82.3, 41.1, 20.6 and 10.3 μg/mL) and an internal standard stock solution of phenol in saline (3 mg/mL) were prepared. Each standard stock solution (22.8 μL) was mixed with 1.2 μL of the internal stock solution and filtered. A 20 μL aliquot of each filtrate was injected to HPLC. The final concentration of **2·HCl** was 312.5, 156.3, 78.1, 39.1, 19.5 and 9.77 μg/mL, each contained 150 μg/mL phenol. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min. A calibration curve was constructed using the ratio of HPLC peak areas and concentrations of **2·HCl** to demonstrate the linearity as shown in SD Figure S5. The linear regression equation is $Y = 0.034X$ ($R^2 = 0.9927$).

To construct a calibration curve for the adsorption experiments, standard stock solutions in saline of **2·HCl**; 1000, 500, 250, 100, 50 μg/mL and an internal standard stock solution of 12 mg/mL phenol in saline were prepared. Each standard stock solution (228 μL) was mixed with 12 μL of the internal stock solution and filtered. A 200 μL aliquot of each filtrate was injected to HPLC by an autosampler. The final concentration of **2·HCl** was 950, 475, 238, 95.0 and 47.5 μg/mL and contained 600 μg/mL phenol. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min. A calibration curve was constructed using the ratio of HPLC peak areas and concentrations of **2·HCl** to demonstrate the linearity shown in SD Figure S7. The linear regression equation is $Y = 0.0553X$ ($R^2 = 0.9263$).

4.3. Pharmacokinetics in rats

Experiments using rats were conducted in an animal facility under specific pathogen-free conditions, in compliance with institutional regulations approved by the Committee of Tokyo Medical and Dental University (Tokyo, Japan). Five-week-old Jcl:SD rats purchased from CLEA Japan, Inc. (Tokyo, Japan) were maintained for one week before experiments. Compound **2·HCl** (2.5 mg) in 1.0 mL of saline was administered by tail vein injection into 6 week-old Jcl:SD rats. Blood was collected from the tail vein into centrifugal blood collection tubes (E-DS11, Eiken Chemical Co., Ltd., Tokyo, Japan) 15, 30, 45, 60, 120 and 240 min after administration. Blood was centrifuged at 2000*g* for 3 min at room temperature to separate plasma and stored at −80 °C before use. A freeze-thawed plasma sample (50 μL) was mixed with 10 μL of phenol in saline (stock solution: 0.375 mg/mL, giving a final concentration of 62.5 μg/mL) and filtered at 2000*g* for 3 min in a microcentrifuge (MCF-2360, LMS Co., Ltd., Tokyo, Japan). A 50 μL aliquot of each filtrate was injected into an HPLC. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min shown in SD Figure S4. Compound **2** was detected in the HPLC analysis and its peak was characterized by ESI-TOF-MS (m/z calcd for $C_{18}H_{28}N_3O_2$ $[M+H]^+$ 318.22, found 318.19).

4.4. Pharmacokinetics in a rhesus macaque

Experiments using an adult female rhesus macaque, seven years old, were conducted in a biosafety level 3 animal facility, in compliance with institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University (Kyoto, Japan). Compound **2·HCl** (70.6 mg) in 30 mL of 0.1 M sodium phosphate buffer containing NaCl (pH 7.4) was administered by cephalic vein injection. Blood (3.0 mL) was collected from the cephalic vein 0, 0.5, 1, 2, 4 and 24 h after administration using winged needles. Blood was centrifuged at 3600 rpm for 5 min at room temperature to separate plasma then stored at −80 °C before use. A freeze-thawed plasma sample (60 μL) was vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. A 228 μL aliquot of each supernatant was mixed with 12 μL of phenol in saline (stock solution: 0.3 mg/mL, final concentration: 150 μg/mL) and filtered. A 200 μL aliquot of each filtrate was injected into HPLC by an autosampler. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min shown in SD Figure S6. Compound **2** was detected in the HPLC analysis of each filtrate and its peak was characterized by ESI-TOF-MS (m/z calcd for $C_{18}H_{28}N_3O_2$ $[M+H]^+$ 318.22, found 318.19).

