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Endogenously Expressed *nef* Uncouples Cytokine and Chemokine Production from Membrane Phenotypic Maturation in Dendritic Cells¹

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Immature dendritic cells (DCs), unlike mature DCs, require the viral determinant *nef* to drive immunodeficiency virus (SIV and HIV) replication in coculture with CD4⁺ T cells. Since immature DCs may capture and get infected by virus during mucosal transmission, we hypothesized that Nef associated with the virus or produced during early replication might modulate DCs to augment virus dissemination. Adenovirus vectors expressing *nef* were used to introduce *nef* into DCs in the absence of other immunodeficiency virus determinants to examine Nef-induced changes that might activate immature DCs to acquire properties of mature DCs and drive virus replication. *Nef* expression by immature human and macaque DCs triggered IL-6, IL-12, TNF- α , CXCL8, CCL3, and CCL4 release, but without up-regulating costimulatory and other molecules characteristic of mature DCs. Coincident with this, *nef*-expressing immature DCs stimulated stronger autologous CD4⁺ T cell responses. Both SIV and HIV *nef*-expressing DCs complemented defective SIVmac239 delta *nef*, driving replication in autologous immature DC-T cell cultures. In contrast, if DCs were activated after capturing delta *nef*, virus growth was not exacerbated. This highlights one way in which *nef*-defective virus-bearing immature DCs that mature while migrating to draining lymph nodes could induce stronger immune responses in the absence of overwhelming productive infection (unlike *nef*-containing wild-type virus). Therefore, Nef expressed in immature DCs signals a distinct activation program that promotes virus replication and T cell recruitment but without complete DC maturation, thereby lessening the likelihood that wild-type virus-infected immature DCs would activate virus-specific immunity, but facilitating virus dissemination. *The Journal of Immunology*, 2002, 169: 4172–4182.

As the most potent APCs, dendritic cells (DCs)⁵ are central to the immune responses against incoming pathogens (1). In the steady state DCs in the periphery (like those at the body surfaces) are in an immature state and mature upon migration to the draining lymphoid tissues, where they present Ag to naive T cells and initiate Ag-specific immune responses (1). A pathogen must induce DC maturation for effective

adaptive immunity to be activated against the pathogen (reviewed in Ref. 2). Maturation can be induced by two major classes of stimuli; one group signaling through the toll-like receptors (TLRs) and the second via members of the TNF/TNFR family (reviewed in Ref. 2). Changes that occur during DC maturation include up-regulation of CD83, CD25, HLA-DR, and costimulatory molecules such as CD86 and CD80 (1), modulation of chemokine receptor expression (3), secretion of soluble factors such as IL-12 (4, 5) and chemokines (6), and the expression of DC stimulatory and survival molecules such as CD40 and TRANCE-R (7, 8).

In the context of immunodeficiency viruses, however, DCs effectively promote virus amplification and spread especially upon encountering CD4⁺ T cells (reviewed in Ref. 9). Furthermore, immature DCs may be one of the first leukocytes entrapping and possibly replicating immunodeficiency virus crossing a mucosal surface (10, 11) and then (most notably as a more mature cell) transmitting infection to nearby CD4⁺ T cells for amplification (12–14). Thus, a paradox exists between the ability of DCs to present virus for activation of anti-viral immunity vs their ability to exacerbate virus growth. Understanding what drives virus replication in the DC-T cell milieu is critical to identify ways to limit this and favor immune activation.

Nef proteins of SIV and HIV are critical for viral pathogenesis and contain motifs that have been implicated in modulating cellular signaling as well as the trafficking of molecules between the outside and the inside of the cells in ways to facilitate virus dissemination (reviewed in Ref. 15–17). However, the exact mechanism of enhanced virus replication mediated through *nef* is not understood. *Nef* has been shown to exert both positive (15–18) and negative (19–21) effects on T cell activation that might contribute

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⁵ Abbreviations used in this paper: DC, dendritic cell; Adeno, adenovirus; CD40L, CD40 ligand; *gfp*, green fluorescent protein; MCM, monocyte-conditioned medium; MIP, macrophage inflammatory protein; SEB, staphylococcal enterotoxin B; TLR, Toll-like receptor; SIGN, specific ICAM-3-grabbing nonintegrin.

to virus spread. In addition, soluble factors secreted from *nef*-expressing macrophages are sufficient to recruit and activate resting T cells, rendering them permissive to HIV infection (22). Recent studies have provided the first insight into how *nef* may also modify immature DCs to promote virus spread rather than Ag presentation. One report suggests that *nef* modulates DC-specific, ICAM-3-grabbing nonintegrin (DC-SIGN, CD209) expression on the DC surface to favor DC-T cell interactions and subsequent virus spread (23). Other work revealed that *nef* (introduced via a vaccinia recombinant vector) down-modulated class I MHC expression in immature DCs (24), reducing their ability to stimulate class I MHC-restricted CD8⁺ T cell responses. However, this was not supported by a separate study using an adenoviral vector to introduce *nef* (25). Exogenously added recombinant Nef was also shown to activate immature DCs while decreasing class I MHC (26).

SIV (27) and HIV (28) replication in immature DC-T cell co-cultures is dependent on the presence of *nef*. Yet mature DCs readily promote SIV delta *nef* (delta *nef*) growth (27), suggesting that when a mature DC encounters the virus it somehow overrides the need for *nef*. To investigate more closely how *nef* in a wild-type virus could influence immature DC functions to foster virus dissemination, we introduced *nef* into immature DCs using recombinant adenovirus (Adeno). Several groups, including our own, found that Adeno infection had little if any impact on DC membrane phenotype (Fig. 2) (25, 29–35), making it a reliable way to introduce genes of interest. Expression of *nef* in an immature DC triggers the DC to secrete inflammatory cytokines and chemokines much like mature DCs, yet in the absence of the membrane phenotypic changes that are typical of maturation. Furthermore, *nef*-expressing DCs exhibited increased ability to activate autologous CD4⁺ T cells and were able to enhance replication of delta *nef* in immature macaque DC-T cell cultures. However, maturation of the DCs within the virus-loaded cultures was not sufficient to rescue delta *nef* replication. HIV *nef* and SIV *nef* were interchangeable between human and macaque DCs in their ability to trigger these functions. These data suggest that *nef* induces selective or different pathways of the DC maturation signaling network, creating an environment encouraging T cell recruitment and generalized activation of T cells by phenotypically immature DCs. This milieu readily amplifies virus replication, but would less efficiently induce Ag-specific T cell responses, accenting how wild-type virus growth may be driven in the absence of an effective anti-viral immune response.

Materials and Methods

Culture medium

RPMI 1640 (Cellgro; Fisher Scientific, Springfield, NJ) was supplemented with 2 mM L-glutamine (Life Technologies, Grand Island, NY), 50 μ M 2-ME (Sigma-Aldrich, St. Louis, MO), 10 mM HEPES (Life Technologies), penicillin (100 U/ml)-streptomycin (100 μ g/ml; Life Technologies), and 1% human plasma (heparinized).

Animals

Adult macaques (*Macaca mulatta*) were housed in the Tulane Regional Primate Research Center. Animal care operations were in compliance with the regulations detailed under the animal welfare act, and in the Guide for the Care and Use of Laboratory Animals. Before use, all animals used in this study tested negative for Abs to SIV, type D retroviruses, and simian T cell leukemia virus type 1. Male and female adult macaques were used for this study.

SIV isolates

The cloned viruses SIVmac239 (wild type) and SIVmac239 delta *nef* (delta *nef*) (36) were grown as previously described (37).

Generation of DCs

DCs were generated from PBMCs isolated from healthy macaques or HIV seronegative human donors. Donors were not screened for anti-Adeno immunity. Buffy coat units were purchased from the New York Blood Center to generate human DCs. Peripheral blood was collected by standard venipuncture using heparinized Vacutainers (BD Bioscience, San Jose, CA) from healthy SIV-seronegative rhesus macaques (anesthetized with 10 mg/kg ketamine HCl). The mononuclear cell fraction was isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient (38). DCs were generated from either adherent PBMCs as previously described (27) or CD14⁺ monocytes isolated using the anti-human CD14 MACS system (Miltenyi Biotech, Auburn, CA) (6) and were plated at 3×10^6 cells/well in a six-well tray (3 ml/well) in the presence of 100 U/ml recombinant human IL-4 (R&D Systems, Minneapolis, MN) and 1000 U/ml recombinant human GM-CSF (ImmuneX, Seattle, WA). Cytokines were added to the cultures on days 0, 2, 4, and 6 in culture. After 6–7 days in culture, immature DCs were harvested for infection.

