Published in final edited form as:

J Virol Methods. 2009 November ; 161(2): 297–304. doi:10.1016/j.jviromet.2009.07.006.

SIMULTANEOUS ASSESSMENT OF CD4 AND MHC I DOWNREGULATION BY NEF PRIMARY ISOLATES IN THE CONTEXT OF INFECTION

Ayub Ali^{1,2}, Susan Realegeno^{1,3}, Otto O. Yang^{1,2,3}, and Martha J. Lewis^{1,2,*}

¹ Division of Infectious Diseases, Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, California, 90095, USA

² UCLA AIDS Institute, David Geffen School of Medicine at UCLA, Los Angeles, California, 90095, USA

³ Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine at UCLA, Los Angeles, California, 90095, USA

SUMMARY

The HIV-1 Nef protein plays a key role in pathogenesis, as demonstrated by strong selective pressure to maintain its open reading frame, and disease attenuation when it is deleted. Among myriad cellular effects attributed to Nef, downregulation of cell surface CD4 and major histocompatibility complex class I (MHC-I) proteins are the best documented. However, few data regarding primary isolate Nef functions are available, and most studies have been performed using transient transfections to express Nef driven by a non-physiologic promoter. A novel assay system to measure simultaneously the downregulation of CD4 and MHC-I by primary HIV-1 *nef* in a more physiologic viral genomic context is presented. Examination of plasma *nef* mixtures allowed comprehensive profiling of these Nef functions within the quasispecies *in vivo*. Subsets within the circulating *nef* population were observed that are either fully functional or non-functional. These data demonstrated that this assay system allows rapid characterization of bulk and clonal Nef functional profiles that can be used in pathogenesis studies to define further its important role in pathogenesis.

Keywords

HIV-1; Nef; MHC I; CD4

1. INTRODUCTION

Unique to SIV and HIV, Nef is a small myristoylated protein that plays a central role in the pathogenesis of disease, based on *in vivo* observations of humans and primates (Kestler et al., 1991; Piguet and Trono, 1999; Trono, 1995). Macaques infected with an otherwise pathogenic strain of SIV with *nef* deleted tend to have significantly attenuated disease and protection from subsequent challenge with wild type virus (Daniel et al., 1992; Swigut et al., 2004). Similarly,

*Corresponding author: Martha J. Lewis, Division of Infectious Diseases, 37-121 CHS, 10833 LeConte Ave, Los Angeles, CA, 90095, USA, malewis@mednet.ucla.edu, Phone: 310-825-0205, Fax: 310-825-3632.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

slower disease progression has been noted in a cohort of humans infected by blood transfusions from a single HIV-1-infected donor whose virus contained a defect in *nef* (Deacon et al., 1995; Dyer et al., 1997; Kirchhoff et al., 1995; Oelrichs et al., 1998).

Yet the exact mechanism of Nef's contribution to pathogenesis is not completely understood. Among its many reported functions in infected cells that may contribute to viral pathogenesis, Nef is well documented to downregulate CD4 (Bandres et al., 1995; Garcia and Miller, 1991), downregulate major histocompatibility complex class I proteins (MHC-I) (Cohen et al., 1999; Collins et al., 1998; Schwartz et al., 1996), and modulate cellular activation (Du et al., 1995; Glushakova et al., 1999; Simmons et al., 2001; Smith et al., 1996). Elimination of some or all of these functions leads to an attenuated infection (Geffin et al., 2000). Among the best-understood functions of Nef are downregulation of MHC-I and CD4 molecules from the surface of infected cells. MHC-I downregulation has been shown to render infected cells less visible to circulating CD8⁺ cytotoxic T lymphocytes (CTL) and thereby confer resistance of infected cells to CTL killing (Adnan et al., 2006; Collins et al., 1998; Fujiwara and Takiguchi, 2007; Tomiyama et al., 2002; Tomiyama et al., 2005; Ueno et al., 2008; Yang et al., 2002). Nef's downregulation of CD4 prevents CD4 binding to Env on nascent virions thereby enhancing viron release (Lama et al., 1999; Mangasarian et al., 1999; Yang et al., 2002).

Previous studies of Nef function have been limited by the use of a few homogenous strains of HIV-1 and the expression of Nef using non-physiologic promoters (Collins et al., 1998; Noviello et al., 2007; Schindler et al., 2006). Limited data are available regarding the function of Nef proteins from primary viruses, although some studies have shown variation in the MHC-I downregulatory function relative to HIV-1 disease status (Carl et al., 2001; Kirchhoff et al., 1999; Lewis et al., 2008; Noviello et al., 2007). Additionally, initial observations indicate that even within the same infected individual there may be variations in this function of Nef (Lewis et al., 2008). Further complicating the measurement of Nef's function is the fact that Vpu and Env also downregulate CD4 (Lama et al., 1999; Pang et al., 2000). Therefore this function of Nef typically has been studied by expressing *nef* in a eukaryotic expression vector rather than whole HIV-1-infection in order to isolate Nef's contribution to CD4 downregulation.

