

# Effects of HIV-1 Nef on Virus Co-receptor Expression and Cytokine Release in Human Bladder, Laryngeal, and Intestinal Epithelial Cell Lines

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## Abstract

HIV infections are mainly acquired by mucosal transmission, through oral, rectal, or genital mucosa. Epithelial cells (EC) are the first cells encountered by HIV during infection through sexual transmission and breastfeeding. EC express several receptors critical for both primary HIV infection and secondary transmission. The regulation of co-receptor expression correlates with changes in susceptibility to infection by HIV-1 strains with different tropism. Moreover, inflammatory responses at mucosal surfaces after HIV-1 transmission may influence disease outcome. In the present study, we analyzed the effect of the accessory HIV-1 Nef protein on mucosal EC, using unstimulated or IFN- $\gamma$ -stimulated HEP-2, T24, and Caco2 cell lines as models for homeostatic or inflamed mucosal tracts. We found that Nef significantly upregulated the expression of CXCR4 on the Caco-2 cell surface and the expression of galactosylceramide on the T24 cell surface. In addition, Nef significantly upregulated IL-6 production by T24 and Caco-2 cells, and TNF- $\alpha$  release by all three cell lines analyzed. Notably, Nef abrogated the IFN- $\gamma$ -induced modulation of co-receptor expression and cytokine secretion. Our findings suggest that Nef differently regulates co-receptor expression and cytokine secretion at the epithelial level, depending on the anatomical derivation of the cells and the inflammatory status.

## Introduction

HIV TRANSMISSION MOST COMMONLY OCCURS BY MUCOSAL CONTACT, via the oral, rectal, or genital mucosa, and quantitative and qualitative defects of mucosal immunity are consistently present in all stages of HIV infection. Mucosal tissues are major sites for early viral replication and may be the major viral reservoir, even in patients receiving HAART. The rapid and extensive depletion of mucosal CD4<sup>+</sup> T cells occurring during the first few weeks of infection is likely to explain, together with mucosal barrier breakdown, the preponderance of opportunistic infections at mucosal sites (23).

In the genital tract of HIV-infected patients, the simultaneous presence of other genital infections and elevated levels of pro-inflammatory cytokines may increase HIV replication in the genital secretions, thereby increasing secondary sexual transmission (17).

Beyond the gastrointestinal (GI) and genital mucosa, the oral mucosa has been implicated in HIV pathogenesis (20). The tonsil is a reservoir and replication site for HIV and

secondary infection, and viral shedding has been clearly documented within the tonsil epithelium (11,42).

Epithelial cells (EC) are the first cells encountered by HIV during infection via sexual transmission and breastfeeding.

Although various epithelial cell lines may be experimentally infected *in vitro* by various laboratory and primary isolates of HIV-1 (4,13,34), infection of EC *in vivo* remains controversial (10). There are conflicting reports as to whether EC merely behave as a reservoir for HIV-1 by sequestering and releasing virus, whether they are able to actively transcytose virus to submucosal mononucleated target cells, or whether they can develop a productive infection (15). Recently, Dorosko and Connor (8) demonstrated that primary human mammary EC (MEC) endocytose HIV-1 and can facilitate virus infection and replication in CD4<sup>+</sup> target cells. These findings suggest that infection of MEC appears unnecessary for these cells to actively contribute to the transmission of HIV to target cells, and that they may serve as a viral reservoir for HIV-1 *in vivo*.

EC express several receptors critical for both primary HIV infection and secondary transmission (21,22,39), including

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CXCR4, CCR5, and galactosylceramide (GalCer). It has been reported that proinflammatory cytokines (2) and sex hormones (39) regulate chemokine receptors at mucosal sites. The regulation of co-receptor expression correlates with changes in susceptibility to infection by both R5 and X4 tropic strains of HIV-1.

Increased mucosal inflammation is an important feature of acute HIV infection, and this local inflammation is associated with lower systemic CD4<sup>+</sup> cell counts during acute HIV infection.