4.5. Adsorption experiments of compound 2 to blood cells of a rhesus macaque

Compound **2·HCl** in saline (5 mg/mL, 0.25 mL) was added to blood (1.0 mL) collected from a macaque, and incubated at 37 °C for 0, 1 and 2 h. After incubation, plasma samples (730 μL) were separated by centrifugation at 3600 rpm for 5 min at room temperature, and PBS (480 μL) was added to the resulting precipitates, increasing their total volume to 1.0 mL, and producing blood cell samples. In addition, plasma (0.5 mL) was added to the blood cells after 1 h incubation and the mixture was incubated again for 1 h to separate plasma (730 μL) and blood cells. The separated plasma and blood cell samples were stored at −80 °C before use. Freeze-thawed plasma samples (60 μL) were vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. Freeze-thawed blood cell samples (200 μL) were sonicated to disrupt cell membranes (Sonifier 250, Branson Ultrasonics, Emerson Japan Ltd., Kanagawa, Japan), and 60 μL of the sonicated products was vortex-mixed with MeOH (200 μL) and centrifuged at 2000*g* for 3 min at room temperature. A 228 μL aliquot of each supernatant obtained from both plasma and blood cell samples was mixed with 12 μL of phenol in saline (stock solution: 12 mg/mL, final concentration: 600 μg/mL) and filtered. After filtration, a 200 μL aliquot of each filtrate was injected into HPLC by an autosampler. Elution was carried out with a linear gradient of 20–35% CH_3CN (0.1% TFA) over 30 min and is shown in SD Figure S8.

Acknowledgments

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Supplementary data

Supplementary data (figures of changes on body weight and on intake amounts of bait and water in rats, tables of HPLC peak areas and HPLC charts) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.005.

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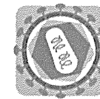

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Attenuation of multiple Nef functions in HIV-1 elite controllers

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Abstract

Background: Impaired HIV-1 Gag, Pol, and Env function has been described in elite controllers (EC) who spontaneously suppress plasma viremia to < 50 RNA copies/mL; however, activity of the accessory protein Nef remains incompletely characterized. We examined the ability of 91 Nef clones, isolated from plasma of 45 EC and 46 chronic progressors (CP), to down-regulate HLA class I and CD4, up-regulate HLA class II invariant chain (CD74), enhance viral infectivity, and stimulate viral replication in PBMC.

Results: In general, EC Nef clones were functional; however, all five activities were significantly lower in EC compared to CP. Nef clones from HLA-B*57-expressing EC exhibited poorer CD4 down-regulation function compared to those from non-B*57 EC, and the number of EC-specific B*57-associated Nef polymorphisms correlated inversely with 4 of 5 Nef functions in these individuals.

Conclusion: Results indicate that decreased HIV-1 Nef function, due in part to host immune selection pressures, may be a hallmark of the EC phenotype.

Keywords: HIV-1, Nef, Elite controllers, Human Leukocyte Antigen (HLA) class I, Immune escape, Replication capacity, HLA-B*57

Background

Elite controllers (EC) are rare (<1%) HIV-1 infected individuals who spontaneously suppress plasma viral loads to undetectable levels in the absence of antiviral therapy. Several factors likely contribute to this phenotype, including host genetics [1], characteristics of HLA-restricted T-cell responses [2], immune-mediated reductions in viral protein function and/or replication [3,4], and acquisition of attenuated viruses [5,6]. Recombinant viruses expressing *gag* and *pol* sequences from EC exhibit reduced *in vitro* replication capacity, due in part to cytotoxic T lymphocyte (CTL) escape mutations selected by certain HLA class I (HLA-I) alleles [3,4], while EC-derived viral envelopes exhibit impaired entry [7]. The *in vitro* function of other viral proteins in EC remains incompletely characterized.

