Nef protein of HIV-1 has B-cell stimulatory activity

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Objective: To examine the B-cell stimulatory properties of the regulatory Nef protein of HIV-1.

Methods: The effect of the HIV-1 regulatory proteins Nef, Tat and Vif, were analyzed for their ability to induce differentiation of normal B lymphocytes into immunoglobulin secreting cells (ISC).

Results: A recombinant Nef protein, but neither Tat or Vif, was able to induce ISC in peripheral blood lymphocyte (PBL) cultures of HIV-1-seronegative donors. Another recombinant Nef protein, d-Nef, with a truncated amino terminal (deletion of 34 amino acids) failed to induce B-cell differentiation. Pretreatment of the Nef protein with a polyclonal anti-Nef-antibody abrogated its B-cell stimulatory activity. The Nef-induced B-cell differentiation was dependent on cell-to-cell contact. Cell surface molecules leukocyte function-associated molecule (LFA)-1, intracellular adhesion molecule (ICAM)-1, human lymphocyte antigen-DR and B7 were involved in the T–B-cell interaction because monoclonal antibodies to these molecules abrogated the Nef-induced B-cell differentiation response. The Nef protein was able to induce interleukin (IL)-6 messenger (m)RNA and IL-6 protein secretion in PBL, with monocytes as the primary source.

Conclusions: These findings indicate that regulatory (Nef) proteins of HIV-1 contribute to the intense B-cell activation that occurs in association with HIV-1 infection. T–B-cell contact-dependent interaction and induction of IL-6 by these proteins appear to play major roles in this process.

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Introduction

AIDS is characterized by a profound loss of cell-mediated immune functions, and the depletion of CD4+ T-helper cells [1,2]. Early in the course of infection, there is intense B-cell activation manifested as elevated levels of serum immunoglobulins, presence of circulating immune complexes, autoantibodies and increased numbers of spontaneous immunoglobulin secreting cells (ISC) [3–5]. The B-cell hyperactivity has been attributed, at least in part, to *in vivo* stimulation of B cells by HIV-1, resulting in polyclonal [6–9] and HIV-1-specific antibody secretion [10,11].

We have demonstrated previously that a soluble protein extract of HIV-1 and its purified native envelope glycoprotein, gp160, both have the ability to induce terminal differentiation of normal peripheral blood B lymphocytes into ISC [6,9].

The mechanism of the gp160-induced B-cell differentiation appears to involve interleukin (IL)-6 secretion and T–B-cell contact. In this study, we examined the HIV-1 regulatory proteins, Nef, Vif and Tat, for their activity on normal peripheral blood B cells from HIV-seronegative healthy volunteers.

The Nef protein has a molecular weight of 27 kD and is encoded by a single open reading frame that overlaps the 3'long terminal repeat. The protein is myristylated and localized in the cytoplasm of HIV-1-infected cells, associated intracellularly with the cell membrane [12]. The *nef* gene product is highly immunogenic as demonstrated by the induction of antibodies to *nef in vivo* preceeding those to structural proteins Env and Gag [13,14], and the devel-

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opment of Nef-specific cytotoxic T cells [15–17]. The function of the Nef protein is not well understood. Its proposed function as a negative regulator of HIV-1 replication [18,19] has been refuted by some investigators [20,21]. Recent studies of the protective effects of live SIV vaccine with Nef-deletion mutants [22] have prompted re-examination of the functional properties of the *nef* gene and its product. In this study we demonstrated that Nef proteins have the ability to induce polyclonal B-cell differentiation; this activity is dependent upon T–B-cell contact-dependent interaction and secretion of IL-6 by monocytes.

Materials and methods

HIV-1 regulatory proteins, recombinant Nef (designated PC25) and Vif proteins from $HTLV_{IIIB}$, were prepared in *Escherichia coli*. d-Nef is a truncated Nef protein with a deletion of 34 amino acids at the N terminal (gift from J. Ghrayeb, Centecor, Malvern, Pennsylvania, USA). Soluble proteins of HIV-1 (IIIB) and Nef-deleted mutants (HIV_{X10-1}) [23] were purified by sucrose density gradient centrifugation from culture supernatants of H9 cell cultures as described previously [6]. All recombinant and synthetic peptides studied were tested for and found to be free of endotoxin using the Limulus amoebocyte lysate assay (E-TOXATE, limit of detection 0.06 EU/ml; Sigma, St Louis, Missouri, USA). The protein content was estimated using the BCA protein estimation kit (Pierce, Rockford, Illinois, USA). Recombinant Nef protein derived in *E. coli* and purified by gel filtration on Superose-12 (FPLC), was also obtained from the AIDS Reference Reagent Program, Bethesda, Maryland, USA. Synthetic Tat peptides (amino acids 1-72), which have been shown to have transactivating properties, were a gift from Dr S. Khan (Wistar Institute, Philadelphia, Pennsylvania, USA). All the experiments were performed with PC25 and initial results were confirmed using the Nef protein obtained from the AIDS Reference Reagent Program as specified.