Cytokine release may also constitute a self-enhancing model of HIV infection in solid tissues. Nevertheless, inflammatory responses at mucosal surfaces may be both harmful and helpful for viral dissemination. While recruiting HIV-susceptible CD4<sup>+</sup> T cells to the site of infection, cytokines/chemokines also lead to increased recruitment of HIV-specific CD8<sup>+</sup> T cells (26,30).

Some effects induced by HIV-1 are mediated by viral factors, such as Nef, an accessory protein involved in AIDS pathogenesis and disease progression (18). To date, most of the activities of Nef have been attributed to the intracellular expression of the protein or its association with virions. However, Nef is known to be secreted from infected cells (12) in association with small membrane-bound vesicles (5). It has been demonstrated that HIV-1 Nef modulates the cell surface expression of several membrane-associated proteins (28), and induces cytokine and chemokine release by macrophages and dendritic cells (DC) (33,25,27,29).

Despite numerous reports describing how HIV-1 Nef affects immune cells, the effect of Nef on epithelial cells remains unknown. In the present study, we investigated the effect of extracellular HIV-1 Nef exposure on human bladder, laryngeal, and intestinal epithelial cell lines, focusing on HIV co-receptor expression and cytokine release. Moreover, we evaluated the effects of Nef on IFN- $\gamma$ -stimulated EC, as a model of inflamed mucosal epithelium.

## Materials and Methods

### Nef protein

Recombinant HIV-1 Nef (BRU variant) obtained from *E. coli* was provided by DIATHEVA s.r.l. (Fano-ITALY). The Nef protein was highly purified (>99%) as assessed by SDS-PAGE, Western blotting, and by analytical HPLC. Lyophilized protein was dissolved in sterile water and aliquots were stored at -70°C. A dose-response titration curve was performed to assess the optimal concentrations of Nef. The biological activity was assessed by induction of phenotypical and functional activation of monocyte-derived DC. Endotoxin content of Nef was determined by Pyrotell *Limulus amoebocyte* lysate assay (Cape Cod Inc., Falmouth, MA). The endotoxin level was <0.03 unit/mL (<0.001 ng/mL).

### Cell cultures

T24 cells, a human epithelial cell line derived from bladder carcinoma, were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, 100  $\mu$ g of streptomycin per mL, and 100 U of penicillin per mL (all from GIBCO-BRL, Gaithersburg, MD).

HEp-2 cells, a human epithelial cell line derived from laryngeal carcinoma, were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% FBS, 1% non-essential amino acids, glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL).

For all experiments, T24 and HEp-2 cells were seeded onto 24-well plates at a density of  $5 \times 10^4$ /well and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Twenty-four hours after the seeding, Nef (0.1  $\mu$ g/mL), IFN- $\gamma$  (600 U/mL; BD Biosciences, Bedford, MA), or their combination were added directly to the culture medium.

Caco-2 human colonic carcinoma cells were grown in a controlled atmosphere of 5% CO<sub>2</sub> at 37°C in Dulbecco's modified essential medium (Lonza, Verviers, Belgium) containing 4.5 g/L glucose, 2 mM L-glutamine, 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin, 1% nonessential amino acids, 1% HEPES, and 10% FBS, as described previously (6). After 21 d, differentiated Caco-2 cells were left untreated or were treated with Nef (0.1  $\mu$ g/mL), IFN- $\gamma$  (600 U/mL), or their combination.

### Flow cytometry

After 24 h of Nef, IFN- $\gamma$ , or IFN- $\gamma$ /Nef treatment, T24, HEp-2, and Caco-2 cells were harvested and washed twice with ice cold PBS. Analysis of cell surface markers was performed using fluorescein isothiocyanate (FITC)-conjugated anti-CCR5 and phycoerythrin (PE)-conjugated CXCR4 (all from BD Biosciences) in direct immunofluorescence assays. For indirect immunofluorescence staining anti-GalCer mAb (Chemicon, Temecula, CA) was added, followed by FITC-conjugated isotype-specific goat anti-mouse Ab (BD Biosciences). Negative controls included directly labeled or unlabeled isotype-matched irrelevant mAb. The cells were analyzed with a FACScan flow cytometer and CellQuest software (Becton Dickinson).

