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Dynamic range of Nef functions in chronic HIV-1 infection

Philip Mwimanzi [a,b,1], Tristan J. Markle [b,1], Yoko Ogata [a], Eric Martin [b], Michiyo Tokunaga [a], Macdonald Mahiti [a], Xiaomei T. Kuang [b], Bruce D. Walker [c], Mark A. Brockman [b,d], Zabrina L. Brumme [b,d,**,1], Takamasa Ueno [a,*,1]

[a] Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[b] Simon Fraser University, Burnaby, BC, Canada V5A 1S6
[c] Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology and Harvard University, Boston, MA, USA
[d] British Columbia Centre for Excellence in HIV/AIDS, Vancouver BC, Canada V6Z 1Y6

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ABSTRACT

HIV-1 Nef is required for efficient viral replication and pathogenesis. However, the extent to which Nef's functions are maintained in natural sequences during chronic infection, and their clinical relevance, remains incompletely characterized. Relative to a control Nef from HIV-1 strain SF2, HLA class I and CD4 down-regulation activities of 46 plasma RNA Nef sequences derived from unique chronic infected individuals were generally high and displayed narrow dynamic ranges, whereas Nef-mediated virion infectivity, PBMC replication and CD74 up-regulation exhibited broader dynamic ranges. 80% of patient-derived Nefs were active for at least three functions examined. Functional co-dependencies were identified, including positive correlations between CD4 down-regulation and virion infectivity, replication, and CD74 up-regulation, and between CD74 up-regulation and PBMC replication. Nef-mediated virion infectivity inversely correlated with patient $CD4^{\pm}$ T-cell count. Strong functional co-dependencies and the polyfunctional nature of patient-derived Nef sequences suggest a phenotypic requirement to maintain multiple Nef functions during chronic infection.

Introduction

The highly variable HIV-1 Nef protein is required for efficient viral replication and disease progression *in vivo* (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). Nef exhibits multiple functions *in vitro*, including enhancement of virion infectivity and replication (Münch et al., 2007; Miller et al., 1994), down-regulation of cell-surface CD4 (Aiken et al., 1994; Garcia and Miller, 1991) and HLA class I (HLA-I) (Collins et al., 1998; Schwartz et al., 1996), up-regulation of HLA class II associated invariant chain (CD74) (Schindler et al., 2003; Stumptner-Cuvelette et al., 2001), and others (Das and Jameel, 2005; Heigele et al., 2012; Kirchhoff et al., 2008). Variation in Nef activity has been demonstrated for laboratory-adapted viral strains (Fackler et al., 2006; Keppler et al., 2006), viral quasispecies within a single individual (Ali et al., 2009; Lewis et al., 2008), and small numbers of clinically isolated sequences (Na et al., 2004; Zuo et al., 2012), including those from long-term nonprogressors (Corro et al., 2012; Premkumar et al., 1996; Tobiume et al., 2002) and patients with advanced infection (Carl et al., 2001). However, the functional breadth of naturally occurring Nef variants have not been comprehensively assessed using panels of clinically derived sequences. Here, we assessed five key Nef functions (enhancement of virion infectivity and replication capacity in PBMC, down-regulation of cell surface CD4 and HLA-I, and up-regulation of CD74) using 46 clonal *nef* sequences from unique chronic HIV-1-infected individuals. We examined the dynamic ranges, co-dependence, and clinical correlates of these five Nef activities.

Results and discussion

Genotypic and phenotypic profile of patient-derived Nef sequences

We analyzed plasma HIV-1 RNA sequences, as these represent the current replicating virus better than proviral DNA (Crotti et al., 2006). Patient Nef sequences displayed no major phylogenetic clustering (Supplemental Fig. 1). Codon-specific

* Correspondence to: Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, JAPAN. Fax: +81 96 373 6825.
** Corresponding author at: Faculty of Health Sciences, Simon Fraser University, Burnaby, BC, Canada V5A 1S6. Fax: +1 778 782 5927.
E-mail addresses: zbrumme@sfu.ca (Z.L. Brumme), uenotaka@kumamoto-u.ac.jp (T. Ueno).
[1] They contributed equally as first and corresponding authors, respectively.

