from the ability to down regulate CD4 [51]. Interestingly, although reverse transcriptase activity was not affected, reverse transcription in HIV-infected cells produced significantly lower numbers of proviral DNA copies in the absence of Nef. Using a Hela-CD4 βGal indicator cell line it was revealed that the effect of Nef on virion infectivity was already apparent in a single cycle infection [52, 53]. Infection of mitogen-activated peripheral blood mononuclear cells (PBMC) with Nef+ and Nef- HIV strains demonstrated enhanced replication of the Nef+ viruses. This stimulatory effect on viral replication was even more pronounced when unstimulated PBMC were infected with Nef+ or Nef- HIV, subsequently followed by mitogenic stimulation, showing that Nef- viruses required much more time to replicate to appreciable levels *in vitro*. A similar stimulatory effect of Nef on viral replication was shown in primary macrophages.

Although the data from Schwartz *et al.* [51] suggests a role for Nef early during replication (before the completion of reverse transcription), the normalization of PBMC infections to equivalent levels of infectivity between Nef+ and Nef- viruses continued to reveal a slowed replication, arguing that Nef also contributes in secondary spread in PBMC cultures [52]. Aiken and Trono studied how Nef enhances viral replication and demonstrated, using MLV pseudotyped viruses, that this process is independent of HIV envelope glycoproteins and likely results from increased proviral DNA synthesis [53] (Fig. **2**). Furthermore, they could

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                          10        20        30        40        50        60
                 ....|....|....|....|....|....|....|....|....|....|....|....|
NL4.3            MGGKWSKSSVIGWPAVRERMRRAEPAADGVGAVSRDLEKHGAITSSNTAANNAACAWLEA
Na7              ........R.AG..S...K..EQ....................Y.........T...D......
SIVcpzptt        ..N......IV...Q....L..TQE..E...E..Q..ARR.....RH.PQT.QTL....E
                 Myristoylation site    Charged a-helix
                          70        80        90       100       110       120
                 ....|....|....|....|....|....|....|....|....|....|....|....|
NL4.3            QEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGY
Na7              ..........R.........R.............I.........................F
SIVcpzptt        M.Q.D.....R...........F...........VY.K...E.....V........
                 Acidic    PxxP motif                            di-Arg motif
                 cluster  130       140       150       160       170       180
                 ....|....|....|....|....|....|....|....|....|....|....|....|
NL4.3            FPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREV
Na7              ..............I.........F......AEQ....T...N....M.....E.A....
SIVcpzptt        ...N.........Y...F....S.EA.....DNL....LCT..FE.E.K..
                 Oligomerization              di-Acidic motif  di-Leu motif  di-Acidic motif
                         190       200
                 ....|....|....|....|....|....|.
NL4.3            LEWRFDSRLAFHHVARELHPEYFKNC
Na7              .Q.K........M........Y.D.
SIVcpzptt        .I.K...Q..LR.L...K....YRD-
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B. Functional domains in Nef from HIV-2, SIVsmm and SIVmac239

