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The Nef Protein of HIV-1 Induces Loss of Cell Surface Costimulatory Molecules CD80 and CD86 in APCs¹

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The Nef protein of HIV-1 is essential for its pathogenicity and is known to down-regulate MHC expression on infected cell surfaces. We now show that Nef also redistributes the costimulatory molecules CD80 and CD86 away from the cell surface in the human monocytic U937 cell line as well as in mouse macrophages and dendritic cells. Furthermore, HIV-1-infected U937 cells and human blood-derived macrophages show a similar loss of cell surface CD80 and CD86. Nef colocalizes with MHC class I (MHCI), CD80, and CD86 in intracellular compartments, and binds to both mouse and human CD80 and CD86. Some Nef mutants defective in MHCI down-modulation, including one from a clinical isolate, remain capable of down-modulating CD80 and CD86. Nef-mediated loss of surface CD80/CD86 is functionally significant, because it leads to compromised activation of naive T cells. This novel immunomodulatory role of Nef may be of potential importance in explaining the correlations of macrophage-tropism and Nef with HIV-1 pathogenicity and immune evasion. *The Journal of Immunology*, 2005, 175: 4566–4574.

Establishment of infection in vivo by HIV-1 is more efficient for macrophage-tropic viral strains (1). HIV-1 also infects dendritic cells (DCs)⁴ (2). Strains of the related SIV that induce lytic infection in macrophages show less efficient infection in vivo than strains that cause persistent productive infection in macrophages (3). HIV/SIV strains defective in the *nef* gene, encoding the multifunctional accessory Nef protein, are also attenuated in their ability to cause persistent infection and disease (4–6). Nef inhibits the death of infected macrophages and DCs to create a cellular reservoir of persistent infection (7). It also induces pathways for attracting and activating T cells (8–10) to prime them for viral infection, leading to in vivo amplification of infection. In such a situation, a viral strategy to inhibit the resulting inevitable antiviral T cell priming would be crucial. Because it efficiently removes both MHC class I (MHCI) and MHC class II (MHCII) from the cell surface (11–13), Nef is likely to contribute to immune evasion. However, these effects alone may not mediate efficient immune evasion, because Nef does not affect surface levels of all

MHCI isotypes or nonclassical MHC molecules (14, 15). Furthermore, Nef down-modulates MHC molecules after they reach the cell surface, potentially allowing some presentation of antigenic peptides. Because costimulatory signals are also essential for effective priming of naive T cells (16), we have assessed the effect of Nef on the cell surface levels of the major costimulatory molecules on APCs such as macrophages or DCs, namely, the proteins of the B7 family, CD80 and CD86 (17). We find that Nef also mediates a loss of CD80 and CD86 from the APC surface. This effect does not involve residues in Nef that are crucial for MHCI down-regulation. Nef associates in cells with CD80 and CD86. Significantly, a clinical isolate of HIV-1 carries a mutant Nef that retains MHCI at the cell surface, but efficiently down-modulates CD80 and CD86, with functional consequences for naive T cell activation.

Materials and Methods

Wild-type (WT) and mutant *nef* genes and plasmids

The *F2-nef* and *D1-nef* genes from Indian HIV-1 subtype C primary isolates have been described earlier (18). These and other *nef* mutant genes described below were subcloned into the bicistronic mammalian expression vector *pIRES2-eGFP* (BD Clontech), or expressed as *nef-eGFP* fusion genes by ligating the *nef* gene inserts into the plasmid *peGFP-N3* (BD Clontech), or were expressed in the *pMT3* expression vector (BD Clontech) after tagging with the influenza virus hemagglutinin epitope (HAp). The primers used for HAp tagging were: forward, CTGCAGATGTACCCAT ACGATGTTCCAGATTACGCT; reverse, CGGAATTCAGCAGTCTT TGTAAGAACTC.

Truncation mutants of *nef* were made by PCR amplification of the $\Delta 100$ and $\Delta 125$ fragments from the cloned *F2-nef* gene. The primers used were: WT *F2-nef* CGGGATCCAATGGGTGGCAAGTGGTCAA (outer forward), CGGAATTCAGCAGTCTTGTAGAACTC (outer reverse), CG GAATTCATGGGTGGCAAGTGGTCAA (inner forward), CGGG TACCTCAGCAGTCTTGTAGAACTCCG (inner reverse); $\Delta 100$ *F2-nef* CGGGATCCAATGGGTGGCAAGTGGTCAA (forward), GCGGATC CAATTTACCCTTCCAGTCCCCC (reverse); $\Delta 125$ *F2-nef* CGGGATC CAATGGGTGGCAAGTGGTCAA (forward), GCGGATCCAATTTA CCCTTCCAGTCCCCC (reverse).

The PCR-amplified fragments were subsequently cloned into the *pIRES2-eGFP* or *peGFP-N3* vectors (BD Clontech). The G2A mutant of *F2-nef* was made by PCR amplification using the primers: forward, CG GAATTCATGGCTGCGAAGTGGTCAAATGCAGC; reverse, CGGA ATTCAGCAGTCTTGTAGAACTC.

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⁴ Abbreviations used in this paper: DC, dendritic cell; β -gal, β -galactosidase; eGFP, enhanced GFP; PACS-1, phosphofurin acidic cluster-sorting protein-1; SH3, Src homology 3; TfR, transferrin receptor; WT, wild type.

Other site-directed, single amino acid mutants were generated using the In Vitro Mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. The panel of HAp-tagged NL4-3 *nef* mutants (7) was gifted by Dr. W. C. Greene (University of California, San Francisco, CA).

OVA plasmid

A 1.9-kb *Bam*HI/*Hind*III fragment containing the *c-myc*-p-LCMVp-OVA-coding sequence described earlier (19) was cloned into the *Bgl*II-*Hind*III site of the expression vector pMD312 to generate a construct expressing a GFP-*c-myc*-p-LCMVp-OVA fusion protein.

Animals

C57BL/6 and OT-I mice were obtained from The Jackson Laboratory. The OT-II mice were gifted by A. Rudensky (University of Washington, Seattle, WA). Mice were bred and maintained in the animal facilities of the National Institute of Immunology (New Delhi, India). All animal experiments were done under the approval of the Institutional Animal Ethics Committee.

Anti-Nef Abs

Full-length His-tagged F2-Nef protein expressed in *Escherichia coli* was purified, and anti-Nef polyclonal Abs were raised in New Zealand White rabbits using this purified rF2-Nef protein. The IgG fraction was purified from immune sera on protein A-Sepharose (Amersham).

T cell activation assays

Activation of T cell hybridoma cells (13.8 and B3Z) was estimated by colorimetric measurement (A570 nm) of enzymatic activity expressed from an IL-2 promoter-driven β -galactosidase (*β -gal*) reporter gene. The activation-induced proliferation of primary TCR-transgenic OT-I or OT-II cells was measured by [³H]thymidine incorporation. BMC-2 cells were used as APCs 8 h after transfection at 10⁶ cells/ml (for 13.8 or OT-II T cells), or in titrating numbers (for B3Z and OT-I T cells). Responder T cells were used at 10⁶ cells/ml, and activation was estimated after coinubation with transfected APCs for 24 h. Azide-free anti-CD80 and anti-CD86 mAbs (eBioscience) were used for functional blockade at 10 μ g/ml each.