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Shannon entropy scores of the patient-derived Nef clonal sequences ($N=46$) correlated significantly with those of 1191 subtype *B* sequences retrieved from the Los Alamos database (Spearman $R=0.92$, $p<0.0001$), suggesting them to be representative of subtype B sequence diversity. All patient-derived and SF2 Nef proteins were examined by Western blot using two independent anti-Nef primary antibodies (representative data shown in Fig. 1A). No major differences in

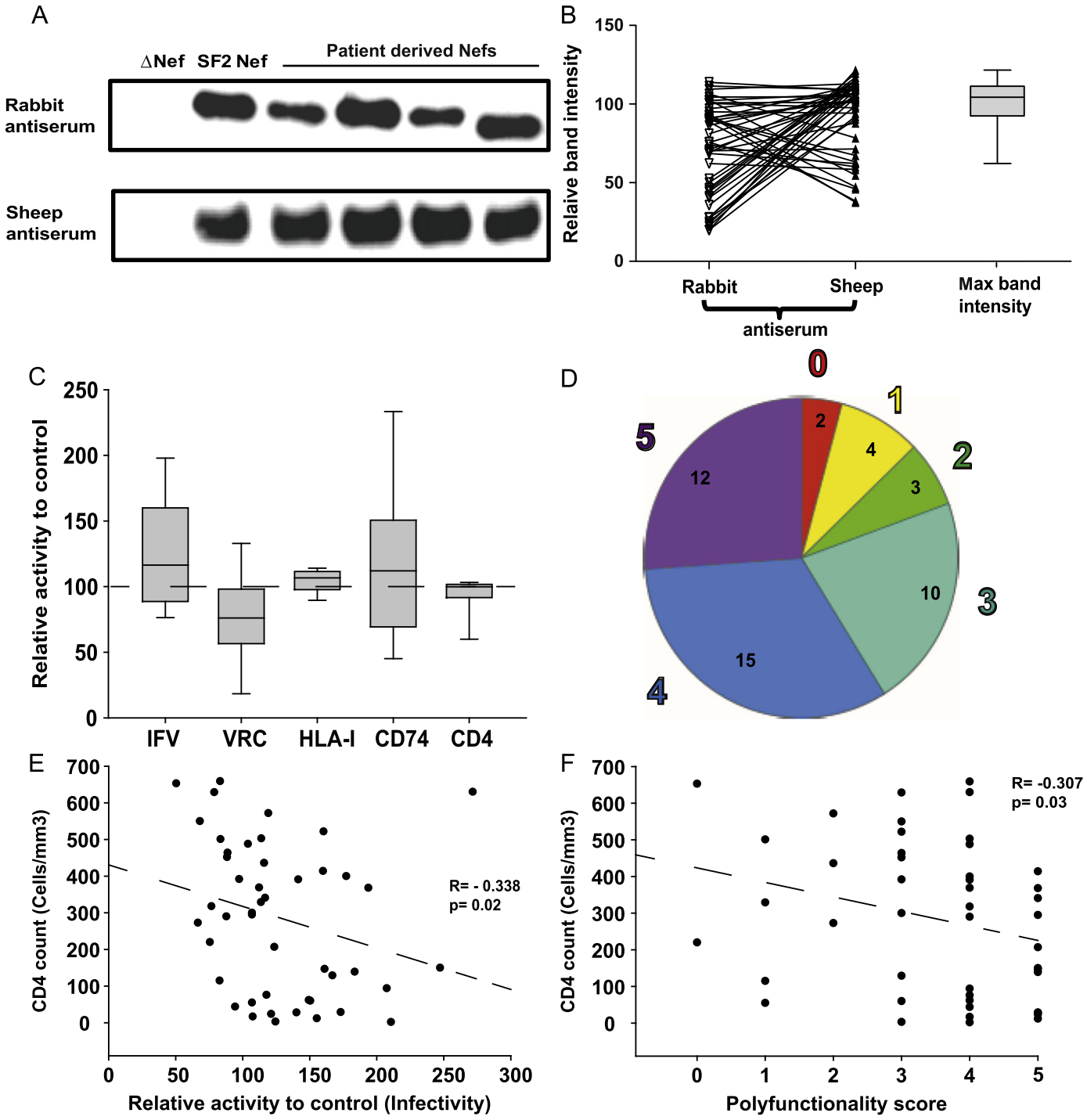

Fig. 1. Western blot and functional profile of 46 patient-derived Nef proteins. (A) Representative blots of ΔNef, SF2 Nef, and four patient-derived Nef clones (B) Band intensities relative to SF2 control, using rabbit (left), sheep (middle), and maximum of rabbit/sheep combined (right), for each patient-derived Nef. (C) *In vitro* dynamic ranges of five Nef-mediated activities: infectivity (IFV), viral replication (VRC), HLA-I down-regulation, CD74 up-regulation, and CD4 down-regulation. Nef function in each assay was normalized to that of control Nef strain SF2, which was considered as 100% (dotted line). Box and whisker plots show the median (horizontal line), interquartile range (edges of box) and range (whiskers) of functions for $N=46$ chronic patient-derived Nef clones. (D) To assess combined functional differences of each patient-derived Nef, a polyfunctionality score was developed. For each of the five Nef activities tested, functions above the 33rd percentile of the population were defined as "adequate" while those below this cutoff were defined as "poor". The number outside the pie chart indicates the "polyfunctionality score", while the number within each slice indicates the number of patient-derived Nef sequences exhibiting this score. (E) Individual Nef functions and the Nef polyfunctionality score were compared to markers of clinical disease in this population of chronic patients. An inverse correlation was observed between Nef-mediated viral infectivity and patient $CD4^+$ T cell count ($R=-0.338$, $p=0.02$; Spearman's correlation). (F) An inverse association was also observed between Nef polyfunctionality score and $CD4^+$ T cell count ($R=-0.307$, $p=0.03$; Spearman's correlation).

steady-state expression levels were observed among Nef proteins (Fig. 1B).

