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A Novel HIV-1 Nef Mutation in a Primary Pediatric Isolate Impairs MHC-Class I Downregulation and Cytopathicity

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Abstract

HIV-1-induced cytopathicity of thymocytes is a major cause of reduced peripheral T cells and rapid disease progression observed in HIV-1-infected infants. Understanding the virulence factors responsible for thymocyte depletion has paramount importance in addressing the pathogenesis of disease progression in children. In this study, thymocyte depletion was analyzed following infection with two primary CXCR4-tropic HIV-1 pediatric isolates (PI), PI-2 and PI-2.1, which were serially derived from an *in utero*-infected infant. Although highly similar to each other, PI-2 showed markedly decreased thymocyte depletion *in vitro* compared with PI-2.1. Further analysis showed a novel deletion in the Nef protein (Nef Δ K7S) of PI-2, which was absent in PI-2.1. This deletion inhibited Nef-mediated major histocompatibility complex class I (MHC-I) downregulation in infected thymocytes *in vitro* and *in vivo*; in contrast, the mutated Nef continued to downregulate CD4 surface expression *in vitro*. These results suggest that HIV-1 Nef contributes to thymic damage in infants through selective functions.

Keywords: HIV-1, Nef, MHC-I downregulation, cytopathicity, thymocyte

Introduction

THE THYMUS IS PARTICULARLY ACTIVE during early life as the immune system develops in infants. Thymocyte infection with HIV-1 leads to severe thymic involution and dysfunction, resulting in declined thymopoiesis and subsequent depletion of peripheral T lymphocytes.¹ Although direct killing of thymocytes has been suggested as a major mechanism responsible for thymic dysfunction,^{2,3} HIV-1 also causes nonspecific activation and proliferation of intrathymic progenitor cells through secretion of proinflammatory cytokines in the thymic microenvironment that result in elevated viral infection, exhaustion of precursor cells, and destruction of thymic architecture.^{4,5} These conditions within the thymus have been observed in children infected with HIV-1 and are suggested to be a leading cause of rapid disease progression in perinatally infected children.^{5,6} Studies to understand the underlying pathological and molecular aspects of thymus dysfunction in HIV-1 infection suggest that the Nef protein of

HIV-1 plays a key role in impairing thymocyte development in addition to interfering with peripheral T cell function.⁷⁻¹⁰

Nef is a phosphorylated and myristoylated 27 kDa protein that is unique to HIV/SIV and is relatively conserved with a 75%–85% similarity within clade B of HIV-1.¹¹ Nef is a multifunctional protein that is suggested to play multiple key roles in HIV-1 and SIV pathogenesis.^{8,12} Deletion of Nef markedly attenuates SIV pathogenicity in the rhesus macaque model of AIDS,^{8,13} and dysfunctional Nef has been associated with delayed disease progression in humans.¹⁴⁻¹⁶ Nef has also been shown to impair T cell development^{10,17} and enhance viral replication and infectivity.^{18,19} Its expression begins early in the viral life cycle and localizes to the plasma membrane, cytoplasm, and within the budding virions. Nef promotes viral infectivity, immune evasion, and replication capacity by altering the activation state of infected cells. It does so by modulating cell signal transduction pathways and altering cell surface expression of receptors. Nef regulates various cell signaling molecules, such as Hck, Lck, Fyn, Lyn,

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Vav, Src, PAK1/2 (NAK), MAPK (Erk1), c-Raf1 kinase, PI3K, and Ask1.^{20,21} Nef downregulates cell surface receptors such as CD4,^{22–24} major histocompatibility complex class I (MHC-I),^{23–25} MHC-II, and MHCII-associated invariant chain (Ii, CD74),²⁶ CD28,²⁷ and host restriction factor SERINC5.²⁸

Nef-mediated downregulation of surface expression of MHC-I and CD4 has been extensively studied *in vitro*. The downregulation of MHC-I A and B molecules protects infected cells from CTL-mediated killing.^{29–31} Previously, we described two closely related CXCR4-tropic HIV-1 primary isolates [pediatric isolates (PI)-2.1 and PI-2] serially derived from an *in utero*-infected infant, and their dissimilar abilities to cause thymocyte depletion.³² PI-2.1 showed aggressive thymocyte depletion *in vitro*, while isolate PI-2 showed diminished cytopathicity. Given the central role of Nef in HIV-1 disease progression, we performed a detailed analysis of Nef sequences of these two pediatric isolates and their corresponding abilities to downregulate MHC-I and CD4.