Cells and culture conditions

Peripheral blood lymphocytes (PBL) from healthy seronegative donors were isolated by Ficoll-Hipaque density gradient centrifugation. T cells were purified from PBL using a double rosetting procedure with neuraminidase-treated sheep red blood cells (sRBC), followed by passage of the rosetting cell fraction through a T-cell column (Beckmann, Fullerton, California, USA). The resulting cell population was >98% CD3+ [T3 monoclonal antibody (MAb), Coulter Immunology, Hialeah, Florida, USA] by flow cytometry. B cells were enriched by treating the non-rosetting cell fractions with anti-CD2 MAb and newborn rabbit complement to remove contaminating T cells.

The enriched B-cell fraction contained 12% myeloperoxidase positive cells and 97% CD19+ (B4 MAb; Coulter Immunology) lymphocytes. In some experiments, PBL were depleted of cells adhering to plastic prior to separation of T and B cells; adherent cells were collected by scraping the plastic with a rubber instrument. The B-cell fraction was designated to be purified B cells. PBL or combinations of enriched/purified B and T cells were cultured in the presence of various concentrations of test proteins for 7 days. To study the T–B-cell interaction, T and B cells were cultured in the upper and lower chambers, respectively, of Transwell plates (Costar, Cambridge, Massachusetts, USA) in the presence of Nef proteins. To prevent active cytokine secretion, T cells pulsed for 24 h with 0.01 μg/ml Nef (TNef) were fixed with 1% paraformaldehyde (PFD) in phosphate-buffered saline (PBS) as described previously [24].

Fixation was stopped by the addition of 0.06% glycylglycine (Sigma) and washing.

Any possible leaching of PFD was accomplished by incubating the cells for 60 min at 37°C, followed by washing with PBS and resuspension in fresh culture medium. ISC were quantified by reverse hemolytic plaque assay (RPA) [6] and IgG secreted in the culture supernatants was quantitated by enzyme-linked immunosorbent assay (ELISA) [9] as described below.

In some experiments Nef proteins were pretreated with a 1 : 1000 dilution of normal rabbit serum (NRS) or various dilutions of polyclonal rabbit anti-Nef antibody (AIDS Reference Reagent Program) for 60 min at 37°C.

To study the effect of blocking protein synthesis, protein synthesis inhibitors, puromycin, or cycloheximide (Sigma), were added to the cultures 24 h prior to termination.

To determine which cell surface molecules are involved in T–B-cell interactions, MAb to specific cell surface molecules were added to cultures of T and B cells in the presence of Nef. MAb to the following molecules were used: human lymphocyte antigen (HLA)-DR (Becton Dickinson, Mountainview, California, USA), LFA-1α (TS/22), LFA-1β (TS/18), ICAM-1 (RR-1; Dr T. Springer, Center for Blood Research, Boston, Massachusetts, USA); B7 (BB-1; Dr E.A. Clark, University of Washington, Seattle, Washington, USA), CD7 (3A1; Dr L. Jung, University of Worcester, Massachusetts, USA), CD44 (Hermes-3; Dr E. Butcher, VA Medical Center, Palo Alto, California, USA), and IL-6 (Genzyme, Boston, Massachusetts, USA). The ability of LFA-1α, LFA-1β, HLA-DR, ICAM-1 and B7 to block T-cell dependent B-cell differentiation has been demonstrated previously [24,25]. Identification of cell surface molecules

on cultured or uncultured cells was performed by staining the cells with fluorescence isothiocyanate (FITC)- and phycoerytherin (PE)-conjugated MAb.

Reagents used included HLA-DR-FITC and IL-2R-PE MAb (Becton Dickinson). Fluorescence was analyzed on an EPICS C flow cytometer (Coulter).

