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Human Immunodeficiency Virus Type 1 Nef-Mediated Downregulation of CD4 Correlates with Nef Enhancement of Viral Pathogenesis

Cheryl A. Stoddart,[1] Romas Geleziunas,[1] Sharon Ferrell,[1] Valerie Linquist-Stepps,[1] Mary E. Moreno,[1] Christopher Bare,[1] Weiduan Xu,[1] Wes Yonemoto,[1] Patricia A. Bresnahan,[1] Joseph M. McCune,[1,2,3] and Warner C. Greene[1,2,3]*

Gladstone Institute of Virology and Immunology[1] and Departments of Medicine[2] and Microbiology and Immunology,[3] University of California, San Francisco, California 94141

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The *nef* gene products encoded by human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus type 1 (SIV-1) increase viral loads in infected hosts and accelerate clinical progression to AIDS. Nef exhibits a spectrum of biological activities, including the ability to downregulate surface expression of CD4 and major histocompatibility complex (MHC) class I antigens, to alter the state of T-cell activation, and to enhance the infectivity of viral particles. To determine which of these in vitro functions most closely correlates with the pathogenic effects of Nef in vivo, we constructed recombinant HIV-1 NL4-3 viruses carrying mutations within the *nef* gene that selectively impair these functions. These mutant viruses were evaluated for pathogenic potential in severe combined immunodeficiency (SCID) mice implanted with human fetal thymus and liver (SCID-hu Thy/Liv mice), in which virus-mediated depletion of thymocytes is known to be Nef dependent. Disruption of the polyproline type II helix $(Pxx)_4$ within Nef (required for binding of Hck and p21-activated kinase-like kinases, downregulation of MHC class I, and enhancement of HIV-1 infectivity in vitro but dispensable for CD4 downregulation) did not impair thymocyte depletion in virus-infected Thy/Liv human thymus implants. Conversely, three separate point mutations in Nef that compromised its ability to downregulate CD4 attenuated thymocyte depletion while not diminishing viral replication. These findings indicate that the functional ability of Nef to downregulate CD4 and not MHC class I downregulation, Hck or PAK binding, or $(Pxx)_4$-associated enhancement of infectivity most closely correlates with Nef-mediated enhancement of HIV-1 pathogenicity in vivo. Nef-mediated CD4 downregulation merits consideration as a new target for the development of small-molecule inhibitors.

Nef, a 25- to 27-kDa regulatory protein encoded by human and simian immunodeficiency viruses, accelerates clinical progression to AIDS in humans infected with either human immunodeficiency virus type 1 (HIV-1) or HIV-2 and in rhesus macaques experimentally infected with simian immunodeficiency virus (SIV) (6, 15, 27, 33, 35, 38, 46, 58, 62, 71). Similarly, Nef enhances the pathogenicity of HIV-1 in severe combined immunodeficiency (SCID) mice implanted with fragments of human fetal thymus and liver (SCID-hu Thy/Liv mice) (3, 4, 16, 31). The molecular mechanisms underlying these effects of Nef remain unknown. However, in newborn rhesus macaques, Nef is dispensable for disease progression (5, 6), indicating that it is not an obligate virulence factor.

Among its numerous properties, Nef can downregulate cell surface expression of CD4 (19) and many major histocompatibility complex (MHC) class I (13, 39, 67) antigens, enhance the infectivity of viral particles (12, 48), and stimulate the replication of HIV-1 in peripheral blood mononuclear cells (PBMC) (48, 69). Nef also perturbs various intracellular signal transduction pathways (14, 20, 47, 53, 65, 72) and impairs Fas- and tumor necrosis factor receptor-mediated apoptosis (21).

Downregulation of CD4 by Nef involves endocytosis and lysosomal degradation of CD4, likely the result of Nef-mediated recruitment of cellular adaptors to the cytoplasmic tail of CD4 (9, 25, 39, 41, 42, 44, 54). These interactions seem to require distinct residues within Nef, situated at both the amino and carboxy termini of this viral regulatory protein. At the amino terminus, residues W57 and L58 of Nef have been proposed to interact with the cytoplasmic tail of CD4 (28, 42, 45). Two pairs of residues situated at the carboxy terminus of Nef participate in CD4 downregulation as well (9, 25, 41). A dileucine motif, L164-L165, is required for the interaction of Nef with clathrin-associated adaptor molecules such as AP-1 and AP-2. The role of the second pair of residues (D174 and D175) is unclear. These residues may either interact with the CD4 cytoplasmic tail or bind to a vacuolar ATPase involved in both adaptor binding and acidification of endosomes (42, 45).

Nef-mediated downregulation of CD4 was first postulated as a mechanism to prevent viral superinfection (7). Additionally, it has been proposed that Nef-mediated removal of CD4 from the cell surface could lead to liberation of the CD4-associated protein tyrosine kinase Lck, in turn promoting enhanced phosphorylation of various substrates such as the T-cell receptor ζ chain. Such events could modify the overall state of T-cell activation, perhaps creating an intracellular milieu more conducive to HIV replication (14, 20). This mechanism might also contribute to the finding that thymocytes from Nef-transgenic mice display surface markers indicative of cellular activation (29, 40, 68).

To increase HIV-1 virion infectivity, Nef must be present

* Corresponding author. Mailing address: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94141-9100. Phone: (415) 695-3800. Fax: (415) 826-1817. E-mail: wgreene@gladstone.ucsf.edu.

during viral particle assembly in the host producer cell (2, 49, 52). The effect of Nef, however, is only manifested in the infected target cell. In terms of enhancement of virion infectivity, both CD4-dependent and CD4-independent components are demonstrable. HIV-1 virion infectivity enhancement occurring in the absence of CD4 was believed to be attributable to Nef's facilitation of proviral DNA synthesis, occurring during the afferent portion of the viral life cycle (2, 11, 66). However, more recent studies also suggest that Nef enhances virion entry into the cytosol (64), perhaps reflecting changes in the lipid composition of the virion membrane (74). Consistent with this notion, when HIV-1 particles are forced to enter cells via acidified endosomes by vesicular stomatitis virus G pseudotyping, Nef no longer enhances infectivity (1). When CD4 is present on the producer cell, Nef may enhance infectivity through CD4 downregulation, leading to either more efficient release of virions or more effective envelope engagement of CD4 receptors present on the subsequent target cells (36, 60).

