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HIV-1 Nef increases T cell activation in a stimulus-dependent manner

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ABSTRACT **Lentiviral Nef increases viral replication *in vivo*, plays a direct role in pathogenesis, and increases viral particle infectivity. We now find that HIV Nef also increases the activation of T cells, a cellular state required for optimal viral replication. This enhancement is stimulant-dependent. As defined by IL-2 generation, activation of T cells stimulated with classical mitogens [phorbol 12-myristate 13-acetate (PMA) + anti-CD3, PMA + phytohemagglutinin, and PMA + ionomycin] is unaffected by the expression of Nef. However, Nef increases IL-2 secretion when cells are stimulated through the T cell receptor and the costimulus receptor (CD28). This increase in activation, which depends on Nef myristylation, is caused by an increase in the number of cells reaching full activation and not by an increase in the amount of IL-2 secreted per cell. These findings demonstrate that Nef lowers the threshold of the dual-receptor T cell activation pathway. The capacity of Nef to increase T cell activity may be very important *in vivo* when Nef is the predominant or the only viral gene product expressed.**

Although the means by which Nef enhances pathogenesis of simian immunodeficiency virus (SIV) in macaques is unknown, infection by Nef-expressing virus leads to higher viral titers (1), and SIV- and HIV-mediated disease is correlated with viral burden and loss of CD4 T cells (2). The lentiviral Nef protein promotes viral replication in T cells (3–5), a process that requires mitogenic activation of the host cell (6–8). Furthermore, HIV infection of the human T cell line Jurkat, as well as primary human T cell cultures, has been shown to enhance T cell activation as mediated by antibody engagement of the T cell receptor and the CD28 coreceptor (9). Although it is known that Nef expression, which evolved as the predominant early transcript (10, 11), optimizes viral replication, the influence of Nef on T cell activation remains unresolved. Furthermore, the effect of Nef expression on primary human CD4 T cell activation has not been examined.

Consistent with the enhancing capacity of Nef for viral replication in T cells, previous T cell murine hybridoma work demonstrated that Nef introduced by retroviral transduction increased IL-2 secretion, a definitive measure of T cell activation, after stimulation of the T cell receptor (12). Culturing of macaque peripheral blood lymphocytes (1) and a herpesvirus saimiri-infected macaque T cell line (13) require the addition of IL-2. Unlike lymphocytes, however, this cell line becomes IL-2-independent when infected by a nef^+ SIV but not by nef^- SIV (13). This finding provides further support that Nef can play a direct, positive role in the T cell activation pathway. Other reported positive, activation-enhanced effects by Nef indicate a role in dysfunctional *in vivo* murine development of T cells, where the transgenic expression of Nef occurs in thymocytes (14), and in CD69 expression leading to apoptosis in Jurkat cells expressing surface CD8-Nef fusion protein (15). Examination of human cells has resulted in a demonstration of either neutral (16–18) or suppressive (15, 19–23) Nef effects on T cell activation. To extend our Nef studies to human T cells, we have transduced various Nef genes and control vectors into the Jurkat line and primary human CD4 T cells. Consistent with *in vivo* effects, we find that Nef can enhance human T cell activation, but only when cells are stimulated by engaging the T cell receptor and the CD28 coreceptor. Relative to chemical mitogens, this form of stimulation more closely approximates *in vivo* T cell activation. By examining activation at the single-cell level, we demonstrate that Nef lowers the T cell activation threshold. Thus, under the conditions used here, Nef displays a capacity to increase the activation state of human CD4 T cells.

MATERIALS AND METHODS

T Cell Cultures and Retroviral Transduction. Jurkat E6–1 T cells (24), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, were grown in complete growth medium (RPMI 1640 supplemented with 10% heat-inactivated FCS/2 g/liter sodium bicarbonate/1 mM nonessential amino acids/10 mM sodium pyruvate/4 μl/liter 2-mercaptoethanol/50 μg/ml gentamicin, adjusted to pH 7.4). Cells were transduced by liposome-mediated (25) PA-317-packaged LXSN retroviral transduction (26) to express neomycin phosphotransferase (Neo) only or Neo and one of the following Nef proteins: nonmyristylated NL4–3 Nef mutant, generated by a glycine-to-alanine switch at residue position 2 (G2A) (27), or myristylated Nef from HIV-1 strain SF2 or from strain NL4–3. The next day cells were treated with 2 mg/ml (active) G418 (Life Technologies, Gaithersburg, MD). After 7–10 days in selection, cells were returned to complete medium and whole populations of G418-resistant cells were used in subsequent studies.