Cells and transfection

BMC-2 cells were maintained in Click's medium, with FCS, 2-ME, L-glutamine, and antibiotics. U937 cells were maintained in RPMI 1640 medium, with FCS, antibiotics, and LPS (0.5 μ g/ml) for maintenance of high MHC and CD80/CD86 levels. Primary human macrophages were grown from PBMC by culturing them with rM-CSF (100 U/ml; Sigma-Aldrich) for 3 days, and live cells were used for infection in the continuing presence of the growth factor. Primary macrophages and DCs were grown from mouse bone marrow by culturing nonadherent mouse bone marrow cells with either M-CSF (30% L929 fibroblast-conditioned medium as M-CSF source) or rGM-CSF (PeproTech), respectively, for 9 days, with periodic growth factor replenishment. Tightly adherent cells were excluded from the GM-CSF-containing cultures on day 7, and live cells were used for transfection on day 9. Transfections were done using Fugene6 (Roche) or Effectene (Qiagen), according to the manufacturers' protocols, with 12 μ g of plasmid DNA for 2 \times 10⁶ cells.

Flow cytometry

Cells were stained with primary and secondary reagents on ice for 30 min, as appropriate. For intracellular staining, cells were permeabilized with 0.03% saponin. Stained cells were analyzed on a BD-LSR (BD Biosciences) flow cytometer. Data were analyzed using FlowJo software (TreeStar).

Antibodies

The human-specific mAbs used were W6/32 for MHC1 and OKT-9 for transferrin receptor (TfR) (used as culture supernatants), and biotinylated anti-CD80 and anti-CD86 (eBiosciences). The mouse-specific mAbs used as culture supernatants were: Y-3 for MHC1 (H-2K^b and H-2D^b) and 3/23 for CD40. Other mouse-specific mAbs used were: anti-CD54 PE, anti-CD80 biotin, anti-CD86 biotin, and anti-CD11b biotin (BD Pharmingen). The secondary reagents used included goat anti-mouse IgG (Fc) PE and donkey anti-rat IgG (Fc) PE (Jackson ImmunoResearch Laboratories), and streptavidin-PE or streptavidin-CyChrome (BD Pharmingen). For studies with HAp-tagged genes, transfected cells were stained for surface molecules and then permeabilized for intracellular HAp staining using an anti-HAp mAb (Cell Signaling Technology). Similarly, gene products with the *myc*-p-tag were detected in permeabilized cells with an anti-*myc*-p mAb (Cell Signaling Technology).

Confocal microscopy

For confocal microscopy, cells were grown on coverslip-bottomed dishes, transfected, and fixed in situ with 3% paraformaldehyde, followed by saponin (0.1%) permeabilization and blocking with 1% BSA. Staining was done on coverslips with primary and secondary reagents, as appropriate. Confocal images were acquired on a Bio-Rad MRC-1024 confocal microscope (Bio-Rad) with factory-set dichroics and a Krypton-Argon laser, using LaserSharp software (Bio-Rad), or using a Zeiss LSM 510 Meta confocal system equipped with Argon, HeNe, and HeCd lasers (Zeiss). Images were processed in MetaMorph (Universal Imaging) and Adobe Photoshop (Adobe Systems) softwares. Image quantitation was done as described (20), using Metamorph software.

Viruses and infection

HIV-1 viral stocks were generated by electroporation of HeLa cells with the infectious molecular clones pNL4-3, pNL4-3 FS Nef (gifted by H. Gottlinger, University of Massachusetts Medical School, Worcester, MA), or pNL4-3 ADA or pNL4-3 ADA Δ Nef (gifted by M. Stevenson, University of Massachusetts Medical School, Worcester, MA). Virus stocks were harvested 72 h later and filter sterilized. U937 cells were serum starved for 1 h before infection. For each infection, 1 \times 10⁶ cells were infected with 100,000–200,000 cpm of reverse-transcriptase counts of virus. After a 4-h adsorption, cells were washed and incubated for 72 h before being stained for surface CD80, CD86, or MHC1, and intracellular p24 gag protein.

Immunoprecipitation and Western blot analyses

Transfected cell lysates were immunoprecipitated with either anti-Nef, anti-CD80, or anti-CD86 Abs and Western blotted for the indicated molecules after 12% SDS-PAGE of 50 μ g of protein/lane and transfer. The secondary reagents were goat anti-rabbit IgG HRP (Cell Signaling Technology) or donkey anti-rat IgG HRP (Jackson ImmunoResearch Laboratories). Blots were developed with the diaminobenzidine reagent (Bio-Rad).

Yeast two-hybrid analysis

The *F2-nef* and *CD80/CD86* genes were fused to the DNA binding or activation domains, respectively, of the yeast Gal4 protein gene. For this, the *F2-nef* gene was transferred as an *Eco*RI-*Bam*HI fragment to pGADT7 (BD Clontech), which expresses inserted genes as fusion proteins together with the yeast Gal4 protein activation domain. The CD80 and CD86 genes were PCR amplified from plasmids pBJCD80 and pBJCD86, containing the human CD80 and CD86 genes, respectively, using the primers: CD80, GAATTCACCATGGGCCACACACGGAGGCAG (forward), CTGCA GTTATACAGGGCGTACACTTTCC (reverse); CD86, GGATCCAC CATGGGACTGAGTACAATTCTCTTTGTG (forward), GTCGACAA TTTAAAACATGTATCACTTTTGTGCGCAT GAA (reverse).

These were cloned as *Eco*RI-*Pst*I and *Bam*HI-*Sal*I fragments, respectively, into pGBKT7 (BD Clontech), which expresses inserted genes as fusion proteins to the yeast Gal4 protein DNA binding domain. The clones were checked by restriction digestion, DNA sequencing, and protein expression using an in vitro coupled transcription-translation system (TNT; Promega). These plasmids were cotransformed into *Saccharomyces cerevisiae* strain AH109 (*MATa trp1-901 his3 leu2-3, 112 ura3-52 ade2 gal4 gal80URA3::GAL-lacZ LYS2::GAL-HIS3*) containing the *HIS3* and *lacZ* reporter genes under the control of GAL4 binding sites. The host strain containing plasmids pAS2-SNF1 and pACT2-SNF4 was used as a positive control (21). Various negative controls that included single or dual transformants were also run in the same assay. The transformed AH109 yeast cells were plated on either complete yeast extract/peptone/dextrose medium or synthetic dextrose in the absence of either leucine or tryptophan, or both. Protein interaction was tested by growth on synthetic dextrose plates without leucine, tryptophan, and histidine, and the specificity of the interaction was tested as growth on these plates containing 20 mM 3-amino-1, 2, 3-triazole. The β -gal filter-lift and liquid assays were conducted, as described elsewhere (22).