HIV-1 Nef is an accessory protein required for maintenance of high viral loads and progression to AIDS [8], as demonstrated by slow or non-progressive disease in hosts infected with *nef*-deleted or otherwise *nef*-defective strains [5,6,9,10]. Nef exhibits a variety of *in vitro* functions that may modulate pathogenesis, including CD4 down-regulation [11], HLA-I down-regulation [12], HLA class II invariant chain (CD74) up-regulation [13], enhancement of virion infectivity [14], and stimulation of viral replication in PBMC [15] (for reviews see [16-18]). Multiple Nef activities may act together to facilitate immune evasion and enhancement of viral spread *in vivo* [19]; however, multi-functional assessments of patient-derived Nef clones from HIV elite controllers are lacking. Although Nef sequence diversity is highly influenced by host HLA-I selection pressures [20], the relationship between HLA-associated polymorphisms and Nef function is largely unknown. Assessing multiple *in vitro* Nef functions in EC, a population that is highly enriched for protective HLA-I alleles such

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as B*57 [1], provides an opportunity to investigate these questions.

Previous analysis of plasma HIV RNA Nef sequences in our cohort of EC revealed no evidence of gross mutational defects [21], suggesting that any impairment in Nef protein function would have a more complex etiology. For this study, we generated recombinant viruses encoding a single representative HIV RNA Nef clone from 45 EC to assess Nef-mediated down-regulation of HLA class I, up-regulation of HLA class II invariant chain (CD74), viral infectivity, and viral replication in PBMC. The same Nef clone was engineered into a GFP-expression vector to assess its ability to down-regulate CD4. Results were compared to the activities of HIV RNA-derived Nef clones from 46 chronic progressors (CP). Finally, we assessed the role of host immune selection pressures, most notably novel polymorphisms associated with HLA-B*57 in EC, on Nef function in these individuals.

Results

Nef protein expression and viral production

For each of 45 EC and 46 CP, a single representative plasma HIV RNA-derived Nef sequence with an intact open reading frame (ORF) was cloned into a recombinant NL4.3 virus construct. Consistent with previous analyses of bulk plasma HIV RNA sequences from our EC cohort [21], clonal Nef sequences from EC showed no evidence of gross defects or recent shared ancestry (Figure 1, Additional file 1: Table S1). Western blots revealed comparable band intensities between EC and CP, indicating that EC Nefs were not markedly diminished in steady-state protein expression levels (Figure 2A, B). Similarly, $p24^{Gag}$ levels in culture supernatants were comparable between groups, indicating that EC Nefs were not significantly impaired in virion production (Figure 2C).

Nef-mediated enhancement of viral infectivity and replication is impaired in EC

All viruses harboring EC Nef displayed infectivity greater than the negative control NL4.3ΔNef, which had 7.3% infectivity relative to NL4.3-Nef_{SF2}. Compared to control strain NL4.3-Nef_{SF2}, median EC Nef infectivity was 55% (IQR 38-76%), values that were significantly lower than CP-derived Nef (median 116, IQR 88-160%) ($p < 0.001$; Figure 3A). Similarly, all viruses harboring EC Nef displayed higher replication capacities than the negative control