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                        Basic cluster
                          10        20        30        40        50        60
                 ....|....|....|....|....|....|....|....|....|....|....|....|
HIV-2            MGASGSKKLSKHSRGLRERLLRARGDGYGKQRDASGGEYSQFQEESGREQNSPSCEGQQY
SIVsmm           ..GVT...QR.RGGN......Q...ETC..LW.G.E..L...DA...GLS.L...P.R.
SIVmac239        ..GAI.MRR.RP.GD..Q......ET.RLLGEVEDG...SPGGLDKGLS.L.....K.
                 Myristoylation site            Y-residues
                          70        80        90       100       110       120
                 ....|....|....|....|....|....|....|....|....|....|....|....|
HIV-2            QQGEYMNSPWRNPATERQKDLYRQQNMDDVDSDDDDLIGVPVTPRVPRREMTYKLAIDMS
SIVsmm           CE.QF..T...........A.LE.........--N...SV.F....T.L.A.........
SIVmac239        N..Q...T......E..E.LA..K....I.E-...V..S.R.K..L.T.S........
                 CD4 down                  Acidic cluster  PxxP motif
                         130       140       150       160       170       180
                 ....|....|....|....|....|....|....|....|....|....|....|....|
HIV-2            HFIKEKGGLQGMFYSRRHRILDIYLEKEEGIIPDWQNYTHGPGVRYPMYFGWLWKLVSV
SIVsmm           ........E.Y.E..K...L..........A...I.........P.
SIVmac239        ........E.IY.A....................D..S...I...KT.........P.
                        di-Arg motif   Thioesterase interaction
                         190       200       210       220       230       240
                 ....|....|....|....|....|....|....|....|....|....|....|....|
HIV-2            ELSQEAEEDEANCLVHPAQTSRHDDEHGETLVWQFDSMLAYNYKAFTLYPEEFGHKSGLP
SIVsmm           DV.D..Q...H......I.QW..RW..V.A.K..PE...K.M..IKH.....S....S
SIVmac239        NV.D..Q...EHY.M......QW..PW..V.A.K..PT...T.E.YVR.....S....S
                 AP interaction  V1H interaction        MHC-I down
                         250       260
                 ....|....|....|....|....|...
HIV-2            EKEWKAKLKARGIPYSE------
SIVsmm           .E.V.RR.T...LLKMADKKETS
SIVmac239        .E.VRRR.T...LLNMADKKETR
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Fig. (1). Amino acid alignment of HIV-1 (**A**) and HIV-2 (**B**) Nef with their respective closely related Simian Immunodeficiency Viruses (SIVcpzptt and SIVsmm). The most important protein interaction domains are shown.
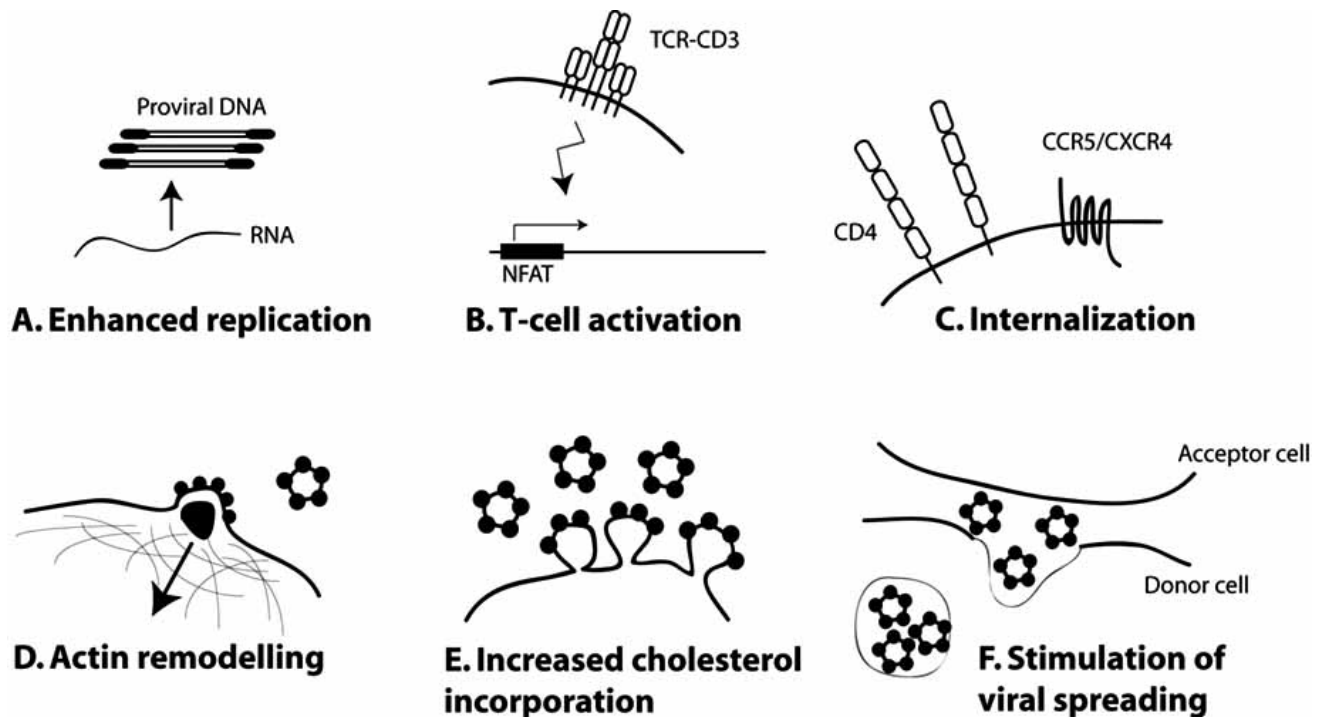
Fig. (2). Schematic overview of Nef functions involved in viral infectivity and replication.