Elutriated lymphocytes were obtained from healthy human donors at the Department of Transfusion Medicine, National Institutes of Health (28). We removed cells positive for CD8, CD19, MHC II, and CD11b by using magnetic beads (Dynal, Great Neck, NY) to obtain a purified population of CD4 T cells. Cells were expanded by using anti-CD3/anti-CD28-cis-coated magnetic beads according to the method published by Levine *et al.* (29). The day after purification, CD4 T cells were transduced as above with modifications described elsewhere (30). Briefly, cells were transduced in protamine sulfate-supplemented medium, and, after an overnight incubation at 37°C, cells were treated with 1 mg/ml (active) G418 for 7 days. Live cells were harvested over Lymphocyte Separation Medium (ICN), and whole populations were used in subsequent

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Abbreviations: ELISPOT assay, enzyme-linked immunosorbent spot assay; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin.
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activation experiments. Proliferation rates were determined by exponential curve fitting of cell counts obtained over 1- to 3-week periods.

Flow cytometry. Analysis of cell surface molecules was performed as reported (31). FITC-conjugated mAbs to CD3, CD4, and CD28 were purchased from Caltag (South San Francisco, CA), and all other antibodies and antibody conjugates were purchased from PharMingen. Analysis of intracellular IL-2 was performed according to protocols supplied by the manufacturer (PharMingen) by using monensin (Calbiochem), 0.1% saponin (Polysciences), and 4% paraformaldehyde (Sigma).

SDS/PAGE and Western Blot. Cell pellets were suspended in 4°C lysis buffer [1% NP-40 in 20 mM Tris, pH 8.0/150 mM NaCl/2.0 mM EDTA; supplemented with 1.0 μg/ml leupeptin/1.0 μg/ml aprotinin/1 μg/ml pepstatin A/250 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride] for 20 min. Clarified lysate then was incubated with a polyclonal anti-Nef antibody (32), obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, for 1 hr and precipitated overnight at 4°C in the presence of protein G-Sepharose beads. The equivalent of 5 million cells was applied to SDS/PAGE, transferred to nitrocellulose, and probed with an anti-Nef mAb (catalog no. 13–160-100; Advanced Biotechnologies, Columbia, MD). The blot then was probed with a secondary horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) and then incubated with chemiluminescence solution (SuperSignal Ultra; Pierce). Radiographic film was exposed to the membrane and developed. By inclusion of recombinant Nef (a kind gift of Paul Wingfield, National Institute of Arthritis and Musculoskeletal and Skin Diseases), cellular quantities were estimated.

Cell Stimulation and ELISA of Supernatants. Anti-CD3 mAb (clone HIT3a; PharMingen) was immobilized on an enzyme immunoassay/RIA 96-well microtiter plate (catalog no. 3590; Costar) in binding buffer (0.2 M sodium bicarbonate, pH 8.0) overnight at 4°C. Wells were washed three times with sterile PBS followed by the addition of 10^5 Jurkat T cells to wells containing phorbol 12-myristate 13-acetate (PMA; Calbiochem) and either immobilized anti-CD3, phytohemagglutinin PHA-P (Sigma), or ionomycin (Calbiochem), or to wells immobilized with anti-CD3 and containing soluble anti-CD28 (clone CD28.2; PharMingen). After 24 hr, supernatants were examined for IL-2 concentration by standard ELISA techniques using paired capture and detection anti-IL-2 antibodies (R&D Systems).

For primary cells, the magnetic beads present in the proliferating cultures were removed and cells were centrifuged over Lymphocyte Separation Medium. Cells were washed and incubated overnight without exogenous stimulation. The next day, 2×10^5 cells negative for trypan blue and 6×10^5 beads—precoated with anti-CD3 and anti-CD28—were added to each well of a U-bottom, 96-well plate. Cells were incubated at 37°C, and supernatants were collected at the indicated times to quantitate IL-2 secretion by standard ELISA methods.

***In Situ* ELISA and Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay.** Both *in situ* ELISA and the ELISPOT assay, which identifies individual activated cells, involved the immobilization of the activating anti-CD3 and anti-CD28 mAbs and the capturing anti-IL-2 mAb (clone 5355.111; R&D Systems), all in the same well. The enzyme immunoassay/RIA microtiter plate was used for the *in situ* ELISA, and, to facilitate counting activated cells, a nitrocellulose-backed microtiter plate (catalog no. MAHA54510; Millipore) was used for the ELISPOT assay. In each well of both plates, 0.33 μg of each mAb was immobilized in 0.2 M sodium bicarbonate, pH 8.0, overnight at 4°C. After rinsing three times with sterile PBS, wells were blocked with complete growth medium for at least 1 hr. Jurkat cells were washed once in serum-free RPMI medium 1640, added to the wells in 100 μl of complete growth medium, and incubated at 37°C in humidified air with 5% CO_2 for 24 hr. During the last 2 hr, known concentrations of rIL-2 standards (Genzyme) were added to the *in situ* ELISA plate to generate a standard curve. Cells were removed by vigorous washing with 0.05% Tween-20 in PBS, and captured IL-2 was quantitated *in situ* by standard ELISA techniques.

