BioFactors

# Immunoregulatory effects of HIV-1 Nef protein

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## Abstract.

During HIV infection, the perturbation of the adaptive and innate immune responses contributes to the progressive immunosuppression leading to an increased susceptibility to opportunistic infections and neoplastic diseases. Several impairments observed in HIV-infected patients include a gradual loss of $CD4^+$ T cells, $CD8^+$ T cell dysfunction, and a decreased number and function of natural killer (NK) cells. Moreover, a functional impairment and variation in the number of DC and B cells were observed during HIV infection. HIV-1 codes for proteins, including the accessory Nef proteins, that interacting with immune cells may contribute to AIDS pathogenesis. Here, we review the recent progress on the immunomodulatory effect of the accessory Nef protein and its role in the pathogenesis of HIV-1 infection.


Volume 35, Number 2, March/April 2009, Pages 169–174 •
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**Keywords:** Nef, HIV-1, AIDS immunopathogenesis

## 1. Introduction

HIV-1 Nef is a 27-kDa protein expressed early after virus infection. Two forms of Nef have been described, a membrane-bound myristoylated form and a nonmyristoylated cytoplasmic form. The nef gene is highly conserved in all primate lentiviruses (HIV-1, HIV-2, and SIV) and the encoded Nef protein appears to be a virulence factor critical for the development of AIDS [1]. *In vitro*, Nef also facilitates virus replication and enhances virions infectivity. HIV/SIV *nef*-defective strains, are attenuated in their ability to cause persistent infection and disease [2] and deletions in the Nef gene have been detected in HIV-1 strains isolated from long-term nonprogressing AIDS patients [3]. Nef exerts pleiotropic effects, involving membrane-bound or cytoplasmic stages, and depending on its intracellular localization, interferes with cellular signal transduction pathways and modulates the cell surface expression of several membrane-associated proteins [4]. Nef properties have mainly been associated with its biochemical activities within infected cells. In addition, Nef can be released by infected cells and found in cell-culture supernatant and in the serum of HIV-1 infected patients [5].

During HIV infection, the perturbation of the adaptive and innate immune responses contributes to the progressive immunosuppression leading to an increased susceptibility to opportunistic infections and neoplastic diseases. Nef is involved in the impairment of the immune response by several possible functional activities. Nef has been shown to exert suppressive as well as enhancing effects depending on the property of target cells and state of cell activation and differentiation [6].

Here, we review the immunoregulatory effects of Nef and we will discuss the role of Nef in the pathogenesis of HIV-1 infection.

## 2. Effects of Nef on T lymphocytes

### 2.1. $CD4^+$ T lymphocytes

$CD4^+$ T cells are the major target of HIV-1 in the host and the magnitude of viral replication in these cells is closely related to their activation state. The hallmark of HIV infection is the gradual depletion of $CD4^+$ T cells that contributes to the dysregulation of the immune system, rendering the host susceptible to opportunistic infections. A plethora of reports demonstrate that Nef performs several functions in virally infected as well as bystander $CD4^+$ T cells [7]. The best-characterized function of Nef is its ability to significantly reduce $CD4$ surface molecules. Nef removes $CD4$ from cell surface by enhancing its endocytosis via recruitment to AP-2 adapter complexes and directing the receptor to lysosomes for degradation [8]. The down-modulation of $CD4$ could be critical for HIV-1 pathogenesis by preventing superinfection, promoting virus release and infectivity, and lowering the antiviral immune response [1].

Another well-conserved and defined property of Nef is its ability to downregulate the cell surface expression of

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Received 26 November 2008; accepted 8 January 2009
DOI: 10.1002/biof.28
Published online 11 March 2009 in Wiley InterScience (www.interscience.wiley.com)

MHC class I molecules [9]. Nef reduces MHC-I expression through the recruitment of AP-1 to the MHC-I cytoplasmic tail to re-route MHC-I from the *trans*-Golgi network to lysosomes. Moreover, Nef favours the endocytosis of MHC-I from the plasma membrane to the *trans*-Golgi network in a PACS-1, AP-1 and clathrin-dependent manner [10,11]. Recently, Lubben et al. demonstrated that AP-1 and clathrin are both needed for MHC-I down-regulation, PACS-1 is dispensable, while AP-2 impedes down-regulation [12]. In this way, Nef might reduce the recognition of HIV-infected cells by cytotoxic T lymphocytes (CTL) and might provide a selective advantage for viral persistence and replication *in vivo* [13,14]. Despite the differences between the Nef induced CD4 and MHC-I initial intracellular trafficking, it has been recently demonstrated that CD4 and MHC-I are ultimately found in the same $Rab7^+$ vesicles and are both targeted for degradation via the activity of the Nef-interacting protein β-COP [15]. Recent data show that Nef not only reduces MHC-I molecules but also downregulates surface expression of mature MHC-II and up-regulates MHC-II associated invariant chain (Ii) [16]. Nef usurps AP-2 complexes to dysregulate Ii trafficking and potentially interfere with antigen presentation in the context of MHC-II [17,18]. This might contribute to the impaired helper T cell responses observed in AIDS patients.