complement the reduced infectivity exhibited by Nef- mutants by expressing Nef *in trans*. Because Nef- particles contain normal amounts of RNA and have wild type levels of RT-activity, it could be suggested that Nef may act by altering the processing of the internalized viral core by rendering it more competent for viral DNA synthesis.

Experiments in which Nef proteins from HIV-1 primary isolates, HIV-2 and SIVmac239 were used to complement the lack of Nef in a HIV-1 Nef- virus, revealed that stimulation of proviral DNA synthesis is a highly conserved characteristic of Nef [53]. Recent work showed that Nef from diverse primate lentiviral lineages is able to mediate the enhancement of virion infectivity and stimulate viral replication [21].

NEF ENHANCES HIV INFECTIVITY BY ACTIVATING CD4+ T CELLS

Although the true mechanism by which Nef increases particle infectivity has remained enigmatic, there is some evidence that Nef stimulates viral infectivity by enhancing the production of infectious HIV particles. One of the ways exploited by Nef in order to promote viral infection and replication is by activating CD4+ T-cells, creating an optimal environment for HIV replication (Fig. **2**). Since Nef is one of the few viral proteins expressed from unintegrated 2-LTR circles, Nef this way preconditions the newly infected cell for massive viral replication. Nef induces T-cell activation by interfering with signal transduction pathways downstream of the T-cell receptor (TCR) [54, 55]. An early report showed that Nef increases IL-2 secretion when T-cells are stimulated through the T-cell receptor and the costimulatory receptor CD28 by an increase in the number of activated cells rather then by an increase in the amount of IL-2 se-

creted per cell [56]. More recently, gene expression profiling studies have revealed that Nef induces a transcriptional program in Jurkat T-cells that is almost identical to that of physiological anti-CD3 induced T-cell activation [57].

Interestingly, while Nef appears to impair the formation of functional immunological synapses (IS) between HIV-1 infected T-cells and antigen presenting cells, by misrouting TCR and Lck from the T-cell-APC contact zone [58, 59] it promotes the formation of virological synapses facilitating direct cell-to-cell spread [60, 61]. As such, Nef may serve as a master switch in managing the intricate balance between these two types of cellular crosstalk in order to improve virus survival and propagation. In conclusion, Nef appears to modulate the intracellular environment to maximize viral replication (by T-cell activation) and cell survival (impair IS formation).

NEF ENHANCES HIV INFECTIVITY BY DOWN REGULATING HIV RECEPTORS

Besides T –cell activation, Nef also stimulates viral infectivity by reducing the surface expression of the HIV receptor CD4 [62, 63] and the HIV coreceptors CXCR4 and CCR5 [64, 65] (Fig. **2**). However, studies by Saksela *et al.* [66] and others [67, 68] showed that the down regulation of CD4 by Nef is genetically separable from its ability to stimulate viral replication in PBMC and to enhance virion infectivity. Nevertheless, it is thought that a reduction in the numbers of CD4 molecules and chemokine coreceptors on the cell membrane would facilitate viral budding from infected cells.

