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Functionally-Impaired HIV-1 *Nef* Alleles from a Mother-Child Transmission Pair

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Abstract: Unusual HIV-1 *nef* alleles were isolated from a woman and her vertically infected child. Both patients eventually progressed to develop AIDS. The child died at age 6.5 years, while the mother is currently alive, 13 years since her diagnosis with HIV-1. Predicted amino acid sequences of both mother and child Nefs diverged from the HIV-1 clade B consensus. In particular, they exhibited two separate 5-amino acid deletions bracketing a C-terminal dileucine regulatory motif and Trp-Gly mutations at the site for cleavage by the HIV-1 protease. The child's Nef showed a modest ability to enhance HIV-1 infectivity in MAGI cells, whereas the mother's Nef did not alter HIV-1 infectivity in the assay. Both Nefs were partially functional for CD4 down-regulation. The child's Nef was fully functional for MHC-1 down-regulation, while the maternal Nef was non-functional. To our knowledge this study is the first to describe a functional divergence between Nef alleles in a case of mother-to-child HIV-1 transmission.

Keywords: HIV-1, Nef, *in vitro*, CD4, MHC, infectivity.

Introduction

The *nef* genes of human immunodeficiency (HIV-1) and simian immunodeficiency (SIV) viruses encode 27-34 kd myristoylated proteins, which are expressed early after establishment of the provirus in host cells [1]. Although largely dispensable for viral growth in cell culture [2], the expression of Nef results in increased viral pathogenicity and higher viral loads both in humans [3,4] and in animal models for HIV/SIV infection [5-7]. Correspondingly, Nef-deleted SIV exhibits reduced pathogenicity when used to infect rhesus macaques [5]. Also, *nef*-deleted HIV-1 variants have been isolated from long-term survivors (LTS) of HIV-1 infection [8-10]. A number of LTS individuals remained healthy more than 10 years after a diagnosis of HIV-1 infection in the absence of ongoing antiretroviral therapy [9]. For many of these patients, the lack of expression of Nef was the only observable attenuation of the HIV-1 virus. Additionally, it was demonstrated in transgenic mouse models for HIV-1 infection that expression of the HIV-1 nef gene increases cytotoxic and pathogenic effects [11,12].

Although the mechanism(s) for Nef's effects on HIV-1 pathogenesis are not fully understood, a considerable amount of information has been obtained from structural, functional and genetic analyses. First, Nef is present in the HIV-1 viral core where it may influence core disassembly and enhance viral entry into the cytosol [13] and may also influence the rate of proviral DNA synthesis [14,15]. Second, production of Nef during the viral infectious cycle results in decreased expression of both the CD4 antigen [16,17] and some major histocompatibility class I (MHC-I) [18] antigens on the surface of HIV-1-infected cells. These functions are genetically separable, as mutations have been generated that abolish either the CD4 or MHC-I down-regulation but do not affect the infectivity enhancement properties of Nef [19-21].

The presence of naturally occurring mutations of *nef* has been examined in several studies of both pediatric and adult HIV-1-infected patients [22-24]. Results of these studies suggest that the accumulation of short deletions in the *nef* gene as well as changes in individual Nef amino acids may result in partial loss of Nef function.

We report here the characterization of two HIV-1 *nef* alleles from a HIV-1-positive mother and her child who acquired the HIV-1 virus by mother-to-child transmission. These *nef* genes encoded 193-amino acid (aa) Nef proteins, shorter than the 206 aa HIV-1 clade B Nef consensus sequence [25]. The Nef proteins expressed by these HIV-1 *nef* alleles were shown to be stable when expressed in mammalian cells, and retained partial function in *in vitro* Nef assays for HIV-1 infectivity and regulation of CD4 and MHC-I antigens on the surface of infected cells.

Methods

Cell cultures and plasmids

293 cells were cultured in DMEM supplemented with 10% Fetal Bovine serum (FBS). MAGI cells [26] were maintained in DMEM supplemented with 5% FBS, 0.1 mg/ml G418, 0.05 mg/ml

Hygromycin B. J-Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1 mM HEPES. All media were supplemented with 0.29 mg/ml glutamine, and 100 units each of penicillin and streptomycin. Cell culture media and supplements were obtained from Gibco/BRL (Life Technologies, Gaithersburg MD). The full-length HIV-1_{HXB2} and HIV-1_{NL4-3} plasmids were obtained through the AIDS Reagent program. The HIV-1_{IIIB} envelope expression plasmid, pENVIII [27], was a gift from Dr. Eric Freed (Laboratory of Molecular Microbiology, NIAID, NIH).

PCR amplification and cloning

The *nef*-encoding portion of HIV-1 was amplified from proviral DNA in crude lysates of mononuclear cells (PBMC), as described in Roth et al. [28], using a nested polymerase chain reaction (PCR) protocol. Primers for the first round PCR were: NEF2 forward outer- 5'-TTC GCC ACA TAC CTA GAA GAA TAA GA-3'; NEF2 reverse outer- 5'-CCG CCC AGG CCA CGC CTC CCT-3'. Each reaction contained 20 µl of PBMC DNA (about 1 µg) in 10 mM TrisCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 200 µM nucleotide triphosphates, 2 µM of each primer, and 2.5 units Taq DNA polymerase per 100 µl reaction. Thermocycler parameters were: 95^{o}C for 30s, 55^{o}C for 45s, 72^{o}C for 2 min, for a total of 35 cycles. Five microliters of the first round reaction was used as template for a second round PCR, with nested primers under identical reaction conditions. Inner primers were: NEF2 forward inner- 5'-TTG CTA TAA GAT GGG TGG CAA GTG-3'; NEF2 reverse inner- 5'-CGG AAA GTC CCT TGT AGC AAG CTC-3'. The Nef PCR products were resolved in 1 % agarose gels, from which the *nef*-specific bands were excised, purified and cloned in the pCR3.1 vector (Invitrogen, Carlsbad, CA). Nef clones were sequenced using fluorescent dideoxy-nucleotide terminators with ABI Prism reagents and the ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequences for HIV-1 *nef* alleles C28B and M29 were submitted to the Genbank sequence database, and have been assigned accession numbers AF397200 and AF397201, respectively.