A new, modified whole-genome assay system to measure simultaneously the ability of Nef to downregulate MHC-I and CD4 is presented. Use of a whole genome vector allows for the expression of Nef at physiologic levels under the control of an HIV-1 LTR promoter. The deletion of segments of Env and Vpu permits the measurement of Nef's independent contribution to CD4 downregulation. Lastly, our approach allows the use of cloned quasiespecies rather than just a single isolate to generate Nef functional profiles more accurately reflecting its *in vivo* function within the circulating swarm of HIV-1. This system provides a rapid assay for the measurement of multiple Nef functions that more closely reflects its function *in vivo* compared to previous assay systems.

2. MATERIALS AND METHODS

2.1 Creation of an *env*- and *vpu*- defective cloning vector to accept primary *nef* genes

The 3' half-genome NL4-3-based plasmid modified to contain the HSA-HA reporter gene within *nef*, p83-10-HA_xNef, served as the starting template for construction of a target vector for cloning primary *nef* sequences (Ali et al., 2003; Ali and Yang, 2006). This construct was chosen to allow detection of parental vector without *nef* insertion by staining for HA. First, a deletion within *env* was created. The plasmid pNL4-3-EGFP-ΔEnv containing a deletion of 581 bp (7039–7620) in *env* (Pang et al., 2000) was digested with Nde I and Bam HI, and the resulting fragment was subcloned into p83-10-HA_xNef replacing wild-type *env*. Next, the deletion within *vpu* was added. The NL4-3-based vector p210–13 (Gibbs et al., 1994) containing a deletion of 127 bp in *vpu* (6063–6180) was digested with EcoRI and Nde I. The

digested product was then inserted into the p83–10-HA_xNef- Δenv plasmid, creating a double deletion, Δenv and *vpu*.

In order to reconstruct a whole genome molecular clone the modified 3' half-genome construct described above, p83–10-HA_xNef- $\Delta env\Delta vpu$, was combined with a modified version of the 5' half-genome construct, p83-2-HSA_xVpr (Ali et al., 2003). Three modifications were made to p83-2-HSA_xVpr. First, a duplicate BspEI site was abolished from the 5' LTR leaving a unique site in the 3' LTR at the 3' end of *nef*. Second, a duplicate Xba I site before *vpr* was removed leaving a unique site at the 5' end of *nef*. Third, an Nru I site was created at the junction of the HIV insert and the plasmid backbone. This plasmid was designated p83-2-HSA_xVpr-Nru⁺Bsp⁻Xba⁻. Finally, the whole genome construct was created by digesting both plasmids with EcoR I and Nru I. The appropriate fragments were gel purified then ligated. The resultant plasmid was named AA1305#18 (Figure 1). All modifications were confirmed by sequencing. The final plasmid sequence is available via GenBank accession number XXX (to be made available upon acceptance).

2.2 Construction of a panel of control *nef* alleles: NL4–3 Nef, Delta Nef, M20A Nef, and LL>AA Nef

All control *nef* alleles were inserted using the restriction sites XbaI and BspEI of AA1305#18 described above. An XbaI restriction site was created at the 5' end of *nef* by PCR using the following primer pair: Nef 8787 XbaIF 5' GCTCTAGAATGGGTGGCAAGTGCTCAA and Nef 9495R 5' TTATATGCAGCATCTGAGGGC. The high fidelity enzyme Phusion (New England Biolabs) and standard reaction conditions as recommended by the manufacturer for a 50 μ L reaction were used for amplification. The following PCR cycle was used: 5 min. at 98°C, 35 cycles of 98°C for 10s, 57°C for 30s, 72°C for 30s, followed by a final extension at 72°C for 10 min. The resulting PCR product contained novel XbaI and native BspEI restriction sites, which were used for cloning into the full-genome construct AA1305#18. The following plasmids were used as templates for control Nefs: p210-5 (Nef) (Gibbs et al., 1994) obtained through the AIDS Research and Reference Reagent Program (Cat. No. 2485), p83-10 (wild-type NL4-3 Nef) (Gibbs et al., 1994) obtained through the AIDS Research and Reference Reagent Program (Cat. No. 2480), p83-10 Nef M20A (Ali et al., 2003) (a mutant specifically defective in Nef's function to downregulate MHC I molecules (Akari et al., 2000)), and a plasmid containing the previously described dileucine to alanine (LL->AA) Nef mutant specifically defective in its CD4-downregulation function kindly provided by John Guatelli (Craig et al., 1998; Riggs et al., 1999). All mutants were confirmed by sequencing.