Effects that require Nef early in the viral life cycle can be mediated by the expression of Nef from viral replication intermediates, such as pre-integration 2-LTR circles, but

may also be mediated by Nef present in the virion. It was shown that approximately 10-100 Nef molecules are present in a single HIV virion [69-71]. The viral protease recognizes a specific and highly conserved region between the N-terminal membrane-anchoring domain and the globular core domain of Nef and cleaves most of the Nef molecules present in HIV particles [69]. Until today, the biological significance of proteolytic cleavage of Nef is not understood. While mutations in Nef that prevent cleavage by HIV protease are no longer able to increase HIV particle infectivity, it was also shown that other mutations in this region, that do not affect cleavage, result in a similar phenotype [72, 73]. Alternatively, Nef incorporation into virions may not directly be related to viral infectivity but could serve to recruit cellular proteins into viral particles, which in turn may stimulate viral infectivity [74].

NEF ENHANCES HIV INFECTIVITY THROUGH INTERACTION WITH THE ACTIN CYTOSKELETON

It has been suggested that virion fusion to the host cell membrane is facilitated by receptor clustering induced by actin microfilaments [75] while other studies suggest that the formation of a functional reverse transcription complex involves the association of the HIV viral core with the actin cytoskeleton [76].

Since there is a body of evidence that Nef interacts with proteins involved in the organization of the actin cytoskeleton, Hope and coworkers [77] studied whether Nef enhances HIV infectivity through a mechanism that involved the actin cytoskeleton. They showed that Nef functions to allow the HIV genome to efficiently penetrate the cortical actin network and that this process takes place at a point after fusion and before completion of reverse transcription (Fig. **2**).

Nef has been shown to associate with and activate Vav1, a Rho GTPase exchange factor (GEF) involved in cytoskeletal rearrangements in cells [78] and PAK2, a serine/threonine kinase that mediates actin cytoskeleton organization and rearrangement in human cells [79-82]. Interestingly, after previous findings suggested that Nef interacts with Vav1 and PAK2 through its SH3 binding domain [68, 78], more recent studies have revealed a novel protein interaction domain in Nef (surrounding the critical F195 residue) that is essential in the recruitment of Vav1 into the Nef-PAK2 complex [83, 84]. The SH3 binding domain is essentially the same region that was found to be implicated in the ability of Nef to increase viral infectivity [67]. However, an evaluation of two F191 mutants (analogous to the F195 residue in HIV-1 SF2) showed that disrupting the Nef-PAK2 interaction is not detrimental for T-cell activation, viral replication and apoptosis [85].

It was also shown that Nef can associate with the viral core, corresponding with the notion that Nef may facilitate trafficking of the viral core after fusion and involving the actin cytoskeleton [86, 87].

Together, these observations suggest that the ability of Nef to locally remodel the actin cytoskeleton may be key to enhance HIV infectivity and stimulate replication.

NEF ENHANCES HIV INFECTIVITY THROUGH LIPID RAFTS

HIV buds *via* specialized microdomains in the plasma membrane, i.e. lipid rafts. Nef also appears to increase the infectivity of HIV by specifically guiding newly formed viral particles to lipid rafts [88]. These lipid rafts are especially enriched in sphingolopids and cholesterol, and contain significant amounts of Nef in HIV infected cells. Nef also increases the lipid content in progeny virions and this correlates directly with virion infectivity. It could be suggested that cholesterol-enriched virus particles dock and fuse better with lipid rafts of recipient cells. More recent work identified a cholesterol recognition motif in Nef's C-terminal part and showed that Nef stimulates the synthesis of cholesterol and subsequently increases its transport into lipid rafts and progeny virions [89, 90] (Fig. **2**).