Analysis of DC phenotype

The DC phenotype was monitored by flow cytometry for each experiment. At least 1×10^4 DCs were resuspended in PBS/5% FCS/0.1% sodium azide (staining buffer; 100 μ l/well of a V-bottom 96-well tray (ICN Bio-medicals, Aurora, OH)). The cultures were stained with the appropriate PE-conjugated IgGs (BD Biosciences; Ancell, Bayport, MN; R&D Systems) or with PE-labeled mAbs against HLA-DR, CD25, CD58, CD80, (BDIS), CD4, CD40, CD86, CD206, CXCR4, CCR5 (BD Pharmingen, San Diego, CA), CD83 (Immunotech, Marseilles, France), HLA-ABC (DAKO, Carpinteria, CA), CD74 (Ancell), CD209 (used for both human and macaque cells; R&D Systems), or CCR5 (used for macaque cells; R&D Systems). Indirect staining with goat F(ab')₂ anti-mouse Ig (BioSource, Camarillo, CA) was used to detect CD205 expression using the mouse anti-human CD205 clone 38-2 (39), CD209 staining using either mAb 507 (provided by Dr. V. KewalRamani) (40) for macaque cells or DC28 for human cells (AIDS Research and Reference Reagent Program, National Institutes of Health, Bethesda, MD). The necessary unconjugated IgGs were included as negative controls for the indirect staining. The cells were incubated with the Abs for at least 20 min at 4°C, then were washed four times with staining buffer and fixed in 10% formalin in PBS (pH 7.2–7.4). For the indirect stains, the cells were washed after the primary Ab staining, incubated with the secondary goat-anti-mouse Ig, and washed again before fixation. Stained cells were examined by flow cytometry using a FACScan (BD Biosciences), and the data were analyzed with CellQuest software (BD Biosciences).

Isolation and phenotyping of CD4⁺ T cells

The CD14-negative fractions of the macaque and human PBMCs were cultured at $0.5\text{--}1 \times 10^7$ /ml for 6–7 days with the DCs, with additional fresh medium being added to the cultures every 2 days. On the day of the assay, the CD4⁺ T cells were further purified by negative selection using anti-CD8 and anti-HLA-DR MACS beads (Miltenyi Biotech) (6). The resulting cell preparations were at least 99% viable by trypan blue dye exclusion. Comparative infection studies (using macaque cells that are usually more fragile than human cells) confirmed that such cultured T cells were functional and behaved just like T cells freshly isolated from PBMCs on the day of the experiment, avoiding the need to re-bleed the donors (data not shown). The purity of the CD3⁺CD4⁺ T cells was verified as >95% by direct staining flow cytometry for membrane expression of CD8 and HLA-DR (vs the simlest isotype control). The activation state of the isolated CD4⁺ T cells was also monitored each time before performing the experiments by direct staining with FITC-conjugated anti-CD3 (BD Pharmingen) combined with PE-conjugated anti-CD69, -CD25, or -HLA-DR (BD Biosciences, Fig. 5F).

Adeno isolates and infection of DCs

Adenovirus expressing the HIV-1 SF2 *nef* allele (Adeno-*nef*/HIV), a mutant in the PxxP region of SF-2 *nef* (Adeno-*nef*/HIV_{PA}), or Adeno expressing the green fluorescent protein (Adeno-*gfp*) were previously described by Swingle et al. (22). Adeno expressing *gfp* and the HIV-1 SF2 *nef* allele was constructed using the Adeno-quest kit from Quantum Biotech (Qbiogene, Carlsbad, CA) and their pQBI-AdCMV5GFP shuttle vector. This virus was used for the extensive FACS analyses, allowing us to gate on *gfp*⁺ cells in both the control and *nef*-loaded populations. Recombinant Adeno encoding the SIVmac239 *nef* allele (Adeno-*nef*/SIV) was derived from Adeno type 5, deleted of E1 and E3 regions to generate a replication-deficient Adeno (29), and purchased from Quantum Biotech (Durham, NC). For Adeno infection, 10^5 immature DCs were infected in 50 μ l medium with 100–1000 PFU of

virus in a 96-well, round-bottom tray (ICN Biomedicals) for 2 h at 37°C. After 2 h fresh medium containing 1% human plasma was added. For T cell activation experiments 10^2 – 10^4 immature DCs were infected in 50 μ l medium, and after 2 h 10^5 autologous T cells were added to the DCs. Adeno-infected DCs or DCs mixed with T cells were cultured for various lengths of time before the indicated parameters were measured. Adeno stocks were monitored for endotoxin contamination using the Single Test *Limulus* Amebocyte Lysate Assay (N289-06; BioWhittaker, Walkersville, MD). Endotoxin levels in the Adeno preparations were routinely <0.06 endotoxin units/ml.

Immunoperoxidase staining of cells for Nef

Cytospins of DCs were prepared using a cytocentrifuge (Shandon, Pittsburgh, PA), loading $\sim 2 \times 10^4$ cells/slide (41). Slides were air-dried for 1 h at room temperature and subsequently fixed in absolute acetone for 10 min at room temperature. The slides were incubated with a mouse mAb recognizing SIVmac251 Nef (17.2, AIDS Research and Reference Reagent Program) for 30 min at room temperature, washed five times with staining buffer, and incubated for 30 min with HRP-conjugated donkey anti-mouse Ig (DAM-HRP; 1/300 dilution; Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. Non bound HRP-conjugated donkey anti-mouse Ig was washed off, and bound HRP was detected with stable 3,3'-diaminobenzidine tetrahydrochloride dihydrate (Research Genetics, Huntsville, AL). The slides were mounted in PBS/glycerol and analyzed using an Olympus AX70 microscope (Melville, NY).

Determination of T cell activation

To assess levels of T cell activation and proliferation, 10^2 – 10^4 immature DCs (infected or not with Adeno) were mixed with 1×10^5 CD4⁺ T cells and cultured in a round-bottom, 96-well tray for up to 6 days. Tritiated thymidine ($[^3\text{H}]\text{TdR}$; 1 μCi /well; NEN, Boston, MA) was added to the cells for the final 8 h of culture, and the $[^3\text{H}]\text{TdR}$ incorporated by proliferating cells was measured using a Wallac 1205 Betaplate liquid scintillation counter (Gaithersburg, MD). Responses are reported as mean counts per minute of $[^3\text{H}]\text{TdR}$ incorporated by duplicate or triplicate cultures (\pm SEM). T cell activation was also monitored by flow cytometry, where additional wells containing replicate DC-T cell mixtures were collected after 3 days and stained for T cell activation markers (PE-CD25, CD69, or HLA-DR) in combination with CD3 (FITC). The T cells were analyzed by gating on small and large activated lymphocytes by forward scatter excluding the DCs and DC-T cell conjugates. The gate marker was set relative to the isotype controls for each condition, and the percentage of cells stained above this cut-off point is given. Expression of the nuclear activation Ag Ki-67 was also monitored as a measure of T cell activation after 1–3 days of DC-T cell coculture by immunoperoxidase staining of acetone-fixed cells (above) using the anti-Ki-67 mAb (MIB-1, AMAC, Westbrook, ME) (41). The percentage of Ki-67⁺ cells was calculated by counting the numbers of Ki-67⁺ cells in five independent fields (averaging 100 total cells in each) and is expressed as mean \pm SEM over the five fields.

Measurement of chemokines and cytokines

The supernatants of Adeno-infected DCs (vs mock controls) were collected, by taking 50 μ l from the 200- μ l cultures and replacing it with 50 μ l fresh medium at the indicated time points. Aliquots were stored at -20°C before analysis of the chemokine and cytokine content by ELISA. Recombinant anti-human CCL3 (macrophage inflammatory protein-1 α) (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), CXCL8 (IL-8), IL-10, and IL-6 ELISAs were obtained from R&D Systems. CCL3, CCL4, CCL5, CXCL8, and IL-6 ELISAs cross-reacted with macaque chemokines and cytokines. Anti-human IL-12 ELISA was purchased from Endogen (Woburn, MA) and cross-reacted with macaque IL-12. Recombinant anti-mouse ELISAs for TNF- α , IL-10, and IFN- γ were purchased from U-CyTech (Utrecht, The Netherlands).