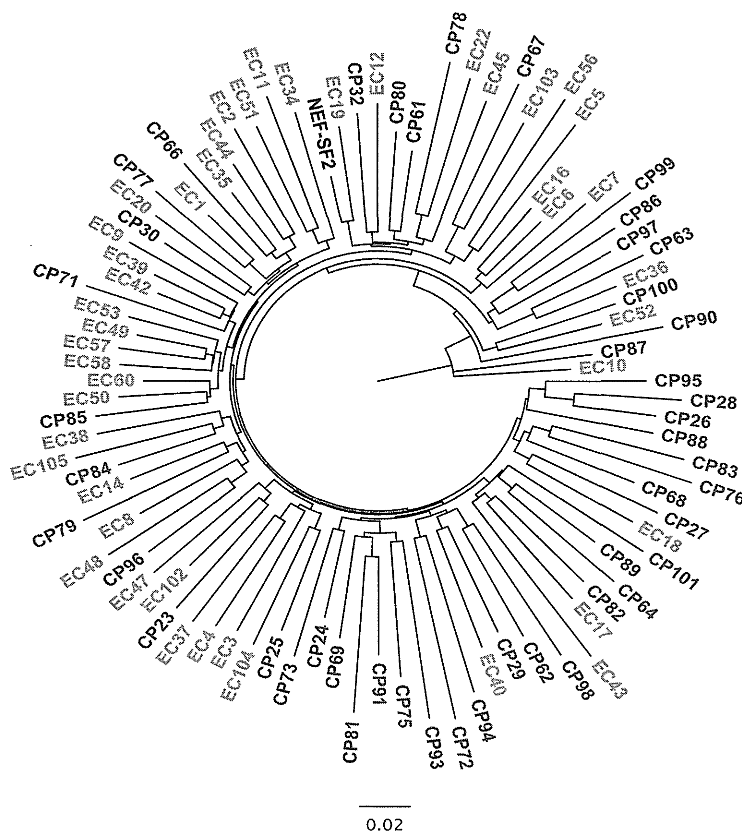

Figure 1 Maximum-likelihood phylogenetic tree of plasma HIV RNA-derived Nef clonal sequences. EC-derived Nefs are red, CP-derived Nefs are blue, and control strain SF2 is black.

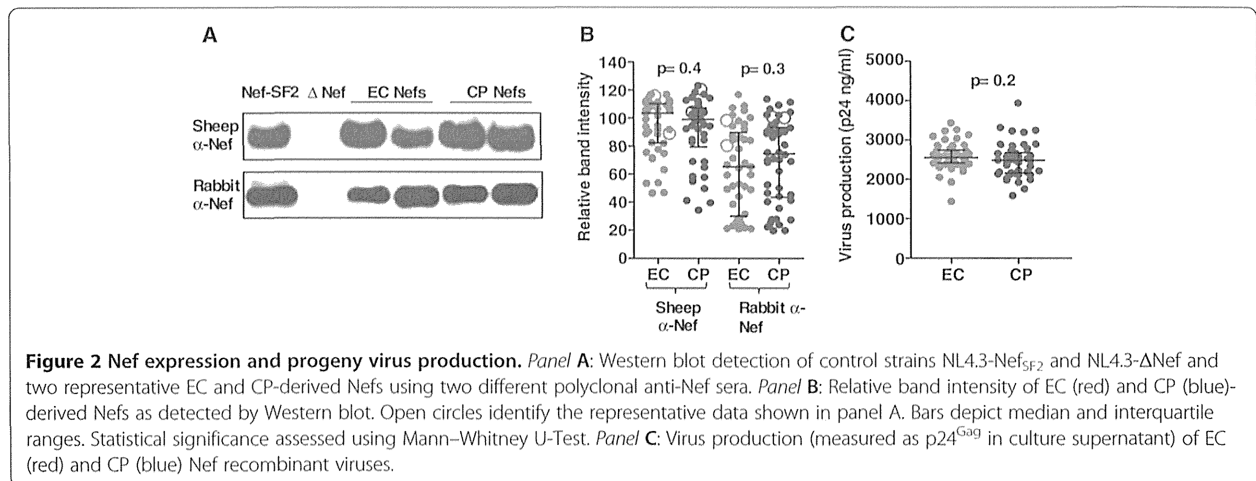

Figure 2 Nef expression and progeny virus production. *Panel* **A**: Western blot detection of control strains NL4.3-Nef_{SF2} and NL4.3-ΔNef and two representative EC and CP-derived Nefs using two different polyclonal anti-Nef sera. *Panel* **B**: Relative band intensity of EC (red) and CP (blue)-derived Nefs as detected by Western blot. Open circles identify the representative data shown in panel A. Bars depict median and interquartile ranges. Statistical significance assessed using Mann–Whitney U-Test. *Panel* **C**: Virus production (measured as $p24^{Gag}$ in culture supernatant) of EC (red) and CP (blue) Nef recombinant viruses.