Results

HIV-1 Nef reduces cell surface CD80 and CD86 levels in human and mouse macrophages

A WT *nef* gene (*F2-nef*) cloned from an Indian clinical isolate (18) was used for transfection studies. In human monocytic U937 cells expressing F2-Nef, the levels of cell surface CD80 and CD86 were reduced 5- to 10-fold at 48 h posttransfection, along with reduction in the surface levels of MHC1 (Fig. 1A). No significant change in the surface levels of the TfR could be detected (Fig. 1A). There

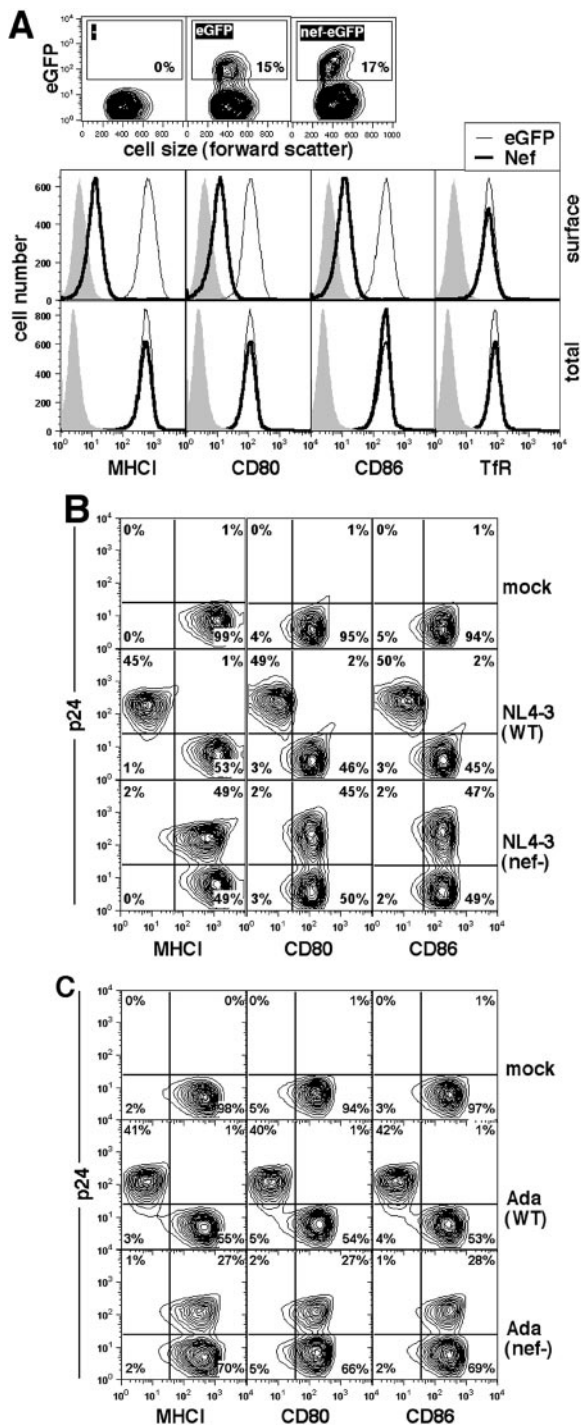


FIGURE 1. Nef causes reduction of surface CD80 and CD86 levels in a human monocytic cell line. **A**, U937 cells were transfected to express eGFP either alone or with F2-Nef, or were mock transfected (—), as indicated. Two-parameter plots show the frequencies of and gates set for eGFP⁺ cells. Histograms show surface-staining levels or postpermeabilization-staining levels for the indicated molecules in cells expressing eGFP alone or with Nef. Gray-shaded curves indicate isotype controls. **B**, Two-color flow cytometric analysis for intracellular p24 protein vs cell surface MHCII, CD80, and CD86 expression levels on U937 cells, either uninfected, or infected with the HIV-1 strains indicated at 72 h after infection. **C**, Two-color flow cytometric analysis for intracellular p24 protein vs cell surface MHCII, CD80, and CD86 expression levels on primary human monocytes, either uninfected or infected with the HIV-1 strains indicated at 72 h after infection.

was no reduction in the total cellular levels of any of these molecules, as measured by staining postpermeabilization (Fig. 1A). The F2-Nef effects were based on comparison with an enhanced GFP (eGFP) control, under conditions of comparable transfection efficiencies of the two plasmids.

We next examined MHCII, CD80, and CD86 levels on U937 cells infected with either WT or *nef*-deficient strains of HIV-1 (NL4-3 strain). In HIV-infected cells, as indicated by p24gag expression, while WT HIV-1 induced a loss of cell surface MHCII, CD80, and CD86 levels, the *nef*-deficient virus did not induce any down-regulation of these molecules (Fig. 1B). Furthermore, when primary human monocytes from peripheral blood were grown in M-CSF and infected with either WT or *nef*-deficient strains of HIV-1 (ADA strain), the WT, but not *nef*-deficient HIV-1 again induced a loss of cell surface MHCII, CD80, and CD86 levels (Fig. 1C).

Nef expression led to a similar 5- to 6-fold reduction of surface CD80 and CD86 in the murine monocytic BMC-2 cell line (Fig. 2A). Surface levels of two other macrophage proteins, CD11b and CD54, were unaffected (Fig. 2A). There was no reduction in the total cellular levels of any of these molecules (Fig. 2A). F2-Nef had similar effects on primary nontransformed mouse bone marrow DCs and macrophages (Fig. 2B) as well. As earlier, the F2-Nef effects were based on comparison with an eGFP control, under

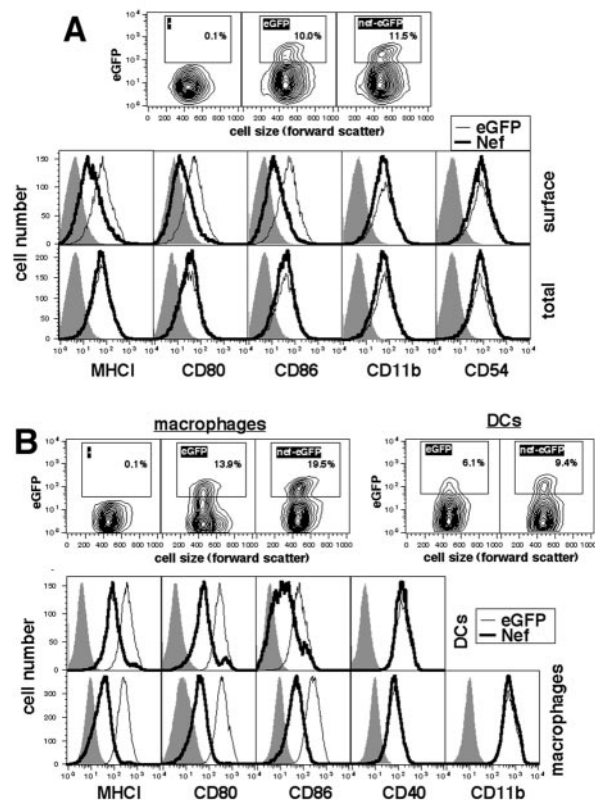


FIGURE 2. Nef causes reduction of surface levels of CD80 and CD86 in mouse myeloid lineage cells. **A**, BMC-2 cells were transfected to express eGFP either alone or with F2-Nef, or were mock transfected (—), as indicated. Two-parameter plots show the frequencies of and gates set for eGFP⁺ cells. Histograms show surface-staining levels or postpermeabilization-staining levels for the indicated molecules in cells expressing eGFP alone or with Nef. **B**, Primary DCs or macrophages were transfected to express eGFP either alone or with F2-Nef. Two-parameter plots show the frequencies of and gates set for eGFP⁺ cells. Histograms show surface-staining levels for the indicated molecules in cells expressing eGFP alone or with Nef. All gray-shaded curves indicate isotype controls.

conditions of comparable transfection efficiencies of the two plasmids.