The list of membrane proteins which intracellular trafficking is affected by Nef now includes the costimulatory CD28 molecule [19] and the chemokine receptor CXCR4 [20]. The down-regulation of CD28 molecules from the surface of infected cells may suppress immune response and cause anergy. The Nef-induced down-modulation of CXCR4 strongly inhibits lymphocyte migration and enhances the resistance of infected cells to superinfection [20,21]. Moreover, it has been demonstrated that Nef interacting with CXCR4 induces $CD4^+$ T cell apoptosis, contributing to the depletion of $CD4^+$ T cells through a bystander effect [22].

Nef interacts with a number of kinases or other cellular proteins involved in signal transduction pathways [4], potentially priming infected cells for activation and protecting them from apoptosis [13]. Moreover, Nef has been suggested to associate with membrane microdomains, known as rafts, priming T cells for activation [23]. Our previous findings indicate that Nef exerts its activating effect principally on suboptimally stimulated cells, rendering them properly activated for the replication of the virus [24].

Besides the ability to induce intracellular signalling pathways, recently it has been demonstrated that Nef is able to inhibit TCR-mediated activation. In fact, Nef impairs the formation of functional immunological synapses between HIV-infected T cells and APC altering the intracellular trafficking of Lck and T cell receptor (TCR) [25]. Thus, Nef manipulates the intracellular milieu to create an optimal environment for HIV replication.

Virtually, all biological activities of Nef require its N-terminal myristoylation for the association with cellular membranes. A recent study has found that myristoilated Nef is more compactly folded than its nonmodified variant, suggesting that the cytosolic form of Nef presents different accessible surfaces than the membrane-bound form of Nef when the myristate and parts of the anchor domain are hidden in the lipid bilayer. Moreover, myr+ and myr- Nef proteins showed different oligomerisation properties. Indeed, myristoylated Nef prevails in a monomeric state and, in this form, might be internalized faster than the nonlipidated protein that forms dimers, trimers, or even oligomers of greater magnitude [26].

### 2.2. $CD8^+$ T lymphocytes

$CD8^+$ T cell antiviral cytotoxicity is known to be crucial for effective immune surveillance in the setting of persistent infection. $CD8^+$ T lymphocytes play a central role in HIV infection by direct cytolysis of infected cells and by the secretion of factors that suppress viral replication and, high levels of $CD8^+$ T cell responses are correlated with long-term nonprogression [27]. However, despite the strong CTL response, HIV generally evade immune attack and induces a lethal outcome through AIDS.

The variability of the Nef protein derived from primary isolates may affect the processing of this viral antigen and impair its presentation to CTL [28]. Moreover, by downregulating the expression of MHC class I molecules Nef might reduce the recognition of HIV-infected cells by CTL [13]. In addition, Nef may cause CTL apoptosis through induction of Fas-L on the infected $CD4^+$ T cells [29,30]. Conversely, Nef inhibits apoptosis in these virally infected host cells through its concomitant suppressive effects on ASK-1, a key intermediate in the Fas and TNF-α death signalling cascade [31].

We previously evaluated the functional competence of $CD8^+$ T cells primed by Nef-pulsed dendritic cells (DC) and we found that Nef down-regulates the cytotoxic activity and IFN-γ production by $CD8^+$ T cells [32]. Therefore, $CD8^+$ T cells primed by Nef-pulsed DC may become anergic, and this closely fits with $CD8^+$ T cell anergy in the setting of HIV infection [33].

## 3. Effects of Nef on dendritic cell biology

DC are instrumental in the development of pathogen-specific immune responses and are well-equipped for activation of both the innate and adaptive immune response [34,35]. DC interaction with HIV is relevant in the pathogenesis of AIDS favouring both the initial establishment and spread of the infection and the development of antiviral immunity [36].