### Cytokine ELISA

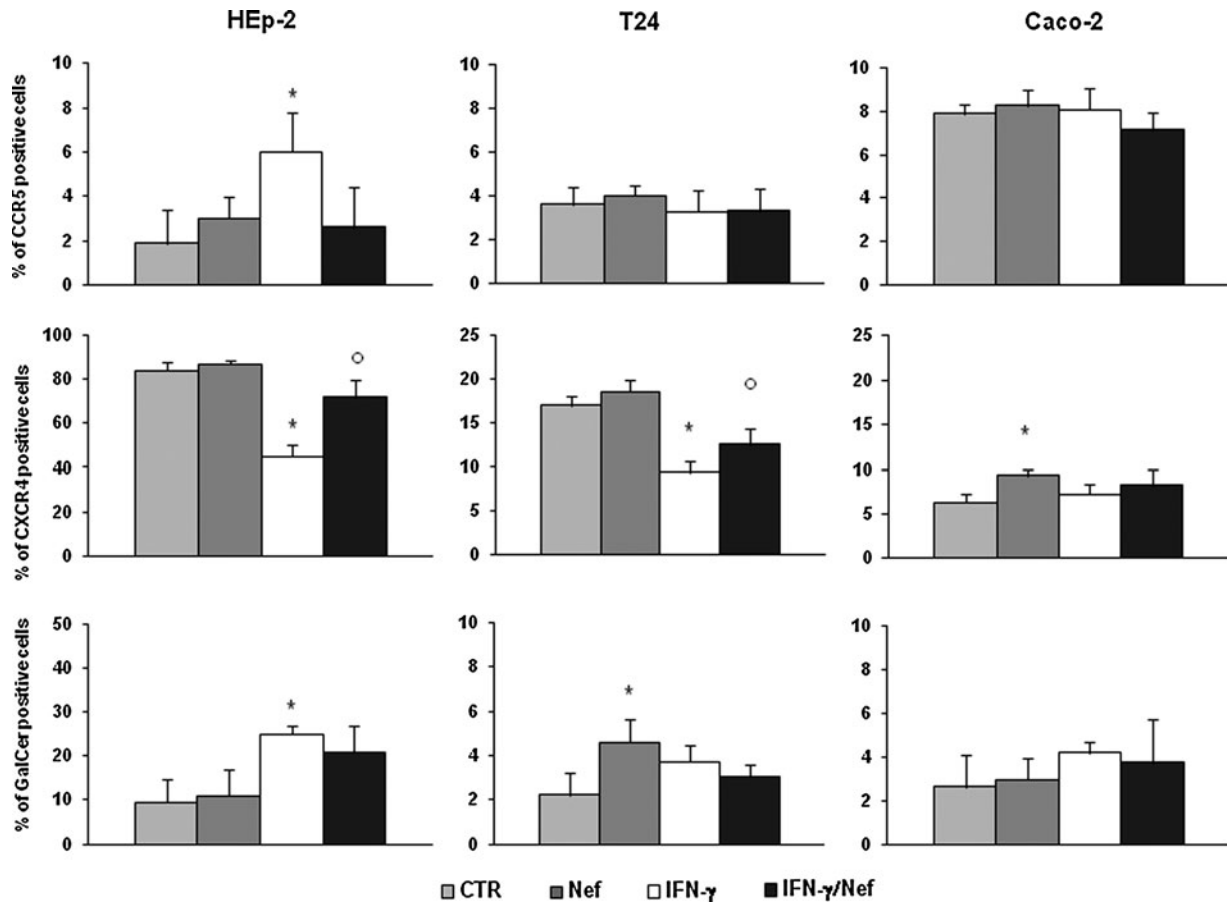
Supernatant aliquots of T24, HEp-2, and Caco-2 cells were collected 48 h after treatment with Nef, IFN- $\gamma$ , or their combination, and IL-6 and TNF- $\alpha$  secretion were measured by a sandwich ELISA (BD OptEIA) according to the manufacturer's instructions.