The influenza hemagglutinin HA-7 epitope (YPYDVPDYASL) was introduced into each clone to provide an additional option for detection of the expressed Nef proteins. First, synthetic oligonucleotides were annealed to form the double-stranded HA-7 sequence with a *Bsi*W I site at the 5' end and a blunt 3' end. This was ligated to pCR 3.1 pre-digested with *Hin*d III and *Eco*R V. The *nef* alleles were then re-amplified with primers containing the appropriate sites (5' *Hin*d III-Nef and 3' *Bsi*W I-HA). PCR amplicons were digested with *Hin*dIII and *Bsi*WI, and ligated into the HA-tagged vector. The resulting construct expressed the HA-7 epitope at the C-terminus of the Nef peptide.

MAGI-cell HIV-1 infectivity assays

Stocks of viral particles pseudotyped with the HIV-1_{IIIB} envelope and trans-complemented with control or patient *nef* alleles were produced by three way transfection in HeLa cells. The HIV-1 clone pNL4-3 KFS (Δnef, Δenv) was transfected with the pENVIII envelope vector and the Nef plasmid

clone to be tested using the Effectene reagent (Qiagen, Valencia, CA). The resulting viral particles were infectious for a single round of replication.

MAGI cells [26] were then infected with equivalent amounts of viral particles (100 pg p24). Dishes containing infected cells were incubated 18 h to allow the expression of proteins from plasmid clones, at which time the supernatants were removed and assayed for HIV-1 p24. The cells were fixed and stained with the β-galactosidase indicator substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside). The number of blue cells in each dish was enumerated as a function of the input virus. Duplicate dishes were plated for each virus stock.

CD4 antigen assays

The assays for CD4 down-regulation were performed as described previously [19], with the following modifications: Nef plasmids were co-transfected into 293 cells with both CD4 and IL-2 receptor α (CD25) plasmids using calcium phosphate. Control and experimental transfections were incubated 6 h before the addition of fresh DMEM and were done in triplicate. After allowing 24-36 hr for expression of protein from transfected DNA, the 293 cells were harvested, washed and stained with antibodies to CD4 (CD4 tricolor, Caltag, Burlingame, CA) and CD25 (FITC-tagged, Becton-Dickinson, San Jose, CA). Cells were analyzed by flow cytometry (FACScan, Becton-Dickinson). CD25-positive cells were scored for relative expression of the CD4 antigen. Aliquots from each transfection experiment were analyzed by SDS/PAGE and Western blotting to nitrocellulose membranes. The expression of Nef was detected by incubation of blots with rabbit polyclonal anti-Nef serum, followed by incubation with horseradish peroxidase-tagged goat anti-rabbit serum (Sigma, St. Louis, MO). Nef bands were detected by chemiluminescence using the ECL reagents from Amersham (Indianapolis, IN).

MHC-I antigen assays

Nef-HA constructs were subsequently transferred into the pCI vector (Promega, Madison WI) for maximum expression in the J-Jurkat T-lymphocyte cell line. Jurkat cells were transfected with 36 μg of each Nef clone by electroporation. As a control to monitor transfection efficiency, we co-transfected 12 μg *Aequoria* (jellyfish) green fluorescent protein (GFP) plasmid. This construct was made by subcloning the *Hin*d III-*Not* I fragment of pEGFP-1 (Clontech, Palo Alto, CA) into pCR3.1, in order to produce pCR3.1-GFP. For transfections, log phase Jurkat cells were resuspended in RPMI (without serum) at a concentration of 8 x 10^6 cells per 400 μl aliquot. The cells were mixed with plasmid DNA, transferred into cuvettes (0.4 cm gap) and electroporated with a 0.25 kV, 960 μF capacitance pulse at a resistance of 200 ohms (Gene Pulser, BioRad, Richmond CA). The Jurkat cells were allowed to rest for 10 min at room temperature, transferred to a microcentrifuge tube containing 400 μl RPMI, and centrifuged at 3000 rpm for 5 min to pellet cells. After careful removal of the medium, the cells were resuspended in 1 ml RPMI and incubated 18 h at 37^o to allow expression of Nef proteins. The Jurkat

cells were stained with phycoerythrin (PE)-conjugated anti-MHC-I serum, (clone W6/32, DAKO, Carpinteria, CA) at a 1:100 dilution, and analyzed by FACscan, scoring for the mean PE-labeled fluorescence of the GFP-positive cell population.

Results

Patients and sample collection

Patient samples were originally collected in 1993-94, as part of a study of mother-to-child transmission of HIV-1. They consisted of two blood samples for the child, taken in September 1993 (sample C28A) and January 1994 (sample C28B), and a maternal blood sample (M29), which was obtained in September 1993, two weeks after the child's first sample. We described sequences for the C2-V5 region of the HIV-1 *env* gene in this mother-child pair in an earlier publication [28].