Materials and Methods

HIV-1 pediatric isolates and virus stocks

HIV-1 primary isolates and plasma samples for virus isolation were obtained as close as possible to birth from 10 of 34 children born to 204 HIV-1-seropositive mothers monitored prospectively in the Los Angeles Pediatric AIDS Consortium between June 1988 and May 1995.^{32–34} The isolates were characterized as syncytium inducing (SI) or nonsyncytium inducing (NSI) by using the MT-2 assay.³⁵ The characteristics of some of the children providing the viral isolates were previously described: PI-2 is an SI HIV-1 isolate obtained 1 week after birth from an infant infected *in utero* who developed AIDS and died within 6 months.³⁴ PI-2.1 was isolated from plasma 1 month after birth from the same infant. PI-2 and PI-2.1 isolation and propagation were done by directly adding serum from an infected infant to a pool of purified activated allogeneic CD4⁺ T cells, prepared as described previously.^{36,37} Briefly, allogeneic CD4⁺ T cells from three normal donors were individually purified by capture in CD4 monoclonal antibody-coated tissue culture flasks (Applied Immune Sciences, Santa Clara, CA) and activated by stimulation with 200 ng/mL anti-human CD3 antibody (OKT3; Ortho Biotech, Inc., Raritan, NJ) plus 5,000 U/mL recombinant human interleukin-2 (IL-2) for 5 days. Cells from three donors were combined, cryopreserved in liquid nitrogen, and then thawed and cultured in medium with IL-2 for 2–3 days before infection. The makeup by phenotype of the CD4⁺ T cell pool at the time of infection by the pediatric isolates was as follows: 95% CD4⁺ CD8⁻, 5% CD4⁺ CD8⁺, <1% CD8⁺ CD4⁻, 60% CD27⁺, and 50% HLA-DR⁺. In addition, 94% of the cells from the stimulated CD4⁺ T cell pool expressed CXCR4 and 30%–40% expressed CCR5 (data not shown).³² The same CD4⁺ T cell pool was used for isolating PI-2 and PI-2.1 virus from plasma (passage 1) and for preparing virus stocks (passage 2) and for preparing the control viruses including CCR5-tropic virus HIV-1 NF-NSX (NF-NSX) and the CXCR4-tropic virus HIV-1 NL4-3 (NL4-3).^{38,39} The infectivity of the pediatric isolates and of the molecular-clone-derived laboratory viruses was determined by limiting dilution assays in PBMC. The CCR5-tropic

pediatric isolate PI-8 was prepared similarly to PI-2 and PI-2.1 and used as control for the Western blot studies.³²

Cloning and sequencing of the nef gene in pediatric HIV-1 isolates

RNA was isolated from plasma using the TRIzol reagent (Invitrogen, Carlsbad, CA) and was reverse transcribed into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Reverse transcription was primed with a random primer (Applied Biosystems) and following nested polymerase chain reaction (PCR), the cDNA of full-length *nef* was amplified. First and second rounds of PCR were carried out using Nef 8687F (5-GTAGCTGAAGGGACAGATAGGGTTAT-3) and Nef 9589R (5-TAGTTAGCCAGAGAGCTCCCA-3) as the outer primer set and Nef 8787 XbaI (5-GCTCTAGAATG GGTGGCAAGTGCTCAA-3) and Nef 9495R (5-TTATATG CAGCATCTGAGGGC-3) as the inner primer set, respectively. Numbering of primers is based on the NL4-3 sequence. High Fidelity Advantage[®] HD Polymerase Mix (Clontech, Mountain View, CA) was used for PCR, according to the manufacturer's instructions. After cloning of PCR products into the Topo TA cloning vector (Invitrogen), plasmid DNAs were isolated from at least six colonies for sequencing with the ABI 3130 Genetic Analyzer (Applied Biosystems).

Production of single-round infectious, pseudotyped HIV-1 reporter virus

The reporter HIV-1 construct AA1305#18 plasmid containing an NL4-3 backbone with reporter antigens heat shock antigen (HSA) or mouse CD24 (mCD24) and deletions in Vpu, Env, and Vpr was used to produce VSV-G pseudotyped virus as described previously.⁴⁰ Insertion of isolated *nef* genes was done using PCR products that were gel purified with Quick Spin Column (Qiagen, Valencia, CA) and digested with *Xba*I and *Bsp*EI restriction enzymes (New England Biolabs, Ipswich, MA). Single-round infectious pseudovirus was produced by cotransfecting 293T cells with the whole-genome plasmid AA1305#18 containing *nef* alleles and a plasmid encoding the envelope glycoprotein from VSV-G using BioT transfection reagent (Bioland, Paramount, CA).

Western blot

HIV-1 *nef* genes from the pediatric isolates (PI) were cloned into a plasmid containing the NL4-3 genome (pNL4-3) to make infectious virus as described previously.⁴¹ To construct NL4-3/PI-*nef*, the *nef* gene of NL4-3 was replaced with either individual *nef* obtained from Topo cloning or quasispecies or *nef* obtained from the nested PCR as described above. T1 cells were infected with NL4-3 virus containing *nef* alleles from either wild-type NL4-3 or alleles from the pediatric isolates (multiplicity of infection [MOI]=1). On day 5, lysates harvested from the infected cells were run onto 10% polyacrylamide gel electrophoresis (PAGE) for protein separation. Following transfer to a PVDF membrane (Millipore, Billerica, MA), HIV-1 proteins were detected by an anti-Nef antibody (Nef#2949; NIH AIDS Reagent Program) or an anti-Gag antibody (Gag#6457; NIH AIDS Reagent Program) followed by detection using the SuperSignal