To enumerate IL-2-secreting cells in the ELISPOT assay, cells were removed from the nitrocellulose-backed plate by vigorous washing, and 200 ng/ml of biotinylated detection anti-IL-2 (R & D Systems) was added to the plate and incubated at 4°C overnight. The plate then was washed and incubated in the presence of streptavidin-horseradish peroxidase (Kirkegaard & Perry Laboratories) for 2 hr at room temperature. The plate was washed and 200 μl of chromogen substrate [10 mg of 3-amino-9-ethylcarbazole (Sigma) dissolved in 1 ml *N*,*N*-dimethylformamide added to 30 ml of 0.1 M sodium acetate buffer, pH 5.0, filtered, and quenched with 15 μl of 30% H_2O_2 immediately before use] was added to each well. This substrate converts to a water-insoluble product to form a red "spot." After 30 min at room temperature the plate was washed with deionized water and allowed to air-dry, and spots were counted with a dissecting microscope.

RESULTS

Nef Expression and Analysis of Surface Markers. Jurkat E6–1 cells were transduced with the LXSN retroviral vector containing the *neo* gene alone or *neo* together with a *nef* gene that expresses one of the following HIV-1 Nef proteins: myristylated Nef from either strain SF2 or NL4–3, or a mutant nonmyristylated NL4–3 Nef (G2A). Cell populations were selected for G418 resistance [conferred by neomycin phosphotransferase (Neo)], and transduced populations were exam-

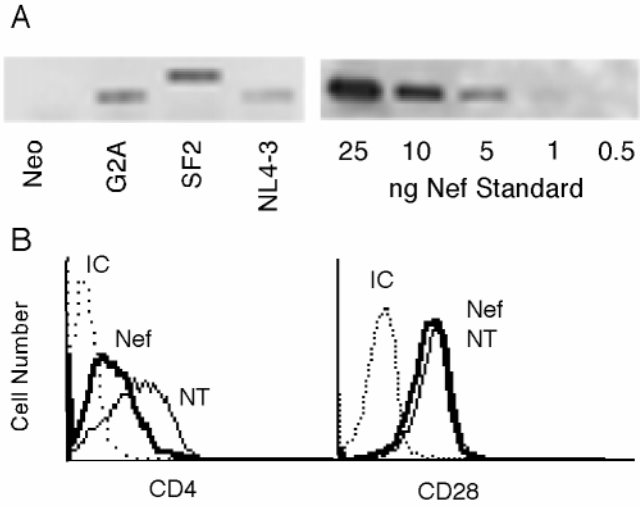

FIG. 1. Physical and functional confirmation of Nef expression. Western blot analysis (*A*) shows Nef expression from Jurkat E6–1 T cells. The HIV-1 *nef* gene was introduced into cells by liposome-mediated LXSN retroviral transduction, followed by selection in G418. Cells were transduced with nonmyristylated NL4–3 Nef (G2A), myristylated NL4–3 Nef (NL4–3), and SF2 Nef (SF2) and by the LXSN control vector (Neo). Cells were lysed and Nef was immunoprecipitated with rabbit anti-Nef polyclonal antibody. Samples from 5 million cells were electrophoresed, and resolved proteins were transferred to nitrocellulose. Nef was detected by monoclonal anti-Nef antibody. A Western blot for recombinant NL4–3 Nef is shown at 0.5–25 ng per lane. (*B*) FITC analysis of cell surface expression of CD28 and CD4 on nontransduced (NT) cells (thin line) or on cells transduced with HIV-1 Nef from strain SF2 (thick line). Cells were stained directly with FITC-conjugated anti-human mAbs or with murine isotype control Abs (IC).

ined directly without cloning. Western analysis demonstrated the specific expression of the Nef protein and, when compared with recombinant protein, suggested cellular levels between 1 and 10 ng per 5 million cells (Fig. 1*A*). As demonstrated previously (33), SF2 Nef migrated slightly slower than NL4–3 Nef.