Isolation of nef alleles

The PCR amplification of the HIV-1 *nef* gene from proviral DNA samples from both mother (M29) and child (C28B), yielded amplicons that were visibly shorter than standard *nef* amplicons (not shown). We failed to amplify *nef* sequences from the C28A sample, possibly due to deterioration of the PBMC lysates during long-term storage at -20^{o} C. The nucleotide sequences for both patient alleles revealed open reading frames of 579 nucleotides with small in-frame deletions, predicting 193 amino acid (aa) Nef proteins, compared with the 206 aa HIV-1 subtype B Nef consensus [25]. When the predicted patient Nef amino acid sequences were aligned with HIV-1_{NL4-3} Nef and the HIV-1 subtype B consensus (Figure 1), two 5-aa deletions in the C-terminal portion of the Nef protein were observed. These short deletions flanked a conserved "dileucine" sequence motif [29] that is necessary for down-regulation of the CD4 antigen by Nef in HIV-1-infected host cells. This sequence, (ENNSLL, residues 160-165 for the HIV-1 subtype B consensus, see Figure 1), was conserved in the maternal M29, but was present in the child's C28B Nef as EDNSLI. There were other areas where the patient Nef proteins differed from the subtype B consensus, notably at the sequence at amino acid residues 57-58 (Fig. 1), which constitutes the cleavage site for the HIV-1 protease and is a potential CD4 cytoplasmic tail interaction site [19]. This dipeptide, Trp-Leu in the consensus Nef sequence, was changed to Gly-Leu in M29 Nef and to Gly-Val in the C28B Nef sequence.

The presence of the dileucine motif in an unusual context, together with additional mutations in the amino acid sequences of both patient Nefs led us to examine whether their activities might be altered in various assays of Nef function, including viral infectivity and down-regulation of cell surface CD4 and MHC-I antigens. Accordingly, the patient *nef* alleles were cloned in plasmid vectors suitable for expression in mammalian cells. Because we were uncertain if polyclonal anti- Nef sera would detect the expressed proteins, a C-terminal influenza virus hemagglutinin epitope (YPYDVPDYASL) was added to each Nef construct.

Figure 1. Clustal alignment of Nef sequences. Amino acid sequences for mother (M29) and child (C28B) Nefs were aligned with HIV-1 subtype B Nef consensus (Bcons) and Nefs HIV-1_{NL4-3} and HIV-1_{HXB2} using Clustal X. Dots indicate gaps inserted to facilitate alignment. Dashes indicate homology with B consensus. The stop codon in HIV-1_{HXB2} Nef is indicated by an asterisk.

HIV-1 infectivity

Nef -deleted HIV-1 virus particles were complemented with the patient Nefs and used to infect MAGI cells, HeLA-CD4 cells expressing β-galactosidase under the control of the HIV-1 LTR promoter, thus enabling colorimetric detection and enumeration of the HIV-1 infected cells. The infectivity of the patient Nef-complemented viruses was compared with that of intact HIV-1_{NL4-3} (Figure 2). The infectivity of intact HIV-1_{NL4-3} virus was arbitrarily set at 100 %. As a control for *nef* complementation the same amount (based on p24 antigen concentration) of *nef* -deleted HIV-1_{NL4-3} virus complemented with HIV-1_{NL4-3} *nef* was included. This virus was 85 % as infectious as intact HIV-1_{NL4-3}. Complementation with HIV-1_{HXB2} Nef (which produces a truncated Nef peptide) resulted in a viral preparation with 30 % infectivity relative to intact HIV-1_{NL4-3} virions. The pediatric Nef (C28B) was capable of a moderate enhancement of the infectivity of HIV-1_{NL4-3} ΔNef virus, whereas the maternal Nef variant M29 failed to display any activity in the assay. In addition, results of HIV-1 p24 assays on supernatants from these MAGI cell cultures were identical to, and confirmed the relative infectivity values for these viral preparations (data not shown).

CD4 antigen regulation

In the next set of experiments, we examined the ability of the patient Nefs to regulate the expression of the CD4 cell surface antigen. The *nef* clones were transiently co-transfected into 293 cells with

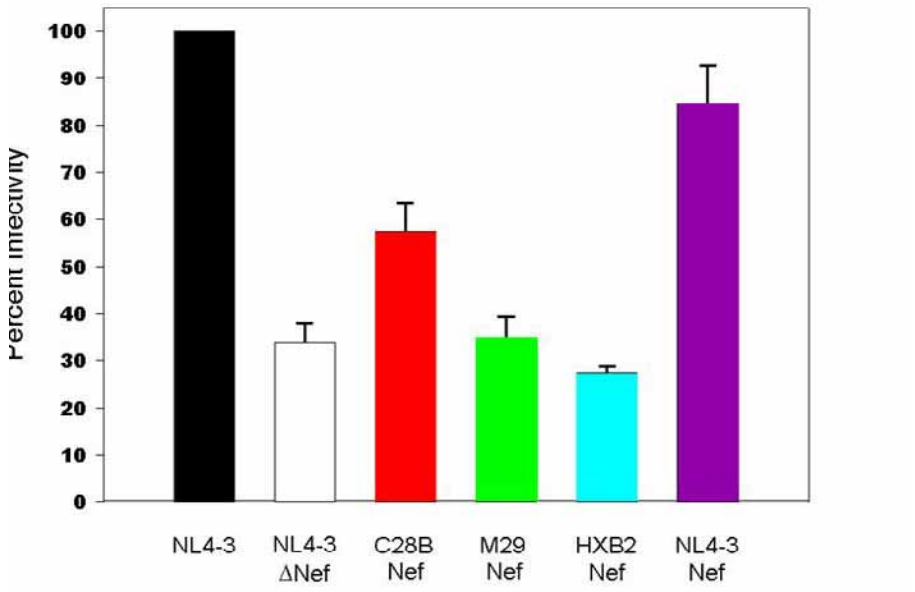

Figure 2. MAGI assay of mother and child Nefs. Average number of blue cells in three separate experiments, compared to values for intact HIV-1_{NL4-3} virions (100%). Error bars indicate standard error. From left to right: Intact NL4-3 virus, NL4-3-ΔNef (nef-deleted), NL4-3-ΔNef complemented with Nef C28B (child), NL4-3-ΔNef complemented with Nef M29 (mother), NL4-3-ΔNef complemented with Nef NL4-3.