B-cell differentiation assays

The reverse hemolytic plaque assay was performed as described previously [6]. Briefly, 0.2 ml test cells (0.5×10^6/ml) were added to 0.7 ml 0.5% noble agar-containing DEAE-dextran, together with 0.05 ml 20% sRBC in Hank's balanced salt solution, and 0.05 ml 1:4 guinea-pig complement. The mixture was placed on petridishes in triplicates and covered with coverslips. The formation of plaques were counted after incubation for 3 h at 37°C. Results are expressed as the number of plaques per 10^6 cells plated.

Polyclonal IgG secreted in the culture supernatants were measured by ELISA as described previously [9]. Briefly, 100 µl of appropriately diluted test supernatants were incubated on anti-human IgG-coated microtiter plates for 2 h at 37°C. After the addition of alkaline phosphatase-conjugated anti-human IgG, color development of the phosphate substrate was measured on the ELISA reader (Molecular Devices, Menlo Park, California, USA), and the amount of Ig was calculated by the softmax ELISA program (Molecular Devices).

Northern blot analysis

PBL cultures were incubated with Nef proteins in the presence or absence of rabbit anti-Nef antibodies. Total cellular RNA was extracted and Northern blot analysis was performed as described previously [26] with cDNA probes for IL-6 (gift of Dr P. Sehgal, Rockefeller University, New York, New York, USA) and 28s RNA probe (American Type Culture Collection, Rockville, Maryland, USA).

IL-6 bioassay

The B lymphoblastoid, IL-6-dependent murine cell line B9, was a kind gift from Dr G. Tosato (Food and Drug association, Bethesda, Maryland, USA). Standard recombinant IL-6 (Genzyme) or test supernatants were added to 2×10^3 B9 cells.

B9-cell proliferation was determined by 14C-thymidine incorporation on day 3 as described previously [26].

Results

Nef proteins have B-cell stimulatory activity

Figure 1 shows that the PC25 Nef protein, but neither Vif nor a synthetic Tat peptide, was capable of inducing an increase in the number of ISC in the PBL cultures in a dose-dependent manner, with a maximal response at 0.01 µg/ml. The results were confirmed with another preparation of recombinant Nef protein, obtained from the AIDS Reference Reagent Program. The specificity of the Nef-induced response was determined using a polyclonal rabbit anti-Nef antiserum. Addition of this antiserum, but not normal rabbit serum, abrogated Nef-induced IgG production in PBL cultures (from 64 + 3 ISC/10^6 cells to 10 + 2 in the presence of a 1:10 dilution of rabbit anti-Nef antibody). Treatment of PBL with inhibitors of protein-synthesis, puromycin and cycloheximide, also abrogated this response (data not shown), suggesting that the Nef protein actively induces Ig secretion in PBL.

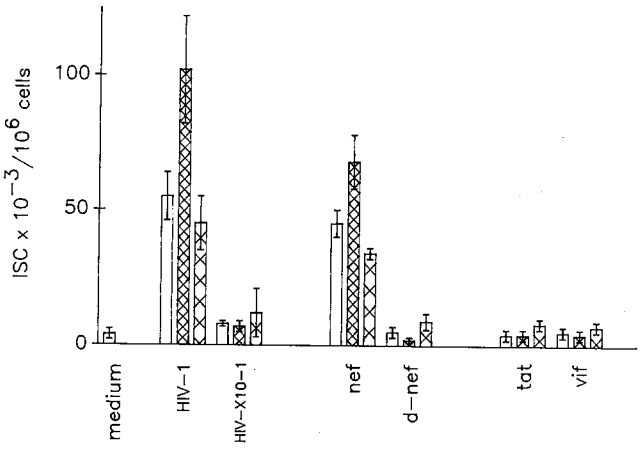

Fig. 1. Nef proteins induce B-cell differentiation. Peripheral blood lymphocytes were cultured in the presence of test proteins at various concentrations for 7 days. Nef indicates the PC25 protein (see Materials and methods). B-cell differentiation was determined by reverse hemolytic plaque assay. Results shown represent mean and SD of eight separate experiments. □, 0.1 µg/ml; ▩, 0.01 µg/ml; ☒, 0.001 µg/ml. ISC, immunoglobulin secreting cells.