West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

Immunophenotyping

Surface expression of MHC-I and CD4 in HIV-1-infected and uninfected cells was done using flow cytometry. Study of MHC-I downregulation by Nef on infected thymocytes^{40,42} with FITC- and PE-conjugated monoclonal antibodies was done as previously described.^{42–45} PE-conjugated or FITC-conjugated KC57 mAb (Beckman Coulter, Brea, CA) was used to determine intracellular HIV-1_{Gag} protein expression in distinct thymocyte subsets^{43,46} and was performed following surface immunophenotyping.^{44,45} Study of CD4 downregulation by Nef on infected cells was described previously.⁴⁰ Briefly, 2×10^6 T1 cells were infected with 100 μ L (10 ng p24) of viral stock. Three days postinfection, surface expression of CD4 and mCD24 was detected with PE-conjugated human anti-CD4 (BioLegend, San Diego, CA) and FITC-conjugated mouse anti-CD24 (BioLegend). Multiparameter data acquisition was done on a dual-laser FACS Calibur flow cytometer (BDIS, San Jose, CA) or an MACSQuant VYB, and data analysis was performed with FCS Express 6.0.

In vitro infection of postnatal thymocytes

Normal human postnatal thymus specimens were obtained from infants and children undergoing corrective cardiac surgery. The tissue was cut into small pieces, red blood cells were removed by NH_4Cl /Tris lysis buffer, and the mechanically disrupted tissue was passed over a cell strainer to generate a single-cell suspension of thymocytes as previously described.^{39,44,46} Thymocytes were infected *in vitro* with CXCR4-tropic pediatric isolates (PI-2 and PI-2.1), the CXCR4-tropic molecular clone NL4-3, the CCR5-tropic molecular clone NF-NSX, or mock infected with the supernatant from the same CD4⁺ T cell pool used for virus stock preparation. The thymocytes were cultured in serum-free medium for 14 days with IL-2 and IL-4 as previously described.³² All thymocyte infections with pediatric isolates were standardized by using equal numbers of virus infectious units/ 10^7 cells. HIV-1 virion production was measured by HIV-1 Gag p24 enzyme-linked immunosorbent assay as previously described.⁴⁴

In vivo infection of SCID-hu mice

C.B-17 SCID mice were bred at UCLA and implanted with human fetal thymus and liver graft (thy/liv) under the murine kidney capsule.⁴⁷ Four to six months postimplantation, the thy/liv grafts of SCID-hu mice implanted with thy/liv grafts from the same donor were infected with different HIV-1 isolates (PI-2, PI-2.1, NL4-3, or NFN-SX) by injecting 10–30 ng of p24 in a 50- μ L volume directly into the graft as previously described.⁴⁴ Mock-infected implants, used as controls in the experiments, were prepared by injecting implants with 50 μ L of appropriate control supernatants. Two different experiments consisting of 10–12 SCID-hu mice per 3-week time point were conducted. MHC-I and CD4 phenotyping in HIV-1-infected cells was done at 3 weeks postinfection as previously described.⁴⁵

Results

PI-2 is less cytopathic to thymocytes *in vitro* than PI-2.1 or NL4-3

HIV-1 strains isolated from *in utero* and perinatally infected children vary in rates of disease progression and exhibit differential effects on thymopoiesis during *in vitro* culture.^{6,32} Beyond the observation that CXCR4-tropic HIV isolates cause more thymocyte depletion than CCR5-tropic HIV isolates,⁴⁸ it is unclear what molecular mechanisms determine differential effects on thymocyte depletion and thymic development by HIV-1. Two CXCR4-tropic HIV-1 isolates (PI-2 and PI-2.1) were isolated from an *in utero*-infected infant and were found to infect CD4⁺ thymocytes differently.³² Primary human thymocytes were infected *in vitro* with PI-2, PI-2.1, or NL4-3 viruses in parallel with a mock-infection control and cultured in serum-free medium supplemented with IL-2 and IL-4 for 14 days. Flow cytometric analysis of surface CD4 and CD8 expression was done on the thymocytes to measure subset depletion. Apoptotic and dead cells were excluded using 7-AAD, and quadrants were set using isotype controls. Following *in vitro* infection of human thymocytes, PI-2 was less efficient in depleting CD4⁺ thymocytes (9% CD4⁺ depletion) compared with PI-2.1 (40% CD4⁺ depletion) or the CXCR4-tropic molecular clone NL4-3 (90% CD4⁺ depletion) as related to the percentage of CD4⁺ cells in mock-infected thymocytes (Fig. 1A). Despite initial infection with equal infectious units, PI-2 *in vitro* replication was also lower than PI-2.1 as measured by p24 levels in the culture supernatants (Fig. 1B).