NEF ENHANCES HIV INFECTIVITY BY STIMULATING VIRAL SPREADING

A more indirect mechanism in which Nef can contribute to viral infectivity is by the up regulation of DC-SIGN (CD209) on dendritic cells [91, 92]. DC-SIGN is expressed on dendritic cells and mediates the clustering of DCs with T-cells, a crucial event in the initiation of immune responses but also pivotal in the spreading of HIV to CD4+ T-cells. DC-SIGN also binds gp120 molecules of the HIV envelope, not only allowing very efficient capturing of HIV particles but also the subsequent transfer of infectious virions during DC-T-cell synaps formation [93-95]. HIV Nef can interfere with the expression of DC-SIGN by inhibiting endocytosis. The up regulation of DC-SIGN significantly increases the clustering of DCs with T-cells and stimulates HIV transmission [91]. A recent study showed that Nef is required for the efficient transmission of HIV from DCs to T-cells [92] (Fig. **2**). Coexpression of CD4 and DC-SIGN appears to enhance HIV internalization and intracellular retention but impairs HIV transmission to T-cells. Nef facilitates the transmission of HIV from DC to T-cells significantly by up regulation of DC-SIGN and down regulation of CD4.

It has been shown that HIV transmission efficiency is related with the maturation status of DCs [94, 96]. Given Nef induces T-cell activation, it was investigated whether Nef had a similar effect on DC maturation that could explain enhanced HIV transmission. However, no Nef-induced changes in maturation markers (HLA-DR, CD83 and CD86) were observed. Although this does not completely exclude that Nef increases HIV transmission by stimulating DC maturation, it does suggest that the differential trafficking of DC-SIGN and CD4 are important mechanisms of Nef-induced stimulation of HIV transmission.

HIV NEF AND REPLICATIVE FITNESS

Since recently, HIV fitness has become a research focus. Viral fitness is defined as the ability to adapt to or reproduce in a specific environment [97]. In the case of HIV, fitness is affected by both host factors and viral factors [5, 98]. In this respect, early studies with HIV strains lacking a functional Nef protein have shown that these viruses replicate very

inefficiently in cell cultures [42]. Thus far, most research on HIV replicative fitness has focussed on the interplay between treatment, development of drug resistance and the impact on viral replicative capacity [99, 100] and has established that a replicative fitness cost is associated with nearly all reverse transcriptase inhibitor (RTI), protease inhibitor (PI) and integrase inhibitor (INI) resistance mutations when the respective drug is absent. Recently, several studies have established the role of viral replicative fitness in HIV disease progression [101-103] and expansion of the epidemic [104-106]. One study compared the replicative fitness of NSI/CCR5 and SI/CXCR4 viruses isolated from slow progressors and normal progressors and showed that slow progressors harbour viruses with decreased replication potential [102]. Furthermore, they found strong and independent correlations between viral load and total relative replicative fitness values of viruses from normal progressors and slow progressors, suggesting that replicative fitness is a strong predictor of disease progression. Another study showed that viral replicative fitness increases during disease and correlates directly with plasma viral load and viral genetic diversity and inversely with CD4+ T-cell count [103]. A loss of viral replicative fitness and genetic diversity was observed when antiretroviral therapy was initiated. Unfortunately, in none of these studies the Nef alleles were analyzed. Although there is a clear correlation between the LTNP status and (1) a low replicative fitness and (2) a defective *nef* gene, studies that show direct evidence for a role of Nef in viral fitness are still lacking.

Direct competitions between isolates of HIV-1 group M subtypes (A-D, CRF01), HIV-1 group O and HIV-2 revealed that HIV-1 group M isolates were typically a hundred fold more fit than group O and HIV-2 strains [105]. These differences in viral replicative fitness reflect the epidemiological success of these diverse human lentiviruses. Interestingly, the lower replicative fitness of HIV-2 also goes hand in hand with the lower pathogenic potential of this virus. Although the clinical manifestations are very similar for HIV-1 and HIV-2 patients, humans infected with HIV-2 progress to AIDS at a much slower pace than individuals infected with HIV-1. These differences have been attributed to a slower viral evolution [107], a lower replicative fitness [105, 108], a more immunosuppressive envelope glycoprotein [109], a better preserved ability to produce IL-2 [110], lower rates of T-cell apoptosis [111-113], a broader anti-HIV-2 neutralizing antibody response and a more efficient cell-mediated immune response [111]. In addition, several studies have reported that the *nef* gene of HIV-2 strains harbours more natural variation and concluded that HIV-2 *nef* alleles contain higher proportions of deletions and truncations [114, 115]. These changes may contribute to the attenuated pathogenic phenotype of HIV-2. Recently, Schindler *et al.* [31] showed that HIV-2 *nef* alleles, unlike HIV-1, can efficiently down regulate the TCR-CD3 complex and as such block HIV-induced T-cell activation and possibly limit disease progression upon infection with HIV-2.