In vitro SIV infection of DC-T cell cultures

After infection of immature DCs with Adeno as described above, 10^5 T cells were added to 10^4 DCs (per well) in a 96-well, round-bottom tray. The cocultures were subsequently pulsed with 5×10^3 50% T cell infectious dose of SIVmac239 wild type or delta *nef*/10⁵ cells for 1.5 h at 37°C as previously described (27). Every 2 days of a 15-day coculture, 50- μ l aliquots were collected and stored at -20°C before the p27 content was analyzed by ELISA (Zeptometrix, Buffalo, NY). To determine whether the addition of maturation stimuli would rescue delta *nef* replication in immature DC-T cell cultures, the cultures were infected (27), after which the indicated maturation stimuli were added. These included 50% monocyte-conditioned medium (MCM) (42), soluble CD40 ligand (CD40L; 1/100 of

a baculovirus stock) (6), or a combination of PGE₂ (10^{-7} M, PGE₂; Sigma P6532) with TNF- α (50 ng/ml; R&D Systems). Two or 3 days postinfection, DC maturation in the infected cultures was verified by FACS, measuring the expression of CD25, CD86, CD83, and HLA-DR. In some experiments 5 ng/ml staphylococcal enterotoxin B (SEB) or allogeneic CD4⁺ T cells were added to provide an activated T cell environment.

Statistical analysis

The statistical significance of the *nef*-induced chemokine and cytokine production was analyzed by comparing the peak values of chemokine or cytokine production in Adeno-*nef*- vs Adeno-*gfp*-infected DCs. The nonparametric SIGN test was used to directly compare the two groups. There were no statistically significant differences between the mock and Adeno-*gfp* controls, and *p* values (to three decimal places) are shown for the Adeno-*gfp* vs Adeno-*nef* cultures. A value of *p* < 0.05 was considered statistically significant.

Results

Nef does not induce changes in the membrane phenotype of immature DCs

To measure *nef*-induced changes in immature DCs, Adeno vectors were used to express *nef* in the DCs (Adeno-*nef*). Infection of immature DCs with Adeno expressing the green fluorescent protein (*gfp*; Adeno-*gfp*) or Adeno expressing *gfp* and the HIV-1 SF2 *nef* allele (Adeno-*gfp-nefHIV*) enabled measurement of infection efficiency by FACS by monitoring the percentage of *gfp*-positive DCs (Fig. 1A). The infection efficiency in different donors ranged from 5–90% (>10 experiments), averaging 40% for human DCs and 53% for macaque DCs. Similarly, the levels of *gfp* expression varied between donors and the various Adeno preparations, with the Adeno-*gfp* infections sometimes appearing more efficient than the Adeno-*gfp-nefHIV* infections, as shown in Fig. 1A. However, this did not alter the *nef* effects observed (below). Both the levels of expression and the numbers of expressing cells typically peaked around 48 h postinfection with either Adeno-*gfp-nefHIV* or Adeno-*gfp*, and therefore *gfp* expression was routinely measured at this time. The levels of Nef expression (immunoperoxidase staining; Fig. 1B) were variable between cells, with some Nef-positive

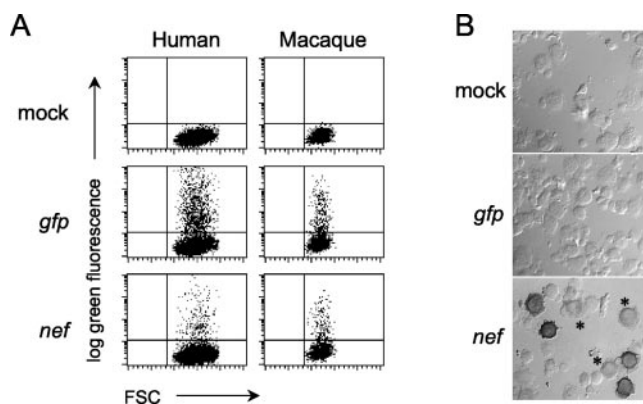


FIGURE 1. Expression of *gfp* and *nef* in immature DCs infected with recombinant Adeno. **A**, FACS analysis of immature human or macaque DCs infected with Adeno-*gfp-nefHIV* (*nef*) or Adeno-*gfp* (*gfp*) or uninfected (mock) monitored 48 h postinfection (peak infection). The dot plots showing forward size scatter (FSC; x-axes) vs log green fluorescence intensity (*gfp*; y-axes) were gated on large cells and indicate the *gfp*-positive Adeno-infected DCs. **B**, Macaque DCs were spun onto glass slides 48 h after mock infection or infection with Adeno-*nefSIV* (*nef*) or Adeno-*gfp* (*gfp*). The cells were immunoperoxidase stained (dark reaction product) for the expression of SIV Nef. Faintly stained Nef-positive cells readily visible above background staining of Adeno-*gfp*-infected or uninfected cells and the isotype controls (not shown) are indicated by asterisks. The original magnification was $\times 40$. Typical results from one of more than five experiments are provided.

cells staining very strongly for the Nef protein (dark reaction product) and others quite faintly, but above background (asterisks). The amount of Nef-expressing DCs and that of *gfp*-expressing DCs correlated closely. Nef expression in Adeno-*nef*HIV-infected DCs was also confirmed by Western blot (data not shown).

To test our hypothesis that *nef* creates a mature DC we first analyzed whether the expression of *nef* in DCs alters the DC membrane phenotype. Our initial studies (>15 experiments) revealed that neither Adeno infection (35) nor the presence of *nef* (Fig. 2) up-regulated the expression of common maturation markers (HLA-DR, CD25, CD83, CD86). The same was true when SIV-mac239 *nef* was tested in both human and macaque DCs (data not shown), but more detailed analyses have been performed with the HIV construct that we have available in house. Advances in the DC field highlight many molecules that are expressed by DCs at different stages of activation, some of which may be important in HIV/SIV replication and/or DC-T cell communication (reviewed in Ref. 9). Thus, a wide panel of markers was monitored to more extensively assess the effects of *nef* on immature DC membrane phenotype (Fig. 2). Uninfected controls run in parallel (not shown) with those cells in Fig. 2 confirmed that Adeno infection has no impact on the expression of these markers by DCs (35). Notably,

the DC phenotype was identical in the Adeno-*gfp*- and Adeno-*gfp-nef*-infected DC cultures (Fig. 2). Despite some variability between both human and macaque donors (Fig. 2B), there was no consistent up- or down-modulation of any markers in response to *nef*, even when the *gfp*-positive fractions (Adeno-infected cells) were directly compared (Fig. 2). Importantly there were no changes in the expression of known HIV-binding molecules such as CD4, CCR5, CXCR4, CD206 (macrophage mannose receptor), or CD209 (DC-SIGN) in response to *nef*.

Nef induces chemokine and inflammatory cytokine production in immature DCs

Another characteristic feature of DC maturation is the secretion of chemokines and inflammatory cytokines (6, 43, 44). It has been shown that *nef* triggers macrophages to secrete CCL3 (MIP-1 α) and CCL4 (MIP-1 β) (22) and that exogenous recombinant Nef induces human DCs to secrete several cytokines and chemokines (26). We specifically investigated whether *nef* expressed in an immature DC could induce changes in chemokine or cytokine expression. Human and macaque DCs were analyzed for HIV *nef*-induced production of chemokines (Figs. 3 and 4). *Nef* expression in DCs induced the secretion of β -chemokines CCL3, CCL4, and