In agreement with previous findings (12, 34), expression of Nef in Jurkat T cells did not alter cell surface levels of CD3 or CD45 (data not shown). There also was no change in CD28 expression (Fig. 1*B*). Additionally, the unactivated parental cell line did not express the IL-2 receptors CD25 or CD122, and Nef expression did not alter these phenotypes (data not shown). Although this Jurkat line stains heterogeneously for surface CD4, down-modulation was evident with Nef expression (Fig. 1*B*), consistent with previous reports (26, 35).

Nef Increases Activation by T Cell Antigen Receptor and CD28 Costimulation But Not by Chemical Mitogenic Stimulation. Because IL-2 secretion is a definitive indicator of T cell activation, we measured IL-2 in supernatants after stimulating cells with various agents (Fig. 2). Expression of Nef (from HIV-1 isolate SF2) had no effect on IL-2 production from cells stimulated with combinations of PMA plus either anti-CD3 or PHA (Fig. 2 *A* and *B*). However, when stimulated with anti-CD3 and anti-CD28, cells expressing Nef showed a marked enhancement of IL-2 production (Fig. 2*C*). Furthermore, the effect of Nef appeared to be specific for IL-2 because secretion of two other activation-induced cytokines (IL-8 or tumor necrosis factor α) were not enhanced (data not shown). Similar to the effect seen with PMA and anti-CD3 or PMA and PHA, Nef had no effect on IL-2 secretion in cells stimulated with PMA and ionomycin (data not shown).

For comparison, cells also were transduced with the NL4–3 Nef and with the nonmyristylated NL4–3 Nef (G2A). The NL4–3 Nef, like the SF2 Nef, enhanced IL-2 expression after mAb costimulation (Fig. 2*D*), whereas the nonmyristylated G2A mutant Nef had no effect. Myristylation of Nef is essential for CD4 modulation (27, 36) and for increased HIV infectivity (37) and, thus, also appears to be essential for this positive effect on T cell activation.

The inability of Nef to enhance CD3 plus PMA- or PHA plus PMA-induced T cell activation (Fig. 2 *A* and *B*) might be the result of a PMA-mediated saturation of the activation pathway, as suggested by the 3-fold increase in IL-2 secretion by CD3 plus PMA over that seen with CD3 plus CD28 antibody stimulation (compare Fig. 2 *A* with *C*). To address this possibility, NL4–3 Nef-expressing and control G2A cells were incubated with PMA at various submaximal concentrations (0.1–1 ng/ml) with a constant level of anti-CD3 mAb. Under these conditions, where the PMA-induction of IL-2 becomes limiting, we did not observe an Nef-mediated enhancement of IL-2 secretion (data not shown). Therefore, the effect of Nef on cellular IL-2 secretion is seen only by stimulation of surface CD3 and CD28 receptors.

Myristylated Nef Increases the Number of Cells Reaching Full Activation. In the simplest terms, the presence of Nef in cells stimulated through the CD3 and CD28 receptors either increases IL-2 generation per cell or increases the number of activated cells. To distinguish between the two, we compared total IL-2 secretion of a cell population to the number of cells within that population that reach full activation and secrete IL-2. This was achieved by examination of the same population of cells in an *in situ* ELISA and ELISPOT assay. Activation studies as performed in Fig. 2 involved incubation of cells in 96-well plates with immobilized anti-CD3 and anti-CD28 followed by standard ELISA estimates for IL-2 in a separate plate containing immobilized anti-IL-2 capture antibody. With both the *in situ* ELISA and ELISPOT assay all three antibodies are immobilized in the same well. Cells that are activated by engagement of the CD3 plus CD28 receptors secrete IL-2, which is captured locally. The *in situ* ELISA, which is developed as a standard ELISA, measures the total IL-2 secreted by a defined cell density; whereas, the ELISPOT assay (38) makes use of a substrate that generates a water-insoluble product that precipitates out of solution and forms a spot at which the activated cell resided. The total number of spots represents the total number of cells that secreted IL-2. To determine the dynamic range of this assay with Jurkat T cells and to exclude cell density effects on IL-2 secretion, we examined various