Nef-mediated downregulation of MHC class I may enable HIV-1-infected cells to more effectively evade cytotoxic T-lymphocyte-mediated recognition and lysis (13). MHC class I downregulation involves different determinants in Nef than CD4 downregulation. Specifically, MHC class I downregulation is dependent on an acidic region (E_{64}EEE) and a polyproline stretch [$(P_{69}xx)_4$] situated within the central portion of the Nef polypeptide (26, 45). This acidic region within Nef appears to bind the cellular protein PACS-1, which participates in relocalization of MHC class I from the cell surface to the trans-Golgi network (55). Downregulation of MHC class I by Nef may also occur through delayed transit from the Golgi complex to the cell surface (70). The polyproline-rich region is also necessary to confer enhanced, CD4-independent infectious potential upon HIV-1 particles (24, 61) and represents a high-affinity binding site for the SH3 domain of the Src family protein tyrosine kinase Hck (61). Additionally, this region is required for Nef interaction with a complex of cellular proteins, including PIX-p95, Vav, and p21-activated kinase-2 (PAK 2) (10, 17, 18, 34, 51, 57, 63, 73). However, thus far, neither Hck nor PAK 2 has been clearly implicated in either Nef-mediated MHC class I downregulation or enhancement of HIV-1 infectivity.

Since Nef accelerates clinical progression to AIDS in hosts infected with primate immunodeficiency viruses, a thorough understanding of Nef function within both the infected host cell and the infected host is needed to delineate the molecular basis for its acceleration of viral pathogenesis in vivo. Because Nef is capable of numerous effector functions, it is important to first determine which function or combination of functions is linked to its pathogenic effects in vivo. We now describe a series of studies that define the molecular determinants in Nef that are required for HIV-1-associated cytopathicity in vivo in the SCID-hu Thy/Liv mouse model.

MATERIALS AND METHODS

Mammalian expression vector preparation. Nef coding sequences were amplified by PCR from the pNL4-3N proviral plasmid (61) (kindly provided by Kalle Saksela, University of Tempere, Tempere, Finland) with the amplimers 5′-GAGAGAAGCTTGACAGGGCTTGGAAAGG-3′, which is situated 30 bp upstream of the start codon of *nef* and introduces a *Hin*dIII site at the 5′ end, and 5′-CTAGTCTAGATTCACAATGATGCATAATCAGGCACGTCGTACGGATAGCAGTCTTTGTAGTACTCCG-3′, which introduces an influenza virus hemagglutinin (HA) epitope tag and an *Xba*I site at the 3′ end of *nef*. These Nef-encoding DNA sequences were next subcloned into the mammalian expression vector pCMV4neo (24) as *Hin*dIII-*Xba*I fragments to create pNL43Nef-HA. Mutations were introduced into *nef* with the Muta-Gene Phagemid in vitro mutagenesis kit (Bio-Rad, Richmond, Calif.) and confirmed by DNA sequencing. Wild-type and mutated *nef* genes were then subcloned into the pCI mammalian expression vector (Promega, Madison, Wis.) to create pCI-NL43Nef-HA. Inserts were removed from pNL43Nef-HA as 5′ *Hin*dIII and 3′ *Xba*I fragments. The 5′ *Hin*dIII site was blunt ended with Klenow polymerase, and the inserts were introduced into pCI at a 5′ blunt-ended *Eco*RI site and a 3′ *Xba*I site.

Proviral plasmid preparation. To create viruses containing select Nef mutations, internal 5′ *Xho*I–3′ *Pml*I fragments of *nef*-bearing targeted point mutations were subcloned from pNL43Nef-HA into the pNL4-3N plasmid. The pNL4-3N *Xho* construct, which fails to express full-length Nef (only 35 N-terminal amino acids of Nef are translated), was produced by digesting pNL4-3N DNA with *Xho*I, filling in the overhanging ends with Klenow polymerase, and religating the plasmid. This mutation introduces a frameshift mutation leading to early termination of the *nef* open reading frame. The presence of each *nef* mutation within the HIV-1 molecular clones was confirmed by DNA sequencing.

Hck-Nef interaction assay. 293T cells transfected with a Hck tyrosine kinase expression vector (59) served as the source of recombinant Hck. Lysates from Hck-transfected 293 cells were incubated with glutathione *S*-transferase (GST)-Nef fusion proteins conjugated to glutathione-agarose beads for 4 h at 4°C. The beads were extensively washed and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Proteins retained on the GST or GST-Nef matrices were analyzed by SDS-PAGE and subsequently immunoblotted with an anti-Hck antibody (Transduction Laboratories, San Diego, Calif.).

CD4 downregulation assays. 293T cells were grown in Dulbecco's modified Eagle's medium (Cellgro, Herndon, Va.) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells were transfected in duplicate by the calcium phosphate precipitation method with 200 ng of a human CD4 expression vector (pCD4-neo) together with 2 μg of a human CD25 expression vector (pCMV4-IL-2Rα) (24) as an internal control and 1 μg of either wild-type or mutant Nef expression plasmid DNA (pCI-NL43Nef-HA). The transfection mixture was added directly to the cells (1×10^6 to 2×10^6) at the time of plating in six-well (35-mm diameter) plates in 2 ml of medium. Eighteen hours later, the transfection mixture was replaced with fresh medium. The cells were harvested 24 h later by scraping in the presence of phosphate-buffered saline–5% heat-inactivated fetal bovine serum (staining buffer). Three-quarters of the harvested cells were stained and analyzed by flow cytometry, while the remainder were lysed in SDS-PAGE sample buffer and analyzed by immunoblotting.

For the flow cytometry studies, the cells were stained with anti-CD4 antibody conjugated to tricolor and an anti-CD25 antibody conjugated to fluorescein isothiocyanate (Caltag, Burlingame, Calif.), followed by analysis on a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, Calif.). To verify Nef expression levels, a portion of the lysates was immunoblotted with an anti-HA epitope tag polyclonal antibody (BabCo, Richmond, Calif.).

MHC class I downregulation assays. Jurkat cells expressing high levels of CD4 (JJK cells) were generously provided by Dan Littman (Skirball Institute, New York, N.Y.). JJK cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. These cells were transfected with pCI-NL43Nef-HA or the Nef mutants by electroporation (250 V and 950 μF) with a Gene Pulser (Bio-Rad). Eighteen hours later, the cells were stained with anti-MHC class I (HLA-ABC) antibodies conjugated to phycoerythrin (Dako, Carpinteria, Calif.) prior to flow cytometric analysis. Equal amounts of total cell lysate (30 μg of protein/lane) were used for immunoblotting with anti-HA epitope antibodies (BabCo) to assess the level of wild-type and mutant Nef protein expression.