plasmids expressing CD4 and CD25 antigens. This allowed us to assay for the relative expression of the CD4 receptor in CD25-positive cells as a function of the amount of the *nef* plasmid introduced. Introduction of the truncated HIV-1_{HXB2} Nef clone (Figure 3, top; open circles) did not affect CD4 expression. Alternatively, when two different versions of the HIV-1_{NL4-3} Nef plasmid were transfected (Figure 3, Nef NL4-3, open squares; and Nef NL4-3HA, filled circles) the level of CD4 was decreased in a dose-dependent fashion. Upon introduction of the patient Nefs (Figure 3, maternal Nef M29, filled squares; child's Nef C28B, diamonds) there was a very modest decrease in CD4 expression. At the highest amount of transfected plasmid (4 μg), both patient Nefs exhibited significant impairment of the ability to down-regulate CD4 (75 % of the Nef-minus control) when compared with the NL4-3 Nefs (12-25 % of the Nef-minus control). Significantly, the levels of Nef protein expressed by the patient alleles were either comparable (C28B) or greater (M29) than HIV-1_{NL4-3} Nef, as shown by Western blotting with anti-Nef (Figure 3, bottom) or anti-HA sera (not shown).

MHC-I antigen regulation

As discussed above, Nef also causes down-regulation of MHC class I antigens in HIV-1-infected cells [18,19,30]. We therefore examined the ability of the patient Nef alleles to cause decreased expression of MHC class I antigens in J-Jurkat lymphocyte cells. Either control or patient *nef* plasmids

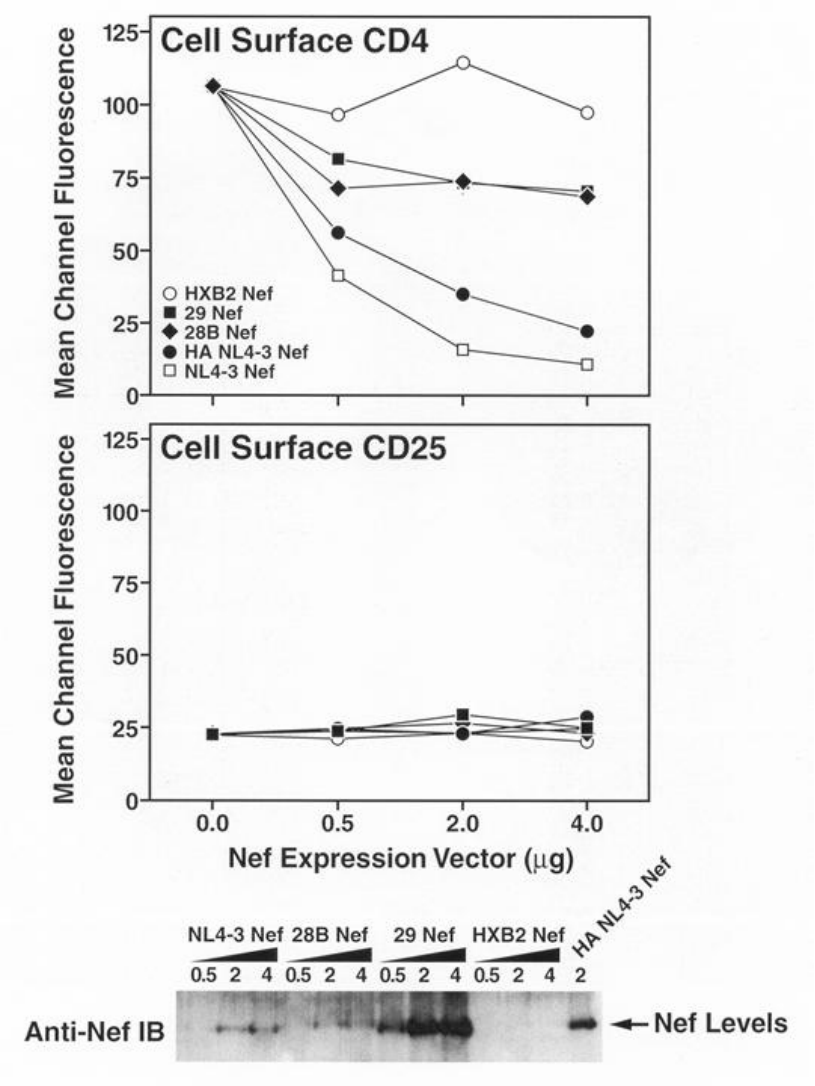

Figure 3. CD4 down-regulation by patient Nefs. Patient *nef* clones were transfected with CD4 and CD25 plasmids. Top: expression of cell surface CD4. Center: expression of cell surface CD25. HIV-1_{NL4-3} Nef, open squares; HIV-1_{NL4-3} Nef-HA, filled circles; HIV-1_{HXB2} Nef, open circles; HIV-$1_{Nef\ C28B}$ (child), diamonds; HIV-$1_{Nef\ M29}$ (mother), filled squares. Bottom: Western blot analysis of expression of Nef in transient transfections shown at top.

were co-transfected with pCR3.1-GFP in Jurkat cells, which endogenously express MHC-I antigens. GFP-positive cells were screened for levels of MHC-I expression on the cell surface (Figure 4). The average of three separate experiments is reported. HIV-1_{HXB2} *nef* was introduced as a non-functional negative control, and the level of endogenous MHC-I expression in the presence of this allele was arbitrarily set at 100%. On transfection of the HIV-1_{NL4-3} clone, a 23% decrease in MHC-I surface expression was observed. The child's Nef C28B was slightly more active, causing a 35 % decrease in MHC-I expression. In contrast, the maternal Nef M29 was unable to cause decreased cell surface expression of MHC-I antigens.