Nef mutants, including a clinical isolate, can differentially affect down-modulation of MHCI vs CD80 and CD86

To further characterize Nef-mediated down-modulation of CD80/CD86 and to identify its possible differences from MHCI down-regulation, we tested a series of Nef mutants. Because the F2-*nef* gene from an HIV-1 subtype C clinical isolate differs from the NL4-3 HIV-1 subtype B *nef* gene (Fig. 3A), we constructed a number of F2-Nef mutants in addition to the available panel of NL4-3-Nef mutants. Furthermore, we tested a variant D1-*nef* gene cloned from an independent HIV-1 isolate from the same patient as F2-*nef*, which has a natural deletion of residues 55–61 (numbered according to the NL4-3 Nef sequence (Fig. 3A)).

The NL4-3-Nef mediated efficient down-regulation of MHCI, CD80, and CD86, but a GG to AA mutation of Nef at residues 2–3 (G2A) was unable to down-regulate any of these surface proteins (Fig. 3B). The G2A mutant protein is known to be myristoylation deficient and is unable to associate with cellular membranes (23). Three other mutants of NL4-3 Nef could not mediate MHCI down-regulation, as shown earlier (24). These include an M to A mutation at residue 20 (M20A), E to A mutations at residues 62 to 65 (E4A) comprising the phosphofurin acidic cluster-sorting protein-1 (PACS-1) binding domain, and P to A mutations at positions 72, 75, and 78 in the Src homology 3 (SH3)-binding PXX motifs (Fig. 3B). However, all three mutants showed normal down-regulation of CD80 and CD86 (Fig. 3B).

Like NL4-3 Nef, the G2A and M20A mutants of F2-Nef were unable to down-regulate surface MHCI, but were fully competent in down-regulating surface levels of CD80 and CD86 (Fig. 3C). We then used C-terminal F2-Nef deletion mutants terminated at residue 100 or 125 (Fig. 3A). F2-Nef truncated from residue 100 onward (F2-Nef-Δ100) was unable to mediate any significant down-regulation of MHCI, CD80, or CD86 (Fig. 3C). However, a smaller C-terminal deletion from residue 125 onward (F2-Nef-Δ125) allowed partial down-modulation of MHCI as well as of CD80 and CD86 (Fig. 3C). Other mutations in F2-Nef that include amino acid residues WL57–58, R77, D86, R106, I109, F121, P130, EE154–155, or LL164–165 did not affect the down-modulation of any of these target molecules by Nef (Table I). Data obtained with Nef mutants were similar in human U937 cells and mouse BMC-2 cells (Table I).

We next tested a variant of the F2-*nef* gene cloned and sequenced from an independent HIV-1 isolate from the same patient. This variant has a natural deletion of residues 55–61 of the Nef protein (D1-*nef*; numbered according to the NL4-3 Nef sequence (Fig. 3A)). The D1-Nef mutant induced no detectable down-modulation of cell surface MHCI, despite remaining as efficient as the WT F2-Nef in inducing the down-modulation of cell surface CD80 and CD86 (Fig. 3D). This result suggested that the CD80/CD86 down-regulation property of Nef is conserved and important for HIV infectivity. To explore this further, we examined the Los Alamos HIV sequence database (www.hiv.lanl.gov/content/hiv-db/ALIGN_CURRENT/ALIGN-IN-DEX.html) for patterns of Nef sequence variation. We focused on the three regions identified by the data shown above. Among 822 Nef sequences, there are six showing alterations in the 62–65 EEEE and the (PXX)3 regions, critical for intracellular relocation of MHCI, but not CD80/CD86. Five of these six alterations are in clinical isolates from progressive HIV-mediated disease, while one is from a long-term nonprogressing case. However, only two sequences show alterations, both as deletions, in the 100–125 region critical for down-regulation of all three molecules. At least one of these is from a long-term nonprogressing case.

