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Nef does not contribute to replication differences between R5 pre-AIDS and AIDS HIV-1 clones from patient ACH142

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Abstract

AIDS-associated, CCR5-tropic (R5) HIV-1 clones, isolated from a patient that never developed CXCR4-tropic HIV-1, replicate to a greater extent and cause greater cytopathic effects than R5 HIV-1 clones isolated before the onset of AIDS. Previously, we showed that HIV-1 Env substantially contributed to the enhanced replication of an AIDS clone. In order to determine if Nef makes a similar contribution, we cloned and phenotypically analyzed *nef* genes from a series of patient ACH142 derived R5 HIV-1 clones. The AIDS-associated Nef contains a series of residues found in Nef proteins from progressors [1]. In contrast to other reports [1-3], this AIDS-associated Nef downmodulated MHC-I to a greater extent and CD4 less than pre-AIDS Nef proteins. Additionally, all Nef proteins enhanced infectivity similarly in a single round of replication. Combined with our previous study, these data show that evolution of the HIV-1 *env* gene, but not the *nef* gene, within patient ACH142 significantly contributed to the enhanced replication and cytopathic effects of the AIDS-associated R5 HIV-1 clone.

Background

The *nef* gene of HIV-1 plays a pivotal role in the pathogenesis of AIDS [4-8]. For example, patients infected with *nef*-deleted HIV-1 exhibited much slower progression to AIDS [6,9-11]. The *nef* gene is important for viral replication in mature T cells [12-16] and macrophages [14,17-19]. When thymocytes are infected, Nef plays a role in increasing the cytopathic nature of the virus [20-24]. The importance of Nef is further corroborated by observations of immune dysfunction in *nef*-transgenic mice [25-28].

Several functions have been assigned to Nef although the role of each in disease progression has not been firmly established (for reviews see: [21,29-37]). We chose to focus on Nef's abilities to downmodulate CD4 [38] and cell surface MHC-I A and B molecules [39,40], and its ability to enhance viral infectivity [12,41,42]. These functions

have been well studied by several labs and in various cell types and systems [17,43-50]. Nef mediated enhancement of infectivity may be due to Nef downmodulation of cell surface CD4, allowing more efficient Env incorporation into HIV-1 particles [51,52]. Enhancement of infectivity may also occur when Nef is present in CD4 negative producer cells [12,53-56]. In this case, enhancement appears to act at a post-entry, pre-integration step in the viral life cycle [57,58] and may be related to interaction of the viral pre-integration complex with the actin cytoskeleton [59]. Downmodulation of MHC-I A and B molecules protects cells from lysis by HIV-1 specific cytotoxic T cells [40]. The ability to avoid the immune system may be important in establishment and maintenance of infection.

Kirchhoff and colleagues compared the predicted amino acid sequences of Nef proteins from progressors with those of non-progressors and found that certain residues characterize Nef sequences from each type of patient [1]. When compared to non-progressor Nefs, progressor Nefs were better able to downmodulate CD4 and less able to downmodulate MHC-I molecules, and also may have an increased ability to enhance HIV-1 infectivity [1,2].

Results

Previously, we demonstrated that the ACH142 AIDS clone *E11 was better able to replicate and cause cytopathic effects in human fetal thymus-liver grafts implanted in severe combined immune deficient mice (SCID-hu thy/liv mice), than the pre-AIDS clones, 8G9 and 32D2 [60]. In an analysis parallel to this study, we examined the phenotypes of the *env* genes from these clones and determined that the AIDS associated *env* likely contributed to the observed replication differences between the AIDS clone and the pre-AIDS clones [61]. In order to determine if *nef* made a similar contribution, ACH142 *nef* genes were amplified from PHA-activated PBMC infected with the HIV-1 clones ACH142-*E11, 32D2, and 8G9. The one kilo base *nef*/LTR products were gel purified and inserted into the pGEM-T vector (Promega). Six *E11, six 32D2 and three 8G9 full-length *nef* genes were sequenced.

Analysis of the predicted amino acid sequences of the consensus Nef proteins revealed a high degree of conservation among the patient ACH142 biological clones (Fig. 1). When compared to sequence/function studies reported in the literature, as reviewed in [31], no lack of function mutations could be found, but three interesting differences were revealed. The AIDS Nef protein contains the rare motif GEEE (amino acids 62–65), whereas the two pre-AIDS proteins contain the more common EEEE sequence at this position. This motif has been reported to be important in MHC-I downmodulation. The *E11 sequence also has a significant lengthening of the N-terminal portion of Nef caused by repetition of the four amino acid sequence, AEPA (amino acids 23–26). Using the analysis of Kirchhoff *et al*, we calculated the Nef progression score of each ACH142 Nef. A +1 score was assigned for residues characteristic of progressors and a -1 score was assigned for those commonly found in non-progressors at the positions denoted by bold italic symbols (Fig. 1). This number therefore reflects the degree of similarity between each ACH142 Nef sequence and Nefs from progressors or non-progressors at particular amino acid positions. We found that the AIDS associated *nef*, *E11, is more similar to progressor Nef sequences with a Nef progression score of +5 than are the pre-AIDS Nefs from the same patient which had Nef progression scores of +2 and +3.