2.3 Primary HIV-1 *nef* quasispecies cloning from plasma

Study subjects provided informed consent according to a protocol approved by the UCLA Institutional Review Board. Plasma samples from three HIV-1-infected persons whose circulating Nef quasispecies had previously been studied for ability to downregulate MHC-I in the whole-genome HIV-1 (Lewis et al., 2008) were utilized. Viral RNA was isolated from 1ml of plasma using the Ultrasens Viral Isolation Kit (Qiagen) according to the manufacture's protocol. Viral RNA was used as a template for cDNA synthesis using Omniscript Reverse Transcriptase (Qiagen) and the following gene-specific primers: Nef 9589R 5' TAGTTAGCCAGAGAGCTCCCA, Nef 8670F 5' AATGCCACAGCCATAGCAGTG, Nef 8675F 5' GCAGTAGCTGAGGGGACAGATAGG, Nef 8687F 5' GTAGCTCAAGGGACAGATAGGGTTA, Nef 8736F 5' AGAGCTATTCGCCACATACC. RT products were then used for nested PCR using Master Taq Kit (Eppendorf). The first PCR used the same primers used for reverse transcription. The nested PCR used the following primers: Nef 8787 XbaIF 5' GCTCTAGAATGGGTGGCAAGTGCTCAA and Nef 9495R 5' TTATATGCAGCATCTGAGGGC. Both PCR reactions were carried out using the following conditions: 5 min. at 95°C, 35 cycles of 95°C for 30s, 54°C for 40s, 72°C for 60s, followed by

a final extension at 72°C for 10 min. PCR products were gel-purified with Quick Spin Columns (Qiagen) and subsequently cloned in bulk by the TA method into pCR2.1-TOPO vector (Invitrogen). Ligation mixtures were grown in liquid culture with ampicillin (rather than individual colony selection on solid media) in order to preserve the quasispecies mixture of cloned PCR products. For subject 00037 ten individual clones were selected for testing in parallel with the cloned quasispecies mixture. Plasmid DNA was isolated and digested with XbaI and BspEI (New England Biolabs) and subsequently subcloned into the *nef* position of the whole-genome construct AA1305#18 described above.

2.4 Sequencing and phylogenetic analysis of *nef* alleles

Multiple single colonies from each cloning reaction were sequenced to confirm cloning success and the preservation of the quasispecies diversity. Sequencing was performed using M13F and M13R vector primers and ABI Big Dye 3.1 Sequencing Kit according to the manufacturer's protocol. Sequences were edited, translated into amino acid sequences and aligned with NL4-3 Nef using BioEdit. A neighbor-joining phylogenetic tree was constructed for all subject 00037 clones using the DNAdist and Neighbor programs of Phylip 3.64.

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

Single-round infectious pseudotyped virus was produced by co-transfecting the whole-genome plasmid AA1305#18 and a plasmid encoding the envelope glycoprotein from VSV (VSVg) (Burns et al., 1993) provided by Dr. Irvin Chen. 293T cells were transfected with 10µg of each plasmid using a standard calcium phosphate technique (2002). Supernatant containing virus was collected on days 2 through 5 post-transfection and filtered through a 0.45µm filter. Virus production was quantified by p24 antigen ELISA (Perkin Elmer, Waltham, MA). Transfections typically produced greater than 200,000 pg/ml of p24.

2.6 Flow cytometric assessment of Nef-mediated downregulation of CD4 and MHC-I on infected cells

T1 lymphocytes (Salter et al., 1985) were infected at 37°C for 4 hours with 1ml of pseudotyped virus stock containing 250 – 500 ng p24 antigen. Parallel infections were performed with viruses carrying either wild-type NL4-3 *nef* (positive control for both CD4 and MHC-I downregulation), M20A *nef* (positive control for CD4 downregulation and negative control for MHC-I downregulation), LL>AA *nef* (negative control for CD4 downregulation and positive control for MHC-I downregulation), or primary *nef* quasispecies described above. On day 4 post-infection 2×10^5 cells were stained with anti-murine CD24/HSA-FITC (BD Pharmingen), anti-human CD4-APC (BD Pharmingen) and anti-human HLA A*02-PE (ProImmune), washed twice and fixed with 1% paraformaldehyde. Uninfected T1 cells stained with isotype control antibodies were used to set the negative quadrants. T1 cells stained with anti-human HLA A*02-PE and WT NL4-3 Nef infected cells stained with anti-murine CD24/HSA-FITC were used to establish appropriate compensation between FL1 and FL2 channels. There is no overlapping signal with PE or FITC and APC, detected in FL4, so no further compensation was required. At least 5×10^4 live cells were counted using a FACScan flow cytometer, and data were analyzed using CellQuest software (Becton Dickinson). Maximum levels of HLA A*02 and CD4 were determined using the delta Nef mutant. Absence of staining with anti-HA-FITC antibody (Roche) confirmed no contamination from the parent cloning plasmid during virus production (data not shown).