Functional characterization of patient-derived Nef sequences

All 46 patient-derived Nef proteins exhibited at least partial activity for all functions tested (Fig. 1C and Supplemental Fig. 2). Relative to a control Nef, derived from HIV-1 strain SF2, patient-derived Nef sequences were generally highly functional with respect to down-regulation of HLA-I and CD4, while dynamic ranges of other Nef functions were broader (Fig. 1C). Median [IQR] Nef activities, normalized to those of SF2 control, were: virion infectivity, 116% [88–160]; viral replication capacity, 76% [57–98]; HLA-I down-regulation, 106% [98–112]; CD74 up-regulation, 112% [69–151]; and CD4 down-regulation, 99% [92–102] (Fig. 1C). Aligned amino acid sequences and functional activities of the 46 patient-derived clonal nef sequences are shown in Supplemental Table 1.

The relatively conserved CD4 down-regulation function observed in our cohort is consistent with most previous studies of chronic Nef sequences (Agopian et al., 2007; Carl et al., 2001; Zuo et al., 2012). Similar preservation of HLA-I down-regulation function has also been reported by some studies (Noviello et al., 2007; Zuo et al., 2012), however others have observed wider ranges in chronic infection (Lewis et al., 2008) or inefficient Nef-mediated HLA-I down modulation in later infection stages

Table 1
Analysis of Nef residues associated with functions.

Nef activity	Codon[a]	AA[b]	No. of subjects[c]		Relative Nef activity		p-value	q-value
			AA+	AA−	AA+	AA−		
Viral infectivity	8	R	18	25	107.0	140.1	0.02	0.2
	10	L	5	34	82.8	116.4	0.01	0.2
	10	V	8	31	146.0	107.0	0.005	0.2
	21	R	32	14	131.9	97.8	0.02	0.2
	49	A	36	8	122.4	85.7	0.008	0.2
	85	F	7	39	94.3	121.2	0.02	0.2
	152	Q	5	40	76.7	120.2	0.002	0.2
Viral replication in PBMC	10	M	8	31	104.9	69.5	0.001	0.1
	135	F	8	38	116.8	69.7	0.008	0.2
	135	Y	38	8	69.7	116.8	0.008	0.2
	182	Q	6	40	26.3	80.4	0.001	0.1
	194	M	17	29	60.7	84.9	0.003	0.1
	194	V	21	25	93.8	69.5	0.005	0.2
CD74 up-regulation	12	E	5	41	195.1	102.9	0.01	0.2
	21	Q	6	40	180.9	98.1	0.01	0.2
	94	K	40	6	124.4	52.9	0.004	0.1
	205	D	21	25	150.5	82.1	0.001	0.02
	205	N	25	21	82.1	150.5	0.001	0.02

[a] HXB2 numbering.
[b] AA, amino acid.
[c] Gaps in the alignment are not counted; as such, amino acid totals do not always add up to 46.