To elucidate the significance of these differences and to assign phenotypes to each Nef protein, we inserted each consensus *nef* gene into an actin promoter driven expression vector (pA-*nef*). Next we analyzed the ability of each Nef to downmodulate cell surface CD4 and MHC-I A2 molecules on the T lymphoblastoid cell line, SupT1. Electroporation and flow cytometric analysis of SupT1 cells bearing pA-*nef* expression vectors was done as previously described [50]. Two μg of pCMV-*EGFP* and 10 μg of each pA-*nef* expression plasmid, or empty pA vector were introduced into SupT1 cells by electroporation. The cells were then plated in 10 cm dishes and cultured for 24 hours. Subsequently, the cells were incubated with CD4-PerCP and MA2.1-PE monoclonal antibodies to detect CD4 and the A2 allele of MHC-I by flow cytometry. Data were collected with a FACSCalibur instrument and the GFP+ population was analyzed for CD4 and MHC-I surface expression with CellQuest software.

Expression of consensus *nef* genes from the two pre-AIDS clones, 8G9 and 32D2, induced the highest level of CD4 downmodulation, similar to that of the NL4-3 *nef* gene (Fig. 2). The late stage *E11 consensus *nef* gene induced significantly less CD4 downmodulation ($p < 0.0001$ by Student's t-test). In contrast, the ability to downmodulate MHC-I A2 molecules was similar for *E11 and NL4-3 *nef* genes, while the *nef* genes from the earlier ACH142 clones exhibited significantly less MHC-I downmodulation ($p < 0.003$ by Student's t-test). When increasing doses of Nef expression plasmid were used in electroporation of SupT1 cells, this difference was heightened. In no case was the *E11 Nef better able to downmodulate CD4 than the two earlier clones' Nefs or NL4-3 Nef. Likewise, in no case were the two pre-AIDS Nef alleles better able to downmodulate MHC-I A2 molecules than the *E11 AIDS Nef. Nef expression levels were similar for all three patient ACH142 derived *nef* genes. The 8G9 and 32D2 Nefs were expressed at 1.5 and 1.6 times higher levels than the *E11 Nef respectively, as determined by radiometric quantitation of a Nef immunoblot (Fig. 2E).

```
                1                                                          58
*E11            MGGKLSKR-MRQ-AVVRERMQRAEPAAEPAAEGVGAVSRDLERHGAITSSNTASTNADCA
32D2            MGGKLSKR-MREWTVVRERMQR----AEPAAEGVGAVSRDLERHGAITSSNTASTNADCA
8G9             MGGKLSKR-VRKWAAVRERMQR----AEPAAEGVGAVSRDLEKHGAITSSNTASTNADCA
NL4-3           MGGKWSKSSVIGWPAVRERMRR----AEPAADGVGAVSRDLEKHGAITSSNTAAnNAACA

               59                                                         118
*E11            WLEAQGEEEVGFPVRPQVPLRPMTYKAALDLSHFLKEKGGLEGLIYSRRRQEILDLWVYH
32D2            WLEAQEEEEVGFPVRPQVPLRPMTYKGALDLSHFLKEKGGLEGLIYSRRRQEILDLWVYH
8G9             WLEAQEEEEVGFPVRPQVPLRPMTYKGALDLSHFLKEKGGLEGLIYSRRRQEILDLWVYH
NL4-3           WLEAQEEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEGLIhSQRRQDILDLWIYH

               119                                                        178
*E11            TQGYFPDWDNYTPGPGIRYPLTFGWCFKLVPVEPEQIERINEGENNCLLHPISQHGMGDP
32D2            TQGFFPDWDNYTPGPGIRYPLTFGWCFKLVPVEPEQIEKANEGENNCLLHPISlHGMEDP
8G9             TQGYFPDWQCYTPGPGIRYPLTFGWCFKLVPVEPEQIEKANEGENNSLLHPMSlHGMEDP
NL4-3           TQGYFPDWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSlHGMDDP

               179                                208      Nef Progression Score
*E11            ERE-VLMWKFDSRLAFHHMAREKHPEYYKDC                    +5
32D2            ERE-VLMWKFDSRLAFHHMAREKHPEYYKDC                    +3
8G9             EREEVLMWKFDSRLAFHHMAREKHPEYYKDC                    +2
NL4-3           ERE-VLEWRFDSRLAFHHVARELHPEYFKNC                    -2
```

Figure 1

The R5 AIDS HIV-1 clone ACH142-*E11 Nef has a higher progression score than the Nefs from two patient ACH142 derived pre-AIDS R5 HIV-1 clones, 32D2 and 8G9. A Clustal W alignment of predicted Nef amino acid sequences is shown. Gaps in the alignment are represented by a dash. Bold amino acid residues represent changes of interest between isolates. Numbers above the alignment represent the amino acid position in this alignment. Bold, italicized residues were used to calculate the Nef progression score according to the method of Kirchhoff *et al* [1]. Upper case bold italicized letters indicate residues that are more common in progressors. Lower case bold italicized letters indicate residues that are more common in non-progressors.