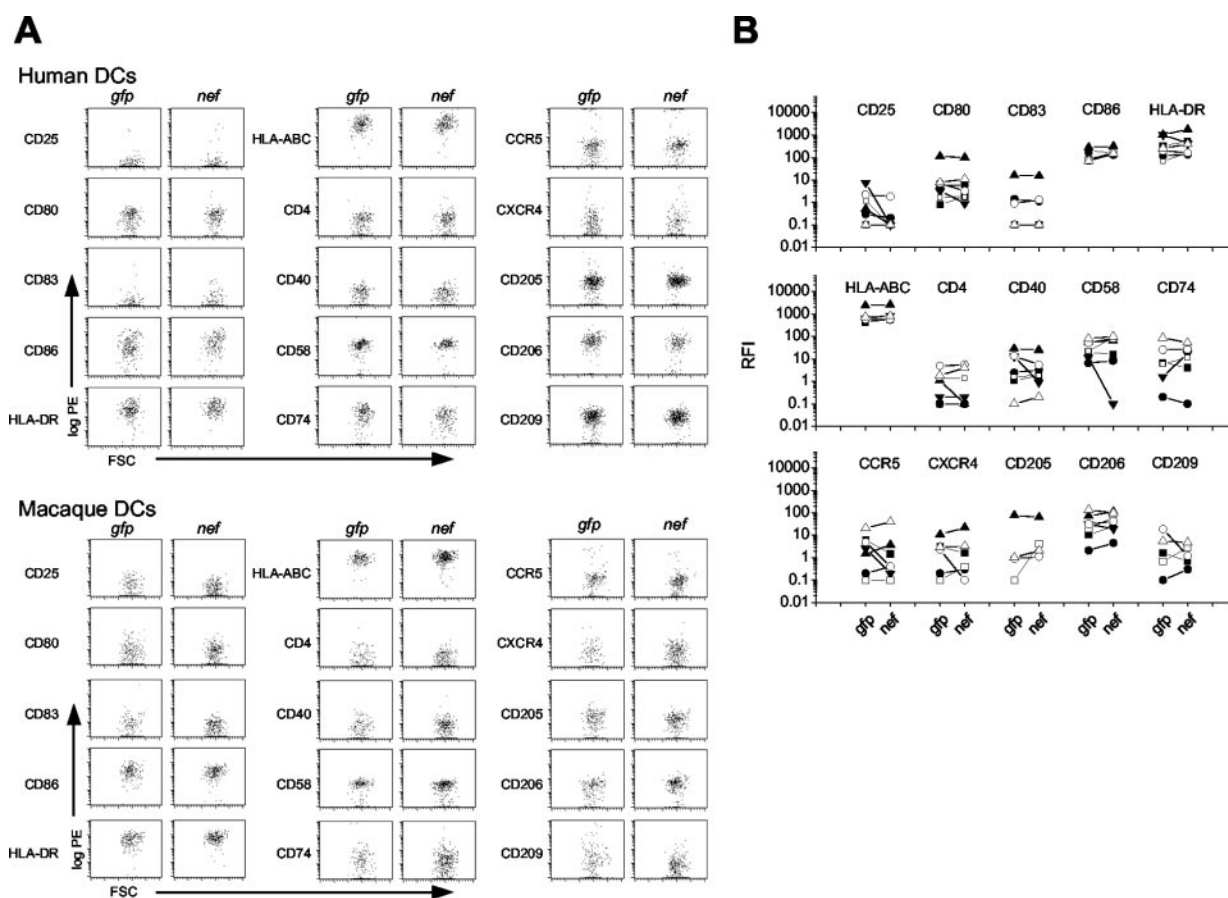


FIGURE 2. Phenotypic analysis of Adeno-infected immature human and macaque DCs. Human and macaque immature DCs were infected with Adeno-*gfp-nef*HIV (*nef*) or Adeno-*gfp* (*gfp*). Forty-eight hours postinfection the DCs were analyzed for expression of the indicated markers by staining with PE-conjugated mAbs or PE-conjugated goat anti-mouse Ig to detect unlabeled primary Abs. Indirect stains for CD205 (human and monkey, mAb 38-2) and CD209 (human, mAb DC28) are shown. Macaque DCs shown were stained with PE-labeled mAb 12507 (anti-CD209), but gave the same results with indirect staining. A, PE staining (log PE) of large *gfp*-positive cells (forward scatter (FSC) vs log PE) data are shown from one representative experiment of at least three similar experiments (different donors) for both monkey and human DCs. Isotype controls for direct stains exhibited mean fluorescence intensities of <1 log and indirect stains 1–1.5 logs. B, The PE relative fluorescence intensity (RFI) of the *gfp*-gated cells was calculated for each donor. The RFI for each phenotypic marker was determined by subtracting the mean fluorescence intensity of the appropriate isotype control. Symbols represent the same donor in each graph. ■, ▲, and ●, Three or four individual macaques; □, △, and ○, three human donors. Staining equivalent to the isotype control was assigned a value of 0.1 to accommodate log-scale analysis.

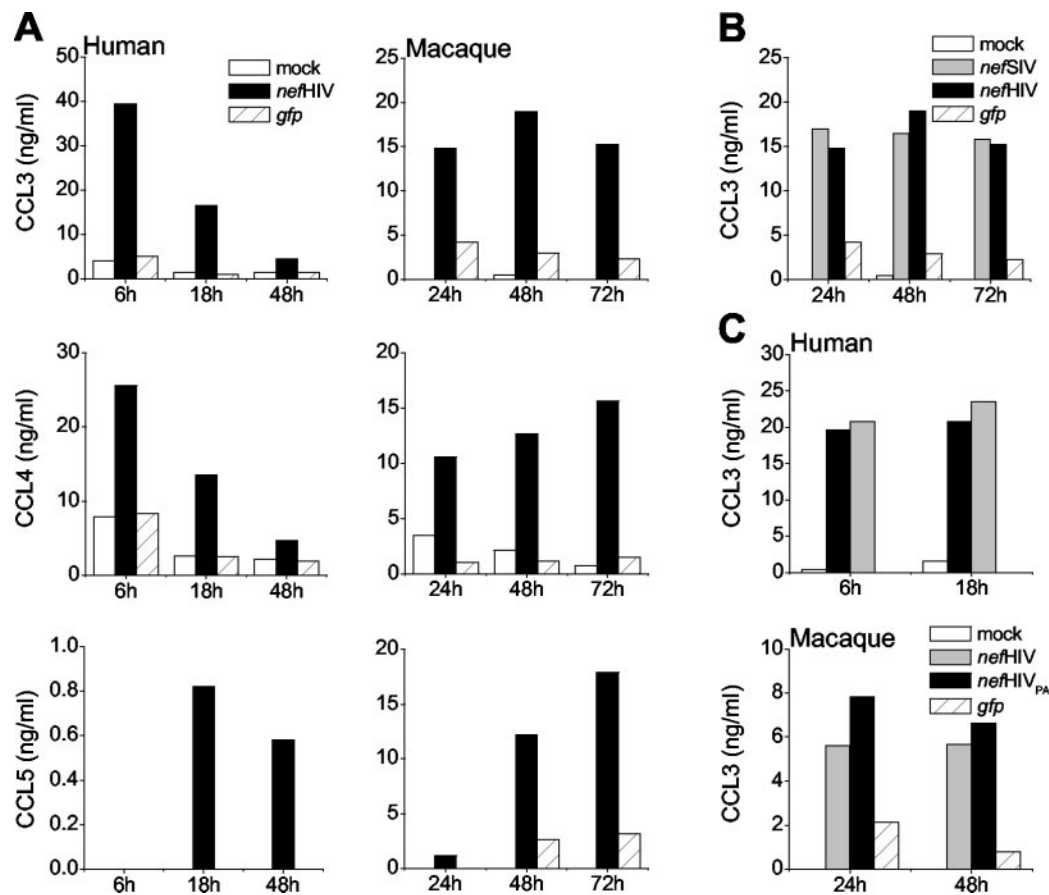


FIGURE 3. SIV and HIV *nef*-induced chemokine production in human and monkey DCs. **A**, Immature human and macaque DC were left uninfected (mock) or were infected with Adeno-*nefHIV* (*nefHIV*) or Adeno-*gfp* (*gfp*). The supernatants were collected at the indicated time points after infection and assayed for CCL3, CCL4, and CCL5. The results represent one of >10 experiments. **B**, Immature macaque DCs were left uninfected (mock) or were infected with Adeno-SIV*nef* (*nefSIV*), Adeno-HIV*nef* (*nefHIV*), and Adeno-*gfp* (*gfp*), and supernatants taken at 24, 48, and 72 h were assayed for CCL3 production. **C**, Supernatants from human and macaque DCs uninfected (mock) or infected with Adeno-*nefHIV* (*nefHIV*), Adeno-*nefHIV_{PA}* (*nefHIV_{PA}*), and Adeno-*gfp* (*gfp*) were sampled at the indicated times, and CCL3 was detected by ELISA. The results shown in **B** and **C** are representative of at least three independent experiments.

CCL5 (RANTES). The secretion of CCL3 and CCL4 in human immature DCs peaked at around 6–18 h. CCL5 secretion tended to peak later (18–48 h) and at consistently lower levels. When immature macaque DCs were infected with Adeno-*nefHIV*, chemokine production was induced, but was delayed compared with that of human DCs, with the peak production occurring after 48–72 h (Fig. 3A). The peak chemokine levels from independent experiments using DCs from 6–12 different human (upper row) or macaque (lower row) donors are summarized in Fig. 4A. The *p* values indicate the significance of the *nef*-induced secretion in each case (Adeno-*nef* vs Adeno-*gfp* populations). While *nef*-dependent CCL3 and CCL4 secretion was statistically significant, CCL5 production was not; it was generally secreted at lower levels and less reproducibly between donors. CCL3 secretion was induced by both HIV and SIV *nef* in macaque (Fig. 3B) and human DCs (data not shown). The kinetics of CCL4 production by HIV or SIV *nef*-bearing DCs were similar to those of CCL3 (data not shown).

Two biochemical interactions of Nef have been described that putatively link Nef to signal transduction pathways: SH3 domains of protein tyrosine kinases of the Src family involving the conserved proline-rich (PxxP)₄ repeat in Nef (45–47), and a serine kinase (48) implicated in the effect of Nef on signal transduction in T cells. Both interactions have been mapped to the highly conserved core of the Nef molecule (49). Human and monkey DCs were infected with Adeno-*nefHIV*, Adeno-*nefHIV_{PA}* (PxxP mu-

tant), and Adeno-*gfp*. Just like wild-type *nef*, the PxxP mutant of *nef* induced CCL3 (Fig. 3C), CCL4, and CCL5 (data not shown) production in immature DCs. Therefore, the proline-rich motif is not required for *nef*-induced chemokine production by DCs.