Table 1. Nef induces B-cell differentiation but does not induce proliferation.

	Proliferation	Differentiation	
Stimuli	^{14}C-thymidine incorporation	ISC 10^6 cells	ELISA IgG (ng/ml)
PWM	12037 ± 601 (3)	403 ± 49 (20)	11295 ± 2067 (3)
EBV	6048 ± 170 (3)	251 ± 31 (7)	4187 ± 210 (3)
Nef (PC25)	32 ± 16 (3)	60 ± 11 (6)	906 ± 107 (3)
Medium	28 ± 17 (3)	4 ± 3 (20)	29 ± 18 (3)

Peripheral blood lymphocytes were cultured in the presence of pokeweed mitogen (PWM), Epstein–Barr virus (EBV), 0.01 µg/ml Nef protein or medium alone for 7 days. Proliferative responses were measured by incorporating ^{14}C-thymidine on day 3; B-cell differentiation was determined by measuring the number of immunoglobulin secreting cells (ISC) by the reverse hemolytic plaque assay or the amount of immunoglobulin (Ig) G secreted in the culture supernatants by enzyme-linked immunosorbent assay (ELISA) on day 7. Results show mean and SD of several experiments, denoted in parenthesis.

Table 2. Separation of T and B cells abrogates Nef-induced B-cell differentiation.

Transwell chamber			Differentiation
Lower	Upper	Nef	IgG (ng/ml)
B	T	–	41±3
B	T	+	40±8
B+T	None	–	104±2
B+T	None	+	846±81

T cells (1×10^5) and enriched B cells (with monocyte macrophages; 1×10^5) were cultured together or in separate chambers of Transwell 24-well plates (membrane pore size, 0.4 μm) in the absence (–) or presence of 0.01 μg/ml Nef (+). The results are a representative of three separate experiments.

Table 1 shows that the PC25 Nef protein could induce an increase in the number of ISC and also increase the IgG secreted in culture supernatants. The Nef-induced B-cell differentiation was 12 and seven times less than that induced by pokeweed mitogen (PWM) and Epstein–Barr virus (EBV), respectively. Both of the Nef protein preparations induced a significant proliferative response. Subsequent experiments were performed only with the PC25 Nef protein.

As we have demonstrated previously [6], soluble proteins of HIV-1 induce *in vitro* B-cell differentiation. However, soluble proteins of a Nef-deleted mutant of HIV [23] failed to induce B-cell differentiation. Furthermore, a recombinant Nef protein (d-Nef) lacking the 34 amino acids from the N terminal also lacked B-cell stimulatory activity (Fig. 1). These observations suggest that the N terminal of Nef may be necessary for B-cell stimulatory activity. However, none of the five synthetic Nef peptides in this region (4–18, 20–31, 10–25, 36–46, 37–50; American Biotechnology, Cambridge, Massachusetts, USA) induced IgG secretion by PBL (data not shown).

Nef-induced B-cell differentiation is dependent on T cells and monocyte-macrophages.

Figure 2a shows that the induction of B-cell differentiation by Nef protein is dependent upon the presence of T cells in the cultures. sRBC non-rosetting, B-cell enriched fractions (containing monocytes/macrophages), were cultured with increasing numbers of purified autologous T cells in the presence of Nef protein. Nef could not induce the B-cell plus macrophage cell fraction alone to secrete IgG. Maximal Ig secretion was obtained at a B and T-cell ratio of 50:1, and no further increase occurred with additional numbers of T cells.

Removal of monocyte/macrophages from PBL by adherence to plastic petridishes significantly lowered the B-cell stimulatory activity of Nef, even in the presence of T cells (data not shown).

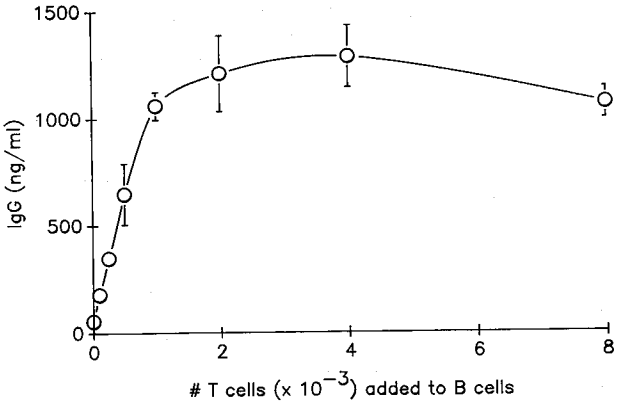

Fig. 2. Nef-induced B-cell differentiation is T-cell dependent. 5×10^4 sheep red blood cell (sRBC) non-rosetting B-cell fractions (containing monocyte macrophages) were cultured with increasing numbers of purified autologous T cells in the presence of Nef proteins for 7 days. Immunoglobulin (Ig) G secreted in the culture supernatants was determined by enzyme-linked immunosorbent assay. Results shown are a representative of four different experiments.

