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

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**References**

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**Correction**

A correction has been published for this article. The contents of the correction have been appended to the original article in this reprint. The correction is also available online at: http://www.jimmunol.org/content/176/4/2670.1.full.html

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

Ashutosh Chaudhry, Suman Ranjan Das, Amjad Hussain, Satyajit Mayor, Anna George, Vineeta Bal, Shahid Jameel, and Satyajit Rath

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.

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 pIRE52-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, CTGCACAGATGTACCTTCTTCTCTC; reverse, CCGGAATTCTGACCTTTCTC. The PCR-amplified fragments were subsequently cloned into the plasmid pIRE52-eGFP or peGFP-N3 vectors (BD Clontech). The G2A mutant of F2-nef was made by PCR amplification using the primers: forward, CGGATATGCGTGGCAGATGTTGAGATC; reverse, CCGGAATTCTGACCTTTCTC.
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 HA-p-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 BamHI/HindIII fragment containing the c- myc-p-LCMVp-OVA-coding sequence described earlier (19) was cloned into the BglII/HindIII 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 coincubation with transfected APCs for 24 h. Azide-free anti-CD80 and anti-CD86 mAbs (eBioscience) were used for functional blockade at 10⁻⁶ cells/ml each. Anti-CD90 and anti-CD11b mAbs (eBioscience) were used for functional blockade at 10⁻⁶ µg/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 µg/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- and rM-CSF-containing cultures on day 7, and live cells were used for infection on day 9. Transfections were done using Fugene6 (Roche) or Effectene (Qiagen), according to the manufacturers’ protocols, with 12 µg of plasmid DNA for 2 × 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 antibodies-specific mAbs used were W6/32 for MHC I and OKT-9 for transferrin receptor (TIR) (used as culture supernatants), and biotinylated anti-CD80 and anti-CD86 (eBiosciences) flow cytometry. Data were analyzed using FlowJo software (Treestar).

**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 monocyte 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 MHC I (Fig. 1A). No significant change in the surface levels of the TIR could be detected (Fig. 1A).
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 MHCI, 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 p24 gag expression, while WT HIV-1 induced a loss of cell surface MHCI, 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 MHCI, 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 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 MHCI, 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 MHCI, CD80, and CD86 expression levels on primary human monocytes, either uninfected or infected with the HIV-1 strains indicated at 72 h after infection.

**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 MHC1 vs CD80 and CD86*

To further characterize Nef-mediated down-modulation of CD80/CD86 and to identify its possible differences from MHC1 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 MHC1, 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 MHC1 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 MHC1, 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 MHC1, CD80, or CD86 (Fig. 3C). However, a smaller C-terminal deletion from residue 125 onward (F2-Nef-Δ125) allowed partial down-modulation of MHC1 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 MHC1 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-INDEX.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 MHC1, 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 MHC1, 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 MHC1, 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 grafted 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-PPPAAA. C. Histograms are shown for surface levels of indicated molecules on grafted 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-Δ100), or F2-Nef-Δ125 (F2-Nef-Δ125). D. Histograms are shown for surface levels of indicated molecules on grafted eGFP+ U937 cells after transfection to express eGFP alone, or with F2-Nef or D1-Nef. All gray-shaded curves indicate isotype controls.
Table 1. Effects of Nef mutations on Nef-mediated down-modulation of cell surface MHCI, CD80, and CD86 in monocytic cell lines

<table>
<thead>
<tr>
<th>Mutants</th>
<th>U937 (Human Cells)</th>
<th>BMC-2 (Mouse Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHCI*</td>
<td>CD80*</td>
</tr>
<tr>
<td>NL-4-3-nef (WT)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NL-4-3-nef-G2A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NL-4-3-nef-WL57AA</td>
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<td>+</td>
</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>NL-4-3-nef-R77A</td>
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</tr>
<tr>
<td>NL-4-3-nef-PPPAAA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NL-4-3-nef-R86A</td>
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<td>+</td>
</tr>
<tr>
<td>NL-4-3-nef-R106A</td>
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<td>+</td>
</tr>
<tr>
<td>NL-4-3-nef-H109A</td>
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<td>+</td>
</tr>
<tr>
<td>F2-nef (WT)</td>
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</tr>
<tr>
<td>F2-nef-G2A</td>
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<td>-</td>
</tr>
<tr>
<td>F2-nef-M20A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F2-nef-F121G</td>
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<td>+</td>
</tr>
<tr>
<td>F2-nef-P130A</td>
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<tr>
<td>F2-nef-E155A</td>
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<td>+</td>
</tr>
<tr>
<td>F2-nef-L164A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F2-nef-D100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F2-nef-D125</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>D1-nef</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, Persistence of down-modulation ability; -, loss of down-modulation ability.

** WT, wild type.

a, (+), 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 MHCI (H-2Kb). 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 MHCI levels (Fig. 4E). As a control, the G2A-Nef mutant that is incapable of down-regulating MHCI, CD80, or CD86 did not affect this response (Fig. 4E). Similarly, stimulation of an OVA-specific MHCI-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 MHCI-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 nefΔ100 genes fused in-frame to the eGFP gene. Transfection with an eGFP-expressing vector was used as a control. All three proteins, MHCI, 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 MHCI (Fig. 5A). Three-color confocal analysis of
these cells showed that Nef, MHCI, 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 MHCI, 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 co-immunoprecipitated 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
with the truncated version of F2-Nef with residues from 100 onward deleted (F2-NefΔ100; Nef100). 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 MHC-I and MHC-II molecules are removed from the cell surface with a t1/2 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 MHC-I 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 on 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 MHC-I 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, cell surface MHC-I and MHC-II molecules to intracellular compartments (13, 24).

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 MHC-I and MHC-II 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 MHC-I and MHC-II molecules are removed from the cell surface with a t1/2 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 MHC-I 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 MHC-I 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 TFR, CD11b, CD54, or CD40. Furthermore, the total cellular levels of MHCII, 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 MHCII, 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 MHCII down-modulation alone is somewhat more permissive than in a region critical for down-modulation of MHCII as well as CD80 and CD86, suggesting the likely importance of CD80/86 down-modulation in HIV pathology.

All relocation effects of Nef on MHCII, 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 MHCII, CD80, and CD86 levels, residues beyond 125 are also needed for optimal function of the Nef protein. Mutations at R77, D86, R106, I109, or L164–165 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 API2/3, 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 MHCII down-regulation (24). In F2-Nef, which remains competent for MHCII relocation, the corresponding residues are EDEGE. However, their modification to DEDGE in D1-Nef prevents MHCII 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 MHCII vs CD80 or CD86. Specifically, the E4A (PACS-1 recruitment domain) and the PPPAEEA (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 MHCII, but efficiently reduce surface levels of CD80 and CD86, again emphasizing the separation between MHCII-directed vs CD80/CD86-directed effects of Nef. Although Nef appears to interact weakly and transiently with specific MHCII 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 MHCII-restricted and MHCII-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 MHCII 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

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References