PI-2 contains amino acid mutations in the Nef protein

The Nef protein was previously shown to affect the pathogenicity of HIV-1 in cultured thymocytes¹⁷ and the thy/liv implant in the SCID-hu mouse model.⁴⁹ Sequencing of the *nef* alleles found in PI-2 and PI-2.1 was done to examine a possible cause of their discrepancy in thymic cytopathicity. Analysis of the two *nef* sequences revealed that the amino acids (aa) of these two isolates were highly similar, and the majority of reported functional motifs were conserved in both isolates (Fig. 2A). The myristoylation residue (G2) required for membrane localization, the oligomerization domain needed for all functions, and motifs such as WL₅₈, LL₁₆₅, DD₁₇₅, and EE₁₅₅, which are required for CD4 binding and lysosomal degradation function, were found to be preserved in both isolates. However, a deletion of seven amino acids (Nef Δ K7S) from amino acid positions 82 to 88 (₈₂KGALDLS₈₈) was present in PI-2 but not in PI-2.1. This sequence falls within the core domain of Nef (aa 72–203), which is a relatively conserved and structured type II polyproline helix (aa 72–77) followed by two α -helices (aa 81–120).⁵⁰ This novel deletion is also 4 amino acids downstream of a polyproline helix (₇₂PXXPXR/K₇₇) binding site for Src-homology domain 3 (SH3) that mediates cellular activation and MHC-I downregulation.^{15,29} In addition to Nef Δ K7S, a single amino acid mutation at position 71 from K to R (K71R), adjacent to the SH3 binding site, was observed in PI-2.

Nef mutations in PI-2 do not inhibit expression of structural proteins

To assess if the identified mutations in the *nef* gene of PI-2 inhibited Nef protein expression, Western blot analysis was

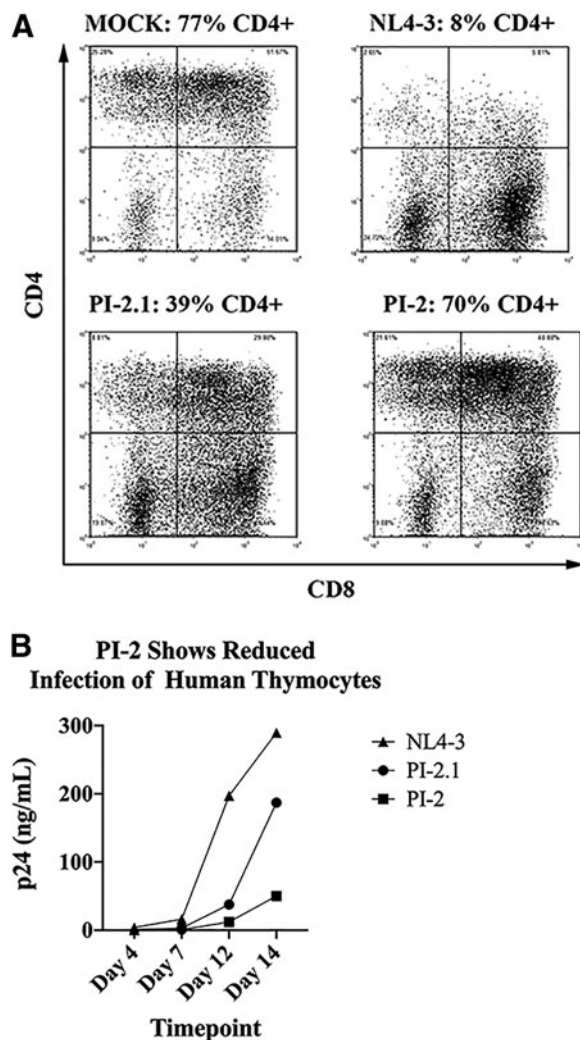


FIG. 1. Thymocytes infected with the CXCR4-tropic HIV isolate PI-2 *in vitro* show less CD4 depletion and lower HIV-Gag p24 levels than thymocytes infected with PI-2.1 or NL4-3. Thymocytes were infected *in vitro* with CXCR4-tropic pediatric isolates (PI-2 and PI-2.1), the CXCR4-tropic NL4-3 molecular clone NL4-3, or mock infected with the supernatant from the same cells (CD4⁺ T cell pool). (A) Fourteen days postinfection, thymocytes were stained for CD4 and CD8. Apoptotic and dead cells were excluded using 7-AAD, and quadrants were set using isotype controls. (B) Viral replication was followed by measuring HIV Gag p24 antigen in the supernatants. PI, pediatric isolates.

done on cells infected with CXCR4-tropic molecular clone NL4-3 (lane 1), CCR5-tropic pediatric isolate PI-8³² (lane 2), and CXCR4-tropic isolate PI-2 (lane 3). The results show that PI-2-infected cells expressed similar levels of HIV-1 Gag (Fig. 2B) and Nef (Fig. 2C) as cells infected with PI-8 or NL4-3 and used as controls. Accordingly, PI-2-infected cells express HIV-1 Nef despite mutations.