The contribution of each consensus *nef* gene to HIV-1 infectivity was determined using the CD4 negative HIV-1 packaging cell line, 5BD.1 and the hygromycin resistance gene-bearing HIV-1 derived vector, TR167 [56]. Cells were co-transfected with pTR167 Δ*nef* (5 μg), pCMV-Tat (2 μg) and either the *E11, 32D2, 8G9 or NL4-3 pCMV-*nef* expression vector (5 μg) to produce hygromycin resistance-transducing HIV-1 vector particles. Vector stocks were used to infect HeLa-CD4 cells; after two weeks of selection with hygromycin, colonies were stained with crystal violet and counted (Fig. 3). All *nef* genes studied here significantly enhanced the infectivity of the vector when compared to *nef* negative vectors ($p < 0.001$ by Student's t-test). The 32D2 pre-AIDS *nef* enhanced infectivity significantly more than the *E11 AIDS *nef* ($p < 0.01$ by Student's t-test). Similar infectivity enhancement was mediated by the 8G9 pre-AIDS *nef*, the *E11 AIDS *nef* and NL4-3 *nef*. Nearly identical results were observed when each patient *nef* was used to complement vectors created with *env* genes from the same HIV-1 biological clone (data not shown).

Discussion

Our results suggest that the AIDS associated *nef* gene studied here does not significantly contribute to the enhanced replication and cytopathic effects of the AIDS associated *E11 R5 HIV-1 clone for the following three reasons. It is highly conserved at almost all known sites within the Nef sequence that are implicated in functional interactions. It does not downmodulate CD4 to a greater extent than pre-AIDS Nefs, nor does it more greatly enhance infectivity in a single round assay compared to the pre-AIDS Nefs from the same patient. Combined with our previous study, we conclude that Env, but not Nef contributes to the enhanced replication of the R5, AIDS-associated HIV-1 clone ACH142-*E11 compared to two pre-AIDS R5 HIV-1 clones derived from the same patient [61].

Previous studies indicated that Nef proteins with high progression scores had enhanced ability to downmodulate CD4, reduced ability to downmodulate MHC-I and increased ability to enhance HIV-1 replication compared to Nef proteins with lower progression scores [1-3]. The

