The Nef Protein of Human Immunodeficiency Virus is a Broad-Spectrum Modulator of Chemokine Receptor Cell Surface Levels That Acts Independently of Classical Motifs for Receptor Endocytosis and $G\alpha_i$-Signaling

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SUPPLEMENTARY MATERIAL AND METHODS

Kinetic Endocytosis Assay

Endocytosis assays were performed essentially as described (Schwartz *et al.*, 1996; Blagoveshchenskaya *et al.*, 2002; Michel *et al.*, 2005). Briefly, CHO CCR3 cells, which stably express amino-terminally HA-tagged human CCR3 receptors, were transfected with a GFP or Nef/GFP expression vector. One day later cells were stained at 4°C with anti-HA-tag mAb (clone 4C12, Abcam) in binding medium (BM: RPMI medium 2% FBS, 20 mM Hepes, pH 7.2). Excess antibody was washed away, and cells were resuspended in cold BM. Where indicated, the natural CCR3 ligand CCL11 (1 μM, BD Pharmingen) was added to the cell suspension as a reference control (Ponath *et al.*, 1996). Cell suspensions were shifted to 37°C and, at time points between 0 and 40 min, aliquots were placed on ice. Non-internalized, antibody-labeled CCR3 molecules were indirectly stained with APC-conjugated goat anti-mouse mAb (Jackson ImmunoResearch). The endocytosis rate of CCR3 was assessed by analyzing the relative decrease of the MFI of the CCR3 staining on viable cells of identical GFP intensity (corresponds to gate R3, Figure 1A) in the course of the kinetic. Percentages shown in supplementary Figure 2 represent the MFI at different time points relative to the MFI at the beginning of the kinetic, and the latter values were set to 100%.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Variable correlation of the degree of CD4 or CKR downmodulation in relation to HIV-1 Nef expression levels. Data from experiment shown in Figure 1 were analyzed for the degree of receptor cell surface staining in relation to the GFP or Nef/GFP expression level. The receptor cell surface staining intensity of cells within four distinct gates with ascending mean GFP expression levels were plotted as percentage of the corresponding receptor cell surface level on GFP-negative cells. Data points represent the arithmetic mean of triplicates ± SD of one representative of three independent experiments. For the Nef/GFP dependent receptor downregulation, a curve was calculated that approximates the measured data points using the software SigmaPlot 9.0 (Systat Software). Data points for the Nef-dependent CD4 downregulation are best fitted with a polynomial function, while data points for the CCR5 and CCR3 downregulation are best fitted with a logarithmic function.

Supplementary Figure 2. Nef moderately enhances the endocytosis rate of human CCR3. A kinetic receptor endocytosis assay was performed as described (Schwartz *et al.*, 1996; Michel *et al.*, 2005) for surface-exposed CCR3 in CHO CCR3 cells expressing Nef.GFP (○), GFP in the absence of the CCR3 ligand CCL11 (●), or GFP in the presence of CCL11 (1 μM) (▼). Each data point represents the mean of triplicates, and the values at time point 0 were set to 100%. The experiment shown is representative for three separate experiments.

Supplementary Figure 3. Nef localizes in a perinuclear compartment in the absence of human CKRs. Parental CHO cells that do not express any human CKRs were transfected with an expression construct for Nef.GFP. 20 h later cells were fixed and permeabilized with the same protocol as used for the immunostaining shown in Figure 5. Nuclei were stained with Hoechst 33258 (Sigma). Images were taken with a Zeiss LSM510 confocal microscope with a 100x PLAN-APO objective. Shown are individual sections (GFP (left panels); Hoechst33258 (middle panels); merge (right panels)) from the center of representative cells. White bars in merged images represent 10 μm.

SUPPLEMENTARY REFERENCES

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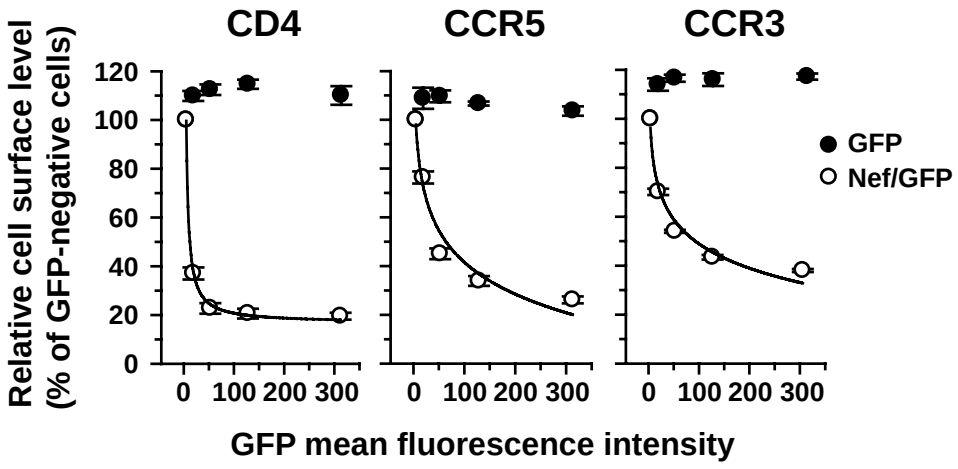
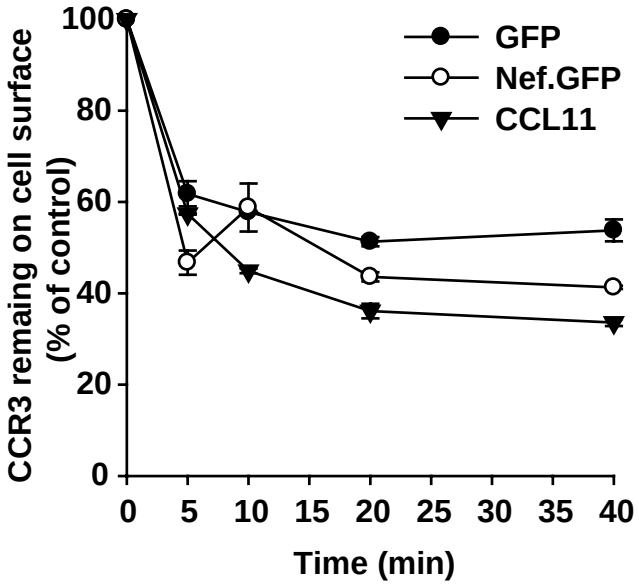
Michel *et al.*, Supplementary Figure 1



Michel *et al.*, Supplementary Figure 2



Michel *et al.*, Supplementary Figure 3