Several reports have suggested the possible influence of Nef on DC function and initial studies have demonstrated deficient replication of Δ*nef* strains in DC-T cell cultures [37,38]. Nef downregulates the expression of MHC-I molecules on DC surface, indicating this to be a global effect on different cell types [39]. The Nef-induced downregulation of MHC-I coincides with the reduced capacity of DC to prime alloreactive $CD8^+$ T cell responses [32]. Beside MHC class I, endogenously expressed HIV-1 *nef* selectively down-regulates CD1a, a molecule in charge of glycolipid/lipid antigens presentation [39]. Exposure of DC to exogenous Nef leads to

an up-regulation of MHC-II molecules, favouring nonspecific $CD4^+$ T cell activation [40]. The up-regulation of $CD4^+$ T cell stimulatory capacity of DC induced by Nef may also be mediated by the increased expression of costimulatory/signalling molecules as well as by the up-regulation of cytokines and chemokines production [40,41]. To boost virion infectivity, Nef also increases surface expression of DC-SIGN resulting in increased clustering of DC with T cells [42]. Thus, Nef strategically promotes bystander $CD4^+$ T cell activation increasing the "pool" of lymphocytes permissive to infection.

Contrary to the earlier described effects exerted on T lymphocytes [20], exogenous Nef up-regulates CXCR4 expression on immature DC [40]. Since CXCR4 drives DC towards lymphonodes, Nef might favour their migration resulting in HIV transfer to T cells. Moreover, Nef may allow DC infection with T-tropic strains and, because the tissue distribution of CXCR4 is much broader than CCR5, this may allow the virus access to a wider range of potential target cells, or alternatively may permit fusion with more permissive target cells.

Although few reports investigate the signalling pathways triggered by Nef in DC, similar molecular partners of Nef has been reported in T cells and DC. Among these, Vav interact with Nef in both human T cell lines and DC [43,44]. Moreover, Nef-induced activation of STAT3 and NF-kB could play an important role in triggering DC to facilitate viral spread [44,45].

Thus, the effect of Nef in subverting DC biology may constitute a viral tool underlying AIDS pathogenesis [46].

## 4. Effects of Nef on monocytes/ macrophages

Monocytes/macrophages play a crucial role in AIDS pathogenesis as well as in viral transmission and represent a major source of virus in late-state infection. Human macrophages represent an HIV reservoir *in vivo* and differently from lymphocytes, survive virus infection [47]. It has been reported that Nef in human monocytes/macrophages triggers secretion of an array of inflammatory chemokines/cytokines promoting recruitment and activation of resting T lymphocytes at site of virus replication [48] and T-tropic strain spread [49]. Accordingly, the Nef-induced down-regulation of CCR5 expression [50] may account for the T-tropic emergence in AIDS. Moreover, HIV may benefit from the Nef-induced CCR5 down-modulation interfering with superinfection, ensuring proper viral morphogenesis, inducing apoptosis, and inhibiting chemotaxis of infected cells toward β-chemokine-secreting CTL [51]. Similarly, to the earlier described effect exerted on T lymphocytes, Nef up-regulates MHC-II associated Ii chain in the monocytic cell line THP-1 and monocyte-derived macrophages [52]. The capacity of Nef to modulate the surface expression of many receptors is likely to contribute to efficient viral spread, immune escape, and disease progression.

We previously demonstrated that Nef is efficiently internalized by monocytes and induces IL-15 synthesis [24]. Moreover, Olivetta et al. demonstrated that the Nef-dependent release of inflammatory factors correlated with the activation of the NF-κB transcription factor [53].

Nef protects human monocyte-derived macrophages from HIV-1-induced apoptosis and this may be a relevant part of the mechanism of the formation of HIV reservoirs [54]. In addition Nef, increasing the migration of monocytes, may contribute to the infiltration of HIV-1-infected and uninfected monocyte/macrophages in organs and tissues contributing to disease progression [55].

## 5. Effects of Nef on NK cells

NK cells provide a first line of defence in the early phases of HIV-1 infection. Studies assessing NK cell number and function in HIV-1 infection indicated both qualitative and quantitative defects [56]. HIV-1 has developed several mechanisms to avoid NK cell-mediated lysis. Among these, Nef selectively decreases HLA-A and HLA-B on the surface of infected cells, whilst leaving the levels of HLA-C and HLA-E unchanged. This selective HLA class I down-regulation may represent for HIV-1 a balance between escape from CTL and maintenance of protection from NK cells expressing HLA-C and -E inhibitory receptors [14]. In addition, Nef down-modulates cell surface expression of the NKG2D ligands MICA, ULBP1 and ULBP2 [57]. Given that all human NK cells express the activating receptor NKG2D, down-modulation of NKG2DLs provides a general mechanism that could explain the resistance of HIV-infected cells to NK cell-mediated lysis, independently from the repertoire of HLA-I-specific inhibitory receptors. Moreover, decreased expression of the non-classical MHC-I like CD1d molecule induced by Nef reduces the visibility of infected cell not only to MHC-I-restricted T cells but also to CD1d-restricted NKT cells [58].