Production of HIV-1 viral stocks. Viral stocks were produced by transfecting 293T cells with pNL4-3N plasmids by the calcium phosphate precipitation method. 293T cells were maintained as described in the previous section. To produce 2 ml of viral stock, 1×10^6 to 2×10^6 293T cells were transfected with 1 μg of pNL4-3N proviral DNA carrying either wild-type or mutated *nef* genes and 3 μg of pCMV4neo vector DNA in six-well plates. Mock viral stocks were prepared by transfecting only pCMV4neo vector. Eighteen hours later, the medium was changed to remove the calcium phosphate precipitate. Virus-containing supernatants were collected 24 h later and centrifuged to remove cellular debris. Large viral stocks were made by pooling multiple individual transfections, and 1-ml aliquots of clarified culture supernatants were frozen in liquid nitrogen. Viral stocks were quantified by the p24 enzyme-linked immunosorbent assay (ELISA) (NEN Life Sciences, Boston, Mass.).

Titration of viral stocks by $TCID_{50}$ determination. Cryopreserved phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) pooled from at least seven donors were thawed and cultured for 2 days in RPMI 1640 (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum and 10 U of human interleukin-2 (Boehringer Mannheim, Indianapolis, Ind.) per ml (complete RPMI). Phytohemagglutinin-stimulated PBMC (10^5) were plated in each well of a 96-well plate. Quadruplicate infections were performed with half-log serial dilutions. The infections were maintained for 7 days. The plates were centrifuged, and the supernatant was removed, diluted twofold, and subjected to a p24 ELISA (NEN Life Sciences). Wells with more than 50 pg of p24 per ml were scored as positive, and the 50% tissue culture infectious dose ($TCID_{50}$) value was determined by Reed-Muench calculations (32).

Spreading PBMC infections. Cryopreserved, pooled, phytohemagglutinin-stimulated PBMC from at least seven donors were thawed and cultured for 2 days in complete RPMI. Triplicate infections were performed with 4×10^6 phytohemagglutinin-stimulated PBMC in 2.5 ml of medium in six-well plates. Culture supernatants containing virus (25 ng of p24) or culture supernatants from mock transfections were added to each well, and the cells were cultured for 22 days. One-half of the medium was replaced twice weekly, and the removed supernatant was frozen for subsequent p24 ELISAs (NEN Life Sciences).

Construction and infection of SCID-hu Thy/Liv mice. Homozygous C.B-17 *scid*/*scid* (SCID) mice were bred and implanted with human fetal liver and human fetal thymus to create SCID-hu Thy/Liv mice as previously described (50). The human thymus implants of these mice were inoculated with HIV-1 as described before (56). Each experiment was performed in a single cohort made with tissue from the same donor.

Thy/Liv implant processing. Thy/Liv implants were collected from SCID-hu mice as previously described (23). For the p24 ELISA, pellets of 2.5×10^6 cells were resuspended in 400 μl of p24 lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 25 mM Tris-Cl, 250 mM NaCl, and 1% aprotinin), rotated overnight at 4°C, and stored at −20°C. Thawed samples were transferred into HIV p24 antibody-coated microplates (Dupont-NEN) for quantitative ELISA. Flow cytometry analysis was performed to assess thymocyte depletion. Specifically, pellets containing 10^6 cells were resuspended in 50 μl of a monoclonal antibody mixture containing phycoerythrin-conjugated anti-CD4 (Becton Dickinson, San Jose, Calif.) and tricolor-conjugated anti-CD8 (Caltag, Burlingame, Calif.). Cells from one implant were also stained with conjugated, isotype-matched antibodies to control for nonspecific antibody binding. Cells were incubated for 30 min in the dark, washed twice with phosphate-buffered saline–2% fetal bovine serum, resuspended in 200 μl of phosphate-buffered saline–2% fetal bovine serum containing 1% paraformaldehyde in 1.5-ml flow cytometry tubes, and analyzed on a FACScan (Becton Dickinson, San Jose, Calif.). After collecting 10,000 events, percentages of marker-positive ($CD4^+$, $CD8^+$, and $CD4^+$ $CD8^+$) thymocytes in the implant samples were determined by gating on a lymphoid cell population identified by forward- and side-scatter properties.

RESULTS

Confirmation of functional phenotypes of HIV-1 *nef* mutants. To identify regions within the NL4-3 *nef* allele that are important for Nef-mediated enhancement of HIV-1-induced pathogenesis in SCID-hu Thy/Liv mice, we created a panel of viruses containing specific point mutations in *nef* that compromised various in vitro functions of this regulatory protein. Mutations disrupting the ability of Nef to downregulate cell surface CD4 included WL57/58AA, LL164/165AA, and DD174/175AA (9, 25, 41, 42, 45). Mutations interrupting the polyproline type II helix within Nef, which serves as an Hck SH3-binding domain (61) and is important for MHC class I downregulation (26, 45) and CD4-independent enhancement of viral infectivity in vitro (24, 61), were also prepared. This mutant contains alanine substitutions at P69, P72, and P75 ($P_1P_2P_3AAA$). Finally, an acidic region within Nef corresponding to four contiguous glutamic acid residues at positions 64 to 67 was replaced with four alanines (E_4A). This acidic region has been implicated in MHC class I downregulation (26, 55).

To verify the phenotype of these Nef mutants with respect to CD4 downregulation, we tested each for the ability to downregulate ectopically expressed CD4 in 293T human embryonic kidney cells. These cells lack expression of the Src family protein tyrosine kinase Lck, which can associate with the cytoplasmic tail of CD4 in T cells (reviewed in reference 8). To ensure that the Nef-mediated effects were specific for CD4, a control expression vector encoding the α chain of the human interleukin-2 receptor complex (CD25) was cotransfected together with CD4 and the various Nef mutants. The Nef mutants LL164AA, DD174AA, and WL57AA each displayed impaired downregulation of surface CD4 (Fig. 1A). In contrast, disruption of the polyproline type II helix within Nef ($P_1P_2P_3AAA$) or mutation of the acidic region (E_4A) within Nef did not impair CD4 downregulation in 293T cells. None of the Nef mutants altered surface expression of CD25 in these cultures (Fig. 1A, middle panel).

To assess the expression levels of these Nef mutants, an aliquot of the cell lysates from these cultures was immunoblotted with an antibody reacting with the C-terminal HA epitope tag present on the proteins (Fig. 1A, lower panel). These studies revealed that the mutant Nefs defective for CD4 downregulation were expressed at levels comparable to wild-type Nef. Of note, we consistently observed that the $P_1P_2P_3AAA$ Nef was expressed at significantly lower levels than the other Nef constructs. Additionally, this mutant and the E_4A mutant displayed slightly more rapid electrophoretic migration, while WL57AA was slightly retarded.