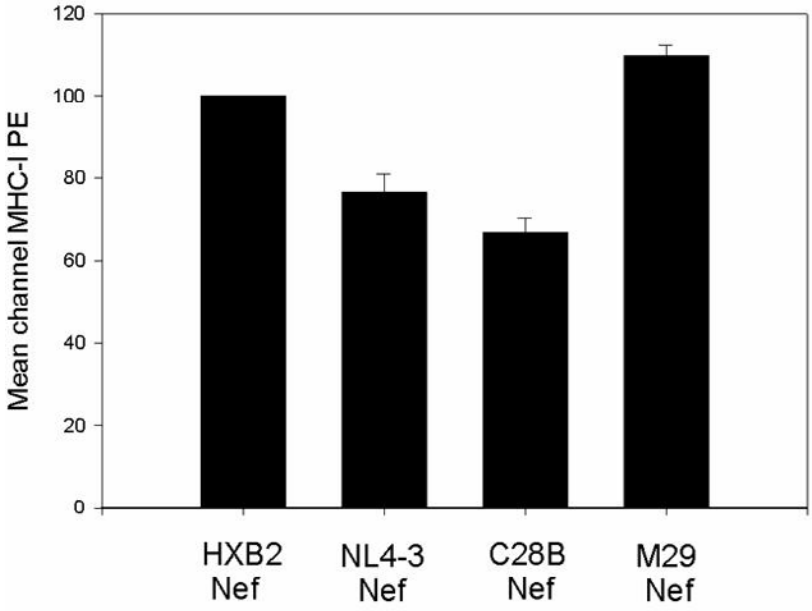

Figure 4. MHC class I down-regulation by patient Nefs. Mean channel fluorescence for MHC-I was measured in three separate experiments. MHC-I expression in the presence of HIV-1_{HXB2} Nef was set at 100 %. Error bars indicate the standard error in three separate experiments.

Discussion

The observation of partially deleted *nef* PCR amplicons from a HIV-1-positive mother-child transmission pair led us to investigate the functions of the cloned *nef* alleles. Therefore, we performed assays of Nef function using the cloned patient *nef* alleles, transfected and expressed in human cell lines or used to trans-complement a *nef*-deleted HIV-1_{NL4-3} viral clone. Both maternal and child Nef proteins showed partial activity in Nef assays (Table 1). The child's Nef C28B exhibited 55 % of the infectivity enhancement of HIV-1_{NL4-3} Nef in the MAGI-cell assay and 33 % of the CD4 down-regulation activity of HIV-1_{NL4-3} Nef. Yet, though the Nef C28B was impaired in infectivity and CD4 down-regulation, there was no impairment of the MHC-I down-regulation activity. In contrast, the maternal Nef M29 gave 35 % of the infectivity enhancement of HIV-1_{NL4-3} Nef, which was equivalent to the HIV-1_{HXB2} Nef negative control. Nef M29 also retained 30 % of the CD4 down-regulation activity of HIV-1_{NL4-3} Nef. Finally, the maternal Nef was non-functional for MHC-I down-regulation.

The results of these assays may yield further clues to the function of the Nef protein. Since the maternal and child Nef sequences are 90 % homologous (Figure 1), it is not surprising that both alleles are equally impaired for CD4 down-regulation. There are two likely explanations for this deficiency in our patient Nefs, the first being the influence of the 5-aa deletions flanking the dileucine motif (Figure 1, residues 160-165). Deletions in this region would have the effect of shortening the C-terminal loop in the Nef protein [31], which may alter the interaction of Nef with either the AP-2 adaptor complex or the cytoplasmic tail of the CD4 receptor. Secondly, the amino acids Trp-56, Leu-57 and Glu-58 have

Table 1. Activity of C28B and M29 Nefs in Functional Assays*.

Assay	C28B Nef	M29 Nef	Nef_{HXB2}
CD4 down-regulation	33	33	0
MAGI-cell infectivity	55	35	30
MHC-I down-regulation	110	0	0

*The activities of patient Nefs are expressed as a percentage of the activity of the functional Nef allele from HIV-$1_{NL4.3}$, which was arbitrarily set at 100% activity. The Nef allele from HIV-1_{HXB2}, expresses a truncated Nef protein which is not stable in mammalian cells.

been shown to be required for efficient CD4 down-regulation [19,30], possibly because they contact the cytoplasmic tail of CD4. The tryptophan residue, Trp-57, has been changed to glycine in both our patient Nefs (Figure 1).

The differences in function between the two patient Nef alleles are noteworthy. The maternal Nef is essentially non-functional in MHC-I and MAGI infectivity assays, whereas the pediatric Nef retains partial MAGI-cell infectivity and is functional for MHC-1 down-regulation. As discussed above, the MAGI-cell infectivity and MHC-I down-regulation functions of Nef can be genetically separated [21], but we note that overlapping determinants for these phenotypes are contained within the central core region of the Nef protein. Activity in the MAGI-cell infectivity assay requires several Nef core motifs, including the acidic region at aa 61-65 and the polyproline domain (Pxx4), aa 69-80; as well as the highly conserved region from aa 108-124 [32]. Mutations in the former two regions impair both infectivity enhancement and MHC-I down-regulation, while mutations in the latter region, especially Asp-123 [32], impair both infectivity enhancement and CD4 down-regulation. Recently it was shown that the amino-terminal portion of Nef, particularly the helical domain from aa 16-22, is also necessary for MHC-I down-regulation [19,21]. Mutation of Met-20 in this domain abolished the MHC-I down-regulation activity of Nef, but did not affect either kinase binding or CD4 down-regulation activity. [21].