### Statistical analysis

Statistical significance of differences was calculated using the Wilcoxon matched-pairs signed-ranks test. A *p* value <0.05 was considered statistically significant.

## Results and Discussion

EC, the most abundant cell type lining the mucosa, represents a potential route for HIV-1 entry. They express CCR5 and CXCR4, the physiologically relevant receptors used *in vivo* (41) by macrophage-tropic (M-tropic) and lymphocyte-tropic (T-tropic) strains, respectively. Since the regulation of co-receptor expression correlates with changes in susceptibility to infection by both R5 and X4 tropic strains of HIV-1, we investigated whether HIV-1 Nef is able to modulate *in vitro* the expression of CCR5 and CXCR4 on the surface of EC of bladder (T24), laryngeal (HEp-2), and intestinal (Caco2) origin. In addition, we analyzed the expression of GalCer, an



**FIG. 1.** Effect of HIV-1 Nef on virus co-receptor expression on untreated or IFN- $\gamma$ -treated EC. HEp-2, T24, and differentiated Caco-2 cells were treated with Nef (0.1  $\mu\text{g}/\text{mL}$ ), IFN- $\gamma$  (600 U/mL), or IFN- $\gamma$ /Nef for 24 h. Analysis of CCR5, CXCR4, and GalCer surface expression was performed by flow cytometry. Results are expressed as the percentage of positive cells, and are means  $\pm$  SEM of 8 independent experiments (\* $p < 0.05$  versus medium;  $\circ p < 0.05$  versus IFN- $\gamma$ ).

alternative primary receptor for HIV-1, known to be expressed on the EC surface.

As shown in Fig. 1, a higher percentage of HEp-2 cells expressed CXCR4 and GalCer with respect to the T24 and Caco-2 cell lines, while CCR5 was similarly expressed on the surface of all three cell lines analyzed. We found that Nef did not alter the percentage of CCR5 expressing HEp-2, T24, and Caco-2 cells, while significantly upregulating ( $p < 0.05$ ) the percentage of CXCR4-expressing Caco-2 cells. In addition, Nef significantly upregulated ( $p < 0.05$ ) the percentage of GalCer-expressing T24 cells.

Since the advanced stages of HIV infection are characterized by chronic inflammation, we also evaluated the effect of Nef on IFN- $\gamma$ -treated cells. We used IFN- $\gamma$  because it is a major factor in the inflammatory and immune responses (7,14,3). We found that IFN- $\gamma$  significantly upregulated ( $p < 0.05$ ) the percentage of CCR5-expressing HEp-2 cells, while it did not modulate the percentage of CCR5-expressing T24 and Caco-2 cells. In addition, IFN- $\gamma$  significantly downregulated ( $p < 0.05$ ) the percentage of CXCR4-expressing HEp-2 and T24 cells, and significantly upregulated ( $p < 0.05$ ) the percentage of GalCer-expressing HEp-2 cells. Nef, when used in combination with IFN- $\gamma$ , did not modulate the expression of any of the receptors analyzed. Notably, Nef abrogated the IFN- $\gamma$ -induced upregulation of CCR5-expressing

HEp-2 cells, and induced downregulation of CXCR4-expressing HEp-2 and T24 cells. Similarly, IFN- $\gamma$  abrogated the Nef-induced upregulation of CXCR4-expressing Caco-2 cells and GalCer-expressing HEp-2 cells.

Published data have reported either an IFN- $\gamma$ -induced increase of CCR5 in cells of monocyte lineage (12,40), or no effect, but concomitant IFN- $\gamma$  induced CXCR4 downregulation (24,32,19). Moreover, it has been reported that co-receptor modulation also occurs in epithelial cells of cervical (HeLa-T4) and colonic (HT-29) origin (2). The differential regulation of co-receptors may help explain the selection of HIV strains with different tropism in the GI, genital, rectal, and oral tracts.