3. RESULTS

3.1 Construction of an Env- and Vpu-deleted HIV-1 vector

A modified full-genome, NL4-3-based plasmid vector was created to allow expression of Nef under the control of the HIV-1 LTR (Figure 1) to evaluate its ability to downregulate CD4 and MHC-I from the surface of infected cells. Since both HIV-1 Env and Vpu also downregulate CD4, portions of both were deleted in order to isolate Nef's contribution to CD4 downregulation (Gibbs et al., 1994; Lama et al., 1999; Pang et al., 2000). In addition to deletions in *env* and *vpu*, XbaI and BspEI restriction sites were modified to create a unique cloning site for *nef* genes. Lastly, the vector contained the reporter gene HSA in the *Vpr* reading frame and the reporter HSA-HA (Ali and Yang, 2006) in the Nef reading frame, allowing detection of infected cells (HSA-expressing) and exclusion of those with vector not containing inserted *nef* (HA-expressing).

3.2 Insertion of HIV-1 *nef* alleles into the vector

In order to assess the ability of *ex vivo* Nef to downregulate CD4 and MHC-I, *nef* alleles were amplified by RT-PCR from the plasma of three chronically infected individuals. The forward primer for the nested PCR created an XbaI restriction site at the 5' end of *nef* and the PCR product included a native BspEI restriction site at the 3' end of *nef* (Figure 2). Rather than selecting single *nef* isolates, the entire bulk quasispecies mixture amplified by PCR was utilized for subsequent insertion into the vector. Both the full-genome vector AA1305#18 and the amplified *nef* quasispecies were digested with XbaI and BspEI. The *nef* locus from AA1305#18 (containing the reporter HSA-HA) was removed by gel-purification prior to ligation of the patient-derived *nef* quasispecies. In addition, four control plasmids were constructed each containing Nef with defined properties. The panel of control Nefs included the following: 1) NL4-3 Nef, which downregulates both MHC I and CD4, 2) Nef with a deletion after the first 12 amino acids (Δ Nef, which downregulates neither), 3) Nef with a methionine to alanine substitution at position 20 (M20A Nef, selectively deficient in MHC I downregulation (Akari et al., 2000)), and 4) Nef with a substitution of alanines for a di-leucine motif at positions 164–165 (LL>AA Nef, selectively deficient in CD4 downregulation (Riggs et al., 1999)).

3.3 Production of VSVg pseudotyped recombinant reporter virus

To rescue the replication defective recombinant reporter virus, the constructs were co-transfected with a plasmid expressing VSV envelope glycoprotein gene, complementing the defective HIV-1 *env* in trans. 293T cells transfected with both plasmids therefore produced VSVg-pseudotyped single-round infectious virus carrying the Δ *env-vpu* genome with patient-derived or control *nef*, and the mCD24/HSA reporter gene (in the *vpr* locus). The supernatant typically contained over 200 ng/ml HIV-1 p24 by day 4 post-transfection (not shown).

3.4 Assessment of CD4 and MHC-I downregulation by control Nefs

These viruses were used to infect T1 cells, a CD4 and MHC-I A*02-expressing HIV-1-permissive cell line (Salter et al., 1985). Four days after infection, the cells were stained with monoclonal antibodies against HSA (FITC-labeled), human A*02 (PE-labeled) and human CD4 (APC-labeled). Flow cytometry demonstrated that Nef mutants expressed at physiologic levels can clearly vary in downregulation of CD4 and MHC-I (Figure 3), whose expression could be examined on infected cells by gating on HSA-expressing cells. Simultaneous assessment of CD4 and MHC-I expression on cells infected with virus containing different control Nef proteins (Figure 3A) revealed functional profiles consistent with the known effects of the Nef mutations. NL4-3 Nef downregulated both CD4 and A*02 compared to Δ Nef (Figure 3B and C), while LL>AA Nef lost the ability to downregulate CD4 but not A*02, and M20A

Nef lost the ability to downregulate A*02 but not CD4. These results validated the ability of the assay to distinguish these functions of Nef.

3.5 Assessment of CD4 and MHC-I downregulation by patient-derived Nef quasispecies

Cells infected with the viruses carrying *nef* quasispecies from the three chronically-infected individuals showed variability in their functions of downregulate CD4 and A*02 downregulation (Figure 4). The Nef swarms from Subjects 00021 and 00022 appeared to contain a majority of alleles fully capable of downregulating both (65 and 71%, respectively), whereas Nefs from Subject 00037 appeared to have two functional subpopulations, one which downregulates both (45%) and the other which downregulates neither (31%). Clonal sequencing of these *nef* mixtures showed that no more than 5% of sequences from these individuals had non-sense or frameshift mutations ((Lewis et al., 2008) and Figure 6). Thus, variation in Nef function occurred in the Nef quasispecies with fully intact *nef* reading frames. These data demonstrated the capacity of this new assay system to examine the functional profile of circulating Nef quasispecies in a more physiologic context, thus more accurately reflecting the overall functionality of Nef *in vivo*.