We also observed that *nef* triggered statistically significant secretion of IL-12, TNF- α , CXCL8 (IL-8), and IL-6 in immature human (Fig. 4) and macaque (Fig. 4A and data not shown) DCs. On the average, cytokine release by macaque DCs was lower than that produced by human DCs (e.g., IL-12 (Fig. 4A) and TNF- α (data not shown)) and in the case of TNF- α did not reach statistical significance. There was almost no background cytokine secretion in uninfected or Adeno-*gfp*-infected immature DCs of TNF- α , IL-6, or IL-12. In contrast, high levels of CXCL8 secretion were observed in uninfected immature DCs, as previously described (6). However, the *nef*-induced CXCL8 production was statistically significant. No *nef*-induced IL-1 β , IL-10, or IFN- γ production was detected (data not shown).

Adeno-nef-infected DCs activate autologous T cells in coculture

The secretion of inflammatory cytokines and chemokines by *nef*-expressing DCs (Figs. 3 and 4) is a characteristic feature of DC maturation, yet the membrane phenotype of *nef*-expressing DCs remains that of an immature DC (Fig. 2). Since mature DCs are more potent in T cell stimulation than immature DCs, and this usually coincides with elevated costimulatory molecule expression

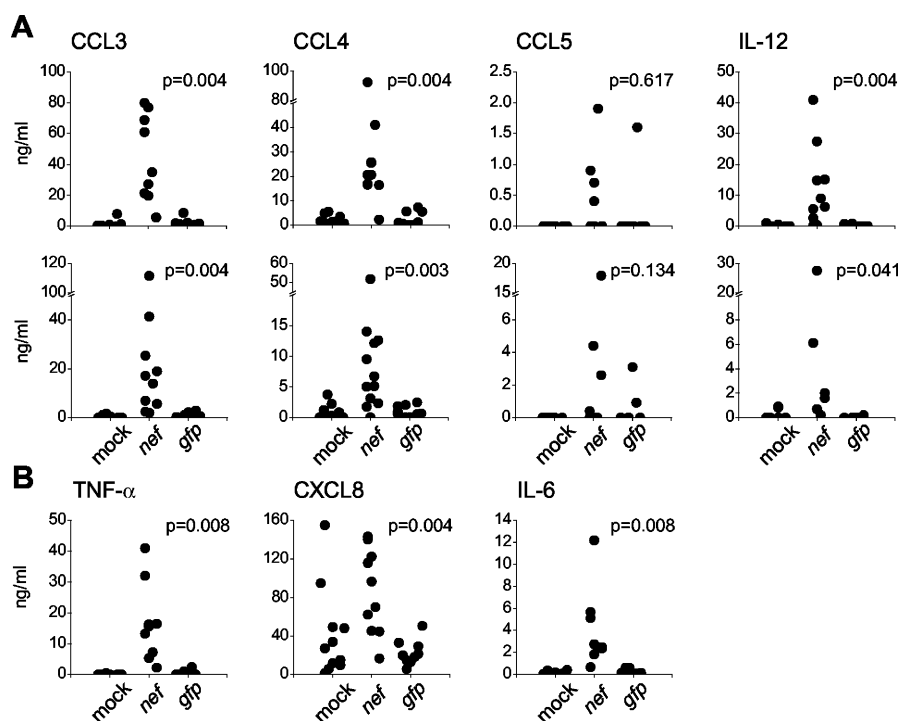


FIGURE 4. Peak cytokine and chemokine production induced by *nef* in immature DCs. Data represent the concentrations of chemokines and cytokines at the peak of production for 10^5 DCs. Values are plotted for 6–12 independent experiments, where each dot represents the results from an individual donor. **A**, Immature Human (upper row) or macaque (lower row) DCs were mock-infected or infected with Adeno-*nef*HIV (*nef*) or Adeno-*gfp* (*gfp*). The supernatants were analyzed for CCL3, CCL4, CCL5, and IL-12 levels by ELISA. **B**, Supernatants from immature human DCs after mock infection or infection with Adeno-*nef*HIV or Adeno-*gfp* were analyzed for TNF- α , CXCL8, and IL-6 production by ELISA. The *p* values shown in each graph indicate the significance of the difference between *nef*- and *gfp*-expressing DCs cultures.

(1), we were interested to ascertain what impact *nef* had (if any) on the ability of immature DCs to activate autologous CD4⁺ T cells. Cocultures of *nef*-expressing DCs (vs control DCs) and autologous T cells were analyzed for T cell activation by several methods. T cell proliferation was monitored by [³H]TdR incorporation (Fig. 5, A–D), expression of the cell cycle nuclear Ag Ki-67 (Fig. 5E), and up-regulation of activation markers by FACS (Fig. 5F). The negligible expression of CD69 and CD25 by the purified T cells before culture with *nef*-bearing vs control DCs is evident in Fig. 5F (top row).

Infection of immature DCs with either Adeno-*nef*HIV or Adeno-*nef*SIV provoked CD4⁺ T cell proliferation in human and macaque autologous DC-T cell cultures (Fig. 5, A and C). In the human system T cells cultured with Adeno-*nef*SIV-infected DCs showed lower [³H]TdR uptake compared with Adeno-*nef*HIV-infected DCs. This was not apparent, however, in the macaque system. These levels of increased autologous T cell proliferation stimulated by the *nef*-expressing immature DCs reflected responses induced by mature DCs, but were significantly lower than responses seen in allogeneic systems (Fig. 5B). The increased autologous T cell proliferation observed in the presence of *nef*-expressing DCs was dependent on the numbers of DCs added to the cocultures (Fig. 5C). Differences in T cell activation were still evident at a dose of 1 DC/30 T cells, but not at the 1/100 ratio. The background autologous MLRs induced by the mock and *gfp*-loaded DCs similarly decreased with lower DC numbers. Further evidence that DCs are needed to augment T cell activation was provided by the fact that addition of *nef*-triggered DC supernatants to purified CD4⁺ T cells resulted in minimal T cell proliferation (<500 cpm [³H]TdR uptake compared with 100–200 cpm for the untreated, mock, or *gfp*-DC supernatant controls vs the stronger responses seen in PMA/ionomycin-positive controls; Fig. 5D). Thus, soluble factors released by *nef*-triggered DCs alone are not sufficient to drive the T cell proliferation seen in the *nef*-DC-T cell cultures.

Expression of the nuclear activation Ag Ki-67 was also increased in *nef*-bearing DC-T cell cocultures (Fig. 5E). In addition,

FACS analysis revealed an increase in the expression of the activation markers CD69 and CD25 by T cells cocultured with *nef*-bearing autologous DCs (Fig. 5F). The percentage of CD69⁺ T cells went from 26–29%, and that of CD25⁺ T cells from 14–18% in the mock or *gfp*-infected DC-T cell cultures to ~45 and 26%, respectively, in the *nef*-loaded DC-T cell mixtures (Fig. 5F). Peak T cell activation levels are shown (Fig. 5). Hence, paralleling the elevated secretion of chemokines and cytokines induced by *nef*, *nef*-loaded immature DCs also stimulate greater autologous MLRs.

Nef, but not activation with maturation stimuli, drives delta nef replication in immature DC-T cell cultures

Mature, but not immature, DCs facilitate delta *nef* amplification in concert with autologous CD4⁺ T cells, whereas wild-type virus replicates strongly in the presence of either DC subset (27). We have hypothesized that *nef* in the wild-type virus can modulate immature DCs to act more like their mature counterparts to drive virus growth. While we detected *nef*-induced secretion of soluble factors by immature DCs as well as a *nef*-dependent augmentation of their ability to stimulate autologous MLRs, this did not coincide with changes in DC membrane phenotype. Thus, we set out to delineate whether activating DCs via traditional maturation stimuli vs *nef* would promote delta *nef* replication in the immature DC-T cell milieu.