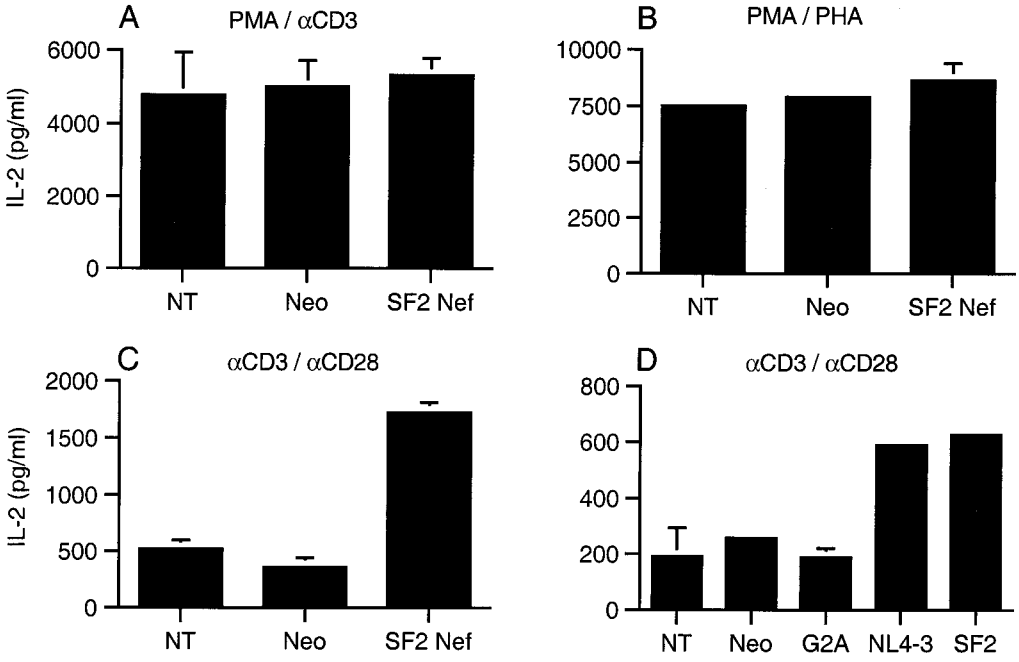

FIG. 2. Myristylated Nef enhances T cell activation in a stimulus-dependent manner. Jurkat cells (10^5; transduced as described in Fig. 1) were activated by either PMA (10 ng/ml) and immobilized anti-CD3 (coated at 1 μg/well) (*A*), PMA and PHA (10 μg/ml) (*B*), or immobilized anti-CD3 (coated at 0.33 μg/well) and soluble anti-CD28 (0.33 μg/well) in 96-well plates (*C* and *D*). After 24 hr at 37°C, IL-2 secreted in the supernatant was measured by standard ELISA techniques. Bars represent mean IL-2 concentration from quadruplicate (*A* and *B*) or duplicate (*C* and *D*) wells. IL-2 secretion from unstimulated cells was undetectable.

input cell numbers. Typically, we carried out these assays at input cell densities of 5,000–50,000 cells per well for the *in situ* ELISA and at 1,000–10,000 cells per well for the ELISPOT assay. Under these conditions the number of activated cells and the amount of IL-2 secreted were found to be linear with respect to cell input (data not shown). The lower limit consistently yielded positive results above background, and cell input densities above the upper limit resulted in saturation of the *in situ* ELISA or the loss of resolution of single cells in the ELISPOT assay.

From the ELISPOT assay, myristylated NL4–3 and SF2 Nef isolates increased the number of CD3/CD28-stimulated cells that became activated and secreted IL-2, relative to the Neo control, but the nonmyristylated mutant G2A did not (Fig. 3*A*). The percentage of these stimulated Jurkat cells that became activated was 0.53 ± 0.14% for Neo-transduced cells and 2.38 ± 0.14% for SF2 Nef-transduced cells, a 4.5-fold increase. By plotting two input cell densities common to both the *in situ* ELISA and the ELISPOT assay, we found that the increase in total IL-2 secretion by Nef-expressing cells, compared with the Neo control and G2A mutant, was directly proportional to the increase in the number of activated cells (Fig. 3*B*). Although triggering T cell activation by antibody engagement of the TCR and CD28 costimulus receptor of the Nef-expressing populations resulted in a 3- to 4-fold increase in the number of IL-2-secreting cells, Nef did not increase the ratio of total IL-2 secretion to the number of activated cells (derived by dividing the results of the *in situ* ELISA by those of the ELISPOT assay). The calculated amount of IL-2 secreted per CD3 plus CD28-activated cell from the experiment in Fig. 3 was 792 ± 236 fg IL-2 per activated cell for Neo control cells and 547 ± 60.5 fg IL-2 per activated cell for SF2 Nef-expressing cells. The correlation of IL-2 generation with number of activated cells is consistent with the published concepts defining T cell activation as a threshold phenomenon leading to an all-or-nothing event (activation) (39, 40).

Thus, Nef enhances the commitment for cellular activation but does not increase the level of cellular IL-2 secretion once activation is achieved. These data demonstrate that Nef affects the rate-limiting step of T cell activation by lowering the threshold.