To examine the ability of these Nef mutants to downregulate cell surface expression of MHC class I, the human JJK Jurkat T-cell line, which expresses high levels of MHC class I and CD4, was electroporated with the various Nef constructs. Mutation of either the polyproline stretch ($P_1P_2P_3AAA$) or the acidic region (E_4A) within Nef significantly impaired the ability of Nef to downregulate surface MHC class I (Fig. 1B). Conversely, mutations of Nef that compromised CD4 downregulation (WL57AA, LL164AA, and DD174AA) did not affect MHC class I downregulation. Expression levels of the Nef mutants in lysates of the transfected Jurkat T cells were assessed (Fig. 1B, lower panel). Again, the $P_1P_2P_3AAA$ Nef was expressed at lower levels than the other Nef mutants. Thus, the MHC class I downregulation results with this mutant must be interpreted with caution.

Finally, the Nef mutants expressed as GST fusion proteins were tested for their ability to interact with the Src family kinase Hck. Consistent with the prior finding that Nef interaction with Hck requires an intact polyproline stretch within Nef (61), the GST-$P_1P_2P_3AAA$ mutant failed to bind Hck (Fig. 1C). Conversely, each of the mutants that impaired CD4 downregulation and the E_4A mutant retained the capacity to interact with Hck in vitro.

Together, these results confirm the in vitro phenotypes of the Nef mutants and are consistent with the previous finding that Nef-mediated CD4 downregulation versus class I MHC downregulation and Hck binding involve different molecular determinants (26, 45, 61). These studies also highlight the overall instability of the $P_1P_2P_3AAA$ Nef mutant in two different cellular environments.

HIV-1 containing mutant *nef* alleles that impair CD4 downregulation are less infectious than wild-type HIV-1. Each of the various Nef mutations was introduced into NL4-3N, a CXCR4-utilizing infectious molecular clone of HIV-1 (61).

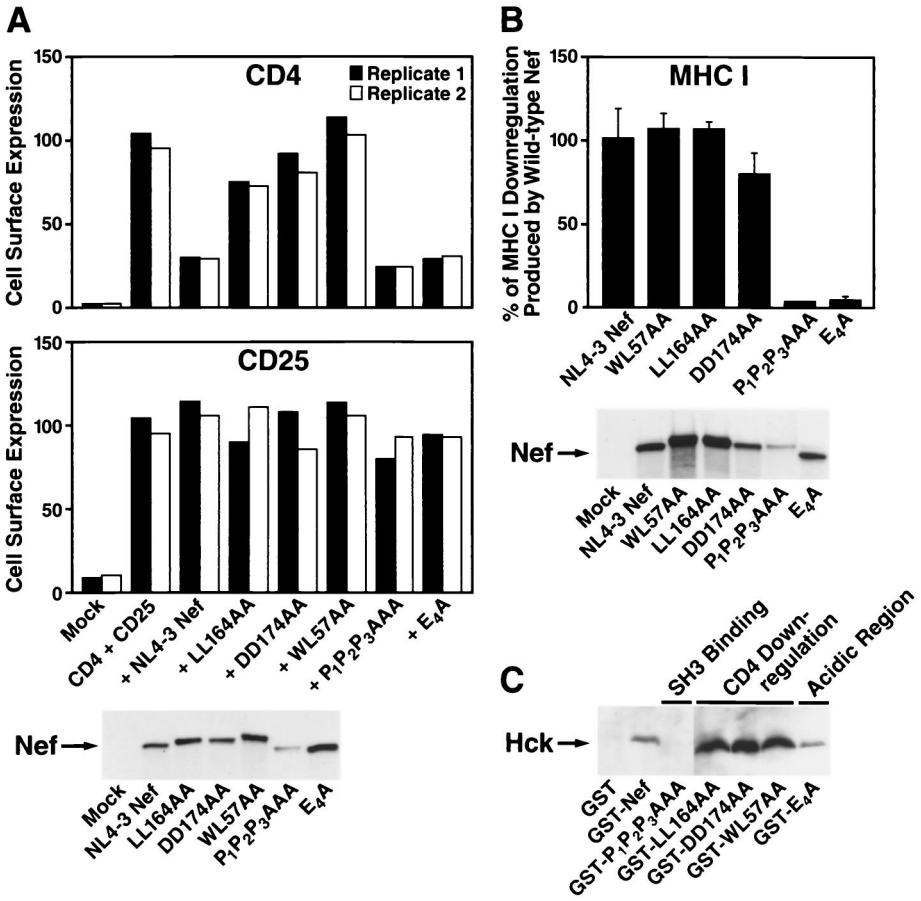

FIG. 1. Downregulation of CD4 and MHC class I and Hck binding by HIV-1 Nef and various mutants of Nef. (A) 293T cells were cotransfected with 0.2 μg of CD4, 2 μg of CD25, and 0.4 μg of expression vector DNA encoding the various Nef constructs. Cell surface expression of CD4 (top) and CD25 (middle) was evaluated 24 h after transfection. Results of replicate transfections are expressed as the percentage of positive cells obtained with CD4 or CD25 alone. The bottom panel represents levels of Nef expression after pooling of the replicate lysates. Immunoblotting was performed with an antibody recognizing the C-terminal HA epitope present in all of the Nef mutants. (B) JJK Jurkat T cells were transfected with 30 μg of pCI-NL43Nef-HA or the various Nef mutants. Cells were analyzed for the mean fluorescence intensity of cell surface expression of MHC class I antigens 18 h after transfection. The results are expressed as a percentage of the response obtained with wild-type Nef. The lower panel shows the relative levels of expression of the various Nef constructs as assessed by immunoblotting of the cell lysates with an antibody specific for the C-terminal HA tag. (C) Lysates from 293T cells transfected with an Hck expression vector were incubated with either wild-type Nef or mutant Nefs fused to GST. To reveal Hck binding, proteins retained by the GST-Nef fusion proteins were immunoblotted with an Hck-specific antibody.

Following the transfection of molecular clones expressing Nef mutants impaired in CD4 downregulation (WL57AA, LL164AA, and DD174AA) or the $P_1P_2P_3$AAA mutant into 293T cells, the overall level of virus production was assessed by measurement of p24 accumulation 48 h later in the culture supernatants. These studies revealed that none of these Nef mutations altered virus production (Fig. 2A).