VIRAL GENETIC ELEMENTS IMPLICATED IN HIV FITNESS: A ROLE FOR NEF?

Recent work, aimed to identify viral genetic factors implicated in HIV replicative fitness, has suggested a prominent role for the polymerase [99, 116, 117] and envelope

genes [104, 118, 119]. During HAART treatment, the pressure applied on the viral RT and/or PRO reduces viral replication, but also selects for escape variants that exhibit a reduced sensitivity to this pressure. These drug resistant variants are likely present in the quasispecies long before treatment initiation, but remain at low frequency due to their low replicative fitness and are rapidly selected upon treatment initiation. These observations suggest that during treatment the HIV *pol* gene is under strong selective pressure and is the primary determinant of replicative fitness.

The situation appears to be different in untreated individuals. Ball *et al.* [104] performed time-course competitions between HIV-1 isolates and detected viral products at different steps in the lentiviral lifecycle and found that entry is controlling replicative fitness whereas subsequent events (such as reverse transcription, integration and transcription) have minimal impact. Another study compared competitions between viral isolates and autologous env-recombinant strains and showed a strong and significant correlation between *ex vivo* fitness of WT virus and env-recombinants in the absence of antiviral drugs [114]. By using competitive binding assays it was established that the relative binding avidity of the virus particle to the host cell, i.e. binding to CD4 and CCR5 or CXCR4, is predictive for the viral replicative fitness [119]. In this respect, it was previously shown that the Env V3-loop is implicated in determining the sensitivity to CCR5 entry inhibitors [120, 121] and that this sensitivity may be related to HIV-1 fitness [119, 122]. Finally, comparative replication studies with CCR5- and CXCR4-tropic biological clones have also suggested a possible role for the V3-loop in HIV fitness [123]. The effects of variations in post transcription steps (such as translation, post-translational modification and assembly) cannot be analyzed since there are no monoclonal antibodies that can clearly differentiate between proteins of two, only slightly different, HIV-1 primary isolates in dual infections.

Replication studies with a subtype B molecular clone (LAI) in which the Long Terminal Repeat (LTR) transcriptional promoters of different HIV-1 subtypes were inserted, revealed significant differences in replication rates between subtypes. These differences in viral fitness depended on both the host cell type and the activation state and suggested that the multidimensional host environment may have shaped the genetic composition of the subtype specific LTRs [124].

Although the contribution of a single genetic element at specific steps during the viral lifecycle or stages of disease may be more pronounced than that of others, this view is too simplistic. The *in vivo* fitness of a pathogen such as HIV is intrinsically determined by the complete viral genetic constitution and how efficiently each pathogenic entity interacts with its environment, i.e. the human cell. As described above, Nef is immanently involved in processes that govern HIV infectivity and replication and relies therefore on direct and indirect interactions with a myriad of cellular proteins. In future studies, it will be key to first identify all cellular molecular partners of Nef and to subsequently disentangle if and how these interactions impact viral replicative fitness. We have engaged in such studies and are currently also evaluating the effect of diverse HIV and SIV *nef* alleles on viral fitness using Nef-recombinant replication-competent reporter viruses in a dual infection/competition assay.

In conclusion, besides *env*, *pol* and the LTR, *nef* is certainly a major candidate-determinant of HIV fitness. Unfortunately, no studies have currently been done to determine the direct contribution of Nef to the overall fitness of HIV. We believe that a better understanding of Nef and its role in HIV replicative fitness and pathogenesis may help to gain better insight in HIV disease and could bring about new therapeutic alternatives.

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