Nef Expression and Examination of Surface Markers in Primary T Cells. Although HIV-infected primary cells are hypersensitized to T cell receptor and CD28 stimulation (9), the effect of Nef on primary human T cells has not been examined. As an extension of our work in the Jurkat line, we wished to determine the effect of Nef on activation in primary cells. In the presence of beads coated with anti-CD3 plus anti-CD28, purified human CD4 T cells were expanded exponentially as described previously (29). Cells were transduced as described in *Materials and Methods*. Western analysis demonstrated Nef expression that was found to be similar in transduced primary and Jurkat cells (Fig. 4*A*). Flow cytometry of Nef-expressing primary cells demonstrated that CD3 and CD28 were not modulated, whereas CD4 was decreased in cells transduced with myristylated Nef (Fig. 4*B*). CD4 expression in parental cells was identical to levels seen in G2A-transduced cells (data not shown). Viability and proliferation rates were unaffected by Nef expression (see Fig. 4 legend).

Nef Enhances Activation of Primary CD4 T Cells. Transduced and parental primary cells were stimulated by beads

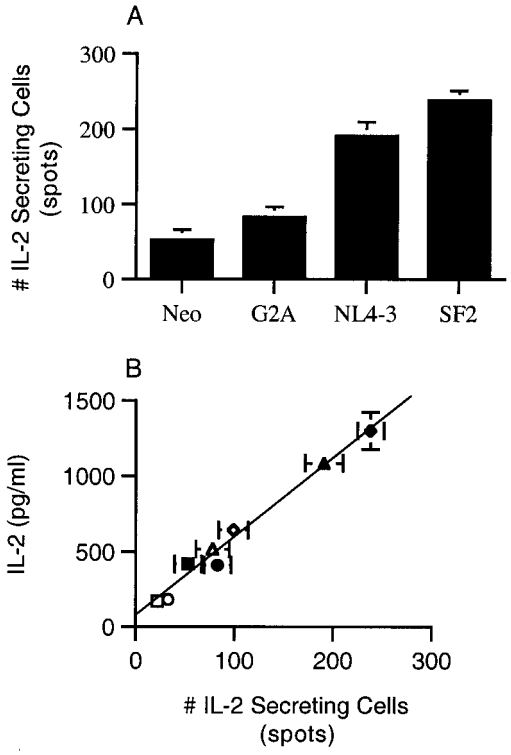

FIG. 3. Nef expression increases the number of IL-2-generating cells. Jurkat cells transduced with NL4–3 (triangles) and SF2 nef (diamonds), and neo (squares) and G2A (circles) controls were stimulated with anti-CD3 plus anti-CD28. Cell activation was examined by ELISPOT assay (*A*) and by *in situ* ELISA (not shown). Data from two input cell densities common to both the ELISPOT and *in situ* ELISA [5×10^3 cells (open symbols) and 10^4 cells (solid symbols)] are shown (*B*). The relationship between the number of activated cells and the total amount of IL-2 secreted for the various transductants at these cell densities was linear, $r^2 = 0.98$. Cells were transduced as in Fig. 1. Data represent mean determinations and SDs from triplicate wells.

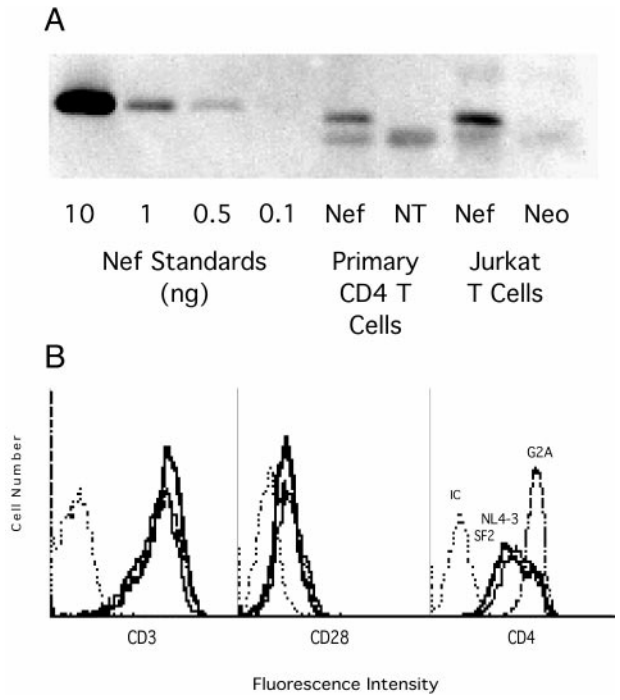

FIG. 4. Physical and functional confirmation of Nef expression in primary CD4 T cells. Western blot analysis (*A*) compares NL4–3 Nef expression in transduced primary CD4 T cells and Jurkat cells, in addition to control non-Nef (NT or Neo) cells. The blot also includes recombinant NL4–3 Nef at 0.1–10 ng/lane. (*B*) Flow cytometric analysis of cell surface expression of CD3, CD28, and CD4 on cells transduced with HIV-1 Nef from strain SF2, NL4–3, or the nonmyristylated NL4–3 (G2A) as shown. Cells were stained directly with FITC-conjugated anti-human mAbs or with murine isotype control Abs (IC). Viability (dye exclusion) of SF2, NL4–3, G2A, and NT cells was 88, 91, 92, and 83%, respectively. Proliferation (doubling time) of cell populations was 2.9, 2.6, 3.5, and 2.6 days, respectively.