Similarly, each of these viruses replicated at very similar levels in spreading infections of phytohemagglutinin-stimulated PBMC cultures (Fig. 2B). Prior studies demonstrated that Nef enhancement of infectivity of HIV-1 particles is considerably diminished when mitogen-stimulated peripheral blood cells serve as targets of infection in the presence of large quantities of virus (48, 69). These studies confirmed that each of the constructed molecular HIV-1 clones was capable of producing infectious virus that could spread effectively in mitogen-stimulated primary T-cell cultures.

To examine whether $P_1P_2P_3$AAA displayed diminished expression levels in infected phytohemagglutinin-stimulated PBMC as found in transfected 293T cells (Fig. 1A) and JJK cells (Fig. 1B), equal numbers of PBMC infected with wild-type NL4-3N or the $P_1P_2P_3$AAA mutant were lysed and immunoblotted with rabbit anti-Nef antibodies (Fig. 2C). The intensity of the $P_1P_2P_3$AAA Nef mutant band (Fig. 2C, lane 2), visualized from 8×10^5 cells, was approximately 33% of the intensity of the wild-type Nef band present in a comparable number of cells (Fig. 2C, lane 5). Dilution of the NL4-3N-infected cell lysate to 2×10^5 or 4×10^5 cell equivalents (Fig. 2C, lanes 3 and 4) was required to obtain a signal comparable in intensity to that produced by 8×10^5 $P_1P_2P_3$AAA-infected PBMC (Fig. 2C, lane 2). Therefore, when expressed in infected primary T cells, in which expression of Nef is controlled by the HIV-1 long terminal repeat and subjected to appropriate splic-

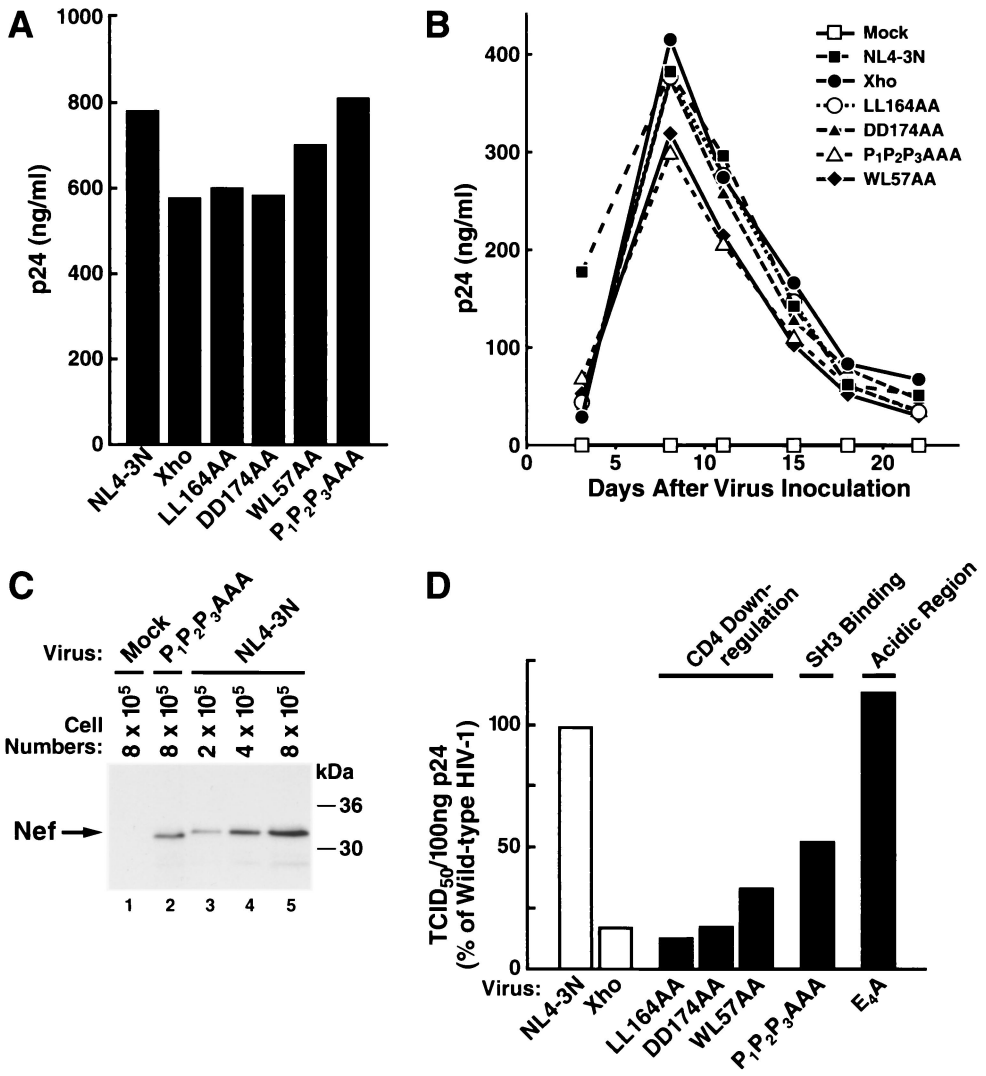

FIG. 2. Virus production, spreading infection, and infectivity of HIV-1 expressing wild-type Nef or the various Nef mutants. (A) Culture supernatants from 293T cells transfected with a molecular clone of HIV-1 carrying the various Nef mutations were analyzed for virus production by p24 antigen-capture ELISA. (B) Phytohemagglutinin-stimulated PBMC were infected (25 ng of p24 per 4×10^6 cells in 2.5 ml of medium), and the culture supernatants were monitored by p24 antigen-capture ELISA. Results show that viral infections peaked on day 8 after virus inoculation. (C) Lysates from NL4-3N- and $P_1P_2P_3$AAA-infected PBMC were immunoblotted with an anti-Nef rabbit serum. (D) The $TCID_{50}$ for each virus was determined by limiting dilution with phytohemagglutinin-stimulated PBMC as cellular targets. The data are expressed as a percentage of the infectivity of wild-type NL4-3N.

ing, the $P_1P_2P_3$AAA Nef is consistently expressed at levels between 25 and 50% of that of wild-type Nef.

We next tested the infectivity of the recombinant viruses carrying the various Nef mutations. The infectivity titer ($TCID_{50}$) was determined for each virus by limiting dilution in phytohemagglutinin-stimulated PBMC (Fig. 2D). NL4-3N carrying Nef mutations making it defective for CD4 downregulation (LL164AA, DD174AA, or WL57AA) displayed diminished infectivity in PBMC; this reduction was comparable to that observed with NL4-3N lacking Nef expression (Xho) and more pronounced than that observed with the NL4-3N clone expressing the $P_1P_2P_3$AAA Nef mutant (24, 61) (Fig. 2D). In contrast, the E_4A Nef mutant, which is impaired in MHC class I downregulation, displayed a level of infectivity similar to that detected with the wild-type virus. These results confirm the correlation between Nef-mediated CD4 downregulation and Nef-mediated enhancement of HIV-1 infectivity in primary T cells expressing CD4 and serving as virus producers (36, 43, 60).