PI-2 is impaired in MHC-I downregulation in vitro and in vivo

While a myriad of cellular effects have been shown for Nef, two of its most clearly defined functions are to downregulate cell surface expression of MHC-I and CD4 mole-

cules. These functions were assessed in thymocyte cultures infected with PI-2 and PI-2.1 and compared with cultures infected with the HIV-1 molecular clones NL4-3 or NFN-SX (NL4-3 with a substituted CCR5-tropic Env from strain JR-FL). Mock-infected cultures were prepared by adding supernatants of uninfected activated allogeneic CD4⁺ T cell pools. Thymocytes infected *in vitro* (Fig. 3) were analyzed for surface expression of MHC-I. Following infection, MHC-I downregulation was assessed by the mean fluorescence intensity (MFI) of MHC-I in HIV-1 Gag⁺ cells (KC57⁺). The cells were surface stained with antibodies to MHC-I (HLA-ABC-FITC) and CD3-APC followed with intracellular staining for HIV-1 Gag proteins using the KC57-PE antibody. Isotype controls were used to set the quadrants. MHC-I expression on mock-infected thymocytes is shown in Figure 3A. Cells productively infected (KC57⁺) with CXCR4-tropic NL4-3 containing a functional Nef (Fig. 3B) had decreased expression of MHC-I (MFI=263) compared with uninfected (KC57⁻) cells (MFI=745). Cells infected with the CCR5-tropic virus NF-NSX containing a functional Nef (Fig. 3C) had a decreased expression of MHC-I (MFI=217) compared with uninfected cells (MFI=551). Cells infected with the pediatric isolate PI-2.1 containing a functional Nef (Fig. 3D) also had decreased MHC-I expression (MFI=220) compared with uninfected cells (MFI=639). However, cells infected with the pediatric isolate PI-2 containing the Nef mutation (Fig. 3E) showed a smaller decrease in MHC-I surface expression (MFI=417) compared with uninfected cells (MFI=573).

The ability of the pediatric isolate Nef proteins to downregulate MHC-I was also tested *in vivo* using an SCID-hu mouse model implanted with human fetal thy/liv tissue that forms a thymus-like organoid as previously described.^{44,45} SCID-hu mice were infected with NL4-3, PI-2, PI-2.1, or mock-negative control supernatant. MHC-I downregulation in productively infected (KC57⁺) thymocytes was measured *ex vivo* at 3 weeks postinfection by measuring the HLA-ABC MFI. As shown in Figure 4A, cells infected with NL4-3 ($n=5$ mice) and PI-2.1 ($n=5$ mice) showed decreased mean surface expression of HLA-ABC compared with cells infected with PI-2 ($n=6$ mice). PI-2 expressing thymocytes had similar levels of MHC-I expression as mock infection ($n=5$ mice). Statistical analysis was done using two-tailed unpaired *t* tests. Similar to the *in vitro* experiments, infections of thy/liv implants with PI-2, PI-2.1, or NL4-3 again demonstrated diminished capabilities of PI-2 to downregulate MHC-I following infection, whereas PI-2.1 exhibited comparable MHC-I downregulation with NL4-3 (Fig. 4A). The percentages of CD4⁺ expression on thymocytes in thy/liv implants following infection with PI-2 were the same as in mock-infected implants, contrary to our earlier *in vitro* experiments. However, PI-2.1-infected implants showed a similar decrease in the percentage of CD4⁺ thymocytes as NL4-3-infected implants (Fig. 4B).

HIV-1 PI-2 Nef downregulates CD4 but not MHC-I expression in the absence of Vpu and Env in vitro

To investigate the small discrepancy in CD4 downregulation between our *in vitro* and *in vivo* experiments, we analyzed the Nef proteins separately from other HIV-1 proteins, Env and Vpu, which have previously been reported

A Myristoylation Site CD4 Binding

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PI-2      1 10 20 30 40 50 60
MGNIWSKRSLSGLGWPVKVRERMRQAEPAAEGVGEVSRDLEKHGAITSS--TANSTACTWLEA
PI-2.1   MGNIWSKRSLSGLGWPVKVRERMRQAEPAAEGVGEVSRDLEKHGAITSS--TANSTACTWLEA
NL4-3    MGGKWSKSSVI GWPAVRERMRRAEPAADGVGAVSRDLEKHGAITSSNTAANNAACAWLEA
**      ***  *; *** *****;*****;*** ********** *;..: *;****

PACS Binding  SH3 Binding  PAK Binding
70 80 90 100 110 120
PI-2  QEDGEEVGF PVRPQVPLRPMTF-----HFLKEKGGLEGLIYSQORQDILD LWVYHTQG
PI-2.1 QEDGEEVGF PVRPQVPLRPMTFK GALDLSHFLKEKGGLEGLIYSQORQDILD LWVYHTQG
NL4-3  QEE-EEVGF PVPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIHSORRQDILD LWIYHTQG
**; ***** *****; ***** ***** *****; *****; *****

Oligomerization  COP-I Binding  AP1/2 Binding  VLH Binding
130 140 150 160 170 180
PI-2  YFPDQNYTQGPVRYPLCFGWCFKLPVEKEKVEEATERENTSL LHPINLHG MEDPEKE
PI-2.1 YFPDQNYTQGPVRYPLCFGWCFKLPVEKEKVEEATERENTSL LHPINLHG MEDPEKE
NL4-3  YFPDQNYTPGPVRYPLTFGWICYKLPVEPDKVEEANKGENTSL LHPVSLHG MDPERE
***** **; ***** *****; ***** ***** *****; *****; *****

190 200
PI-2  VLMWKFD SRLFH HMARELHPEYFKN-
PI-2.1 VLMWKFD SRLFH HMARELHPEYFKN-
NL4-3  VLEWRFD SRLFH HVARELHPEYFKN
** *; *****; *****; *****; *****