3.6 Comparison of Nef's function by Quasispecies versus Individual Clones

In order to validate the testing of *nef* quasispecies, 10 individual *nef* clones from subject 00037 were tested for their ability to downregulate CD4 and MHCI and these results compared to those obtained from quasispecies testing. Ten individual clones were selected at random and sequenced. These sequences were aligned with NL4-3 Nef and 21 other subject 00037 *nef* clones from the quasispecies population. Only one of the 31 sequences contained a deletion resulting in a defective protein sequence. A neighbor-joining phylogenetic tree demonstrated that the individual clones were distributed throughout the population (Figure 5). Pseudotyped recombinant reporter viruses encoding the individual *nef* alleles were used to measure the simultaneous downregulation of CD4 and HLA-A*02 as described above. All of the individual alleles displayed one of 3 distinct functional profiles (Figure 6). Five of 10 clones fully downregulated both CD4 and HLA-A*02, 3 of 10 downregulated neither, and 2 of 10 downregulated CD4 but not HLA-A*02. These results were in excellent agreement with the quasispecies testing where 45% downregulated both, 31% downregulated neither, and 14% downregulated CD4 but not HLA-A*02. As had been previously shown (Lewis et al., 2008), the downregulation function was either fully present or absent for each individual alleles, and the overall level of function of the quasispecies population was determined by the proportion of functional alleles in the population.

4. DISCUSSION

Nef clearly holds a central position in HIV-1 pathogenesis, likely through multiple functions including CD4 and MHC-I downregulation (reviewed in (Fackler and Baur, 2002)). However, the relative contribution of each of these functions to pathogenesis is unknown. Additionally it is not known if all functions are present at all disease stages and in all primary isolates. There is the suggestion that different functions of Nef are optimized at different disease stages. Initial data from primary isolates indicates that both CD4 and MHC-I downregulatory functions are intact in acute and early infection (Kirchhoff et al., 1999; Noviello et al., 2007). Yet it appears that Nef function tends to be lost by the time an individual has progressed to end-stage AIDS (Carl et al., 2001; Kirchhoff et al., 1999; Lewis et al., 2008). Additionally, it has been shown that some chronically infected individuals harbor two functionally distinct populations with respect to MHC-I downregulation (Lewis et al., 2008). Indeed there may be an evolution or trade off between different functions over time and in different *in vivo* contexts (Altes and Jansen, 2000). For example, Nef may optimize different functions in different tissue compartments or in response to differing immune pressure (Ali et al., 2005; Salemi et al.,

2005). Understanding this balance in primary isolates is crucial for a better understanding of Nef's role in pathogenesis.

Previous assays of Nef function have suffered from several limitations including the use of exogenous promoters that therefore express Nef at non-physiologic levels, the expression of Nef in isolation and not in the context of viral infection, and the testing of only single homogenous isolates, primarily standard laboratory strains. Altered expression of Nef may alternatively overestimate or underestimate its functions. Additionally, expression of Nef in non-lymphocyte cell lines that may not accurately reflect the degree of downregulation of these cell surface molecules as their levels and/or intracellular pathways may vary from lymphocytes. Our vector system expresses Nef under the control of the HIV-1 LTR as promoter in a T lymphocyte cell line, more closely resembling the *in vivo* situation. Also, the use of the modified whole genome vector for the expression of Nef places its function in the context of viral infection. Although several reading frames (*env*, *vpr*, and *vpu*) have been modified, the physiologic expression of Nef via LTR-driven transcription is preserved in this NL4-3-based system and the insertion of the reporter gene in *vpr* has been shown not to affect pathogenicity (Jamieson and Zack, 1998).

The use of single, homogenous Nef isolates or testing only laboratory isolates is not necessarily an accurate reflection of the overall function of Nef in infected individuals. *In vivo*, HIV-1 exists as a quasispecies, a swarm of related but not identical sequences. Testing a single primary isolate may not be reflective of the function of the entire circulating quasispecies, even if it appears to be a dominant clone. The use of laboratory strains may also yield misleading conclusions about Nef's functions. Most of these strains, although originally isolated from chronically infected individuals, subsequently have been adapted for growth in cell lines under selective conditions that differ from those *in vivo*, and hence they may have gained or lost functions relative to primary isolates. Although there is the possibility of bias introduced through the process of PCR cloning from plasma, it appears that this method can reliably reflect the degree of diversity present in the quasispecies swarm *in vivo* (Lewis et al., 2008). Comparison of results obtained from testing individual clones with results obtained from testing the quasispecies population showed excellent concordance. By testing the whole population, a profile of the overall level of Nef function both within and between individuals can be generated without the selection bias introduced by testing individual clones.