Determination of minimal inoculum of HIV-1 NL4-3N required to initiate a productive infection in the implants of SCID-hu Thy/Liv mice. To compare the replication kinetics and pathogenic properties of the *nef* mutants in vivo, we employed the SCID-hu Thy/Liv mouse model of HIV-1 infection (50). Prior studies have shown that while HIV-1 Nef is required for efficient in vivo viral replication and pathogenicity, the infectivity and pathogenicity of HIV-1 lacking Nef are attenuated but not abolished in this model. Viruses lacking *nef* retain the ability to induce thymocyte depletion if implants are inoculated with larger amounts of virus (1,000 infectious

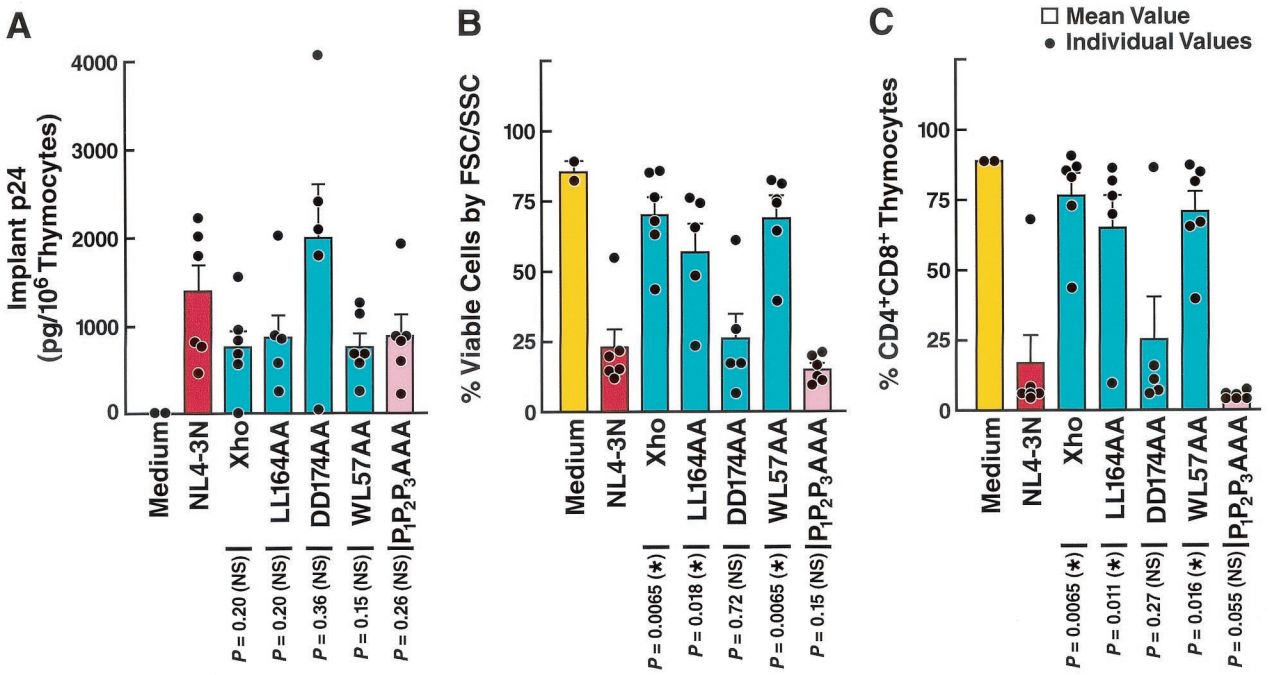

FIG. 3. HIV-1 containing *nef* mutations that impair CD4 downregulation produces significantly less depletion of thymocytes in SCID-hu Thy/Liv mice. (A) Cell-associated p24 accumulation was measured to assess the relative level of viral replication at 6 weeks after inoculation of the Thy/Liv implant mice. (B) At 6 weeks after inoculation of Thy/Liv implant mice with either wild-type NL4-3N or NL4-3N expressing the various Nef mutants, the percentage of live cells was determined by flow cytometric forward- and side-scatter analysis. (C) In cells derived from the same infected implants, the percentage of immature double-positive ($CD4^+$ $CD8^+$) thymocytes present was determined. The height of each bar indicates the mean of the experimental values shown by the solid circles. Error bars indicate standard errors of the mean. *P* values from the Mann-Whitney *U* test were calculated for each mutant relative to wild-type NL4-3N with StatView 5.0 (SAS Institute Inc., Cary, N.C.). NS, not significant. We did not exclude the Xho-infected implant that had no detectable p24 because it was successfully inoculated with 20,000 $TCID_{50}$ and appeared to be of good quality.

units) and observed for longer periods (6 to 9 weeks) (3, 4, 16, 31).

To establish the minimal inoculum of wild-type NL4-3N required for productive infection, 4×10^3 to 4×10^7 $TCID_{50}$ (in 50 μl) were inoculated into each implant of SCID-hu Thy/Liv mice by direct injection. Productive infection of the implants was assessed by p24 ELISA on lysates of dispersed thymocytes from implants collected 4 weeks after inoculation. Only one of four implants inoculated with 4×10^3 $TCID_{50}$ contained detectable p24 (410 pg/10^6 cells), while all implants inoculated with higher doses became productively infected (510 to 2,900 pg of p24/10^6 cells). (Data are expressed as picograms of p24 per 10^6 cells rather than p24 per implant for a measure of infectivity on a per-cell basis because virus-mediated thymocyte depletion reduces overall implant viral loads.) For subsequent studies, we therefore used 20,000 $TCID_{50}$ per implant of NL4-3N and NL4-3N containing the various Nef mutations (all generated by transfection of 293T cells) and collected the implants 6 weeks after inoculation.