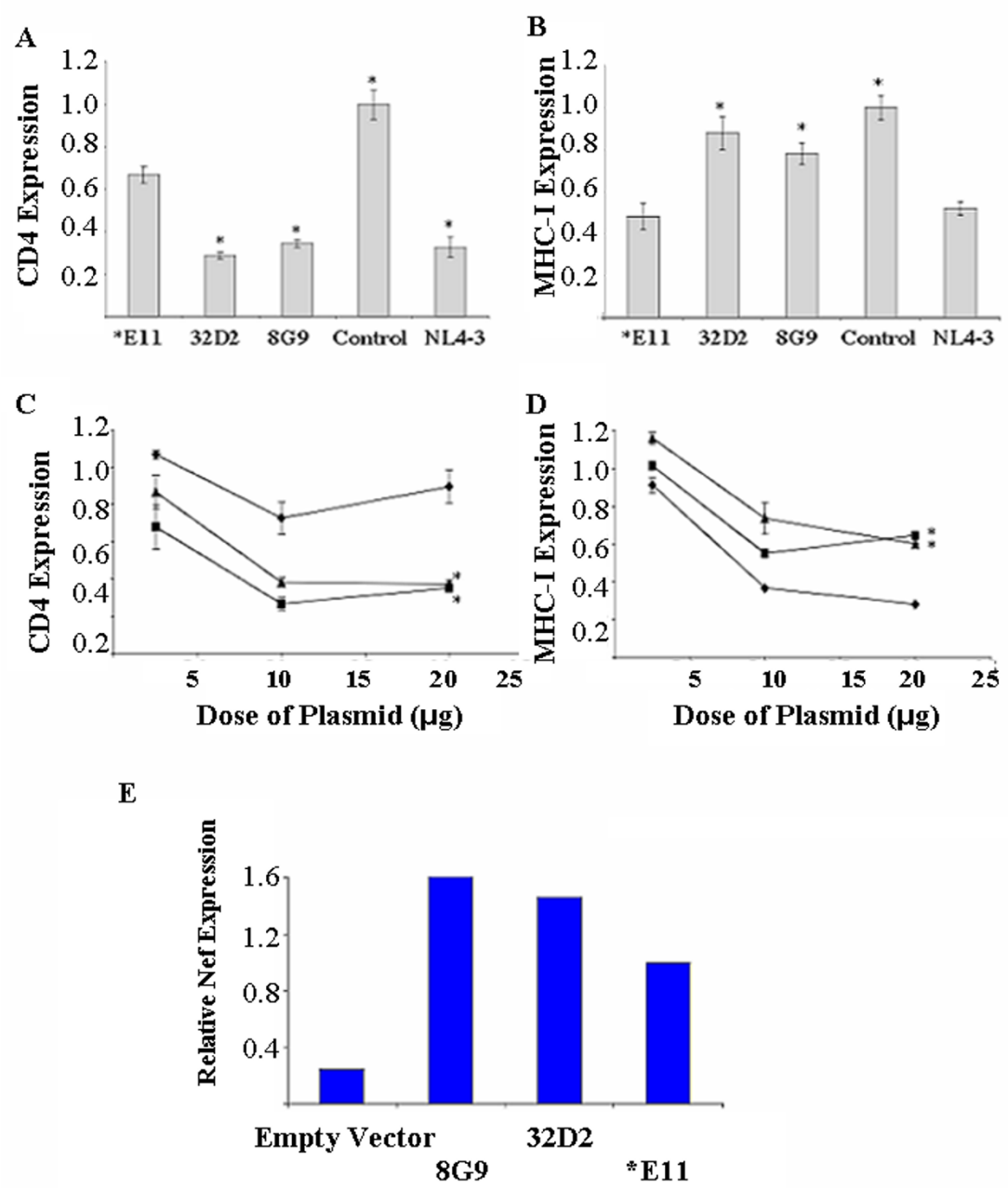

Figure 2

The R5 AIDS Nef from HIV-1 clone *E11 does not down regulate CD4 more than the two pre-AIDS alleles from 32D2 and 8G9, but does downregulate MHC Class I more than the pre-AIDS Nefs. SupT1 cells were electroporated with 10 μg of pA-Nef expression vectors and 2 μg of pCMV-EGFP expression vector. Cells were analyzed by flow cytometry 24-hrs post-electroporation. The fraction of control levels of cell surface CD4 (**A** and **C**) or MHC Class I A2 (**B** and **D**) expression in GFP+ cells are reported for each allele. **C** and **D** 2.5, 10 or 20 μg of pA-*E11 Nef (diamonds), pA-32D2 Nef (squares), or pA-8G9 Nef (triangles) were transferred to SupT1 cells by electroporation. The average of eight transfections for **A** and **B** or two transfections for **C** and **D** is shown. Error bars represent the standard errors of the mean. Samples denoted with asterisks were significantly different from the *E11 sample as determined by the Student's unpaired t-test (**A** and **B**) or by the Student's paired t-test (**C** and **D**). **E** Twenty μg of pA-Nef expression vectors were used for electroporation of SupT1 cells with 2 μg of pCMV-EGFP. Cells were lysed in sample buffer and analyzed by SDS-PAGE and western blot. The blot was probed with a polyclonal rabbit anti-Nef serum followed by ^{125}I-Protein A. The blot was then analyzed by phosphorimager and quantitated using ImageQuant software. Results from a representative experiment of three experiments performed are shown.