Contrary to the effects exerted on T lymphocytes (35), we found that Nef upregulated CXCR4 expression on Caco-2 cells. Similarly, we have previously demonstrated that Nef upregulates CXCR4 expression on immature DC (29). Hence, Nef may favor the selection of T-tropic strains in the GI tract, and because the tissue distribution of CXCR4 is much broader than that of 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.

It has been reported that mucosal infection with opportunistic pathogens may contribute to HIV-1 transmission through co-receptor modulation (37). *Mycobacterium avium*

complex (MAC), which frequently infects mucosal cells, upregulates monocyte expression of CCR5, potentially augmenting HIV-1 entry into CCR5<sup>+</sup> mucosal cells. In addition, MAC-induced secretion of CCR5 ligands, such as macrophage-inflammatory protein-1 (MIP-1), as well as other chemokines, likely promotes the recruitment of additional target monocytes to sites of mucosal MAC infection (36).

EC are known to secrete a variety of cytokines at constitutive levels. In response to tissue damage, infection, or antigen exposure, EC release inflammatory cytokines that can directly affect mucosal integrity (9,16). Because equilibrium between viral replication, immune control, and CD4<sup>+</sup> T-cell depletion is set early during HIV infection, information regarding the pattern of early mucosal cytokine responses, especially at the site of virus entry and initial replication, could substantially contribute to our understanding of the immunopathology of AIDS.

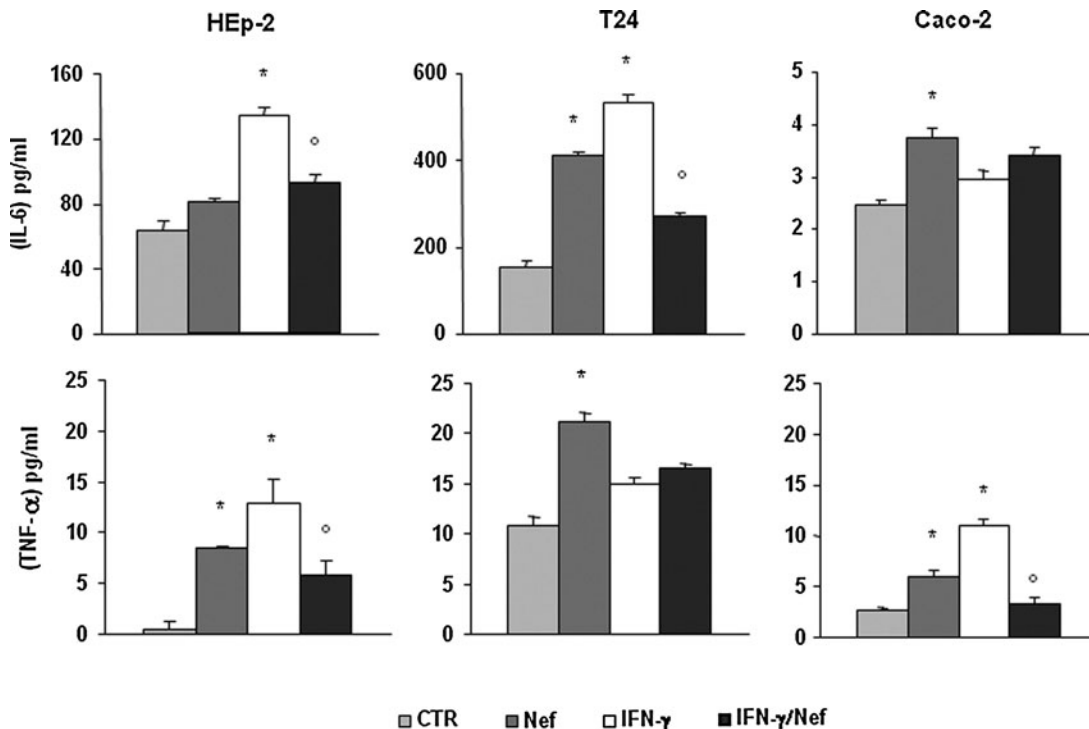
We therefore examined the secretion of TNF- $\alpha$  and IL-6, two inflammatory cytokines known to be potent inducers of HIV-1 expression by HEp-2, T24, and Caco-2 cells following exposure to Nef, IFN- $\gamma$ , or their combination. As shown in Fig. 2, HEp-2, T24, and Caco-2 cells constitutively secrete low levels of TNF- $\alpha$ . On the other hand, EC, except for Caco-2 cells, constitutively secrete higher levels of IL-6. Following Nef treatment, there was a significant increase ( $p < 0.05$ ) in IL-6 release by T24 and Caco-2 cells, and a significant increase ( $p < 0.05$ ) in TNF- $\alpha$  release by all three cell lines analyzed. IFN- $\gamma$  induced significant upregulation ( $p < 0.05$ ) of IL-6 production by HEp-2 and T24 cells, and significant up-