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FIG. 2. Nef mutations from HIV isolate PI-2 do not inhibit the expression of structural proteins. (A) Amino acid sequence comparison of Nef protein from CXCR4-tropic molecular clone NL4-3 and pediatric isolates PI-2 and PI-2.1. Asterisks indicate common amino acids; hyphen indicates a deletion; colon indicates a similar amino acid substitution; space indicates nonsimilar substitution. (B, C) Western blot of virions from CXCR4-tropic molecular clone NL4-3 (lane 1), and pediatric isolates CCR5-tropic PI-8 (lane 2) and CXCR4-tropic PI-2 (lane 3) containing the Nef mutation were screened for expression of HIV-1 viral proteins Gag (B) and Nef (C). Molecular-weight markers are displayed on the left side of the Western blots. Pediatric isolate PI-2 expresses viral protein HIV-1 Nef despite mutations.

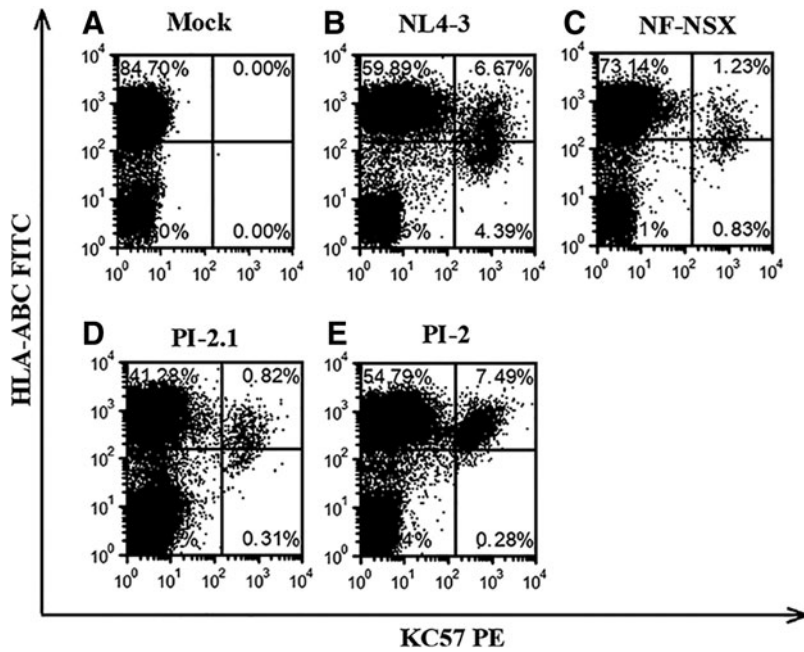
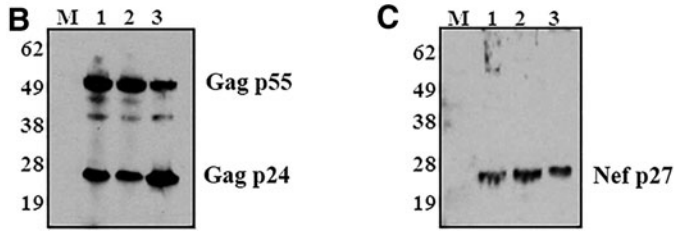


FIG. 3. Pediatric HIV isolate 2 (PI-2) is impaired in MHC-I downregulation *in vitro*. Thymocytes were infected *in vitro* with CXCR4-tropic pediatric isolates (PI-2 and PI-2.1), the CXCR4-tropic NL4-3 and CCR5-tropic NF-NSX molecularly cloned HIV-1 isolates, or mock infected with the supernatant from the same cells (CD4⁺ T cell pool). (A–E) MHC-I downregulation was measured to assess Nef function by using the geometric MFI of MHC-I (HLA-ABC) in KC57⁺ (HIV-1 Gag⁺) cells. (A) Mock infected negative control; (B) CXCR4-tropic virus NL4-3; (C) CCR5-tropic virus NF-NSX; (D) pediatric isolate PI-2.1; and (E) pediatric isolate PI-2. MFI, mean fluorescence intensity; MHC-I, major histocompatibility complex class I.