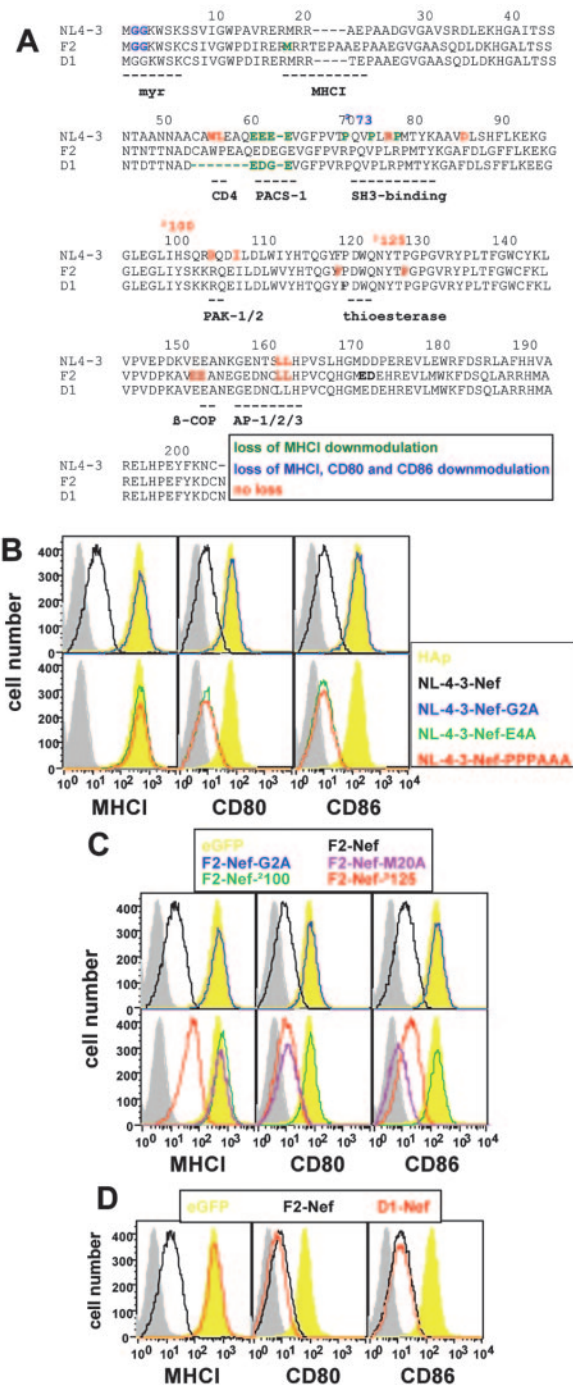


FIGURE 3. Distinct domains of Nef are crucial for down-modulation of MHCI, CD80, and CD86. *A*, Amino acid sequence alignment and identification of the various Nef alleles used: NL4-3, F2-Nef, and D1-Nef. The point mutations used are mapped in the appropriate sequence by color coding, as shown for changes in the abilities to down-modulate MHCI, CD80, and CD86. The deletion mutants constructed for F2-Nef are shown as Δ. Putative functional regions of Nef are indicated at the bottom of each alignment set. Deleted residues are also identified (–). All residues are numbered according to the NL4-3 Nef sequence. *B*, Histograms are shown for surface levels of indicated molecules on gated HAp⁺ U937 cells after transfection to express HAp alone, or with WT NL4-3-Nef, NL4-3-Nef-G2A, NL4-3-Nef-E4A, or NL4-3-Nef-PPPPAAA. *C*, Histograms are shown for surface levels of indicated molecules on gated eGFP⁺ U937 cells after transfection to express eGFP alone, or with WT F2-Nef, F2-Nef-G2A, F2-Nef-M20A, F2-Nef-Δ100 (F2-Nef-²100), or F2-Nef-Δ125 (F2-Nef-²125). *D*, Histograms are shown for surface levels of indicated molecules on gated eGFP⁺ U937 cells after transfection to express eGFP alone, or with F2-Nef or D1-Nef. All gray-shaded curves indicate isotype controls.

Table I. Effects of Nef mutations on Nef-mediated down-modulation of cell surface MHC I, CD80, and CD86 in monocytic cell lines

Mutants	U937 (Human Cells)			BMC-2 (Mouse Cells)		
	MHCI ^a	CD80 ^a	CD86 ^a	MHCI ^a	CD80 ^a	CD86 ^a
NL-4-3- <i>nef</i> (WT ^b)	+	+	+	+	+	+
NL-4-3- <i>nef</i> -G2A	-	-	-	-	-	-
NL-4-3- <i>nef</i> -WL57AA	+	+	+	+	+	+
NL-4-3- <i>nef</i> -E4A	-	+	+	-	+	+
NL-4-3- <i>nef</i> -R77A	+	+	+	+	+	+
NL-4-3- <i>nef</i> -PPPAAA	-	+	+	-	+	+
NL-4-3- <i>nef</i> -D86A	+	+	+	+	+	+
NL-4-3- <i>nef</i> -R106A	+	+	+	+	+	+
NL-4-3- <i>nef</i> -I109A	+	+	+	+	+	+
F2- <i>nef</i> (WT)	+	+	+	+	+	+
F2- <i>nef</i> -G2A	-	-	-	-	-	-
F2- <i>nef</i> -M20A	-	+	+	-	+	+
F2- <i>nef</i> -F121G	+	+	+	ND	ND	ND
F2- <i>nef</i> -P130A	+	+	+	ND	ND	ND
F2- <i>nef</i> -EE155AA	+	+	+	ND	ND	ND
F2- <i>nef</i> -LL164AA	+	+	+	ND	ND	ND
F2- <i>nef</i> -Δ100	-	-	-	-	-	-
F2- <i>nef</i> -Δ125	(+) ^c	(+) ^c	(+) ^c	(+) ^c	(+) ^c	(+) ^c
D1- <i>nef</i>	-	+	+	-	+	+

^a +, Persistence of down-modulation ability; -, loss of down-modulation ability.

^b WT, wild type.

^c (+), Weak persistence of down-modulation ability.

Down-modulation of CD80/CD86 is crucial for Nef-mediated inhibition of naive T cell activation

Using a mouse cell system, we next tested whether the reduction in cell surface CD80/CD86 levels by Nef is functionally significant for the activation of naive T cells. For this, we cotransfected plasmids carrying F2-Nef and *myc*-p-tagged OVA into BMC-2 cells to ascertain that a majority of OVA-*myc*-p-expressing transfected cells also expressed Nef (Fig. 4, A and B). These cotransfected BMC-2 cells were then used as stimulator APCs for either a T cell

hybridoma, B3Z, or for primary splenic cells from TCR-transgenic mice (OT-I). Both B3Z and OT-I CD8 T cells express the same TCR specific for an OVA peptide on MHC I (H-2K^b). A mixture of anti-CD80 and anti-CD86 mAbs blocks the activation of OT-I cells, but not of B3Z cells, showing their differential dependence on costimulation (Fig. 4C). The presence of either F2-Nef or its mutants did not affect the activation of B3Z cells (Fig. 4D). However, the response of OT-I cells was reduced not only by F2-Nef, but also by D1-Nef, which is unable to affect MHC I levels (Fig. 4E). As a control, the G2A-Nef mutant that is incapable of down-regulating MHC I, CD80, or CD86 did not affect this response (Fig. 4E). Similarly, stimulation of an OVA-specific MHC II-restricted T cell hybridoma (13.8) by such transfected BMC-2 APCs was unaffected by Nef (Fig. 4F). However, Nef expression reduced the response induced by these APCs from naive T cells of OT-II mice transgenic for an OVA-specific MHC II-restricted TCR (Fig. 4G). Under these conditions, the OT-II cell proliferative responses require costimulation, while the 13.8 T cell hybridoma does not need any costimulation (data not shown).

Nef associates with CD80 and CD86

The lower surface levels, but unchanged total cellular levels of CD80 and CD86 coincident with Nef expression suggested that Nef redistributes these proteins. We therefore examined whether Nef colocalized subcellularly with these proteins in U937 and BMC-2 cells by confocal microscopy, using expression vectors containing the F2-*nef*, G2A-*nef*, D1-*nef*, and *nef*Δ100 genes fused in-frame to the *eGFP* gene. Transfection with an eGFP-expressing vector was used as a control. All three proteins, MHC I, CD80, and CD86, were predominantly redistributed intracellularly and colocalized with F2-Nef-eGFP, but not with control eGFP, G2A-Nef-eGFP, or NefΔ100-eGFP. The NefΔ100-eGFP protein showed a pattern of intracellular distribution similar to F2-Nef (Fig. 5A). The D1-Nef protein colocalized intracellularly with CD80 and CD86, but not with MHC I (Fig. 5A). Three-color confocal analysis of

FIGURE 4. CD80 and CD86 down-modulation by Nef impairs activation of naive T cells. A and B, Show the coexpression of OVA and Nef in transfected BMC-2 cells. Two-parameter plots (A) show frequencies of OVA-*myc*-p expression in untransfected cells (-), or cells transfected to express OVA-*myc* alone or OVA-*myc* + F2-Nef, as indicated. The gates for OVA-*myc*-p⁺ cells are also shown. Histograms (B) show staining levels for F2-Nef-HAp in OVA-*myc*-p⁺ cells from OVA-*myc*-transfected (thin line) or OVA-*myc* + F2-Nef-HAp-transfected (thick broken line) cultures. The gray-shaded curve is the isotype control. C, Shows OVA-specific MHC I-restricted responses of either the T cell hybridoma B3Z or primary TCR-transgenic OT-I splenic T cells to OVA-transfected BMC-2 cells in the presence (+) or absence (-) of Abs against CD80 and CD86. Lines indicate relevant background values. D and E, Show OVA-specific MHC I-restricted responses of either the T cell hybridoma B3Z (D) or primary TCR-transgenic OT-I splenic T cells (E) to BMC-2 cells variously transfected, as indicated. F and G, Show the OVA-specific MHC II-restricted responses of either the T cell hybridoma 13.8 or the primary TCR-transgenic OT-II splenic T cells, respectively, to BMC-2 cells variously transfected, as indicated. All data are mean ± SE of triplicate cultures.