Nef-induced B-cell differentiation is dependent upon T–B-cell contact

Physical contact between T and B cells was required for Nef to induce polyclonal B-cell differentiation. Purified T cells were cultured in the upper chambers and enriched B cells (containing adherent cells) in the lower chambers of Transwell plates (Costar). Addition of Nef protein resulted in Ig secretion only if T and enriched B cells were cultured in the same chamber (Table 2).

To determine whether Nef-activated T cells were capable of inducing B cells to secrete Ig, purified T cells were cultured with Nef proteins for 12–14 h overnight at 37°C and washed. Flow cytometric analysis of T cells cultured with Nef protein demonstrated an increase in the percentage of cells expressing IL-2 receptor (from 4.6 to 8.3%; n = 3) and HLA-DR (3.8 to 10.6%; n = 3) molecules. No change in the expression of the CD4 molecule was observed at the concentrations of Nef protein used (data not shown).

Nef-activated T cells (T-Nef) failed to induce B cells to secrete IgG. Ig secretion was induced in cultures of B-enriched cells plus Nef-activated T cells when Nef protein was continuously present in the culture (Table 3).

Cell surface molecules involved in contact-dependent T–B-cell interaction

Figure 3 shows experiments in which T cells were cultured with B cells in the presence of Nef and MAb to various cell surface molecules. Addition of appropriate concentrations of MAb to LFA-1α, LFA-1β, ICAM-1, HLA-DR to the cultures abrogated B-cell differentiation responses of B cells; MAb to B7 partially diminished the response, while MAb to CD44, CD7 and normal mouse IgG had no effect on the B-cell response elicited by Nef.

Table 3. Fixation of Nef-acativated T cells with paraformaldehyde abrogates Nef-induced B-cell differentiation.

B cells cultured with	Additions*	Differentiation IgG (ng/ml)
T cells	Medium	30±16
T cells	Nef	498±27
TNef†	Medium	68±3
TNef	Nef	536±42
TNef-PFD‡	Nef	40±3
TNef-PFD	Nef+IL-6	565±24

Enriched B cells (1×10^5) were cultured with T cells (1×10^5) that were untreated or treated with 0.01 μg Nef protein for 12–14 h at 37°C and washed; these T cells are designated as TNef cells. *Medium/Nef (0.01 μg/ml)/interleukin (IL)-6 (100 units/ml); †B cells cultured with TNef alone did not induce immunoglobulin (Ig) G secretion, unless Nef was present continuously in the culture; ‡TNef cells fixed with paraformaldehyde (PFD) as indicated in Materials and methods.

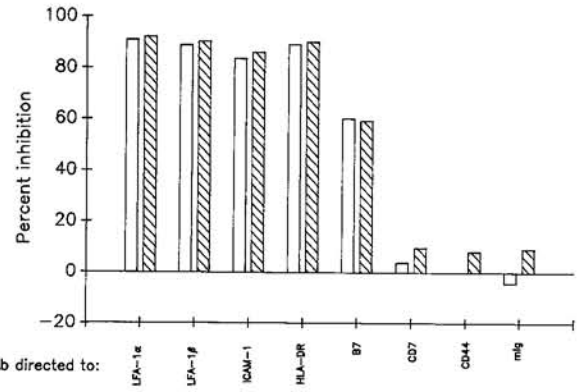

Fig. 3. Cell surface molecules involved in Nef-induced B-cell differentiation: T cells were cocultured with sheep red blood cell (sRBC) non-rosetting B-cell enriched cell population (containing monocyte macrophages) in the presence of 0.01 μg/ml Nef. The involvement of various cell surface molecules was investigated by adding monoclonal antibodies (MAb) to the cell surface and secretory molecules (see Materials and methods). Immunoglobulin (Ig) G secretion was measured by enzyme-linked immunosorbent assay. □, experiment 1; ▨, experiment 2. LFA-1, leukocyte function associated molecule-1; ICAM-1, intracellular adhesion molecule-1; HLA, human lymphocyte antigen.