As noted above, defective *nef* alleles were described from LTS patients [8,9], containing either large deletions or clusters of deletions which disrupt the *nef* reading frame, thus preventing the production of Nef protein. In another study, *nef* alleles from a LTS patient were described in which a 36-nucleotide deletion was compensated by a downstream 33-nucleotide duplication [33]. This follows an established pattern for the repair of small deletions in *nef* that was also observed when *nef*-deleted SIV constructs were used to infect rhesus macaques [34]. In that study, a 12-nucleotide *nef* deletion was compensated by nucleotide insertions; thus, functional Nef protein synthesis was restored. This indicates a strong selective genetic pressure for maintenance of a functional HIV-1 Nef. The deleted/duplicated HIV-1 Nef clone from the human LTS patient [33] was partially functional in cell-culture based assays. Our patients' *nef* alleles also encode partially functional Nef proteins, although

the maternal Nef protein was only partially functional for CD4 down-regulation and was nonfunctional in the other Nef assays.

Since a lack of Nef function has been correlated with long-term survival of HIV-1 infection in many patients, we re-examined the patients' records to see if there was any indication that impairment of Nef activity might have influenced their clinical responses to HIV-1 infection. Briefly, the mother was diagnosed HIV-positive in 1988. She received antiretroviral treatment (ART) on an intermittent basis, including several different nucleoside RT inhibitors. During this period her CD4+ T-lymphocyte values ranged from 180-350 cells/mm^3. This resulted in a diagnosis of AIDS, as any CD4+ value below 200 cells/mm^3 is an AIDS-defining condition. She was on ART when re-contacted in March 2000, at which time her CD4+ level was 350 cells/mm^3, with a viral load of 1700 particles/mm^3. Subsequently she returned to ART with a cocktail that includes a non-nucleoside RT inhibitor. She had not received HIV-1 protease inhibitors at last contact in July 2001. Her son was diagnosed HIV-positive at 15 months, at which time ART was initiated. The child was diagnosed with AIDS at 18 months (28B sample), and was continued on ART (nucleosides), and was eventually deceased at 6.5 yrs of age.

Clearly the child's clinical course is typical for many cases of pediatric HIV-1 infection. The mother has survived for more than 12 years since her HIV-1-positive diagnosis. She has received ART, with several interruptions due to patient non-compliance. Notably, there were periods of relatively stable CD4+ values in the absence of ART. This patient may not be described accurately as a non-progressor, as she had a diagnosis of AIDS. However, her continued survival in the absence of aggressive ART raises the possibility of a role for attenuated HIV-1 replication, which may or may not be due to changes in HIV-1 Nef function.

An examination of the mother and child patient Nef sequences (Figure 1) shows that they encode proteins which have 78 and 75 % homology, respectively, with the fully-functional HIV-1_{NL4-3} Nef. The most dramatic differences have been discussed above, namely, the C-terminal deletions and the W57G mutation at the HIV-1 protease cleavage site. The remaining differences between HIV-1_{NL4-3} Nef and the patient Nef protein sequences result from additional mutations in their nucleotide sequences. Notably, each of the amino acids or sequence motifs thus far identified as essential for MHC-I down-regulation [piguet] is present in both patient Nef proteins, suggesting that there may be additional Nef amino acid residues that influence down-regulation of MHC-I antigens.

Examination of differences in the sequences of the patient Nefs should assist us in the identification of additional Nef elements involved in MHC-I down-regulation. There are 15 individual amino acid differences between the C28B and M29 Nefs (Figure 1), seven of which are located in regions not specifically identified with Nef functions [32,35]. Premkumar et al.[36] isolated a series of *nef* alleles from a single LTS patient, and tested their ability to promote HIV-1 replication when substituted for the *nef* gene of $_{HIV\text{-}1\ NL4.3}$. They observed that patient *nef* alleles encoding an "extra" cysteine at position 138 were deficient in enhancing viral replication. The M29 Nef, which is inactive in the

MHC-I assay has cysteine at position138, while Nef C28B has threonine, identical to the Nef consensus (Fig.1). Mutagenesis experiments are underway to test the effect of the Thr-to-Cys mutation on MHC-I regulation.

Finally, we would like to point out that the obeservation of HIV-1 Nef deletions in a mother-child transmission pair suggests that these particular Nef deletions were stably transmitted, at least in this case. Similarly, we note the comparison between mother-child Nef sequences reported in this study and those reported by Saksena et al. [37] who also described HIV-*1 nef* genes from a mother-child transmission pair. There were unusual sequence features including the lack of C-terminal cyteine residues, in the *nef* alleles studied. Co-culture of HIV-1-infected lymphocytes demonstrated replication-deficient viruses, but functional studies with individual nef alleles were not done. Clearly, in order to understand more fully the effects of Nef expression on HIV-1 pathogenesis, it will be necessary to continue to analyze the interactions of the Nef protein with cellular proteins. Undoubtedly the study of patient *nef* alleles will play an important role in that process.