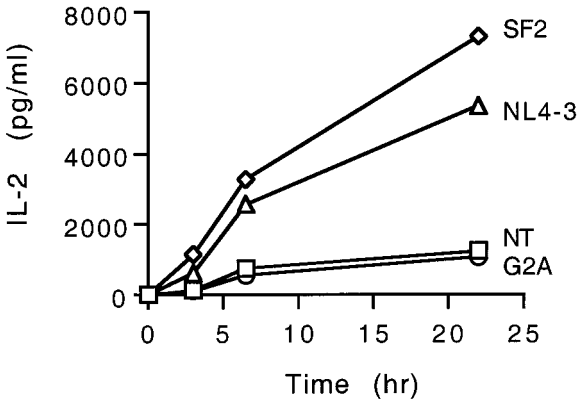

FIG. 5. Nef enhances activation in primary CD4 T cells. Purified CD4 T cells were transduced or not (NT) with the retroviral vectors as shown. Seven days after selection in G418, cells were incubated in the absence of exogenous stimulation overnight. Two hundred thousand rested cells and 6×10^5 beads—precoated with anti-CD3 and anti-CD28 mAbs—were added to each well of a U-bottom, 96-well plate. Cells were incubated at 37°C, and supernatants were collected at various times to quantitate IL-2 secretion by standard ELISA methods. Determinations at 22 hr were averages from triplicate wells with a SD of 6.4% or less.

coated with CD3 and CD28 antibodies as described in *Materials and Methods*. Stimulation of cells expressing the myristylated Nef proteins generated IL-2 levels 5-fold higher than from stimulation of cells expressing the nonmyristylated Nef protein or cells transduced with the empty (Neo only) vector (Fig. 5). In six transductions of primary CD4 T cells from different donors, the level of increased activation resulting from myristylated Nef ranged from a 4- to 10-fold enhancement in IL-2 levels.

To characterize the activation of the Nef^+ T cell population, we performed dual staining of surface CD4 with generated intracellular IL-2 (see *Materials and Methods*). CD4 surface levels vary inversely with Nef expression levels (41). Staining of surface CD4 in unstimulated, nontransduced, G2A mutant NL4–3 Nef and myristylated NL4–3 Nef cells yielded fluorescence intensities of 90.49, 95.87, and 54.13 (geometric mean) fluorescence units, respectively. After stimulation with CD3 plus CD28 antibodies, cells identified as activated ($IL\text{-}2^+$) yielded similar CD4 levels of 97.02, 96.65, and 58.25 fluorescence units, respectively, for nontransduced, G2A, and NL4–3. This finding provides evidence that IL-2 synthesis is occurring coincidentally with Nef-mediated CD4 modulation and that activation is uniform across the Nef^+ cell population.

DISCUSSION

Nef expression facilitates lentiviral pathogenicity. Its role in this process is unknown, but the development of AIDS is preceded by a rampant HIV infection of the lymphoid tissue (42, 43). Nef enhances HIV replication in T cells (3–5), where cellular activation is critical for viral proliferation. *In vivo* T cell activation is believed to be caused by ligation of the surface CD3 and CD28 receptors. The finding that the Nef enhancement is limited to surface receptor engagement suggests that the Nef molecular interaction is early in the T cell receptor activation pathway. The inclusion of PMA bypasses (and excludes the necessity of) these early molecular events. Our work suggests that Nef can play a positive role in T cell activity when cellular stimulation occurs through these surface receptor pathways.

Previous examinations of Nef activity in T cells have generated diverse conclusions, but these experimental approaches included differences in the variable measured to define activation, the choice of mitogen, the choice of expression vector, and more subtle factors, such as limiting the examination to clonal isolates. That the Nef effect on T cell activation in our system, as defined specifically by IL-2 secretion, can be affected either neutrally or positively by the choice of mitogen (Fig. 2) exemplifies only one of the difficulties in comparing published works. The one previous report that examined CD3 and CD28 costimulation defined Nef as a neutral factor in affecting IL-2 secretion (16), but the four CD4-positive cell lines transduced with the cytomegalovirus (CMV) promoter-based Nef-expression vector used in that report did not display CD4 modulation, suggesting the bioactivity of Nef was very low. More recently, the CMV promoter in Jurkat cells has been shown to be inactive in the absence of inducers such as PMA (22).