Nef proteins induce IL-6 mRNA induction and IL-6 secretion

The ability of Nef proteins to induce B-cell stimulatory cytokine IL-6 was examined. Addition of Nef proteins to PBL cultures resulted in the expression of IL-6 mRNA, 6 h after stimulation (Fig. 4). The Nef-induced expression of IL-6 mRNA could be abrogated by pretreating Nef proteins with a polyclonal rabbit anti-Nef antibody (Fig. 4, lane 4). Analysis of IL-6 secretion by the proliferation of B9 cells, showed that PBL rested for 72 h secreted <0.1 units/ml IL-6, which increased to 3.8±0.2 units/ml IL-6 (mean±SD of three experiments) in the presence of 0.01 μg/ml Nef protein. To identify the cell source of IL-6 secretion, purified T, B and adherent cells were cultured with Nef protein. The adherent cell fraction was most active in secreting IL-6 in the presence of Nef proteins. IL-6 secretion was undetectable in culture supernatants of purified T cells or purified B cells stimulated with Nef proteins (data not shown). Addition of antibodies to IL-6 abrogated the Nef-induced B-cell differentiation (Fig. 3); normal rabbit serum had no effect (data not shown). Finally, Table 3 shows that fixation of Nef-activated T cells abrogates B-cell differentiation; the addition of exogenous IL-6 restores IgG secretion.

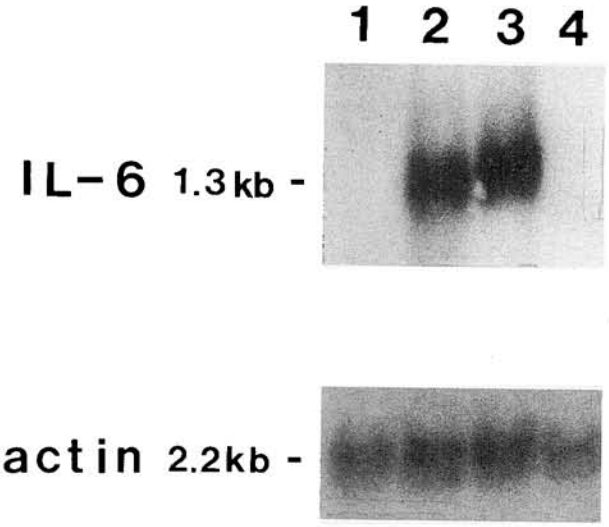

Fig. 4. Induction of interleukin (IL)-6 mRNA by Nef proteins: peripheral blood lymphocytes (PBL) were cultured for 72 h in RPMI 1640 plus 10% fetal calf serum before addition of Nef proteins. Northern blot analysis was performed on PBL cultures incubated with Nef proteins in the presence or absence of rabbit anti-Nef antibodies. Lane 1: unstimulated (medium alone); lane 2: 0.1 μg/ml nef; lane 3: 0.01 μg/ml Nef; lane 4: 0.01 μg/ml Nef pretreated with 1:1000 dilution of rabbit anti-Nef antibody.

Discussion

We have demonstrated that proteins encoded by the *nef* gene of HIV induce differentiation responses in normal B lymphocytes. The other regulatory proteins of HIV Vif and Tat did not manifest this activity. The mechanism involves T–B-cell contact-dependent interaction and IL-6 secretion by macrophages.

The function of the *nef* gene, which encodes for a 27 kD protein in regulating viral replication is at present undetermined. It has been proposed that Nef is involved in negative regulation of viral replication by slowing the transcription of viral genome [18,19] and that expression of the *nef* gene product maintains the latency of HIV-1 provirus *in vivo*; however, this function of Nef has not been universally accepted [20,21]. The influence of Nef protein

on host immunological responses is not well understood. Although the amount of Nef *in vivo* is not known, it has been suggested that this regulatory protein plays a significant role in the pathogenesis of HIV infection because circulating antibodies to Nef [13,14] have been identified in HIV-1-seropositive individuals prior to the appearance of antibodies to Env and Gag. In addition, several investigators [15–17] have demonstrated the presence of MHC class I-restricted Nef-specific cytotoxic T cells in seropositive individuals. *In vitro* experiments with the *nef* gene have also demonstrated the possible role of Nef in modulating immune response. To this effect, transfection of the *nef* gene in certain CD4+ T-cell lines has been shown to down-modulate CD4 expression [27], inhibit T-cell receptor-induced IL-2 mRNA induction [28], and inhibit the induction of nuclear binding proteins, NF-kB [29]. This study demonstrates that Nef proteins are also capable of inducing terminal differentiation of normal B cells into ISC. Other HIV-regulatory proteins tested, Vif and a synthetic Tat peptide, failed to induce B-cell differentiation.