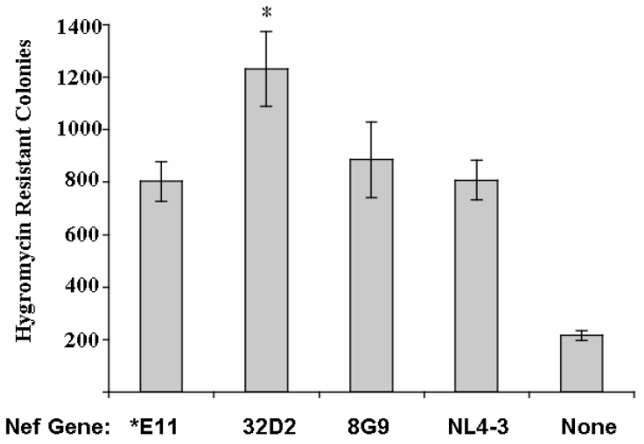

Figure 3
The R5 pre-AIDS HIV-1 clone 32D2 Nef protein enhances infectivity more than the R5 AIDS allele. The 5BD.1 HIV-1 vector packaging cell line was co-transfected with pTR167 ΔNef (5 μg), pCMV-Tat (2 μg) and *E11, 32D2, NL4-3, or 8G9 pCMV Nef plasmid (2.5 μg). Three days post-transfection, 100 μl of the cell supernatants were used to infect 2 × 10^5 HeLa CD4 cells in the presence of 8 μg/ml DEAE-dextran. Viral vectors and cells were incubated together at 37°C for 24 hours. At that time, infectious media was removed and replaced with IMDM plus 10% BCS. At 48 hours post-infection, IMDM + 10% BCS and hygromycin (200 μg/ml) was added. After two weeks of selection, the resultant colonies were stained with crystal violet and manually counted. The average of nine infections from two different viral vector stocks for each Nef is shown. Error bars represent standard errors of the mean (SEM). Asterisked 32D2 samples were significantly different from each of the three other Nef positive samples by the Student's unpaired t-test.

Nef proteins of patient ACH142 derived R5 HIV-1 clones displayed a chronological increase in Nef progression score as predicted, but the phenotype of these genes differed from the predicted phenotypes described above. The AIDS associated Nef protein studied here had a higher progression score (+5) than the pre-AIDS Nef proteins derived from the same patient (+2 and +3), but did not show an increased ability to downmodulate CD4 or to enhance infectivity. Moreover, the AIDS associated Nef protein had greater ability to downmodulate MHC-I A2 molecules. This is likely not explained by differences in Nef expression because the *E11 Nef downmodulated MHC-I to a greater extent despite being expressed at a slightly lower level. One explanation for the discrepancy between our results and those previously reported by others may be that *nef* genes from CXCR4-tropic (X4) HIV-1 isolates have the phenotypes previously reported [2,3] but *nefs* from R5 HIV-1 clones show the phenotypes demonstrated here. Most of the progressor *nefs* used in previous studies were likely derived from X4 HIV-1 because patients with X4 HIV-1 progress to AIDS more rapidly. In contrast, all three *nef* genes studied here were derived from patient ACH142, who never developed X4 HIV-1 [60,61]. More analyses of *nef* genes from R5 HIV-1 clones derived from progressors are needed to test the generality of our observations.

Previous studies have shown that X4 HIV-1 isolates are more sensitive to neutralization by soluble CD4 than R5 HIV-1 [62-64] and that X4 HIV-1 clones incorporate less Env into their virions when cellular CD4 is not downmodulated than R5 HIV-1 clones [52]. It is therefore likely that downmodulation of CD4 has a greater impact on X4 HIV-1 replication than on R5 HIV-1 replication. R5 HIV-1 Nef may therefore have a greater positive effect on viral replication by down-modulating cell surface MHC-I molecules and thereby protecting infected cells from lysis by anti-HIV-1 cytotoxic T lymphocytes.

Moreover, because R5 HIV-1 clones preferentially infect effector memory T cells and macrophages while X4 HIV-1 clones preferentially infect naïve T cells, *nef* genes in each type of HIV-1 may evolve over the course of an infection to better enhance replication in each respective cell type. Determining whether such an evolution occurs may allow us to find specific Nef interactions that occur preferentially in macrophages or in memory or naïve T cells. In particular, Nef's ability to activate T cells may be more essential for X4 HIV-1, since naïve T cells are less easily activated than memory T cells.

Our data lend support to the notion that Nef cannot evolve over the course of disease to enhance all of its functions. This is true for our study in which an AIDS-associated Nef was reduced in its ability to downmodulate CD4 and enhanced in its ability to downmodulate MHC I and is also true for other studies where the reverse was found. This supports a paradigm whereby increased Nef mediated downmodulation of CD4 or MHC Class I molecules correlates with a loss in the other function. This paradigm may be created by structural constraints that limit the ability of Nef to perform both functions optimally or by competition for limiting cellular factors necessary for both processes. Determination of these constraints and/or factors will shed light on the role of Nef in HIV-1 replication and pathogenesis.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KCO carried out most of the experiments and wrote the paper, RMS and BB cloned the nef genes and initiated the calculation of Nef progression scores, MP helped perform the downmodulation and infectivity experiments, MAA

developed the protocol for the downmodulation assays and advised on their use, DC, M-LH and DR designed the study and DC edited and revised the manuscript.