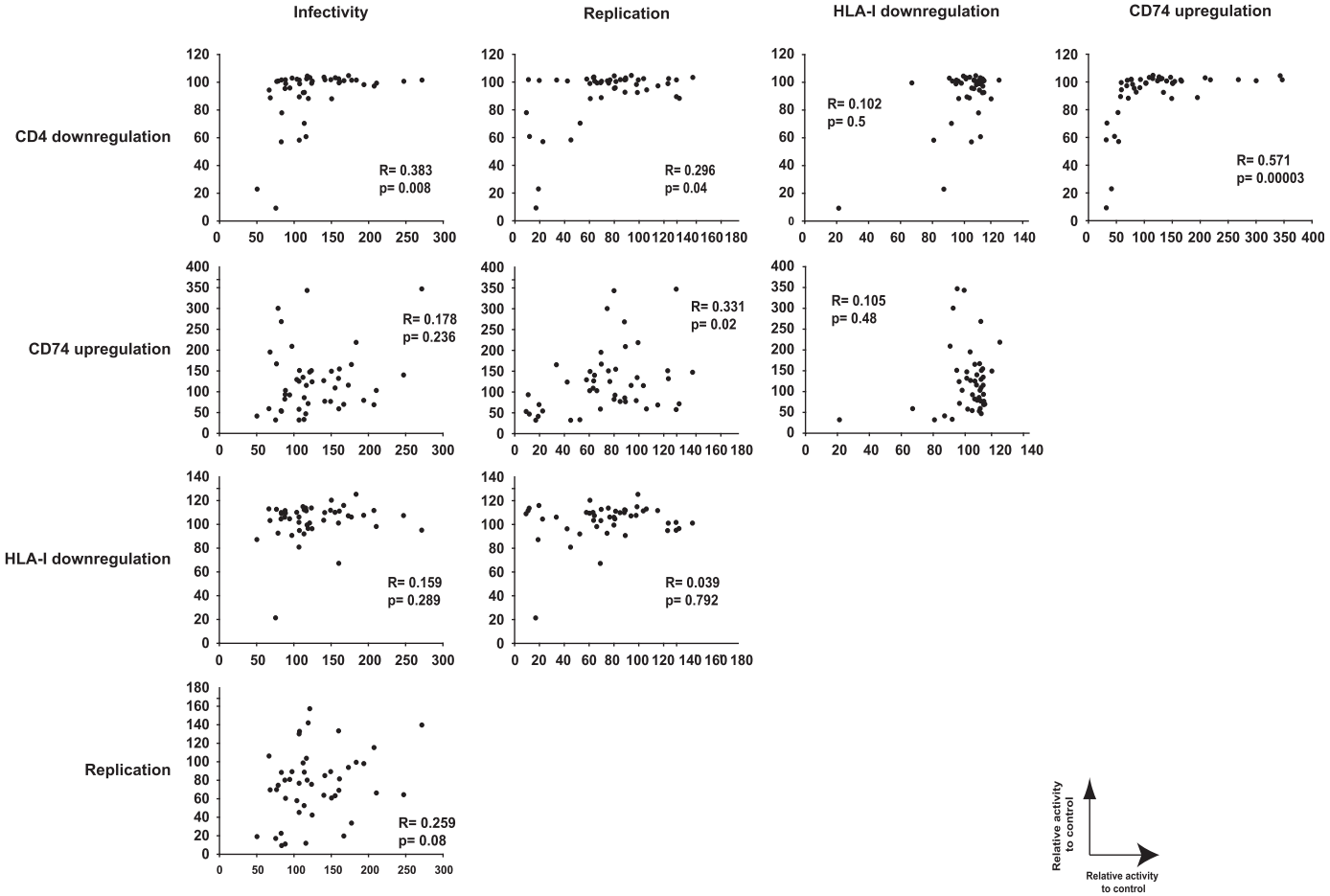

Fig. 2. Co-dependence between *in vitro* Nef activities. Pairwise associations between each of the five *in vitro* functions were examined for the patient-derived Nef clones. Significant correlations were observed between CD4 down-regulation and infectivity, viral replication, and CD74 up-regulation; and between CD74 up-regulation and viral replication (all $p < 0.05$, Spearman's correlation).