Immature macaque DC-T cell cocultures were pulsed with delta *nef* (27), and the maturation stimulus, MCM, was added after the infection to mature the DCs in the culture. After 2–3 days the DCs had matured (FACS confirmed up-regulated CD25, CD80, CD83, and CD86; data not shown); however, replication of delta *nef* was consistently lower than that of wild-type virus (Fig. 6A). Since MCM contains chemokines (50) that may interfere with the spread of infection in the DC-T cell mixtures, other maturation stimuli that are known to activate macaque DCs (PGE₂/TNF- α or CD40L) (6) were examined. Just as with MCM-activated DCs, maturation of the DC with either PGE₂/TNF- α or CD40L (confirmed by FACS analysis after 2–3 days; data not shown) did not rescue the

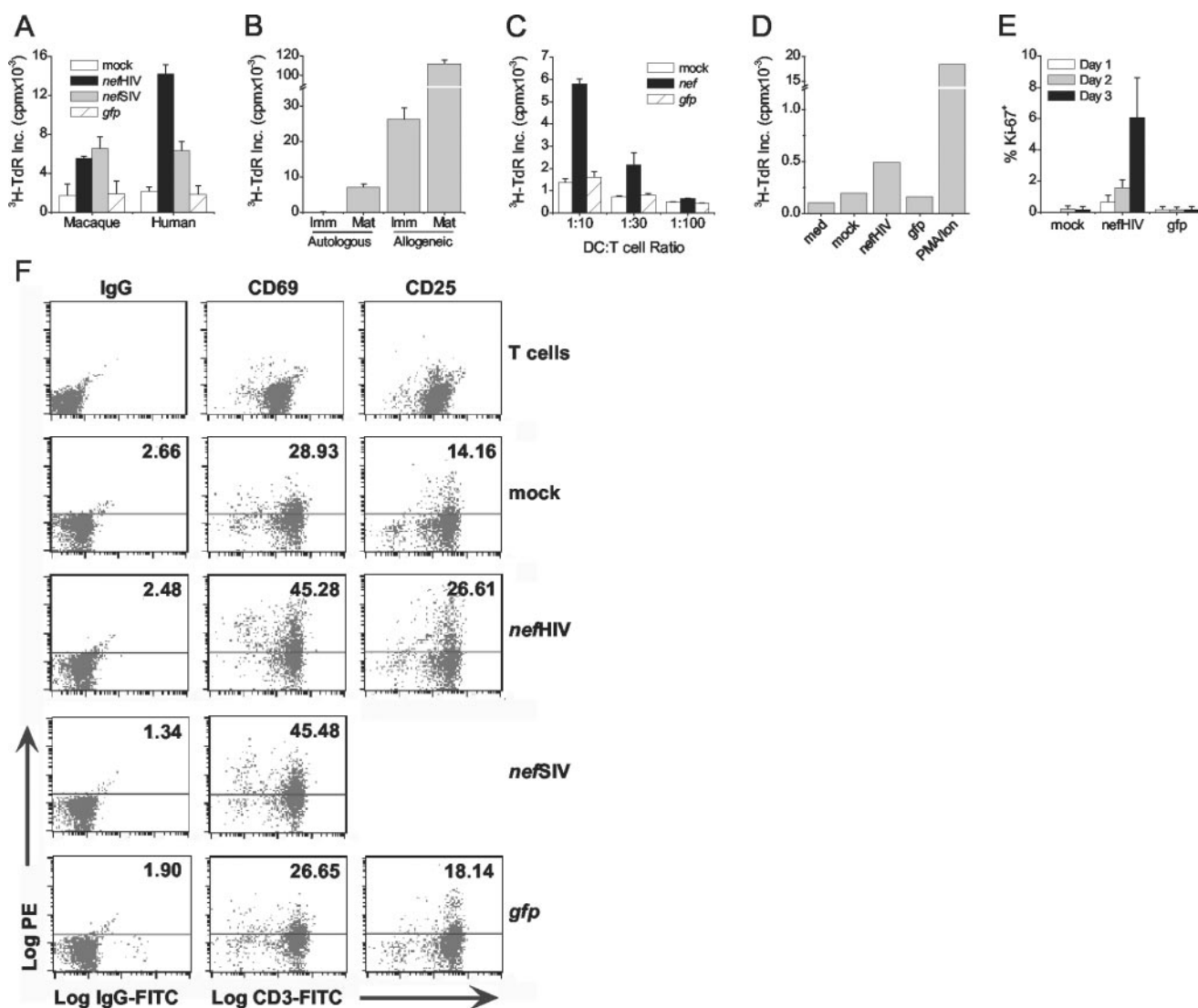


FIGURE 5. Nef expression in immature DCs induces T cell activation. **A**, Immature human or macaque DCs (10^4 cells/well) were mock-infected (mock) or infected with Adeno-*nef*HIV (*nef*HIV), Adeno-*nef*SIV (*nef*SIV), or Adeno-*gfp* (*gfp*). After infection, resting autologous 10^5 CD4 $^{+}$ T cells were added. T cell proliferation was assessed after 3 days (human) or 6 days (macaque) by measuring the amount of [3 H]TdR incorporated. The mean counts per minute \pm SEM [3 H]TdR incorporated in triplicate cultures of one experiment that is representative of three are shown. **B**, Immature (Imm) and mature (Mat) DCs were generated from healthy macaque blood and cultured with CD4 $^{+}$ T cells isolated from the same (Autologous) or a different (Allogeneic) animal (10^4 DC with 10^5 T cells). The amount of [3 H]TdR incorporated during the last 8 h of a 5-day culture is shown (mean counts per minute \pm SEM from triplicate cultures from one of at least four similar experiments). **C**, Graded doses of mock-, *nef*HIV-, or *gfp*-infected immature human DCs were added to autologous CD4 $^{+}$ T cells (10^5 cells/well) to obtain DC/T cell ratios of 1/10, 1/30, and 1/100. Three days after culture T cell proliferation was analyzed by measuring [3 H] TdR uptake. Results are representative of two experiments, and values shown are the means (\pm SEM) of duplicate wells. **D**, Purified CD4 $^{+}$ T cells (10^5 /well) were cultured in medium (med) or in the presence of DC-conditioned supernatants (mock, *nef*HIV, or *gfp*). Supernatants were collected from mock-, *nef*HIV-, or *gfp*-infected immature DCs, and each was diluted relative to adding the *nef*-DC supernatant at a final of 1 ng/ml CCL3 (MIP-1 α). CD4 $^{+}$ T cells were cultured with PMA (10 ng/ml; Sigma) and ionomycin (1 μ M; Sigma) as a positive control for T cell activation (PMA/ion). [3 H]TdR incorporation was measured after 3 days of culture, and data from one of two identical experiments are shown. **E**, Mock-, *nef*HIV-, or *gfp*-loaded immature DCs were mixed with autologous CD4 $^{+}$ T cells (1 DC/10 T cells) and cultured for 1–3 days. At each time point the cells were collected, and cytopins were prepared. The acetone-fixed cells were then immunoperoxidase-stained for Ki-67 expression, and the numbers of Ki-67 $^{+}$ cells were counted. The mean percentages (\pm SEM) of Ki-67 $^{+}$ cells were calculated by counting five separate fields of at least 100 cells each. Data are shown from one of three comparable experiments. **F**, CD4 $^{+}$ T cells were purified (>95%) by depleting CD8 $^{+}$ HLA-DR $^{+}$ cells, and their activation status was monitored before coculture with the DCs (T cells). Mock-, *nef*HIV-, *nef*SIV-, or *gfp*-loaded immature human DCs were cultured for 3 days with autologous CD4 $^{+}$ T cells (1 DC/10 T cells). Before and after culture, the T cells were assayed for expression of the early activation marker CD69 as well as CD25 (for *nef*HIV where more extensive analyses were performed) by staining with FITC-conjugated anti-CD3 and PE-conjugated anti-CD69 or anti-CD25. The FITC-Ig and PE-Ig controls were included and used to set the marker gates shown in the DC-T cell cocultures. Fluorescence data are shown for cells gated on forward scatter and side scatter to encompass resting and activated T cells excluding DCs or DC-T cell conjugates. The percentages of PE $^{+}$ cells above the indicated markers are shown in each DC-T cell panel. One example, representative of three independent experiments, is shown. Identical results were obtained with human and macaque cells.

impaired replication of the delta *nef* virus (Fig. 6B). As expected, wild-type virus grew normally in the immature DC-T cell mixtures, and activation of T cells with SEB rescued the delta *nef* replicative capacity. The slight decrease in virus production upon

addition of the maturation stimuli was seen at most time points in all experiments, but was not significant.