A previous examination of the effect of Nef on a cloned human CD4 T cell demonstrated that the presence of a functional *nef* gene in an infecting HIV did not alter antigen stimulation as defined by cytokine (tumor necrosis factor α, IL-6, granulocyte/macrophage colony-stimulating factor, and IFN-γ) production (18). Because the clone depended on exogenous IL-2, production of this cytokine was not measurable. Our finding that Jurkat production of tumor necrosis factor α (and IL-8) was unchanged by Nef expression is in agreement with this previous human T cell study and suggests that Nef affects a specific pathway involved with IL-2 expression.

Several reports have demonstrated that Nef can suppress T cell activity (15, 19, 22, 23), which may depend on higher levels of Nef (22) and cytosolic location (15). Thus, the viral protein's capacity to suppress or enhance T cell activation may be related to different intracellular levels of Nef. This report includes an estimate for Nef concentration, around 1 ng per 5 million cells; however, no previous report on Nef-mediated effects has estimated Nef concentrations, so comparison of this variable is not possible at this time. The previous reports of T cell inactivation by Nef have used the cytomegalovirus (with PMA induction) (22), the simian virus 40 (15), SRα (SV40/HTLV) (19), and CD3-β (23) promoters. In contrast, this work made use of the Moloney retroviral long terminal repeat. Although HIV infection leads to dysfunction of peripheral CD4 T cells, it occurs in the absence of direct infection of the cell (44); thus, there is no anticipated direct role for Nef in this process. Although we do not know at this time the mechanisms for the different effects of Nef on T cell activity, the *in vivo* and *in vitro* characterization of Nef-positive virus replication suggests that suppression of cell activation is not a dominating feature.

The ability of a cytosolic protein to enhance and to suppress an activation pathway is not unprecedented. The cloning and overexpression of the JNK (c-jun amino-terminal kinase)-interacting protein 1 (JIP-1) was found to sequester and inhibit JNK activity (45). Physiological levels of the JIP-1 adapter protein were found later to enhance JNK activity (46). Whether this example serves as a model for Nef is unknown. Furthermore, what constitutes a physiologically relevant Nef concentration is problematic. Primary T cells inoculated with HIV in the absence of mitogen fail to replicate virus; however, a minor population of S phase cells express the Nef transcript (47). Under these conditions Nef protein will not be expressed in most cells, and the concentration may not be determinable by today's methods. An assay that can determine Nef concentrations at the cellular level will be necessary to test this hypothesis. Typically, studies of HIV infection of primary cells require the use of mitogens such as PHA. Under these conditions the enhancing activity of Nef in T cells may not be evident (see Fig. 2).

Infection of human peripheral blood lymphocytes by HIV-1 renders T cells hypersensitive to T cell receptor and CD28 stimulation (9). Presently, two HIV proteins other than Nef have been shown to enhance T cell activation. Association of the viral envelope with surface CD4 has been demonstrated to

activate pathways in both primary T cells and T cell lines (48–50). Another HIV regulatory protein, Tat, enhances IL-2 secretion in both Jurkat and primary T cells (9, 51). Therefore, a system that expresses only Nef would be important to delineate its specific biochemical capacity. Such a system is also physiologically relevant. Early in HIV infection after mitogenic stimulation, Nef is the predominant transcript (10, 11). Furthermore, as mentioned above, a minor subpopulation of primary T cells inoculated with HIV in the absence of mitogen expresses the Nef transcript. For these reasons, to identify the effect of Nef on T cell activity, we have restricted expression to this one HIV protein.

How could the Nef expression, a proviral gene product, mediate enhancement of T cell activation to promote viral replication? (*i*) Nef is a virion protein (52, 53) and, thus, could affect cells during the entry process. (*ii*) Nef transcription may occur in the absence of integration (47, 54, 55), that is, at a time when T cell activation would lead to integration and viral production. (*iii*) The existence of a fully functional but silent HIV provirus in CD4 T cells (8) has been demonstrated in patients on highly active antiretroviral therapy (56, 57). It is of interest that CD3 plus CD28 stimulation was found to perform recovery of virus from these cells better than PHA.

Our findings suggest a specific interaction of Nef with the T cell receptor and costimulus receptor-signaling pathways. Additionally, these findings suggest a positive role for Nef in affecting a T cell state supportive of viral replication.

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