(Carl et al., 2001). Our observation that Nef-mediated enhancement of virion infectivity was relatively well preserved among chronic patient-derived sequences, while Nef-mediated viral replication capacity was on average lower than the SF2 control strain, is perhaps notable since previous studies of these Nef activities have failed to observe consistent associations with clinical status (Carl et al., 2000; Crotti et al., 2006; Tobiume et al., 2002). Nef function can be influenced by the choice of assay systems, cell lines, and control strain used (Kirchhoff et al., 2008; Mwimanzi et al., 2011; Suzu et al., 2005); these factors, combined with the smaller number of patients previously studied, may explain some of these divergent results.

Taken together, our data support CD4 and HLA-I down-regulation as essential *in vivo* functions during chronic HIV-1 infection. In contrast, the broader dynamic ranges of virion infectivity, replication capacity in PBMC, and CD74 up-regulation may suggest differential requirements for these activities in maintaining viral fitness during chronic infection. Alternatively, some functions may serve as surrogates of other Nef activities not assessed, such as modulation of cellular activation. Indeed, an association between CD74 up-regulation and polyclonal T-cell activation was recently demonstrated in HIV-infected subjects, suggesting that Nef could mediate this effect directly or indirectly through CD74 up-regulation in virus-infected cells (Ghiglione et al., 2012). Nonetheless, our results extend our understanding of Nef functions that facilitate viral replication and immune evasion in naturally occurring sequences (Brambilla et al., 1999; Casartelli et al., 2003; Crotti et al., 2006; Foster et al., 2001).

Combined functional analyses: Nef polyfunctionality score

To investigate the extent to which individual patient-derived Nef proteins maintained multiple functions simultaneously, we defined a "polyfunctionality" score ranging from 0 (all functions relatively poor) to 5 (all functions adequate) where the 33rd percentile of each function was defined as the cutoff between these two categories (Fig. 1D). More than half (27 of 46) of patient-derived Nefs exhibited a polyfunctionality score ≥ 4 whereas 19.6% (9 of 46) exhibited a score ≤ 2. Two Nef clones scored 0 although both had functional activities >10th percentile for all five activities (Supplemental Table 1), indicating that they were not completely defective. These results support the importance of maintaining multiple Nef functions during chronic infection.

Correlation of Nef functions with HIV-1 clinical parameters

A significant inverse relationship was observed between Nef-mediated virion infectivity and $CD4^+$ T-cell count in our cohort (Spearman's $R=-0.338$, $p=0.02$) (Fig. 1E). To our knowledge, this is a novel observation in chronic infected individuals. Nef polyfunctionality score was also inversely related to $CD4^+$ T-cell count in our cohort ($R=-0.307$, $p=0.03$) (Fig. 1F), although this did not remain significant after removing infectivity from the scoring scheme (not shown). Of note, Lewis et al. (2008) previously reported a relatively broad range of Nef-mediated HLA-I down-regulation function in eleven chronic infected patients and positive correlations with $CD4^+$ T-cell counts, whereas our results showed no relationship between these two parameters. This difference may be due to the fact that Nef-mediated HLA-I down-regulation activity was relatively highly preserved in our cohort (Fig. 1C). No correlation was observed between plasma viral load and any Nef function or the polyfunctionality score. Although further studies will be required to elucidate the underlying mechanism(s) of our observations, these results suggest an important role for Nef-mediated virion infectivity in HIV-1 pathogenesis.

Nef functional co-dependencies

Mutational studies indicate that the genetic determinants of Nef's various functions are largely distinct from one another, and that these functions may therefore be considered largely independent (Dai and Stevenson, 2010). For instance, CD4 down-regulation is determined by the highly conserved Nef motifs $LL_{163,164}$ and $DD_{174,175,}$ while HLA-I down-regulation is mediated by other motifs including M_{20} and $PxxP_{72}$ (Akari et al., 2000; Geyer et al., 2001). However, the extent to which secondary genetic polymorphisms contribute to Nef function, and thus the extent to which the various activities of patient-derived Nef sequences are functionally independent, remains incompletely known (Mwimanzi et al., 2012).