Our new assay system allows the examination of clonal or mixed Nef populations for simultaneous assessment of MHC-I and CD4 downregulation. Our pilot testing of circulating plasma *nef* quasispecies in three chronically HIV-1-infected individuals confirms that function is not uniform across the population, as previously observed for MHC-I downregulation alone (Lewis et al., 2008). Two of the subjects, 00022 and 00037, both had high viral loads and low CD4 counts. However, the functional profile of the *nef* quasispecies was not the same; the majority of *nef* alleles from subject 00022 were functional, while only about half of the alleles from subject 00037 were functional. Subject 00037 had suffered multiple opportunistic infections while subject 00022 had not. The loss of Nef functions in the quasispecies of subject 00037, an individual with end-stage AIDS, is consistent with previous studies reporting loss of Nef function with progression to end-stage disease. Further characterization of *nef* alleles from infected individuals, as well as longitudinal study of Nef function within the same individual will help clarify whether and which Nef functions are associated with clinical disease indicators.

Results from chronically infected subjects also demonstrated that Nef-mediated downregulation of CD4 and MHC-I appeared concordant; that is, for the majority of Nef isolates both functions were either present or absent. Further work will be required to determine whether this trend holds true in different tissue compartments, and across more individuals

spanning varying disease states. Because the mechanisms and domains of Nef involved in downregulating CD4 and MHC-I are distinct, these functions certainly could be differentially regulated. Another interesting observation is that individual Nef sequences appear to be either fully functional or nonfunctional, and this is not explained by the low frequency of defective reading frames. Again, further work will be required to determine the generality of this observation.

In summary, this assay allows the simultaneous assessment of CD4 and MHC-I downregulation by Nef. This assay is amenable to quantification of these functions in primary isolate Nef proteins, either as bulk quasispecies or clones, under controlled expression under a physiologic promoter in the HIV-1 genomic context. This tool will allow examination of these Nef functions for their contribution to disease in pathogenesis studies.

References

- Ausubel, F.; Brent, R.; Kingston, R.; Moore, D.; Seidman, J.; Smith, J.; Struhl, K., editors. Calcium Phosphate Transfection. Short Protocols in Molecular Biology. Vol. 1. Wiley; 2002. p. 9.6-9.7.
- Adnan S, Balamurugan A, Trocha A, Bennett MS, Ng HL, Ali A, Brander C, Yang OO. Nef interference with HIV-1-specific CTL antiviral activity is epitope specific. *Blood* 2006;108:3414–9. [PubMed: 16882705]
- Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. *J Virol* 2000;74:2907–12. [PubMed: 10684310]
- Ali A, Jamieson BD, Yang OO. Half-genome human immunodeficiency virus type 1 constructs for rapid production of reporter viruses. *J Virol Methods* 2003;110:137–42. [PubMed: 12798240]
- Ali A, Ng HL, Dagarag MD, Yang OO. Evasion of cytotoxic T lymphocytes is a functional constraint maintaining HIV-1 Nef expression. *Eur J Immunol* 2005;35:3221–8. [PubMed: 16206233]
- Ali A, Yang OO. A novel small reporter gene and HIV-1 fitness assay. *J Virol Methods* 2006;133:41–7. [PubMed: 16307801]
- Altes HK, Jansen VA. Intra-host competition between nef-defective escape mutants and wild-type human immunodeficiency virus type 1. *Proc R Soc Lond B Biol Sci* 2000;267:183–9.
- Bandres JC, Shaw AS, Ratner L. HIV-1 Nef protein downregulation of CD4 surface expression: relevance of the Ick binding domain of CD4. *Virology* 1995;207:338–41. [PubMed: 7871751]
- Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci U S A* 1993;90:8033–7. [PubMed: 8396259]
- 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;75:3657–65. [PubMed: 11264355]
- 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;10:661–71. [PubMed: 10403641]
- 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. [PubMed: 9450757]
- Craig HM, Pandori MW, Guatelli JC. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. *Proc Natl Acad Sci U S A* 1998;95:11229–34. [PubMed: 9736718]
- Daniel MD, Kirchhoff F, Czajak SC, Sehgal PK, Desrosiers RC. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* 1992;258:1938–41. [PubMed: 1470917]
- 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, recipients. *Science* 1995;270:988–91. [PubMed: 7481804]