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References

1. Kirchhoff F, Easterbrook PJ, Douglas N, Troop M, Greenough TC, Weber J, Carl S, Sullivan JL, Daniels RS: **Sequence variations in human immunodeficiency virus type 1 Nef are associated with different stages of disease.** *J Virol* 1999, **73:**5497-5508.
2. Arganaraz ER, Schindler M, Kirchhoff F, Cortes MJ, Lama J: **Enhanced CD4 down-modulation by late stage HIV-1 nef alleles is associated with increased Env incorporation and viral replication.** *J Biol Chem* 2003, **278:**33912-33919.
3. Carl S, Greenough TC, Krumbiegel M, Greenberg M, Skowronski J, Sullivan JL, Kirchhoff F: **Modulation of different human immunodeficiency virus type 1 Nef functions during progression to AIDS.** *J Virol* 2001, **75:**3657-3665.
4. Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J: **Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients.[see comment].** *Science* 1995, **270:**988-991.
5. Brambilla A, Turchetto L, Gatti A, Bovolenta C, Veglia F, Santagostino E, Gringeri A, Clementi M, Poli G, Bagnarelli P, Vicenzi E: **Defective nef alleles in a cohort of hemophiliacs with progressing and nonprogressing HIV-1 infection.** *Virology* 1999, **259:**349-368.
6. Learmont J, Tindall B, Evans L, Cunningham A, Cunningham P, Wells J, Penny R, Kaldor J, Cooper DA: **Long-term symptomless HIV-1 infection in recipients of blood products from a single donor.** *Lancet* 1992, **340:**863-867.
7. Casartelli N, Di Matteo G, Argentini C, Cancrini C, Bernardi S, Castelli G, Scarlatti G, Plebani A, Rossi P, Doria M: **Structural defects and variations in the HIV-1 nef gene from rapid, slow and non-progressor children.** *AIDS* 2003, **17:**1291-1301.
8. Rhodes DI, Ashton L, Solomon A, Carr A, Cooper D, Kaldor J, Deacon N: **Characterization of three nef-defective human immunodeficiency virus type 1 strains associated with long-term nonprogression. Australian Long-Term Nonprogressor Study Group.** *Journal of Virology* 2000, **74:**10581-10588.
9. Chakraborty R, Morel AS, Sutton JK, Appay V, Ripley RM, Dong T, Rostron T, Ogola S, Palakudy T, Musoke R, D'Agostino A, Ritter M, Rowland-Jones SL: **Correlates of delayed disease progression in HIV-1-infected Kenyan children.** *J Immunol* 2005, **174(12):**8191-8199.
10. Kestler HW 3rd, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC: **Importance of the nef gene for maintenance of high virus loads and for development of AIDS.** *Cell* 1991, **65:**651-662.
11. Rodes B, Toro C, Paxinos E, Poveda E, Martinez-Padial M, Benito JM, Jimenez V, Wrin T, Bassani S, Soriano V: **Differences in disease progression in a cohort of long-term non-progressors after more than 16 years of HIV-1 infection.** *AIDS* 2004, **18:**1109-1116.
12. Aiken C, Trono D: **Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis.** *J Virol* 1995, **69:**5048-5056.
13. Chowers MY, Pandori MW, Spina CA, Richman DD, Guatelli JC: **The growth advantage conferred by HIV-1 nef is determined at the level of viral DNA formation and is independent of CD4 downregulation.** *Virology* 1995, **212:**451-457.
14. Miller MD, Warmerdam MT, Gaston I, Greene WC, Feinberg MB: **The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages.** *Journal of Experimental Medicine* 1994, **179:**101-113.
15. Wu Y, Marsh JW: **Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA.** *Science* 2001, **293:**1503-1506.
16. Papkalla A, Munch J, Otto C, Kirchhoff F: **Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism.** *Journal of Virology* 2002, **76:**8455-8459.