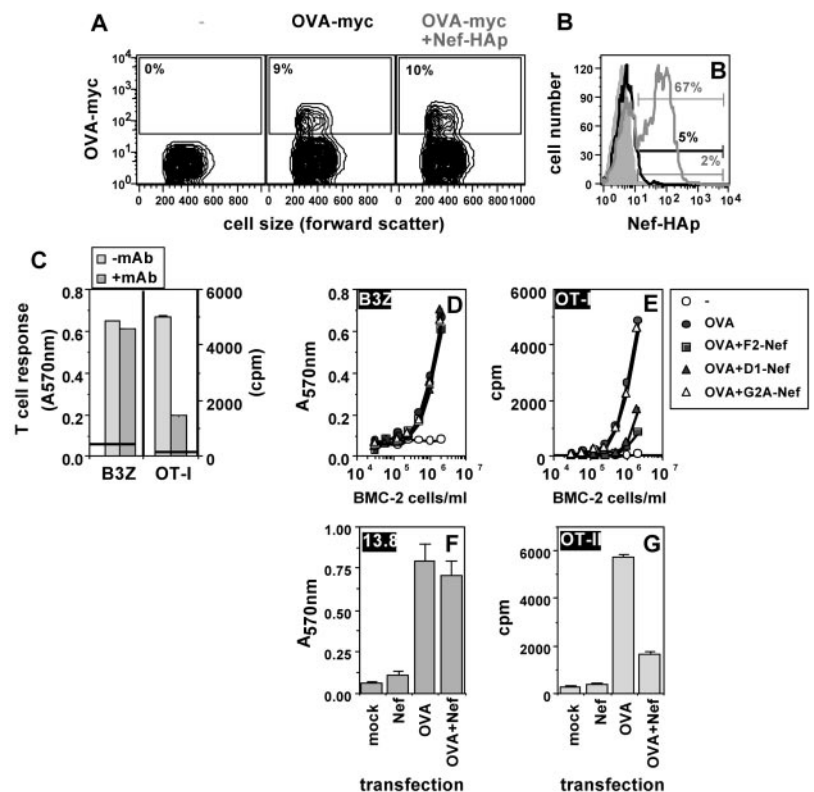
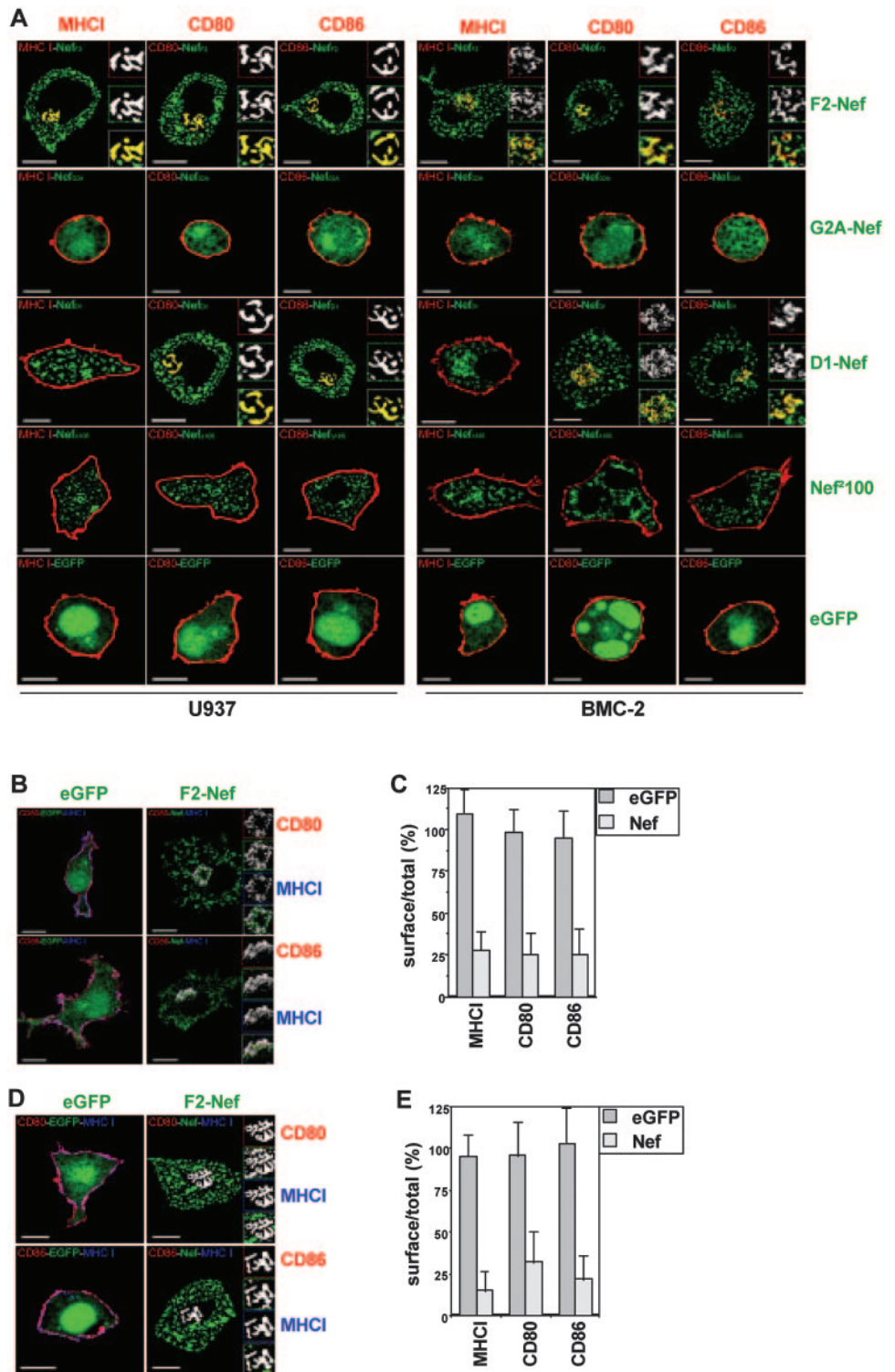


FIGURE 5. HIV-1 Nef protein colocalizes with CD80 and CD86 in transfected cells. *A*, U937 or BMC-2 cells were transfected to express eGFP, F2-Nef-eGFP, G2A-Nef-eGFP, D1-Nef-eGFP, or Nef Δ 100-eGFP (Nef²¹⁰⁰) proteins. The cells were fixed and stained for the various molecules shown in the colors indicated. *B*, BMC-2 cells were transfected to express eGFP or F2-Nef-eGFP proteins, fixed, and stained for the various molecules shown in the colors indicated. *C*, Images of cell populations as represented in *B* were analyzed quantitatively for the fraction of total cellular MHC I, CD80, or CD86 present at the cell surface in either eGFP-expressing or F2-Nef-eGFP-expressing BMC-2 cells. Data are shown as percentage of total cellular protein present at the cell surface (mean \pm SD; $n = 200$). *D*, U937 cells were transfected to express eGFP or F2-Nef-eGFP proteins, and fixed and stained for the various molecules shown in the colors indicated. *E*, Images of cell populations as represented in *D* were analyzed quantitatively for the fraction of total cellular MHC I, CD80, or CD86 present at the cell surface in either eGFP-expressing or F2-Nef-eGFP-expressing U937 cells. Data are shown as percentage of total cellular protein present at the cell surface (mean \pm SD; $n = 200$). In all images, scale bars are shown for each image (10 μ m), and *insets* are at $\times 2$ magnification. *Insets*, Show grayscale images for each color as well as for merged colors.