Besides the functional consequences on NK cell recognition, causing a decreased susceptibility to NK cell-mediated lysis, Nef targeting DC, is also able to modulate NK cell functions. In particular, we found that Nef up-regulates the ability of DC to stimulate the immunoregulatory NK cells ($CD56^{bright}$) while Nef-pulsed DC inhibit cytotoxic NK cells ($CD56^{dim}$) [59].

Nef also affects NK cell development, interfering with the differentiation of a primitive lymphoid precursor with a T/NK potential, decreasing the generation of $CD56^+$ NK cells [60].

Overall, these findings suggest that Nef may favour the escape of HIV-1 from innate immune surveillance.

## 6. Effects of Nef on B cells

B lymphocyte hyperactivation and hypergammaglobulinemia are hallmarks of HIV infection. B cell polyclonal hyperactivity is also accompanied by impaired IgG and IgA specific responses to pathogens and vaccines [61]. Several cofactors have been proposed as mediators of these functional alterations. Given its inability to infect B cells, HIV-1 must alter their function in an indirect way. However, Qiao et al. [62] found that uninfected B cells might accumulate Nef in the

**Table 1**
**Overview on the main functions exerted by HIV-1 Nef and its role in AIDS pathogenesis**

| | Mechanism of action | | | |
|---|---|---|---|---|
| Cell type | Direct | Indirect mediated by: | Type of effect | Outcome |
| DC | endogenous protein<br>exogenous protein | | -modulation of surface markers, morphological and functional differentiation | Viral spread |
| Mo/MΦ | endogenous protein<br>exogenous protein | | -cytokines/chemokines production<br>-cell migration<br>-infected cell survival | Viral spread |
| $CD4^+$ T cell | endogenous protein | DC/monocyte (cytokines/ chemokines, stimulatory capacity) | -downregulation of CD4, MHC-I, CD28<br>-interaction with signal transduction pathways<br>-infected cell survival<br>-cell activation | Viral spread |
| $CD8^+$ T cell | | DC/monocyte (cytokines/ chemokines, modulation of death receptor and MHC-I surface expression) | -decreased recognition of infected cells by CTL<br>-apoptosis<br>-anergy | Immune escape |
| NK cell | | -DC/monocyte (cytokines, modulation of MHC-I surface expression)<br>-down-modulation of NKG2DL on target cell surface | -inhibition of cytotoxic activity and IFN-$\gamma$ production<br>-decreased susceptibility to NK cell mediated lysis<br>-interference with NK cell development | Immune escape |
| B cell | exogenous protein | Monocyte (IL-6, IL-15, ferritin, sICAM, sCD23) | -inhibition of class-switching and specific Ab-response<br>-hyperactivation<br>-hypergammaglobulinemia | Immune escape |

germinal centres of infected lymphoid follicles as a result of internalization from the extracellular environment. Nef inhibits switching to IgG, IgA, and IgE by inducing IkBa and SOCS proteins, which block CD154 rendering Nef-containing B cell less responsive to $CD4^+$ T cell help. Thus Nef, manipulating negative feedback pathways in bystander B cells, may favour HIV-1 evasion from protective T cell-dependent antibody responses. Moreover, the Nef-induced down-regulation of the antigen capture function of immature DC [40] together with the Nef-induced down-regulation of specific antibody response [63] may contribute to immune evasion.

Cytokines induced by Nef as a result of perturbed signalling in macrophages can account, in part, for some aspect of B cell dysfunction. In this context, it has been demonstrated that Nef promotes B cell activation and differentiation by a mechanism involving induction of IL-6 [64] and ferritin [65] secretion by macrophages. Conversely, the Nef-induced up-regulation of IL-15 production may account for the observed inhibition of the *in vitro* induction of a specific antibody response [63].

Furthermore, it has been reported that Nef induces the release of sICAM-a and sCD23 from macrophages that stimulate B cells to render resting T lymphocytes more permissive to HIV-1 infection [66].

Thus, Nef could be responsible for B cell defects both directly, due to its accumulation in B cell, and indirectly through a mechanism involving B cell interaction with T cells and macrophages.

## 7. Conclusions

Our findings and other reported data, strongly suggest that accessory Nef protein should be considered as a master manipulator of host immune system. Nef may contribute to the AIDS pathogenesis by impairing both the humoral and cellular (innate and adaptive) immune responses. Nef, not only affects the activation status of the infected cells to generate a suitable environment for virus production, but also increases the permissiveness of the bystander cells to HIV-1 infection (Table 1).

A better understanding of the function, the mechanism of action, and the cellular partners of Nef, may aid the discovery of therapeutic alternatives based on anti-Nef drugs. Such molecule inhibitors or vaccine-directed CTL responses targeted at blocking Nef functions could improve host immune control.

## Acknowledgements

This work was supported by a grant from the National Research Project on AIDS to M.V.

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