- Du Z, Lang SM, Sasseville VG, Lackner AA, Ilyinskii PO, Daniel MD, Jung JU, Desrosiers RC. Identification of a nef allele that causes lymphocyte activation and acute disease in macaque monkeys. *Cell* 1995;82:665–74. [PubMed: 7664345]
- Dyer WB, Geczy AF, Kent SJ, McIntyre LB, Blasdall SA, Learmont JC, Sullivan JS. Lymphoproliferative immune function in the Sydney Blood Bank Cohort, infected with natural nef/long terminal repeat mutants, and in other long-term survivors of transfusion-acquired HIV-1 infection. *Aids* 1997;11:1565–74. [PubMed: 9365760]
- Fackler OT, Baur AS. Live and let die: Nef functions beyond HIV replication. *Immunity* 2002;16:493–7. [PubMed: 11970873]
- Fujiwara M, Takiguchi M. HIV-1-specific CTLs effectively suppress replication of HIV-1 in HIV-1-infected macrophages. *Blood* 2007;109:4832–8. [PubMed: 17303696]
- Garcia JV, Miller AD. Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature* 1991;350:508–11. [PubMed: 2014052]
- Geffin R, Wolf D, Muller R, Hill MD, Stellwag E, Freitag M, Sass G, Scott GB, Baur AS. Functional and structural defects in HIV type 1 nef genes derived from pediatric long-term survivors. *AIDS Res Hum Retroviruses* 2000;16:1855–68. [PubMed: 11118071]
- Gibbs JS, Regier DA, Desrosiers RC. Construction and in vitro properties of HIV-1 mutants with deletions in “nonessential” genes. *AIDS Res Hum Retroviruses* 1994;10:343–50. [PubMed: 8068414]
- Glushakova S, Grivel JC, Suryanarayana K, Meylan P, Lifson JD, Desrosiers R, Margolis L. Nef enhances human immunodeficiency virus replication and responsiveness to interleukin-2 in human lymphoid tissue ex vivo. *J Virol* 1999;73:3968–74. [PubMed: 10196292]
- Jamieson BD, Zack JA. In vivo pathogenesis of a human immunodeficiency virus type 1 reporter virus. *J Virol* 1998;72:6520–6. [PubMed: 9658095]
- 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;65:651–62. [PubMed: 2032289]
- 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;73:5497–508. [PubMed: 10364298]
- Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med* 1995;332:228–32. [PubMed: 7808489]
- 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;9:622–31. [PubMed: 10375528]
- Lewis MJ, Balamurugan A, Ohno A, Kilpatrick S, Ng HL, Yang OO. Functional adaptation of Nef to the immune milieu of HIV-1 infection in vivo. *J Immunol* 2008;180:4075–81. [PubMed: 18322217]
- Mangasarian A, Piguet V, Wang JK, Chen YL, Trono D. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. *J Virol* 1999;73:1964–73. [PubMed: 9971776]
- Noviello CM, Pond SL, Lewis MJ, Richman DD, Pillai SK, Yang OO, Little SJ, Smith DM, Guatelli JC. Maintenance of Nef-mediated modulation of major histocompatibility complex class I and CD4 after sexual transmission of human immunodeficiency virus type 1. *J Virol* 2007;81:4776–86. [PubMed: 17329339]
- Oelrichs R, Tsykin A, Rhodes D, Solomon A, Ellett A, McPhee D, Deacon N. Genomic sequence of HIV type 1 from four members of the Sydney Blood Bank Cohort of long-term nonprogressors. *AIDS Res Hum Retroviruses* 1998;14:811–4. [PubMed: 9643382]
- Pang S, Yu D, An DS, Baldwin GC, Xie Y, Poon B, Chow YH, Park NH, Chen IS. Human immunodeficiency virus Env-independent infection of human CD4(–) cells. *J Virol* 2000;74:10994–1000. [PubMed: 11069994]
- Piguet V, Trono D. The Nef protein of primate lentiviruses. *Rev Med Virol* 1999;9:111–20. [PubMed: 10386338]