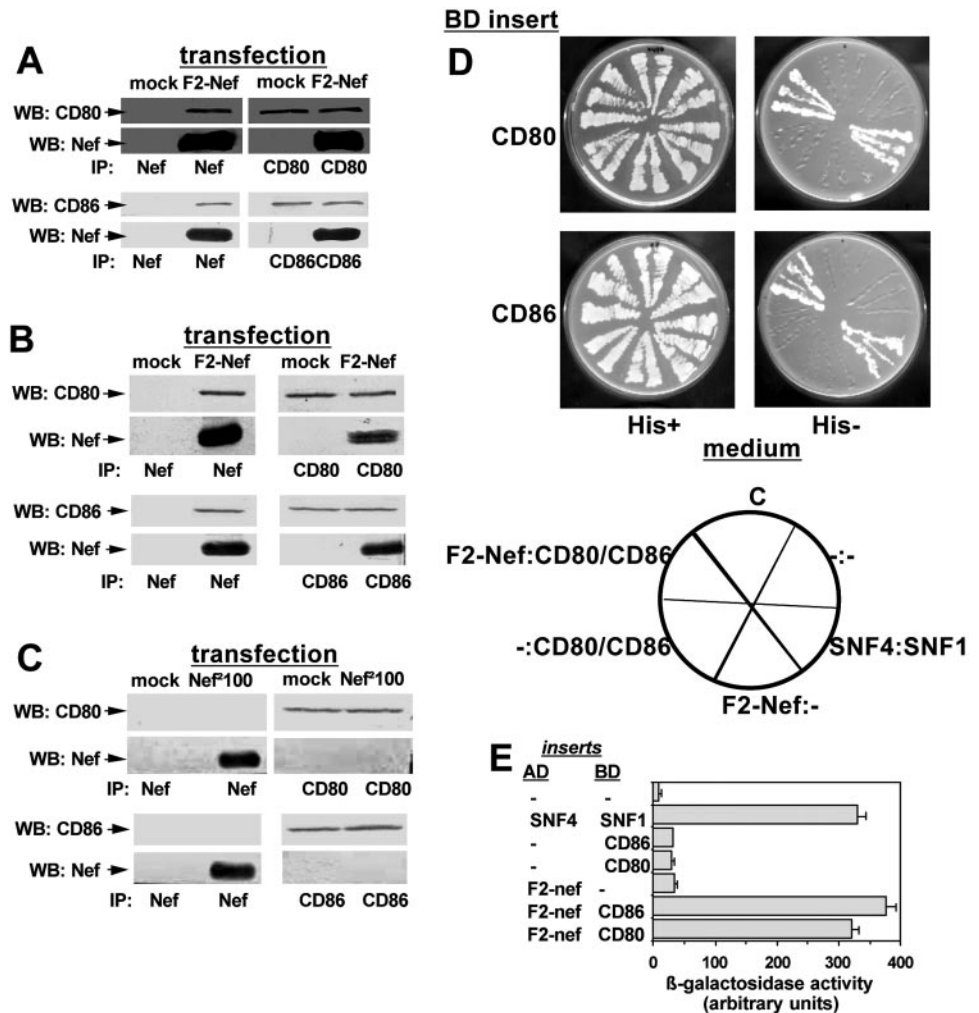


these cells showed that Nef, MHC I, and CD80/CD86 colocalized together at the same intracellular site in Nef-expressing BMC-2 or U937 cells (Fig. 5, *B* and *D*). A quantitative analysis of the imaging data showed a ~ 5 -fold loss of MHC I, CD80, and CD86 target molecules from the cell surface due to Nef (Fig. 5, *C* and *E*). This is comparable to the loss observed on flow cytometric analyses (Figs. 1 and 2).

These colocalization results suggested that while inducing their redistribution, Nef might bind to CD80 and CD86, either directly or in a supramolecular complex. We examined this possibility by

immunoprecipitating F2-Nef from transfected BMC-2 cells and Western blotting the immunoprecipitates for the presence of coprecipitated mouse CD80 and CD86. Both CD80 and CD86 were readily detectable to be coimmunoprecipitating with F2-Nef (Fig. 6A). Similarly, when CD80 and CD86 were immunoprecipitated, Nef was found by Western blotting to be coimmunoprecipitating with them in transfected cells (Fig. 6A). In U937 cells too, immunoprecipitating WT F2-Nef led to coprecipitation of CD80 and CD86, and conversely, immunoprecipitation of CD80 or CD86 also brought down Nef (Fig. 6B). U937 cells were also transfected

FIGURE 6. HIV-1 Nef protein binds to CD80 and CD86 in transfected cells. *A–C*, Western blot analyses of immunoprecipitates from lysates of BMC-2 cells (*A*) or U937 cells (*B* and *C*) transfected, as indicated. The Ab specificities used for immunoprecipitation (IP) and Western blotting (WB) are shown (F2-Nef- Δ 100: Nef²100). *D*, Yeast two-hybrid analysis in AH109 cells using the *F2-nef* gene in the activation domain (AD) vector plasmid and full-length human *CD80* or *CD86* genes in the DNA binding domain (BD) vector plasmid, or control plasmids with no inserts (–), or positive control plasmids containing the *SNF1-SNF4* genes. AH109 yeast cells used for transformation were grown on plates of rich yeast extract/peptone/dextrose medium (*His*⁺) or His-deficient medium without leucine, tryptophan, and histidine (*His*[–]). The growth pattern on plates is shown with plating done according to the template shown, which indicates the inserts in the AD:BD. The activation of the reporter β -gal gene in *His*[–] medium is shown in *E* as enzymatic activity in triplicate cultures (mean \pm SE).



with the truncated version of F2-Nef with residues from 100 onward deleted (F2-Nef- Δ 100), which was unable to mediate any significant down-regulation of MHCI, CD80, or CD86 (Fig. 3C). Although the polyclonal anti-Nef Ab used for immunoprecipitation and Western blotting could efficiently detect the smaller band of F2-Nef- Δ 100, there was no coprecipitation seen between F2-Nef Δ 100 and either CD80 or CD86 (Fig. 6C).