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References

1. Harris, M. From Negative Factor to a Critical Role in Virus Pathogenesis: The Changing Fortunes of Nef. *J. Gen. Virol.* **1996**, *77*, 2379-2392.
2. Cheng-Mayer, C.; Iannello, P.; Shaw, K; Luciw, P; Levy, J. A. Differential Effects of *nef* on HIV Replication: Implications for Viral Pathogenesis in the Host. *Science* **1989**, *246*, 1629-1632.
3. Chowers, M. Y.; Spina, C. A.; Kwoh, T.J.; Fitch, N. J.; Richman, D. D.; and Guatelli, J.C. Optimal Infectivity In Vitro of Human Immunodeficiency Virus Type 1 Requires an Intact *nef* Gene. *J. Virol.* **1994**, *68*, 2906-2914.
4. Miller, M. D.; Warmerdam, M. T.; Gaston, I.; Greene, W. C.; Feinberg, M.B. The Human Immunodeficiency Virus-1 *nef* Gene Product: A Positive Factor for Viral Infection and Replication in Primary Lymphocytes and Macrophages. *J. Exp. Med.* **1994**, *179*, 101-113.
5. Kestler, H. W. III; Ringler, D. J.; Mori, K.; Panicali, D. L.; Sehgal, P. K.; Daniel, M. D.; Desrosiers RC: Importance of the *nef* Gene for Maintenance of High Virus Loads and for Development of AIDS. *Cell* **1991**, *65*, 651-662.
6. Du, Z.; Lang, S. M.; Sasseville, V. G.; Lackner, A. A.; Ilyinskii, P.O.; Daniel, M. D.; Jung, J. U.; Desrosiers, R. C. Identification of a *nef* Allele That Causes Lymphocyte Activation and Acute Disease in Macaque Monkeys. *Cell* **1995**, *82*, 665-674.

7. Baba, T. W.; Jeong, Y. S.; Pennick, D.; Bronson, R.; Greene, M. F.; Ruprecht, R. M. Pathogenicity of Live, Attenuated SIV After Mucosal Infection of Neonatal Macaques. *Science* **1995**, *267*, 1820-1825.
8. Kirchhoff, F.; Greenough, T. C.; Brettler, D. B.; Sullivan, J. L.; Desrosiers, R. C. Brief Report: Absence of Intact *nef* Sequences in a Long-Term Survivor With Nonprogressive HIV-1 Infection. *N. Engl. J. Med.* **1995**, *332*, 228-232.
9. Deacon, N. J.; Tsykin, A.; Solomon A, Smith, K.; Ludford-Menting, M.; Hooker, D. J.; McPhee, D. A.; Greenway, A. L.; Ellett, A.; Chatfield, C.; Lawson, V. A.; Crowe, S.; Maerz, A.; Sonza, S.; Learmont, J.; Sullivan, J. S.; Cunningham, A.; Dwyer, D.; Dowton, D.; Mills, J. Genomic Structure of an Attenuated Quasispecies of HIV-1 From a Blood Transfusion Donor and Recipients. *Science* **1995**, *270*, 988-991.
10. Salvi, R.; Garbuglia, A.R.; Di Caro, A.; Pulciani, S.; Montella, F.; Benedetto, A. Grossly Defective *nef* Gene Sequences in a Human Immunodeficiency Virus Type 1-Seropositive Long-Term Nonprogressor. *J. Virol.* **1998**, *72*, 3646-3657.
11. Hanna, Z.; Kay, D. G.; Rebai, N.; Guimond, A.; Jothy, S.; Joliceur, P. Nef Harbors a Major Determinant of Pathogenicity for an AIDS-like Disease Induced by HIV-1 in Transgenic Mice. *Cell* **1998**, *95*, 163-175.
12. Dickie, P. Nef Modulation of HIV Type 1 Gene Expression and Cytopathicity in Tissues of HIV Transgenic Mice. *Aids Res. Hum. Retrovir.* **2000**, *16*, 777-790.
13. Schaeffer, E.; Geleziunas, R.; and Greene, W.C.; Human Immunodeiciency Virus Type 1 Nef Functions at the Level of Virus Entry by Enhancing Cytoplasmic Delivery of Virions. *J. Virol.* **2001**, *75*, 2993-3000.
14. Kotov, A.; Zhou, J.; Flicker, P.; Aiken, C. Association of Nef with the Human Immunodeficiency Virus Type 1 Core. *J. Virol*. **1999**, *73*, 8824-8830.
15. Aiken, C.; Trono, D. Nef Stimulates Human Immunodeficiency Virus Type 1 Proviral DNA Synthesis. *J. Virol*. **1995**, *69*, 5048-5056.
16. Aiken, C.; Konner, J.; Landau, N. R.; Lenburg, M. E.; Trono, D. Nef Induces CD4 Endocytosis: Requirement for a Critical Dileucine Motif in the Membrane-Proximal CD4 Cytoplasmic Domain. *Cell* **1994**, *76*, 853-864.
17. Chen, B. K.; Gandhi, R.T.; Baltimore, D. CD4 Down-Modulation During Infection of Human T-Cells With Human Immunodeficiency Virus Type 1 Involves Independent Activities of *vpu*, *env* and *nef*. *J. Virol.* **1996**, *70*, 6044-6053.
18. Schwartz, O.; Marechal, V.; Le Gall, S.; Lemonnier, F.; Heard, J.M. Endocytosis of Major Histocompatibility Complex Class I Molecules is Induced by the HIV-1 Nef Protein. *Nat. Med.* **1996**, *2*, 338-342.
19. Mangasarian, A.; Piguet, V.; Wang, J. K.; Chen, Y. L.; Trono, D. Nef-Induced CD4 and Major Histocompatibility Complex Class I (MHC-I) Down-Regulation are Governed by Distinct Determinants: N-Terminal Alpha Helix and Proline Pepeat of Nef Selectively Regulate MHC-I Trafficking. *J. Virol.* **1999**, *73*, 1964-1973.