- Riggs NL, Craig HM, Pandori MW, Guatelli JC. The dileucine-based sorting motif in HIV-1 Nef is not required for down-regulation of class I MHC. *Virology* 1999;258:203–7. [PubMed: 10366557]
- Salemi M, Lamers SL, Yu S, de Oliveira T, Fitch WM, McGrath MS. Phylodynamic analysis of human immunodeficiency virus type 1 in distinct brain compartments provides a model for the neuropathogenesis of AIDS. *J Virol* 2005;79:11343–52. [PubMed: 16103186]
- Salter RD, Howell DN, Cresswell P. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics* 1985;21:235–46. [PubMed: 3872841]
- Schindler M, Munch J, Kutsch O, Li H, Santiago ML, Bibollet-Ruche F, Muller-Trutwin MC, Novembre FJ, Peeters M, Cournaud V, Bailes E, Roques P, Sodora DL, Silvestri G, Sharp PM, Hahn BH, Kirchhoff F. Nef-mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to HIV-1. *Cell* 2006;125:1055–67. [PubMed: 16777597]
- Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nat Med* 1996;2:338–42. [PubMed: 8612235]
- Simmons A, Aluvihare V, McMichael A. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* 2001;14:763–77. [PubMed: 11420046]
- Smith BL, Krushelnycky BW, Mochly-Rosen D, Berg P. The HIV nef protein associates with protein kinase C theta. *J Biol Chem* 1996;271:16753–7. [PubMed: 8663223]
- 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;78:13335–44. [PubMed: 15542684]
- Tomiyama H, Akari H, Adachi A, Takiguchi M. Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8(+) T-cell cytolytic activity and cytokine production. *J Virol* 2002;76:7535–43. [PubMed: 12097566]
- Tomiyama H, Fujiwara M, Oka S, Takiguchi M. Cutting Edge: Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication. *J Immunol* 2005;174:36–40. [PubMed: 15611225]
- Trono D. HIV accessory proteins: leading roles for the supporting cast. *Cell* 1995;82:189–92. [PubMed: 7628010]
- Ueno T, Motozono C, Dohki S, Mwimanzu P, Rauch S, Fackler OT, Oka S, Takiguchi M. CTL-mediated selective pressure influences dynamic evolution and pathogenic functions of HIV-1 Nef. *J Immunol* 2008;180:1107–16. [PubMed: 18178851]
- Yang OO, Nguyen PT, Kalams SA, Dorfman T, Gottlinger HG, Stewart S, Chen IS, Threlkeld S, Walker BD. Nef-mediated resistance of human immunodeficiency virus type 1 to antiviral cytotoxic T lymphocytes. *J Virol* 2002;76:1626–31. [PubMed: 11799157]

Figure 1. Construction of a modified NL4-3-based whole genome plasmid with deletions in *env* and *vpu*, and unique cloning sites for the insertion of unique *nef* alleles

Parent NL4-3-based half-genome plasmids, p83-10 and p83-2.1 with HA and HSA reporters, respectively, are shown on the left. The middle panel shows the deletions in *env*(7039–7620) and *vpu*(6063–6180) in p83-10 and the modifications made to specific restriction enzyme sites (BspEI and XbaI) in both parent plasmids. The right panel shows the final reconstructed whole genome plasmid created by ligating EcoRI- and NruI-digested half-genome plasmids.

Figure 2. Strategy for cloning primary *nef* quasispecies into the modified whole genome vector
Positions of the nested PCR primers are indicated by arrowheads. The forward primer contains an XbaI restriction site sequence. The BspEI site is naturally occurring within the amplified product. *Nef* quasispecies are amplified from plasma RNA, TA cloned, digested with XbaI and BspEI and inserted into the *nef* position of the modified whole genome vector AA1305#18.

Figure 3. A panel of Nef mutants expressed at physiologic levels in the context of the modified whole genome construct demonstrates the simultaneous measurement of CD4 and MHC-I downregulation by Nef

The Nef variant is indicated in upper right corner of each plot. Gates were set on all live, HSA-positive cells. A. Dot plot of simultaneous A*02 and CD4 expression. B. Histogram plot of A*02 expression; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison. C. Histogram plot of CD4 expression; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison.

Figure 4. Simultaneous measurement of CD4 and MHC-I downregulation by *nef* quasispecies isolated from chronically-infected individuals

The Subject number is in upper right corner of each plot. Gates were set on all live, HSA-positive (infected) cells. A. Dot plot of simultaneous A*02 and CD4 expression. Percent of gated events is noted in each quadrant. B. Histogram plot of A*02; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison. C. Histogram plot of CD4 expression; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison.

Figure 5. A Neighbor-joining phylogenetic tree of *nef* sequences derived from subject 00037 demonstrates the distribution and function of the individually tested clones

Thirty-one full-length *nef* alleles were sequenced and aligned with NL4-3 *nef*. The 10 clones used for individual testing are indicated with labels. The functional profile of each clone tested is indicated after its name: ++ (both CD4 and MHCI downregulation present), -- (both CD4 and MHCI downregulation absent), "CD4" (CD4 downregulation present, MHCI downregulation absent). Whether the reading frame was intact or defective is indicated for the non-functional isolates.

Figure 6. Simultaneous measurement of CD4 and MHC-I downregulation by individual *nef* alleles Ten separate recombinant reporter viruses carrying a single *nef* allele from subject 00037 were used to infect T1 cells in parallel. Three days after infection CD4 and HLA-A*02 were measured on live, HSA reporter-positive cells. Representative examples of each of the 3 types of function profiles observed are shown. A. Dot plot of simultaneous A*02 and CD4 expression. B. Histogram plot of A*02; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison. C. Histogram plot of CD4 expression; the filled histogram represents the indicated Nef variant, while the open histogram represents Δ Nef for comparison.