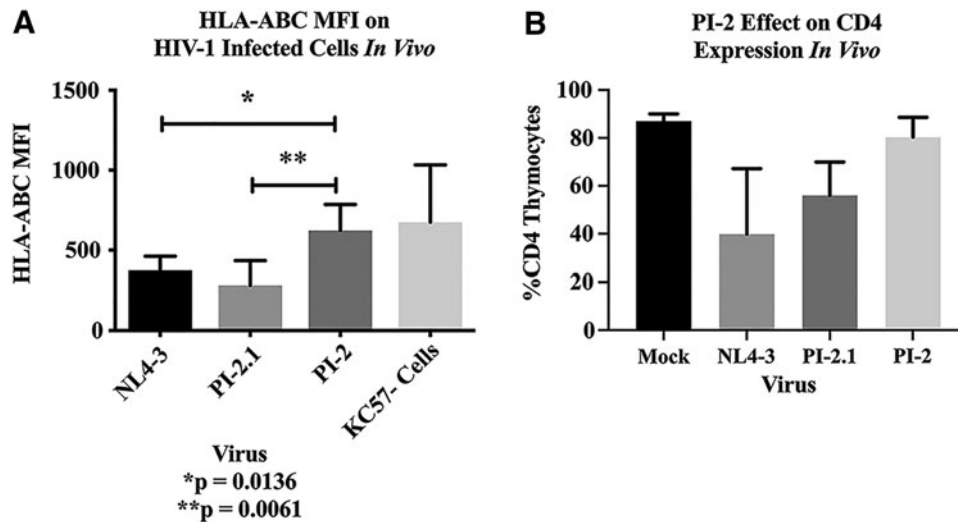


FIG. 4. Pediatric HIV isolate 2 (PI-2) is impaired in MHC-I downregulation *in vivo*, but is less cytopathic than PI-2.1. SCID mice implanted with human fetal thy/liv (SCID-hu mice) were infected with NL4-3 or pediatric isolates PI-2.1 and PI-2. (A) MHC-I downregulation was measured *ex vivo* at 3 weeks postinfection. The MFI values show the levels of MHC-I on productively infected (KC57⁺) thymocytes. Cells infected with NL4-3 ($n=5$ mice), PI-2.1 ($n=5$ mice), and PI-2 ($n=6$ mice). Error bars depict standard error of the mean. Statistical analysis was done using two-tailed unpaired *t* tests. PI-2 expressing thymocytes had similar levels of MHC-I expression as mock infection ($n=5$ mice, data not shown). (B) Percentages of thymocytes expressing CD4 were measured *ex vivo* at 3 weeks postinfection. The percentages of CD4⁺ thymocytes in PI-2 infected are similar to the mock-infected thy/liv implants. thy/liv, thymus/liver.

to contribute to CD4 surface downregulation.^{51,52} To test the effect of Nef separately from Env and Vpu while maintaining their physiologic expression levels, PI-2 and PI-2.1 *nef* genes were inserted into an NL4-3-based genome containing deletions of *env* and *vpu*.⁴⁰ Single-cycle infection was done using VSV-G pseudotyped viruses expressing a CD24 tag and the various *nef* genes. In agree-

ment with our earlier *in vitro* data obtained in thymocytes infected with the original pediatric isolates, PI-2 Nef was unable to downregulate MHC-I in infected cells as much as Nef from PI-2.1 and NL4-3 (Fig. 5A–E). Confirming our previous experiment, PI-2 Nef was still able to downregulate CD4 surface expression *in vitro*, even in the absence of Vpu and Env (Fig. 5F–J).

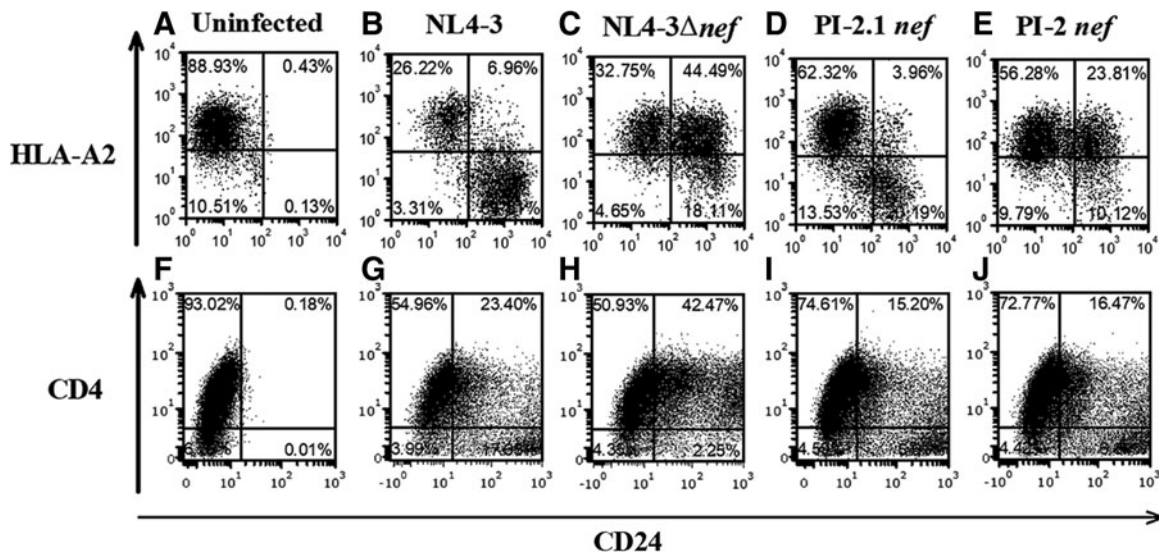


FIG. 5. HIV PI-2 Nef downregulates CD4 but not MHC-I expression in the absence of Vpu, Env, and Vpr. To assess the MHC-class I downregulation of the *nef* genes in pediatric isolates PI-2.1 and PI-2 in the absence of Vpu, Env, and Vpr proteins, the *nef* genes were cloned out of the PI-2.1, PI-2, and NL4-3 isolates and placed in an NL4-3 vector lacking Vpu, Env, and Vpr. To determine the effect of PI-2.1 and PI-2 Nef proteins on MHC-I and CD4 downregulation, VSV-G pseudotyped virus, including a CD24 tag and the various *nef* genes, was used to infect T1 cells. HLA-A2 and CD4 surface expression in T1 cells infected with (A, F) mock control; (B, G) VSV-G Nef pseudotyped defective NL4-3 lacking Vpu, Env, and Vpr; (C, H) NL4-3 ΔNef; (D, I) VSV-G pseudotyped PI-2.1 *nef* lacking *vpu*, *env*, and *vpr*; or (E, J) VSV-G pseudotyped PI-2 *nef* lacking *vpu*, *env*, and *vpr*.