17. Brown A, Moghaddam S, Kawano T, Cheng-Mayer C: **Multiple human immunodeficiency virus type 1 Nef functions contribute to efficient replication in primary human macrophages.** *J Gen Virol* 2004, **85:**1463-1469.
18. Brown A, Wang X, Sawai E, Cheng-Mayer C: **Activation of the PAK-related kinase by human immunodeficiency virus type 1 Nef in primary human peripheral blood lymphocytes and macrophages leads to phosphorylation of a PIX-p95 complex.** *Journal of Virology* 1999, **73:**9899-9907.
19. Balliet JW, Kolson DL, Eiger G, Kim FM, McGann KA, Srinivasan A, Collman R: **Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes vpr, vpu, and nef: mutational analysis of a primary HIV-1 isolate.** *Virology* 1994, **200:**623-631.
20. Duus KM, Miller ED, Smith JA, Kovalev GI, Su L: **Separation of human immunodeficiency virus type 1 replication from nef-mediated pathogenesis in the human thymus.** *J Virol* 2001, **75:**3916-3924.
21. Stove V, Verhasselt B: **Modelling thymic HIV-1 Nef effects.** *Current HIV Research* 2006, **4:**57-64.
22. Stove V, Naessens E, Stove C, Swigut T, Plum J, Verhasselt B: **Signaling but not trafficking function of HIV-1 protein Nef is essential for Nef-induced defects in human intrathymic T-cell development.** *Blood* 2003, **102:**2925-2932.
23. Stoddart CA, Geleziunas R, Ferrell S, Linquist-Stepps V, Moreno ME, Bare C, Xu W, Yonemoto W, Bresnahan PA, McCune JM, Greene WC: **Human immunodeficiency virus type 1 Nef-mediated downregulation of CD4 correlates with Nef enhancement of viral pathogenesis.** *Journal of Virology* 2003, **77:**2124-2133.
24. Jamieson BD, Aldrovandi GM, Planelles V, Jowett JB, Gao L, Bloch LM, Chen IS, Zack JA: **Requirement of human immunodeficiency virus type 1 nef for in vivo replication and pathogenicity.** *Journal of Virology* 1994, **68:**3478-3485.
25. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur 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.
26. Hanna Z, Kay DG, Cool M, Jothy S, Rebai N, Jolicoeur P: **Transgenic mice expressing human immunodeficiency virus type 1 in immune cells develop a severe AIDS-like disease.** *J Virol* 1998, **72:**121-132.
27. Vincent P, Priceputu E, Kay D, Saksela K, Jolicoeur P, Hanna Z: **Activation of p21-activated kinase 2 and its association with Nef are conserved in murine cells but are not sufficient to induce an AIDS-like disease in CD4C/HIV transgenic mice.** *Journal of Biological Chemistry* 2006, **281:**6940-6954.
28. Keppler OT, Allespach I, Schuller L, Fenard D, Greene WC, Fackler OT: **Rodent cells support key functions of the human immunodeficiency virus type 1 pathogenicity factor Nef.** *Journal of Virology* 2005, **79:**1655-1665.
29. Saksela K: **Therapeutic targeting of interactions between Nef and host cell proteins.** *Curr Drug Targets Immune Endocr Metabol Disord* 2004, **4:**315-319.
30. Baur A: **Functions of the HIV-1 Nef protein.** *Curr Drug Targets Immune Endocr Metabol Disord* 2004, **4:**309-313.
31. Arold ST, Baur AS: **Dynamic Nef and Nef dynamics: how structure could explain the complex activities of this small HIV protein.** *Trends Biochem Sci* 2001, **26:**356-363.
32. Fackler OT, Baur AS: **Live and let die: Nef functions beyond HIV replication.** *Immunity* 2002, **16:**493-497.
33. Geyer M, Fackler OT, Peterlin BM: **Structure--function relationships in HIV-1 Nef.** *EMBO Rep* 2001, **2:**580-585.
34. Peterlin BM, Trono D: **Hide, shield and strike back: how HIV-infected cells avoid immune eradication.** *Nature Reviews Immunology* 2003, **3:**97-107.
35. Fackler OT, Alcover A, Schwartz O: **Modulation of the immunological synapse: a key to HIV-1 pathogenesis?** *Nature Reviews Immunology* 2007, **7:**310-317.