HIV-1 expressing *nef* mutations defective for CD4 downregulation are less pathogenic than wild-type HIV-1 in SCID-hu Thy/Liv mice. To determine if the ability of Nef to downregulate CD4 plays a role in the pathogenic events observed in the Thy/Liv mouse model, we analyzed viral replication (Fig. 3A) and thymocyte depletion (Fig. 3B and C) in mice whose implants were inoculated with wild-type NL4-3N, NL4-N (Xho), or the three Nef mutants defective for CD4 downregulation, LL164AA [defective in clathrin adaptor binding (9, 25, 41)], DD174AA [defective in assembly with a cellular vacuolar ATPase responsible for endocytic acidification (42) or in interaction with the cytoplasmic tail of CD4 (45)], and WL57AA [defective in interaction with the cytoplasmic tail of CD4 (42, 45)]. Implants were also inoculated with the $P_1P_2P_3AAA$ mutant, which is capable of CD4 downregulation despite 50 to 75% lower levels of Nef expression.

At 6 weeks after virus inoculation, no statistically significant differences in implant p24 production were observed between wild-type NL4-3N and each of the Nef mutants (Fig. 3A), and most inoculated implants had high levels of p24 (≥500 pg per 10^6 cells) regardless of *nef* genotype. Despite similar levels of p24 production, which indicate comparable infectivity, two of the three Nef mutants defective for CD4 downregulation (LL164AA and WL57AA) were attenuated for thymocyte depletion, as assessed by the percentage of viable cells (events in the live lymphocyte gate as a percentage of total events) based on forward- and side-scatter properties (Fig. 3B), and by the percentage of immature cortical $CD4^+$ $CD8^+$ thymocytes (Fig. 3C). Unlike wild-type NL4-3N, which reduced viable thymocytes from 86% to 23% and $CD4^+$ $CD8^+$ thymocytes from 89% to 17%, LL164AA and WL57AA produced only small declines in these percentages by 6 weeks after inoculation (Fig. 3B and C).

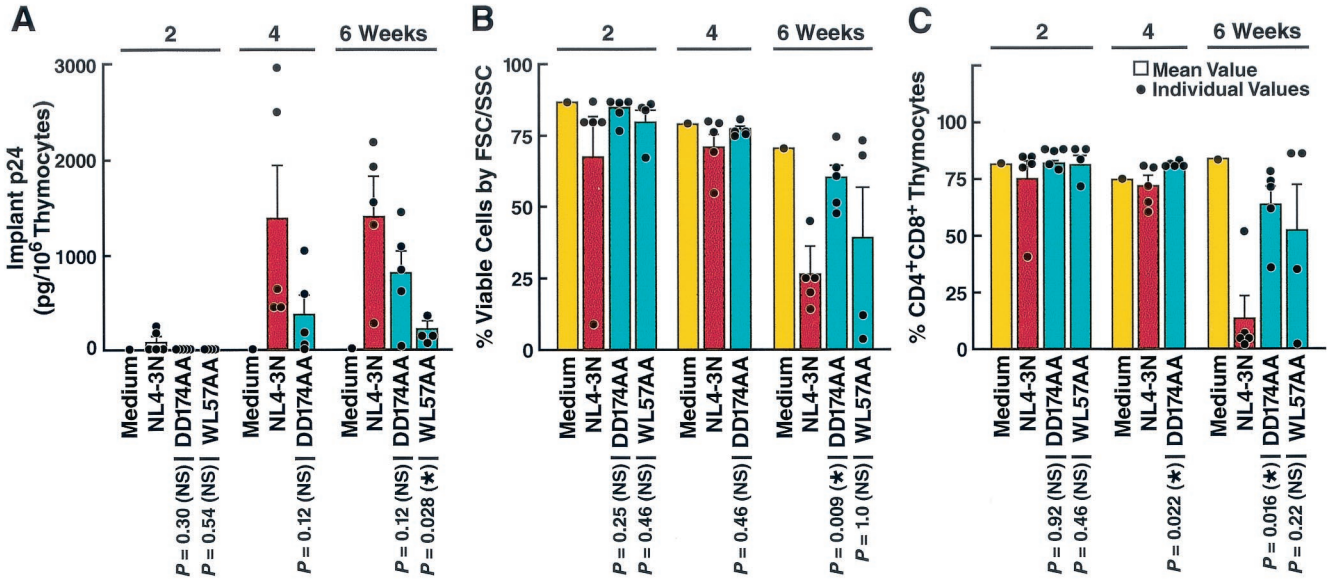

FIG. 4. Time course analyses of viral replication and cytopathicity in SCID-hu Thy/Liv mice infected with wild-type or DD174AA and WL57AA. (A) Viral replication was assessed by analysis of p24 accumulation in infected thymocytes at 2, 4, and 6 weeks after inoculation. (B) Determination of percentage of live cells from human thymus implants assessed after 2, 4, and 6 weeks of infection assessed by flow cytometric forward- and side-scatter measurements. (C) Percentage of viable immature double-positive ($CD4^+$ $CD8^+$) thymocytes remaining at 2, 4, and 6 weeks. Results and statistical analyses were performed as described in the legend to Fig. 3.

The diminished cytopathic properties of these two mutations defective for CD4 downregulation resembled that of NL4-3N (Xho), which encodes only the 35 N-terminal amino acids of Nef. In contrast, and despite its lower level of Nef expression, $P_1P_2P_3$AAA depleted human thymocytes in the SCID-hu Thy/Liv mice as effectively as wild-type NL4-3N did (Fig. 3B and C). This result agrees with previous work in the SCID-hu Thy/Liv mouse model reported by Aldrovandi et al. (3) and suggests that polyproline-dependent enhancement of infectivity (Fig. 2D), MHC class I downregulation (Fig. 1B), and interaction with the cellular kinases PAK and Hck (Fig. 1C) (73) are dispensable for Nef-mediated HIV-1 pathogenesis in SCID-hu Thy/Liv mice. These results thus reveal a correlation between CD4 downregulation and virus-induced depletion of thymocytes in the SCID-hu Thy/Liv mouse model.

Although LL164AA and WL57AA displayed attenuated thymocyte depletion, the DD174AA mutant (which is also defective in CD4 downregulation) reduced thymocyte viability and depleted $CD4^+$ $CD8^+$ thymocytes to the same extent as wild-type NL4-3N in the same experiment (Fig. 3B and C). We attributed this to the observation that the attenuated pathogenicity of Nef mutants in SCID-hu Thy/Liv mice can be overcome by increasing the size of the inoculum and by allowing the infection to proceed for 6 weeks (3, 4, 31). We therefore performed a time course study in a different SCID-hu Thy/Liv cohort, in which implants were collected 2, 4, and 6 weeks after inoculation (Fig. 4) to reveal differences between NL4-3N and DD174AA that were not apparent from a single, relatively late time point.