regulation ( $p < 0.05$ ) of TNF- $\alpha$  production by HEp-2 and Caco-2 cells. Notably, Nef abrogated the IFN- $\gamma$ -induced upregulation of IL-6 production in HEp-2 and T24 cells, and IFN- $\gamma$ -induced upregulation of TNF- $\alpha$  production in HEp-2 and Caco-2 cells. On the other hand, IFN- $\gamma$  abrogated the Nef-induced upregulation of IL-6 secretion by Caco-2 cells and TNF- $\alpha$  secretion by T24 cells.

To exclude any contamination due to bacterial expression or non-specific effects of Nef, we used boiled Nef and anti-Nef mAb as negative controls. Boiled Nef did not exert any effect on co-receptor expression and cytokine release (data not shown), confirming that the observed effects were not due to LPS contaminating the Nef preparation. Pre-incubation of Nef with anti-Nef mAb abrogated the effect of Nef (data not shown), thus confirming that the Nef-induced modulation was indeed a Nef-specific effect.

TNF- $\alpha$  and IL-6 may be both harmful and helpful for viral dissemination. They might favor activation of CD4<sup>+</sup> T cells, rendering them permissive for viral replication and activating HIV replication in latently-infected cells. On the other hand, cytokine modulation might also lead to increased activation of HIV-specific CD8<sup>+</sup> T cells (30,26).

Recent studies have focused on the mechanisms driving the inflammation that enhances HIV replication and dissemination (31). We found that Nef induces IL-6 and TNF- $\alpha$  production by EC, while it upsets IFN- $\gamma$ -induced inflammatory cytokines. This is relevant, considering that most viruses have evolved strategies to defend themselves against host IFN responses (38). Because we observed a mutual



**FIG. 2.** Effect of HIV-1 Nef on cytokine secretion by untreated or IFN- $\gamma$ -treated EC. HEp-2, T24, and differentiated Caco-2 cells were treated with Nef (0.1  $\mu$ g/mL), IFN- $\gamma$  (600 U/mL), or IFN- $\gamma$ /Nef. After 48 h supernatants were harvested and tested for IL-6 and TNF- $\alpha$  by ELISA. Results are expressed as pg/mL and are mean  $\pm$  SEM from 5 independent experiments run in duplicate (\* $p < 0.05$  versus medium;  $\circ p < 0.05$  versus IFN- $\gamma$ ).

regulation of co-receptor expression and cytokine secretion by IFN- $\gamma$  and Nef, we cannot exclude an intersection in their signalling pathways.

Since extracellular Nef has been detected in supernatants from HIV-1-infected cell cultures and in the serum of AIDS patients (12), our results may be relevant to a better understanding of AIDS pathogenesis. Moreover, the study of the events occurring at mucosal sites may aid in the development of preventive or therapeutic vaccines that may be administered by the mucosal route to provide protection against the initial exposure to the virus, and help prevent the subsequent long-lasting mucosal damage.

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### Author Disclosure Statement

No conflicting financial interests exist.

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