36. Roeth JF, Collins KL: **Human immunodeficiency virus type 1 Nef: adapting to intracellular trafficking pathways.** *Microbiology & Molecular Biology Reviews* 2006, **70:**548-563.
37. Quaranta MG, Mattioli B, Giordani L, Viora M: **The immunoregulatory effects of HIV-1 Nef on dendritic cells and the pathogenesis of AIDS.** *FASEB Journal* 2006, **20:**2198-2208.
38. Aiken C, Konner J, Landau NR, Lenburg ME, Trono D: **Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain.** *Cell* 1994, **76:**853-864.
39. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, Baltimore D: **The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells.** *Immunity* 1999, **10:**661-671.
40. Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D: **HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes.** *Nature* 1998, **391:**397-401.
41. Chowers MY, Spina CA, Kwoh TJ, Fitch NJ, Richman DD, Guatelli JC: **Optimal infectivity in vitro of human immunodeficiency virus type 1 requires an intact nef gene.** *J Virol* 1994, **68:**2906-2914.
42. Miller MD, Feinberg MB, Greene WC: **The HIV-1 nef gene acts as a positive viral infectivity factor.** *Trends Microbiol* 1994, **2:**294-298.
43. Cluet D, Bertsch C, Beyer C, Gloeckler L, Erhardt M, Gut JP, Galzi JL, Aubertin AM: **Detection of human immunodeficiency virus type 1 Nef and CD4 physical interaction in living human cells by using bioluminescence resonance energy transfer.** *J Virol* 2005, **79:**8629-8636.
44. Gillim-Ross L, Cara A, Klotman ME: **Nef expressed from human immunodeficiency virus type 1 extrachromosomal DNA downregulates CD4 on primary CD4+ T lymphocytes: implications for integrase inhibitors.** *J Gen Virol* 2005, **86:**765-771.
45. Rose JJ, Janvier K, Chandrasekhar S, Sekaly RP, Bonifacino JS, Venkatesan S: **CD4 down-regulation by HIV-1 and simian immunodeficiency virus (SIV) Nef proteins involves both internalization and intracellular retention mechanisms.** *J Biol Chem* 2005, **280:**7413-7426.
46. Brown A, Gartner S, Kawano T, Benoit N, Cheng-Mayer C: **HLA-A2 down-regulation on primary human macrophages infected with an M-tropic EGFP-tagged HIV-1 reporter virus.** *J Leukoc Biol* 2005, **78(3):**675-685.
47. Kasper MR, Roeth JF, Williams M, Filzen TM, Fleis RI, Collins KL: **HIV-1 Nef disrupts antigen presentation early in the secretory pathway.** *J Biol Chem* 2005, **280:**12840-12848.
48. Swigut T, Alexander L, Morgan J, Lifson J, Mansfield KG, Lang S, Johnson RP, Skowronski J, Desrosiers R: **Impact of Nef-mediated downregulation of major histocompatibility complex class I on immune response to simian immunodeficiency virus.** *J Virol* 2004, **78:**13335-13344.
49. Sol-Foulon N, Esnault C, Percherancier Y, Porrot F, Metais-Cunha P, Bachelerie F, Schwartz O: **The effects of HIV-1 Nef on CD4 surface expression and viral infectivity in lymphoid cells are independent of rafts.** *J Biol Chem* 2004, **279:**31398-31408.
50. Alexander M, Bor YC, Ravichandran KS, Hammarskjold ML, Rekosh D: **Human immunodeficiency virus type 1 Nef associates with lipid rafts to downmodulate cell surface CD4 and class I major histocompatibility complex expression and to increase viral infectivity.** *J Virol* 2004, **78:**1685-1696.
51. Lama J, Mangasarian A, Trono D: **Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner.** *Curr Biol* 1999, **9:**622-631.
52. Lundquist CA, Zhou J, Aiken C: **Nef stimulates human immunodeficiency virus type 1 replication in primary T cells by enhancing virion-associated gp120 levels: coreceptor-dependent requirement for Nef in viral replication.** *J Virol* 2004, **78:**6287-6296.
53. Chazal N, Singer G, Aiken C, Hammarskjold ML, Rekosh D: **Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity.** *J Virol* 2001, **75:**4014-4018.
54. Miller MD, Warmerdam MT, Page KA, Feinberg MB, Greene WC: **Expression of the human immunodeficiency virus type 1 (HIV-1) nef gene during HIV-1 production increases progeny particle infectivity independently of gp160 or viral entry.** *J Virol* 1995, **69:**579-584.
55. Pandori MW, Fitch NJ, Craig HM, Richman DD, Spina CA, Guatelli JC: **Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein.** *J Virol* 1996, **70:**4283-4290.
56. Srinivasakumar N, Chazal N, Helga MC, Prasad S, Hammarskjold ML, Rekosh D: **The effect of viral regulatory protein expression on gene delivery by human immunodeficiency virus type 1 vectors produced in stable packaging cell lines.** *J Virol* 1997, **71:**5841-5848.
57. Tobiume M, Lineberger JE, Lundquist CA, Miller MD, Aiken C: **Nef does not affect the efficiency of human immunodeficiency virus type 1 fusion with target cells.** *J Virol* 2003, **77:**10645-10650.
58. Cavrois M, Neidleman J, Yonemoto W, Fenard D, Greene WC: **HIV-1 virion fusion assay: uncoating not required and no effect of Nef on fusion.** *Virology* 2004, **328:**36-44.
59. Campbell EM, Nunez R, Hope TJ: **Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity.** *J Virol* 2004, **78:**5745-5755.
60. Scoggins RM, Taylor JR Jr., Patrie J, van't Wout AB, Schuitemaker H, Camerini D: **Pathogenesis of primary R5 human immunodeficiency virus type 1 clones in SCID-hu mice.** *Journal of Virology* 2000, **74:**3205-3216.
61. Olivieri K, Scoggins RM, Bor YC, Matthews A, Mark D, Taylor JR Jr., Chernauskas D, Hammarskjold ML, Rekosh D, Camerini D: **The envelope gene is a cytopathic determinant of CCR5 tropic HIV-1.** *Virology* 2007, **358:**23-38.
62. Daar ES, Li XL, Moudgil T, Ho DD: **High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates.** *Proc Natl Acad Sci U S A* 1990, **87:**6574-6578.
63. Ashkenazi A, Smith DH, Marsters SA, Riddle L, Gregory TJ, Ho DD, Capon DJ: **Resistance of primary isolates of human immunodeficiency virus type 1 to soluble CD4 is independent of CD4-rgp120 binding affinity.** *Proc Natl Acad Sci U S A* 1991, **88:**7056-7060.
64. Moore JP, McKeating JA, Huang YX, Ashkenazi A, Ho DD: **Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates.** *J Virol* 1992, **66:**235-243.