Although the DCs matured in response to the stimuli, it was possible that the 1–2 days it took for DCs to fully mature were too

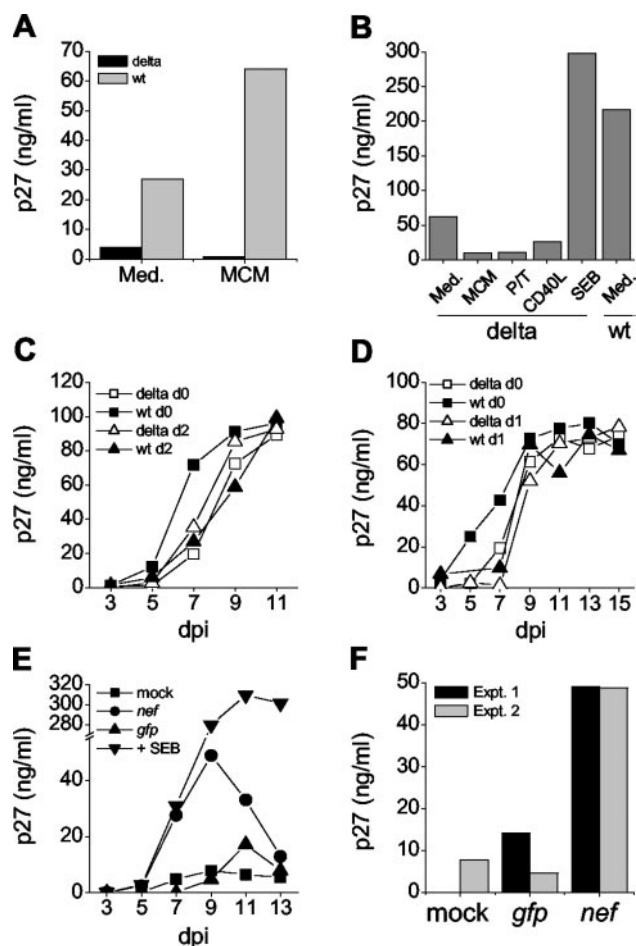


FIGURE 6. Nef expression in DCs enhances delta *nef* replication in immature DC-T cell cocultures. Immature DCs and CD4⁺ T cells were obtained from healthy macaques, and DC-T cell cocultures were infected with wild-type (wt) or delta *nef* (delta) SIVmac239 (27). After infection the cells were washed, and the indicated stimuli were added. The various DC-T cell mixtures were then cultured for up to 15 days, during which time supernatants were collected every 2 days, and productive infection was monitored by p27 ELISA. **A**, Fresh medium (med.) or fresh medium with 50% MCM was added to the cultures after infection. Peak virus production levels detected after 9 days are shown. **B**, After infection, fresh medium (med.), MCM, PGE₂ and TNF- α (P/T), CD40L, and SEB were added to the delta *nef*-infected cultures. Cocultures infected with wild-type virus (wt) were included as a control. The peak p27 levels seen on day 10 of culture are provided. **C**, After infection of immature DC-T cell cocultures with wild-type virus (wt; \blacksquare and \blacktriangle) or delta *nef* (delta; \square and \triangle), SEB was added immediately (day 0 (d0)) or 2 days later (d2) to the cultures. Virus replication was assayed over the following 11 days. **D**, Immature DCs were pulsed with either delta *nef* (\square and \triangle) or wild-type virus (\blacksquare and \blacktriangle), and the virus was washed away before DCs were mixed with allogeneic CD4⁺ T cells immediately (d0) or after an overnight incubation (d1). Productive infection was monitored over the next 15 days. **E**, Immature macaque DCs were infected with Adeno-*nef*SIV (*nef*) or Adeno-*gfp* (*gfp*) or were mock-infected as a control (mock). After 2 h autologous CD4⁺ T cells were added to the DCs, and the cocultures were immediately infected with delta *nef*. As a positive control for virus replication SEB was added to the delta *nef*-infected DC-T cell cocultures. **F**, Immature macaque DCs were pulsed with Adeno-*nef*HIV (Expt. 1), Adeno-*nef*-SIV (Expt. 2), or Adeno-*gfp* (*gfp*) or were mock-infected (mock) as a control. Autologous CD4⁺ T cells were then added just before the cocultures were infected with delta *nef*. The bars show peak p27 levels from two independent experiments. These data are representative of at least three separate experiments.

late to rescue the infectious delta *nef* persisting in these cultures. To test this, we stimulated the T cells after 1–2 days, since immediate activation of the T cells readily reveals the presence of infectious delta *nef* (Fig. 6B) (27). Addition of SEB to delta *nef*-

infected cocultures immediately or 2 days later rapidly amplified delta *nef* in these cultures (Fig. 6C). Similarly, adding delta *nef*-pulsed DCs to allogeneic CD4⁺ T cell immediately or 1 day after the virus pulse also resulted in robust delta *nef* replication and underscored how DCs retain infectious delta *nef* virus for considerable lengths of time (Fig. 6D). Therefore, infectious virus persists for up to 2 days and can be rescued upon encountering activated CD4⁺ T cells, but not simply by maturation of virus-carrying DCs within the cultures.

Since *nef* appears to have unique influence over immature DCs, triggering certain pathways of DC activation, but not others, we were interested in determining whether *nef*-bearing DCs could drive virus growth in the immature DC-T cell milieu. Immature DCs carrying *nef* (or not) were mixed with autologous CD4⁺ T cells and immediately exposed to delta *nef*. The expression of either SIV (Fig. 6, E and F) or HIV (Fig. 6F) *nef* in DCs facilitated SIV delta *nef* replication in immature DC-T cell cocultures (Fig. 6, E and F). Taken together these results stress how HIV and SIV *nef* are interchangeable and signal selective pathways in immature DCs that drive virus replication in immature DC-T cell cocultures.

Discussion

In an attempt to mimic the effects being exerted by virus-associated Nef as it enters the DC and/or the Nef expressed early during replication of DC-trapped virus as probably occurs following mucosal transmission, the studies described herein represent the first extensive investigation into the impact of endogenously derived Nef on the biology of primary immature (human and macaque) DCs. This stems from the observation that the absence of *nef* resulted in defective virus replication in immature DC-T cell mixtures (27). To selectively monitor the impact of Nef on immature DCs in the absence of other immunodeficiency virus determinants we first chose to employ the adenoviral vector system (that we and others have shown has little if any impact on DC function (25, 30–35)) to introduce the *nef* gene into DCs. DC-expressed Nef uniquely activates immature DCs to specifically foster DC-T cell communication benefiting immunodeficiency virus replication instead of immune activation against the virus, emphasizing how the virus may exploit the APC arm of the immune system to ensure its dissemination.

Expression of *nef* in human and macaque DCs induces significant production of inflammatory cytokines and chemokines (Figs. 3 and 4). This probably requires infection and actual expression of the Nef protein, since loading immature DCs with large amounts of inactivated, noninfectious virus (51) does not induce chemokine or cytokine secretion (our unpublished observations). Exogenously applied recombinant Nef was recently shown to trigger similar patterns of cytokine and chemokine release by immature human DCs (26). We similarly saw chemokine release by immature DCs exposed to recombinant Nef (our unpublished observations). However, the levels of cytokine and chemokine production appear to be greater in response to endogenous DC-derived Nef (Fig. 3 and 4), while we similarly observed negligible secretion of IL-1 β and IL-10 (26). How exogenous or endogenous Nef signal DCs still remains to be determined and is being examined in ongoing studies. While macrophages were induced to secrete CCL3 and CCL4 by *nef* (22), immature DCs secreted a much broader panel of factors: CCL3, CCL4, CCL5, TNF- α , CXCL8, IL-6, and IL-12.

Chemokine and cytokine production by DCs normally correlates with complete DC maturation when stimulated with bacteria, CD40L, LPS, dsRNA, or mixtures of cytokines (5, 6, 43, 44, 52). The soluble factors secreted during these responses differ depending on the stimulus (6, 43), suggesting that specific stimuli trigger DCs differently. Notably, *nef* induced the production of a wide range of

chemokines and cytokines, but this did not parallel membrane phenotypic changes typical of DC activation (Fig. 2) that are critical for DCs to acquire potent immunostimulatory activity (reviewed in Ref. 2). This contrasts what was observed when recombinant Nef was provided exogenously (26). Our data suggest that endogenously derived Nef uncouples the secretion of inflammatory cytokines and chemokines from triggering membrane phenotypic maturation in immature primate DCs.

Credence for one stimulus activating distinct pathways in DCs was first provided by Rescigno et al. (53) when they described how LPS regulated two separate pathways in murine DCs: one inducing membrane phenotypic maturation, and the second preventing cell death. Subsequent studies examining LPS triggering through TLR4 (54) or dsRNA via TLR3 (55) further highlight how one stimulus-receptor interaction can signal distinct pathways within DCs, specifically MyD88 adaptor protein-dependent cytokine production vs MyD88-independent membrane phenotypic maturation. Interestingly, we observed *nef*-mediated Stat3 activation in immature DCs (35) that may favor MyD88-dependent cytokine secretion (56). The fact that *nef*, but not maturation stimuli, could rescue the defective replication of SIV delta *nef* in the immature DC-T cell mixtures (Fig. 6), further emphasizes how *nef* exploits DC biology and uniquely triggers immature DCs to favor virus amplification rather than activation of anti-viral immunity. Similarly, membrane phenotypic changes indicative of low level DC activation were comparable in the immature DC-T cell cocultures exposed to delta *nef* or wild-type SIV where only wild-type virus grew effectively (27). Certain DC stimuli have been suggested to protect DCs from viral infection (44), while *nef* probably favors pathways to promote virus amplification.