Pairwise correlations of Nef functions in our patient-derived sequences revealed positive relationships between CD4 down-regulation and all other activities, except HLA-I down-regulation (Fig. 2), suggesting shared molecular mechanisms and/or functional complementarity. Indeed, interaction of Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity (Craig et al., 1998). Nef point mutants impaired in CD4 down-regulation were also most delayed in viral replication (Lundquist et al., 2002). A mechanistic link between Nef-mediated CD4 and CD74 modulation is suggested by the observations that both functions involve interaction of Nef with AP-2 (Chaudhuri et al., 2007; Mitchell et al., 2008; Toussaint et al., 2008), and that mutations $WL_{57,58}AA$ and $LL_{163,164}GG$ lowered both Nef-mediated CD4 down-regulation and CD74 up-regulation functions (Stumptner-Cuvelette et al., 2001), although this remains controversial (Toussaint et al., 2008). Taken together with previous studies, our results suggest that Nef-mediated CD4 down-regulation functions of patient-derived sequences may be, at least in part, mechanistically linked to other Nef functions through common functional motifs and/or interactions with common host proteins *in vivo*.

In contrast, HLA-I down-regulation showed no correlation with any other activity (Fig. 2), suggesting that it may be differentially regulated *in vivo*. This observation is consistent with previous studies of site-directed mutants of laboratory-adapted strains (Akari et al., 2000; Lundquist et al., 2002; Stoddart et al., 2003). Also consistent with previous studies using Nef point mutations undertaken in $CD4^+$ T cells (Lundquist et al., 2002), we observed no correlation between Nef-mediated viral infectivity and viral replication enhancement in PBMCs, supporting distinct genetic determinants of these two functions. A recent study observed that HIV-1 gp41 enhanced viral infection through activation of the CD74 protein-mediated extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (Zhou et al., 2011), raising the intriguing hypothesis that Nef might enhance viral infection through the same mechanism. Of note, no inverse relationships were observed between Nef activities, arguing against functional tradeoffs or the existence of particular substitutions or domains that enhance one function at the expense of another. This is consistent with the maintenance of polyfunctionality for most patient-derived Nef sequences.

Amino acids associated with Nef functions

Identification of highly conserved Nef residues and domains critically important for Nef's various functions has been performed using mutational studies (Lundquist et al., 2002; Neri et al., 2011; Stumptner-Cuvelette et al., 2001) and limited analyses of patient-derived sequences (Glushakova et al., 2001; Lewis et al., 2012). To investigate the contribution of naturally-occurring polymorphisms at Nef's more variable sites on protein function of patient-derived sequences, we performed an exploratory sequence-function analysis restricted to amino acids observed at a minimum frequency of $N=5$

in our dataset. Multiple comparisons were addressed using q-values (Storey and Tibshirani, 2003). Eighteen polymorphisms, occurring at 12 unique codons, were associated with at least one Nef function (all $p < 0.05$, $q < 0.25$) (Table 1). No codon was associated with more than two Nef functions, suggesting that, in general, the secondary (variable) residues and domains that mediate Nef's various activities may also be largely genetically separable. No polymorphisms associated with HLA-I down-regulation activity were identified at $q < 0.25$, therefore we are unable to confirm the novel mutations recently identified in chronic infection by Lewis et al. (2012). However, Y135F, which was previously shown to impair HLA-I down-regulation (Lewis et al., 2012), was associated with higher viral replication in our study. Interestingly, variation at Nef codon 21, (within the highly conserved basic amino acid motif $R_{17} \times R \times RR_{22}$ involved in membrane targeting of Nef (Fackler et al., 2006) and vesicle secretion (Ali et al., 2010)), was associated with lower Nef-mediated viral infectivity and CD74 up-regulation. Future studies will be necessary to elucidate potential mechanisms for these newly identified Nef polymorphisms.