We also used cloned human CD80 and CD86 along with F2-Nef in yeast two-hybrid assays to examine the possibility of direct binding. The F2-Nef and CD80/CD86 genes were fused to the DNA binding or activation domains, respectively, of the yeast Gal4 protein gene. These plasmids were cotransformed into *S. cerevisiae* AH109 cells stably carrying the *His3* and β -gal reporter constructs under control of a Gal4-responsive promoter. Only cotransformation with the F2-Nef/CD80 and F2-Nef/CD86 pairs led to growth of AH109 cells on medium lacking histidine (Fig. 6D). These cells showed substantial induction of β -gal activity over the background for negative controls (Fig. 6E), confirming direct binding of Nef to CD80 and to CD86.

Discussion

In infected macrophages, Nef plays crucial roles, inhibiting cell death to generate cellular reservoirs of persistent infection (7), and inducing pathways for attraction and activation of T cells (9, 10) as targets for further infection. A viral strategy of immune evasion is likely to be useful in such a situation, and Nef is likely to play a central role in such immune evasion as well. Nef down-modulates

cell surface MHCI and MHCII molecules to intracellular compartments (13, 24). However, the effects of Nef on MHC molecules alone cannot be expected to mediate efficient immune evasion. This is because of the means used by Nef to remove MHC molecules from cell surfaces. Because MHCI and MHCII molecules are removed from the cell surface with a $t_{1/2}$ of 3 or 24 h, respectively (25, 26), newly arriving peptide-loaded MHC molecules are likely to remain on the cell surface for an adequate length of time to provide T cell priming. Our functional data indeed suggest this to be the case. Furthermore, Nef does not affect surface levels of all MHCI isotypes (14), including mouse CD1d, a nonclassical MHC molecule that can efficiently prime T cells (data not shown).

Our data now show that Nef also down-regulates the surface expression of CD80 and CD86, major costimulatory molecules on APCs that are crucial for T cell priming. These effects are seen in macrophage lines of both mouse and human origin, as well as in primary cultures of human murine macrophages and DCs, indicating these to be global effects. Human PBL-derived macrophages infected with HIV-1 also show similar effects, establishing that these are relevant consequences of infection rather than being seen only with high Nef levels achieved with transfection. A previous report suggesting that Nef expression in APCs does not affect expression of other cell surface molecules such as MHCI or CD80/CD86 used an adenoviral vector that itself modifies the expression of CD80/CD86, making it impossible to draw any conclusions about the effect of Nef on CD80/CD86 levels (27). Nef is also found as a secreted protein, and extracellular rNef can trigger DCs,

leading to modest up-regulation of surface CD80 and CD86 (28), underlining the pleiotropy of the effects of Nef on APCs. Together, the role of Nef appears to incorporate both APC-mediated bystander T cell activation and immune evasion to ensure that, while the virus has a supply of activated T cells in peripheral lymphoid organs to spread to, the virus-specific T cells among these recruited populations are not allowed to receive Ag-specific priming triggers.

The down-modulation of CD80 and CD86 by Nef is specific, because no changes could be detected in the surface levels of other molecules such as TIR, CD11b, CD54, or CD40. Furthermore, the total cellular levels of MHCI, CD80, and CD86 in Nef-expressing cells are not altered, indicating that the effects of Nef on the cell surface levels of these molecules are due to altered trafficking. Although the *nef* gene used in most of our experiments shown in this study is from a clinical isolate of HIV-1 subtype C, redistribution of CD80 and CD86 is also mediated by the NL4-3 Nef protein from HIV-1 subtype B. Significantly, we have identified a Nef variant (D1-Nef) from a clinical isolate of HIV-1 subtype C that has lost the ability to down-regulate MHCI, but can still down-regulate CD80 or CD86. This observation underlines the importance of Nef-mediated CD80 and CD86 modulation *in vivo*. An analysis of Nef sequence variation in the HIV sequence database is also consistent with this possibility. Sequence variation in regions important for MHCI down-modulation alone is somewhat more permissive than in a region critical for down-modulation of MHCI as well as CD80 and CD86, suggesting the likely importance of CD80/86 down-modulation in HIV pathology.

All relocation effects of Nef on MHCI, CD80, and CD86 are lost with a mutation that prevents Nef myristoylation (G2A (24)), indicating that membrane recruitment of the Nef protein is necessary for its effects on the trafficking of various target cell surface molecules. Serial deletions of the C terminus of the F2-Nef protein indicate that, while the 100- to 125-aa region is critical for reduction in MHCI, CD80, and CD86 levels, residues beyond 125 are also needed for optimal function of the Nef protein. Mutations at R77, D86, R106, I109, or LL164–165 do not affect the down-modulation of any target molecules by Nef, suggesting the potential functions hypothesized at these sites, such as binding of SH3 domains, PAK1/2 or AP1/2/3, are not likely to be involved in these trafficking functions of Nef.

Comparisons between F2-Nef and NL4-3-Nef raise some important issues regarding the role of specific domains of Nef in internalization. The EEEE sequence at residues 62–65 in NL4-3-Nef has been shown to be critical for MHCI down-regulation (24). In F2-Nef, which remains competent for MHCI relocation, the corresponding residues are EDEGE. However, their modification to DEDGE in D1-Nef prevents MHCI relocation, identifying important residues within this critical area. However, this modification does not affect CD80/86 down-modulation.

Further analysis of the effects of Nef mutants reveals distinct roles for different Nef residues in mediating the down-modulation of MHCI vs CD80 or CD86. Specifically, the E4A (PACS-1 recruitment domain) and the PPPAAA (an SH3 binding domain) mutants of NL4-3 Nef, as well as the D1 natural mutant of F2-Nef, which is also devoid of the PACS-1 recruiting domain, do not down-modulate MHCI, but efficiently reduce surface levels of CD80 and CD86, again emphasizing the separation between MHCI-directed vs CD80/CD86-directed effects of Nef. Although Nef appears to interact weakly and transiently with specific MHCI molecules (29), our data indicate that Nef binds strongly and directly to both human and mouse CD80 and CD86, suggesting a possible mechanism for recruitment of cellular signaling and trafficking pathways to achieve the cellular redistribution of CD80 and

CD86. This is supported by the finding that a truncated version of Nef that cannot down-modulate CD80 or CD86 cannot bind to these molecules either.

Importantly, this function of Nef is involved in preventing the activation of naive T cells, providing evidence for the functional significance of our findings in the mechanism of HIV immune evasion. Although Nef down-modulates MHC molecules, this reduction is slow (data not shown). Thus, when T cell activation assays are conducted within 24 h of Nef transfection, presentation of APC endogenous Ag to both MHCI-restricted and MHCI-restricted T cell hybridomas is unaffected by the presence of Nef in these APCs. However, these same APCs are extremely poor activators of naive primary CD4 and CD8 T cells, consistent with Nef-mediated loss of costimulatory function as a major modulator of immune priming. This interpretation is further emphasized when the D1-Nef mutant, which has no effect on MHCI levels, is used in assays with naive CD8 T cells, reinforcing the importance of CD80/86 down-modulation effects of Nef. Together, these data suggest a significant role for Nef-mediated loss of costimulation in immune evasion during HIV infection.

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Disclosures

The authors have no financial conflict of interest.

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