Since Nef failed to induce proliferation of PBL, DNA replication may not be essential for its stimulatory effect; however, because cycloheximide blocks Nef-induced B-cell differentiation, RNA transcription leading to protein synthesis is presumably required.

The induction of B-cell differentiation by Nef protein was specific to the Nef protein in that polyclonal anti-Nef antibodies abrogated the response. In addition, soluble proteins of the Nef-deleted mutant (X10-1) failed to induce B cells to differentiate into ISC. A recombinant protein, produced in the same system as wild type Nef, d-Nef with a deletion of 34 amino acids of the amino terminal of Nef, was unable to induce B-cell differentiation. However, since none of the synthetic linear Nef-peptides including the amino terminal induced B-cell differentiation, it appears that the conformational structure of the Nef protein is important for this activity.

It should be noted that although the Tat synthetic peptide used in this study did not induce B-cell differentiation, it is possible that full-length Tat may function differently.

The induction of B-cell differentiation is a complex process that is initiated and regulated by T lymphocytes. Although terminal B-cell differentiation requires both contact-dependent interaction and cytokine-mediated signals from T cells, intermediate stages of B-cell differentiation can be induced by activation-induced T-cell surface molecules in the absence of soluble factors. In this study, pretreatment of T cells with Nef increased IL-2R and HLA-DR expression, suggesting that Nef could directly activate T cells. However, T cells pretreated with Nef inefficiently aided B cells in the secretion of Ig. In addition to T cells, monocytes were also essential for the B-cell response and depletion of adherent cells abrogated Nef-induced B cell differentiation. Thus, both T cells and macrophages required exposure to Nef for optimal induction of B-cell activity.

This study utilizing semi-permeable membranes to prevent cell-to-cell contact but permitting passage of soluble factors has demonstrated that the Nef-mediated B-cell response is dependent upon T–B-cell contact. It has been suggested that the induction of T-cell dependent B-cell responses involves the occurence of a series of sequential interactions of surface molecules on T and B cells [30–35]. In the Nef-induced B-cell responses, interaction between LFA-1 and ICAM-1 appeared to be essential, because MAb to either molecules abrogated the response. The CD28–B7 interaction also appeared to be involved, but was not essential as MAb to B7 reduced but did not abrogate the B-cell response.

Secretion of IL-6 appeared to be the main mechanism of the monocytes. Increased levels of IL-6 in plasma and spontaneous IL-6 mRNA expression in PBL have been demonstrated in patients with HIV-1 infection [36] and has been suggested that induction of IL-6 by HIV-1 plays a major role in HIV-1-associated hypergammaglobulinemia [37,38]. Addition of Nef proteins to PBL cultures induced expression of IL-6 mRNA in 6 h and IL-6 protein in culture supernatants in 24–48 h. The absence of endotoxin contamination and the ability of anti-Nef antibodies to abolish Nef-induced IL-6 mRNA induction indicates that the Nef effect was specific and not caused by contamination with LPS. The inhibitory effect of anti-IL6 antibodies on Nef-induced B-cell differentiation suggests a direct role for IL-6 in the B-cell stimulatory effects of Nef. Finally, the ability of exogenous IL-6 to restore IgG secretion in enriched B cells in the presence of PFD-fixed Nef-activated T cells implies that IL-6 plays a role in Nef-induced B-cell differentiation.

In conclusion, we have demonstrated that HIV Nef has the ability to induce normal B cells to differentiate into Ig secreting cells *in vitro*. It is possible that the Nef protein contributes to the generalized B-cell hyperactivity in HIV infection. Understanding the mechanism of HIV-1-induced B-cell activation is important for unravelling the pathogenesis of the B-cell defect in HIV-1 infection.

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