20. Goldsmith, M.A.; Warmerdam, M. T.; Atchison, R. E.; Miller, M.D.; Greene, W. C.; Dissociation of the CD4 Down-regulation and Viral Infectivity Enhancement Functions of Human Immunodeficiency Virus Type 1 Nef. *J. Virol.* **1995**, *69*, 4112-4121.
21. Akari, H.; Arold, S.; Fukumori, T.; Okazaki, T.; Strebel, K.; Adachi, A. Nef-Induced Major Histocompatibility Complex Class I Down-Regulation Is Functionally Dissociated from its Virion Incorporation, Enhancement of Viral Infectivity, and CD4 Down-Regulation. *J. Virol.* **2000**, *74*, 2907-2912.
22. Geffin, R.; Wolf, D.; Muller, R.; Hill, M. D.; Stellwag, E.; Frietag, M.; Sass, G.; Scott, G. B.; Baur, A. S. Functional and Structrual Defects in HIV Type 1 *nef* Genes Derived from Pediatric Long-Term Survivors. *Aids Res. Hum. Retrovir.* **2000**, *17*, 1855-1868.
23. Rousseau, C.; Abrams, E.; Lee, M.; Urbano, R.; King, M. C. Long Terminal Repeat and *nef* Gene Variants of Human Immunodeficiency Virus Type 1 in Perinatally Infected Long-Term Survivors and Rapid Progressors. *Aids Res. Hum. Retrovir.* **1997**, *13*, 1611-1623.
24. Kirchoff, F.; Easterbrook, P. J.; Douglas, N.; Troop, M.; Greenough, T. C.; Weber, J.; Carl, S.; Sullivan, J. L.; Daniels, R. S. Sequence Variations in Human Immunodeficiency Virus Type 1 Nef Are Associated with Different Stages of Disease. *J. Virol.* **1999**, *73*, 5497-5508.
25. Kuiken, C.L.; Foley, B.; Hahn, B. H.; Korber, B.; McCutchan, F.; Marx, P. A.; Mellors, J, W.; Mullins, J.I.; Sodroski, J.; Wolinksy, S. *Human Retroviruses and AIDS 1999: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences.* Theoretical Biology and Biophysics Group, Los Alamos National Laboratory: Los Alamos, NM, 1999, p.53.
26. Kimpton, J,; Emerman, M. Detection of Replication-Competent and Pseudotyped Human Immunodeficiency Virus with a Sensitive Cell Line on the Basis of Activation of an Integrated □-Galactosidase Gene. *J. Virol.* **1992**, *66*, 2232-2239.
27. Freed, E. O.; Martin, M.A.; Virion Incorporation of Envelope Glycoproteins with Long but Not Short Cytoplasmic Tails Is Blocked by Specific, Single Amino Acid Substitutions in the Human Immunodeficiency Virus Type 1 Matrix. *J. Virol.* **1995**, *69*, 1984-1989.
28. Roth, W.W.; Zuberi, J.A.; Stringer, H. G. Jr; Davidson, S.K.; Bond, V.C. Examination of HIV Type 1 Variants in Mother-Child pairs. *AIDS Res. Hum. Retrovir.* **1996**, *12*, 925-930.
29. Bresnahan, P. A.; Yonemoto, W.; Ferrell, S.; Williams-Herman, D.;Geleziunas, R.; Greene, W.C. A Dileucine Motif in HIV-1 Nef Acts as an Internalization signal for CD4 Down-regulation and Binds the AP-1 Clathrin Adaptor. *Curr. Biol.* **1998**, *8*, 1235-1238.
30. Piguet, V.; Schwartz, O.; Le Gall, S.; Trono, D.; The Down-regulation of CD4 and MHC-I by Primate Lentiviruses: A Paradigm for the Modulation of Cell Surface Receptors. *Immunol. Rev.* **1999**, *168*, 51-63.
31. Grzesiek, S.; Bax, A.; Hu, J.-S.; Kaufman, J.; Palmer, I.; Stahl, S.J.; Tjandra, N.; Wingfield; P. T. Refined Solution Structure and Backbone Dynamics of HIV-1 Nef. *Protein Science* **1997**, *6*, 1248-1263.
32. Liu, X.L.; Heverker, N.; Fackler, O. T.; Arold, S.; Le Gall, S.; Janvier, K.; Peterlin, B. M.; Dumas, C.; Schwartz, O.; Benichou, S.; Benarous, R. Mutation of a Conserved Residue (D123) Required for Oligomerization of Human Immunodeficiency Virus Type 1 nef Protein Abolishes Interaction

with Human Thioesterase and Results in Impairment of Nef Biological Functions. *J. Virol.* **2000**, *74*, 5310-5319.

33. Carl, S..; Daniles, R.; Iafrate.; A.J. Easterbrook, P. J.; Greenough, T.C.; Skowronski, J.; Kirchhoff. F. Partial Repair of Defective Nef Genes in a Long-Term Nonprogressor with Human Immunodeficiency Virus Type 1 Infection. *J. Infect. Dis.* **2000**, *181*, 132-140.
34. Whatmore, A. M.; Cook, M.; Hall, G. A.; Sharpe, S.; Rud, E. W.; Cranage, M. P.; Repair and Evolution of Nef in vivo Modulates Simian Immunodeficiency Virus Virulence. *J. Virol.* **1995**, *69*, 5117-5123.
35. Piguet, V.; Trono, D. The Nef Protein of Primate Lentiviruses. *Rev. Med. Virol.* **1999**, *9*, 111-120.
36. Premkumar, D. R. D.; Ma, X.-Z.; Maitra, R. K.; Chakrabarty, B. K.; Salkowitz, J.; Yen-Lieberman, B.; Hirsch, M. S.; Kestler, H.W. The *nef* Gene from a Long-Term HIV-1 Nonprogressor. *AIDS Res. Hum. Retrovir.* **1996**, *12*, 337-345.
37. Saksena, N. K.; Ge, Y. C.; Wang, B.; Xiang, S. H.; Ziegler, J.; Palasanthiran, P.; Bolton, W.; Cunningham, A. L. RNA and DNA Sequence Analysis of the *nef* Gene from the First HIV Type 1-Infected Long-Term Nonprogressing Mother-Child Pair. *AIDS Res. Hum. Retrovir.* **1997**, *13*, 729-732.