Discussion

Following comparison of two primary HIV-1 pediatric isolates from the same *in utero*-infected infant, PI-2 and PI-2.1, we identified a unique in-frame deletion Nef Δ K7S in PI-2, which attenuates the depletion of thymocytes *in vitro*. Nef proteins bearing this mutation are defective for MHC-I downregulation, but this effect is not due to reduced Nef expression or global Nef dysfunction. Nef proteins bearing this mutation were still able to downregulate CD4 surface expression *in vitro*. This novel Δ K7S in-frame deletion is in a location that is not known to contain any specific motifs required for previously defined Nef functions. However, it is adjacent to the SH3 binding site that is critical for multiple Nef functions, including cellular activation and MHC-I downregulation.^{53,54} It is possible that Δ K7S alters the tertiary structure of Nef and thus interferes with interactions with host factors. While there is a K versus R polymorphism that falls within the PXXP motif between residues 72–75 when comparing PI-2.1 with PI-2, this is unlikely to contribute to impaired MHC-I downregulation by PI-2. Although the NL4-3 Nef sequence has T71 (Fig. 2A), the subtype B consensus sequence is actually 71R, and thus, PI-2 actually matches “wild-type” Nef more closely than PI-2.1 or NL4-3 at position 71. Moreover, K71R is a conservative substitution. Although highly unlikely, however, we cannot definitively rule out that 71R somehow contributed to the impaired MHC-I downregulation by PI-2.

Previous data show that various mutations in Nef affect oligomerization as well as ternary complex formation with cell molecules such as AP-1 and MHC-I,^{50,55–57} and a survey of *in vivo* Nef sequences demonstrated frequent examples of Nef with impaired MHC-I downregulation despite no identifiable mutations in the defined motifs involved in this function.⁵⁸ Similarly, the significance of the K71R substitution mutation in PI-2 Nef is unclear. This mutation is also located in proximity to the canonical sequence required for SH3 binding. Mutational studies have demonstrated that residues flanking the motif play critical role in determining binding specificity to SH3 domain.⁵⁹

Others have reported that Nef-mediated CD4 downregulation correlates with increased viral pathogenesis in thymocytes, and MHC-I downregulation has diminished influence in cytopathicity.⁶⁰ In contrast, our findings indicate that PI-2-mutated *nef* containing virus was less cytotoxic in thymocytes than PI-2.1, despite having a similar ability to downregulate surface expression of CD4 *in vitro*. PI-2-infected cells did not downregulate MHC-I but did decrease CD4 surface expression *in vitro*, indicating that the novel identified mutations in PI-2 Nef may not have affected binding to several host factors reported to be involved in CD4 downregulation.^{61,62} Point mutations in the *nef* gene (WL57AA, LL164AA, and DD174AA) of the NL4-3 molecular clone abrogate CD4 downregulation and decrease Nef-mediated pathogenesis.⁶⁰ These mutations were not present in PI-2. Van Nuffel *et al.* have previously reported that Nef:PAK2 and an intact VGF motif within Nef are required for reduced thymic output.⁶³ Despite having intact VGF and PAK2 binding motifs and representative CD4 downregulation *in vitro*, PI-2 still lacked the virulence and thymic depletion seen in thymocytes infected with PI-2.1 or molecular clones, indicating a correlation between MHC-I downregulation and Nef's ability to deplete thymocytes.

Given the importance of MHC-I molecules in T cell development, it is unclear whether the defect in MHC-I downregulation by HIV isolate PI-2 is directly related to the attenuated thymic cytopathicity, or if the novel identified mutations disrupt another pathway that potentiates the negative effect of Nef in the thymopoiesis. Further unraveling this phenomenon may provide greater insight into the particularly rapid disease progression seen in HIV-1-infected children, and may offer therapeutic targets for this particularly vulnerable population.

Sequence Data

The nucleotide sequences for PI-2 and PI-2.1 *nef* genes have been deposited in GenBank under accession numbers KC702883 and KC702882, respectively.

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Authors' Contributions

A.A., L.P.-M., R.L.F., O.O.Y., and C.H.U. conceived and designed the experiments; A.A., L.P.-M., A.D.C., and D.A.-P. performed the experiments; A.A., L.P.-M., R.L.F., A.D.C., D.A.-P., and C.H.U. analyzed the data; Y.B. and O.O.Y. contributed reagents/materials/analysis tools and advice; A.A., R.L.F., O.O.Y., and C.H.U. wrote the article.

Author Disclosure Statement

The authors do not have any financial or nonfinancial competing interests.

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