Some limitations of our study merit mention. In contrast to previous reports that evaluated specific Nef functions using quasispecies-derived sequences or multiple clones from smaller numbers of patients (Gray et al., 2011; Lewis et al., 2008), we aimed to evaluate the dynamic range and co-dependencies of a broader array of Nef activities using a larger number of patients. As such, our analysis was limited to a single Nef clone per patient. Although each patient sequence was closely related to the bulk plasma RNA sequence (Supplemental Fig. 1), we cannot rule out selection bias in the clones tested; however, we believe this to be minimal since most clones were polyfunctional. Second, we employed recombinant virus approaches to assess most Nef functions. This method might be limited by incompatibilities between insert and backbone; however, we did not observe significant differences in p24 antigen production among viral stocks (data not shown). Finally, to eliminate potential confounding effects due to other HIV-1 proteins, we assessed CD4 down-regulation function using transient transfection assays. This approach can be affected by Nef expression or cytotoxicity; however, we saw no significant differences in steady-state protein levels by Western blot or in cell death by propidium iodide staining between clones (data not shown). Despite these limitations, our study provides an important quantitative assessment of the dynamic range and functional co-dependencies for five of Nef's activities in naturally occurring patient-derived sequences.

Conclusion

Nef sequences from chronic HIV-1 infection are in general highly polyfunctional with respect to enhancement of virion infectivity, stimulation of viral replication in PBMC, down-regulation of CD4 and HLA-I, and up-regulation of CD74. The dynamic ranges of CD4 and HLA-I down-regulation function were relatively narrow, whereas those for virion infectivity, stimulation of viral replication in PBMC, and up-regulation of CD74 were broader. An inverse association was observed between Nef-mediated enhancement of virion infectivity and $CD4^+$ T-cell count, indicating the potential biological importance of this Nef activity in HIV-1 pathogenesis. Strong functional co-dependencies and the polyfunctional nature of patient-derived Nef sequences suggest a phenotypic requirement to maintain multiple Nef functions *in vivo* during chronic HIV-1 infection.

Methods

Forty-six untreated chronic subtype B infected individuals (median [IQR] plasma viral load 90,850 [28,840–231,000] copies/ml; $CD4^+$ T-cell count 297.5 [72–455] cells/mm^3) were recruited in the Boston area with written informed consent (Brumme et al., 2011; Miura et al., 2009). Nef was amplified from plasma HIV-1 RNA by nested RT-PCR as described (Miura et al., 2008) and cloned into the pIRES2-EGFP vector (Clontech). A median of three Nef clones was sequenced per patient; a single clone with an intact Nef reading frame that clustered with the original bulk sequence was selected for analysis (GenBank accession numbers: JX440926–JX440971).

Nef clones were sub-cloned into a pNL43-ΔNef plasmid as described previously (Ueno et al., 2008). As a control, pNL4.3 harboring *nef* from strain SF2 was used (Ueno et al., 2008). Proviral clones were transfected into HEK-293T cells and culture supernatant containing infectious virions was collected 48 h later. Nef protein expression was verified by Western blot using two different polyclonal primary antibodies as described previously (Mwimanzi et al., 2011, 2013).

With the exception of CD4 down-regulation activity (see below), all Nef functions were determined using this panel of recombinant viruses. Infectivity was determined by exposing TZM-bl cells to virus (3 ng $p24^{Gag}$) followed by chemiluminescence detection as described previously (Wei et al., 2002). Viral replication kinetics were analyzed by infecting 10^6 fresh PBMC from four HIV-seronegative donors with virus (10 ng $p24^{Gag}$), followed by stimulation with phytohemagglutinin three days later. Replication was monitored by $p24^{Gag}$ ELISA over 12 days and results expressed as the Day 9 $p24^{Gag}$ reading (Ueno et al., 2008). To assess Nef-mediated HLA-I down-regulation and CD74 up-regulation, 721.221 cells stably expressing CD4 and HLA-A*24:02 were exposed to virus (300 ng $p24^{Gag}$) for 48 h, followed by staining with PE-labeled anti-HLA-A24 mAb (MBL), Alexa-647 anti-human CD74 mAb (BioLegend), 7-amino-actinomycin *D* (BioLegend), and FITC-labeled anti-$p24^{Gag}$ mAb (KC57-FITC, Beckman Coulter) as previously described (Mwimanzi et al., 2013). Fluorescence intensity of each receptor in $p24^{Gag}$-positive and negative live cells was determined by flow cytometry.

Nef-mediated CD4 down-regulation was assessed by electroporation of CEM T cells with Nef-expression plasmids as previously described (Mwimanzi et al., 2013). At 24 h, transfected cells were stained with allophycocyanin-labeled anti-CD4 antibody (BD Biosciences). Median fluorescence intensity for CD4 was determined by flow cytometry (Millipore Guava 8HT).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.02.005.

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