Unlike earlier reports using primary DCs and transfected cell lines, we observed no consistent down-modulation of class I MHC molecules (24, 26) or up-regulation of CD209 (23) or CD74 (57). While we observed up-regulation of CD74 and CD209 in response to *nef* in two donors, there was no change or even down-regulation of these molecules in all other donors tested (Fig. 2B). This underscores the importance of studying primary cells from multiple donors. It is postulated that the down-modulation of MHC class I or II molecules would reduce immune function in infected settings, while elevated CD209 would foster DC-T cell contact, and each would drive virus growth. The low level CD209 expression present on macaque DCs (Fig. 2) (40) makes it even less likely that this is the sole mechanism at work here, agreeing with the indication that CD209-ICAM-3 interactions are not essential for HIV replication (58).

The reason for the discrepancies in *nef*-mediated effects on immature DCs is not immediately apparent, but may reflect the different viral vectors used to introduce *nef*, the use of recombinant Nef, and/or the *nef* alleles examined in primary cells vs transfected cell lines. The different methodologies used to generate the DCs in each study also cannot be excluded. However, many of our observations were made using both HIV and SIV *nef* expressed in primary macaque and human DCs and not cell lines often used by others. The level of Nef expression in primary DCs vs the variously tested cell lines or the amount of recombinant Nef added may also influence these events. This has been described for T cells, where the intracellular concentrations of Nef dictated whether CD4 vs class I MHC were down-regulated or T cell activation was affected (59). Interestingly, *nef*-induced chemokine production was routinely detected in immature DCs independent of the Adeno infection frequency or the intensity of the *gfp* (and so Nef) expression (data not shown), suggesting that sufficient Nef was being expressed under these conditions. Accurate comparative measurements of DC-associated Nef were not possible in these primary cell cultures. Studies are needed (e.g., using systems in which the *nef* gene is under the control

of inducible promoters) to better define how different levels of Nef may impact primary DC biology.

The *nef* gene can directly activate CD4⁺ T cells (15, 60). HIV or SIV *nef*-expressing immature DCs augmented autologous CD4⁺ T cell activation (much like a mature DC) in both human and macaque systems (Fig. 5), but required fewer *nef*-expressing DCs compared with human DCs exposed to recombinant Nef (26). At this stage it is not clear how DC-derived Nef exactly influenced the T cells in these cultures and whether it was mediated directly or indirectly via the *nef*-carrying DCs. Although we do not see a *nef*-induced increase in CD209 expression that may favor DC-T cell binding (23), it is possible that *nef*-bearing DCs up-regulate other T cell-binding molecules and/or that the T cells become more adhesive as a result of chemokine exposure (reviewed in Refs. 61 and 62), resulting in elevated autologous reactivity (63, 64).

In our previous studies we did not observe T cell activation in autologous immature DC-T cell cultures infected with wild-type SIV (27) or HIV (41, 65). It is quite possible, however, that much smaller subsets of T cells were activated, making them difficult to detect in the infected cultures. This may have been further confounded by preferential infection (and ultimately death) of the activated cells in these cultures. Similarly, significant chemokine or cytokine production was not detected in wild-type vs delta *nef* SIV-infected cultures (our unpublished observations). This is probably largely due to the relatively low frequency of virus-producing DCs in these cultures (9). Even if secreted at lower levels in wild-type-infected cultures, it is possible that cells in the cultures use up the factors being secreted, rendering them even more difficult to detect. Counteractions by other lentiviral determinants cannot be ruled out at this point. As appreciated by others (23, 24), introducing *nef* in a viral vector enables the provision of larger amounts of *nef* into more DCs in the absence of an infectious immunodeficiency virus, allowing us to reveal otherwise subtle *nef*-induced changes (in DCs and T cells) that probably go undetected in wild-type virus infection.

Although the mechanism(s) needs to be elucidated, we have demonstrated that DC-borne *nef* overtly modulates the immature DC-T cell milieu, fostering virus spread. Chemokines have been shown to prevent in vitro infections when added to cultures in recombinant forms (66). While immature DCs (Figs. 3 and 4) (26) and macrophages (22) secrete β -chemokines in response to Nef, HIV (22) and SIV (Fig. 6) still replicate normally. It is possible that macrophage or DC-derived chemokines (secreted in response to Nef) exhibit different biological activities to the recombinant forms and/or that the presence of a variety of factors within such a cellular milieu overrides any blocking effect that a solitary chemokine might otherwise have. Furthermore, the concentration of chemokine(s) used may have quite different effects on the outcome of the infection, since, for example, high dose RANTES has been shown to actually enhance infection (67). Thus, the amounts and combinations of chemokines secreted in these cultures may actually create a more conducive environment for virus growth.

One function of *nef* in a wild-type virus infection may be to modify macrophages (22) and immature DCs (Figs. 3 and 4) (26) to secrete chemokines that would recruit T cells (especially memory T cells (reviewed in Refs. 61, 68, and 69) to the initial site of infection, driving cell-to-cell spread of the virus. T cells in a DC-T cell milieu do not need to enter the cell cycle for virus to replicate in vitro (41, 65) or in vivo (70–72), and cytokines (73) secreted by the DCs (Figs. 3 and 4) and/or DCs themselves (27) might be sufficient to signal the recruited T cells (much like in the autologous MLR) to amplify virus replication. Preferential recruitment of memory T cell subsets would further favor virus amplification in the presence of immature DCs (9).

Recent findings support the idea that both soluble factors and cell contact are essential to drive virus expansion in resting CD4⁺ T cells. Specifically, the factor(s) present in the supernatants of Nef-stimulated macrophages that renders resting CD4⁺ T cells permissive to HIV infection requires contact between the target T cells and B cells (M. Stevenson, unpublished observations). We propose that similar events take place in our DC-T cell cocultures, where Nef-triggered immature DCs are able to provide the necessary soluble and cellular determinants to drive virus growth in the presence of resting CD4⁺ T cells. This is supported by observations that the levels of T cell activation diminished with decreasing DC numbers and that simply adding the supernatants from Nef-expressing immature DCs was insufficient to induce the same levels of T cell proliferation. Studies to elucidate these complex mechanisms are ongoing.

The presence of wild-type virus (*nef*)-carrying immature DCs in this locale would be unlikely to stimulate active anti-viral immunity, since the DCs have not up-regulated MHC and costimulatory molecules needed to appropriately activate strong Ag-specific immune responses. While wild-type virus-bearing DCs (as well as Adeno-*nef*-infected DCs) are responsive to maturation stimuli (our unpublished observations), the robust replication of virus already established before virus-specific T cell activation would be exacerbated by subsequently matured DCs (e.g., Fig. 6A) triggering the T cells and probably simply overwhelm the immune system. In contrast, when confronted with delta *nef* infection, immature DCs could capture virus and begin to process viral Ags in the absence of an all-consuming infection (27). Our in vitro evidence that delta *nef* virus growth was not augmented after maturing the DCs in this milieu (Fig. 6, A and B) suggests that these matured cells could then go on to more effectively activate anti-viral immune responses that might contribute to the lessened viral loads seen during acute delta *nef* infection (74, 75). Once T cells are activated, virus will ultimately begin to spread between the permissive cells. This underscores how without *nef* to exploit the APCs, the immune system can initially cope to somewhat restrict virus dissemination.

Recombinant adenoviral vectors are also being used in HIV vaccine studies (76). Since in vitro studies have indicated that adenoviruses tend not to directly activate DCs (25, 29–35), optimal vaccine efficacy would require that the DCs be additionally activated for maximal presentation of introduced Ags (2). In light of the findings reported herein, we speculate that if an adenoviral vaccine construct contained *nef*, the initial triggering of immature DCs by Nef may favor the recruitment of T cells to the site of immunization, but additional stimuli would be necessary to fully activate the DCs for induction of strong anti-viral immunity.

Encompassing primary DCs from macaques and humans, these studies highlight how the immunodeficiency virus *nef* can manipulate immature DCs, exploiting unique aspects of DC biology to forge virus dissemination while avoiding the activation of virus-specific T cells. A better understanding of how these and potentially other virus-mediated modifications of DCs are manifest will be critical to identify strategies to bias the DC system toward activation of anti-viral immunity instead of facilitating virus spread.

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