The DD174AA virus, like the other Nef mutant viruses, trended toward less replication at 4 and 6 weeks compared to wild-type NL4-3N, but these differences were not statistically significant (Fig. 4A). Despite comparable levels of p24 production, DD174AA (like the other CD4 downregulation mutants) exhibited impaired pathogenicity, producing only slight declines in thymocyte viability and percentage of $CD4^+$ $CD8^+$ thymocytes at 6 weeks compared with significant loss of viability (26% versus 71% for mock infection) and reduction in $CD4^+$ $CD8^+$ thymocytes (14% versus 84% for mock infection) in implants inoculated with NL4-3N (Fig. 4B and C). For NL4-3N, the loss of thymocyte viability and percentage of $CD4^+$ $CD8^+$ cells were paralleled by a 73% reduction in overall cell yield (from 320×10^6 cells per implant at week 4 to 86×10^6 at week 6). Similar findings of impaired replication have been reported in rhesus macaques infected with an SIV molecular clone engineered to express a Nef mutant that is incapable of CD4 downregulation and carries three amino acid substitutions encompassing the analogous D204 residue (30).

The fact that DD174AA depleted thymocytes to the same degree as did NL4-3N by 6 weeks after inoculation in the first SCID-hu study (Fig. 3) but not by 6 weeks in the subsequent time course performed in a different SCID-hu cohort (Fig. 4) is not unexpected, given the well-described (56) donor-to-donor variation in the fetal tissue used for implantation. Indeed, in the time course study, the WL57AA mutant displayed somewhat swifter depletion kinetics than in the previous experiment, reducing cell viability and percentage of $CD4^+$ $CD8^+$ thymocytes to levels intermediate between those caused by wild-type NL4-3N and DD174AA. Moreover, despite the lack of a significant difference in depletion between NL4-3N and DD174AA in the first study, the reduction in the percentage of $CD4^+$ $CD8^+$ thymocytes was significantly greater for $P_1P_2P_3$AAA (wild-type for CD4 downregulation) than for DD174AA (Fig. 3C; 5% versus 25%, $P = 0.0137$), further confirming the impaired phenotype of the DD174AA mutant.

Together, through the analysis of three independent Nef

mutants, our findings reveal a correlation between the inability of Nef to downregulate cell surface expression of CD4 and attenuated HIV-1 pathogenicity in SCID-hu Thy/Liv mice, as evidenced by slower kinetics of thymocyte depletion.

DISCUSSION

In this series of studies, we analyzed which of the various in vitro functions ascribed to HIV-1 Nef are most closely associated with in vivo pathogenesis in the SCID-hu Thy/Liv mouse model. We found that introduction of three different Nef mutations that compromised CD4 downregulation (WL57AA, LL164AA, and DD174AA) into the NL4-3N molecular clone leads to viruses that display attenuated pathogenicity in the human thymus implants of these mice. These changes in cytopathic effect did not significantly correlate with changes in replication of the mutant viruses, although each of these mutant viruses tended to produce lower levels of p24 in the implants. The viruses containing Nef alleles defective for CD4 downregulation did display significantly lower infectivity in phytohemagglutinin-stimulated PBMC, based on limiting-dilution determination of $TCID_{50}$.

More study is required to determine whether cytopathicity and viral replication are indeed separable phenomena in the thymus. In this regard, Glushakova and colleagues recently reported that CD4 downregulation by HIV-1 Nef also correlates with depletion of mature T cells in human lymphoid cell histocultures (22). However, in this system, viral replication was clearly linked to cytopathicity. In contrast to the Nef mutants compromised for CD4 downregulation, NL4-3N that carried a polyproline ($P_1P_2P_3$AAA) mutation and exhibited diminished infectivity and failed to downregulate MHC class I antigens displayed pathogenic potential comparable to that of wild-type virus.

Three recent reports have highlighted the importance of cell surface CD4 downregulation by Nef in augmenting viral production and infectivity (36, 43, 60). The first report indicates that Nef is required to prevent the incorporation of CD4 into nascent HIV-1 particles (36). Viral particles produced in the absence of Nef incorporate fewer envelope proteins and more CD4 molecules. The resulting viral particles are less infectious. A second report shows that in the presence of high levels of CD4, HIV-1 structural proteins accumulate in the host cell, causing a reduction in the release of viral particles (60). Nef reverses this reduction in viral particle release. Finally, Lundquist and colleagues, with a battery of Nef point mutants, reported a strong genetic correlation between replication efficiency and Nef-induced CD4 downregulation (but not Nef-induced enhancement of virion infectivity) in activated primary $CD4^+$ T cells (43). These molecular mechanisms may contribute to our observation that HIV-1 recombinants carrying Nef mutations defective for CD4 downregulation are less pathogenic in SCID-hu Thy/Liv mice.

Our finding that HIV-1 carrying a Nef mutant in which the first three prolines of the $(Pxx)_4$ polyproline stretch were mutated to alanines ($P_1P_2P_3$AAA) displays the same pathogenic potential as wild-type HIV-1 is consistent with a previous study in SCID-hu Thy/Liv mice (3). This result further argues that MHC class I downregulation and Pxx-dependent enhancement of HIV-1 infectivity in phytohemagglutinin-stimulated PBMC are not required for optimal HIV-1 pathogenic potential in human thymus. The dispensability of MHC class I downregulation for pathogenesis in SCID-hu Thy/Liv mice is perhaps not surprising because no immune response is mounted against HIV-1-infected cells in this model.

The $(Pxx)_4$ region within Nef was also suggested to be important for activation of certain cellular signal transduction pathways (72). This function of Nef may also be dispensable in infected thymocytes, as these rapidly dividing and differentiating cells may be in an elevated state of activation compared to quiescent nondividing PBMC. However, it is intriguing that disruption of a similar region within SIV Nef $(Pxx)_2$ did not disrupt pathogenesis in rhesus macaques in one study (37). However, in a second study, this region within SIV Nef appeared to be important, as mutations in this region rapidly reverted to wild-type sequence in infected animals (34). Since the $P_1P_2P_3$AAA Nef mutant was expressed very poorly compared to wild-type Nef yet was capable of comparable induction of pathogenic effects, it seems plausible that a threshold level of Nef (well below that found in cells infected with wild-type HIV-1) is sufficient to promote pathogenesis. However, since this Nef mutant retained the ability to downregulate CD4, it is perhaps this function of Nef that is most crucial for acceleration of pathogenesis.

In summary, our studies demonstrate a correlation between Nef-mediated CD4 downregulation and HIV-1-associated thymocyte depletion in SCID-hu Thy/Liv mice. These studies suggest that the development of small-molecule inhibitors of Nef-mediated downregulation of CD4 might impair HIV-1 pathogenicity.

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