NL4.3ΔNef in PBMCs from four HIV-negative donors. Consistent with previous reports [22,23], replication of patient-derived Nef recombinant viruses in PBMC differed to some extent among donors; however, viruses encoding EC Nef displayed consistently poorer ability to replicate in PBMC relative to those harboring CP Nef in all donors ($p \leq 0.01$; Figure 3B, C). Averaged over all four donors, median [IQR] replication capacities were 34 [23–52]% of NL4.3-Nef_{SF2} for EC-derived viruses and 76 [57–98]% for CP-derived viruses, respectively ($p < 0.001$, not shown).

Modulation of surface HLA-I, CD74, and CD4 by EC Nef

All EC Nef clones displayed greater ability to modulate cell-surface receptors than ΔNef negative controls. Relative to control strain NL4.3-Nef_{SF2}, EC-derived Nef recombinant viruses maintained considerable HLA-I down-regulation activity (median 95 [IQR 79–106]%) that was nevertheless significantly lower compared to CP Nef viruses (median 106 [IQR 96–111]%) ($p<0.001$; Figure 4A, B). The ability of EC Nef viruses to up-regulate CD74 was markedly lower (median 49 [IQR 35–76]%) compared to CP Nef viruses (median 111 [IQR 68–150]%) ($p < 0.001$; Figure 4C, D). HIV-1 Vpu and Env proteins contribute to surface CD4 modulation [24]; therefore, Nef-mediated CD4 down-regulation activity was assessed using DNA expression plasmids. Relative to control Nef_{SF2}, most EC Nef clones maintained substantial CD4 down-regulation activity (median 91 [IQR 76–95]%) that was nevertheless significantly lower compared to CP Nef clones (median 99 [IQR 89–101]%) ($p = 0.002$) (Figure 4E, F). All EC Nef sequences and functional data are provided in Additional file 1: Table S1. Of interest, 32 of 45 (71%) EC Nef viruses displayed replication activity less than 50% of that of control strain Nef_{SF2}, while only one EC Nef virus showed HLA-I down-regulation activity less than 50%. Three EC Nef demonstrated activity less than 50% of Nef_{SF2} for all functions tested, except HLA-I down-regulation (Additional file 1: Table S1).

Host HLA-I allele expression and Nef function in EC

Protective HLA-I alleles, most notably B*57, are over-represented in EC [1,25]. To investigate this as a potential confounder in comparisons between EC and CP, we re-analyzed our data excluding individuals who expressed HLA-B*57 (17/45 of EC and 8/46 of CP). Measures for all five Nef functions remained significantly lower among non-B*57 EC compared to non-B*57 CP (all $p < 0.01$, not shown). Exclusion of individuals expressing any protective allele (defined as B*27, B*57, and B*58:01) yielded similar results (all $p < 0.05$, not shown).

Immune selection by protective HLA-I alleles, including B*57, can modulate the *in vitro* function of certain HIV-1 proteins in EC [3,4]. To examine whether this was also true for Nef, we stratified EC Nef clones by host B*57 expression and observed significantly lower CD4 down-regulation activity in B*57-derived compared to non-B*57-derived EC Nefs (median [IQR] 83 [55–94]% for B*57 vs. 92 [83–97]% for non-B*57 EC, respectively, $p = 0.038$). Significant differences were not seen for the other Nef activities tested (Figure 5). Of 20 HLA-I alleles expressed in a minimum of five EC, correlations with Nef function were also observed for C*06 (in linkage disequilibrium with B*57; median 74 vs 93% CD4 down-regulation activity in C*06 vs. non-C*06 EC) and A*01 (median 83 vs 97% HLA-I down-regulation activity in A*01 vs. non-A*01 EC) (both $p < 0.05$; $q < 0.05$). No HLA-I associations were observed for Nef-mediated infectivity, replication, or CD74 up-regulation activity in EC.

Unique HLA-associated polymorphisms and Nef function in EC

Modulation of viral protein function in EC by protective HLA-I alleles may be due to the selection of unconventional HLA-associated polymorphisms in this patient group [26,27]. To examine this, we applied phylogenetically-corrected methods [28] to identify HLA-B*57-associated Nef polymorphisms in our cohort of 45 EC. Nine

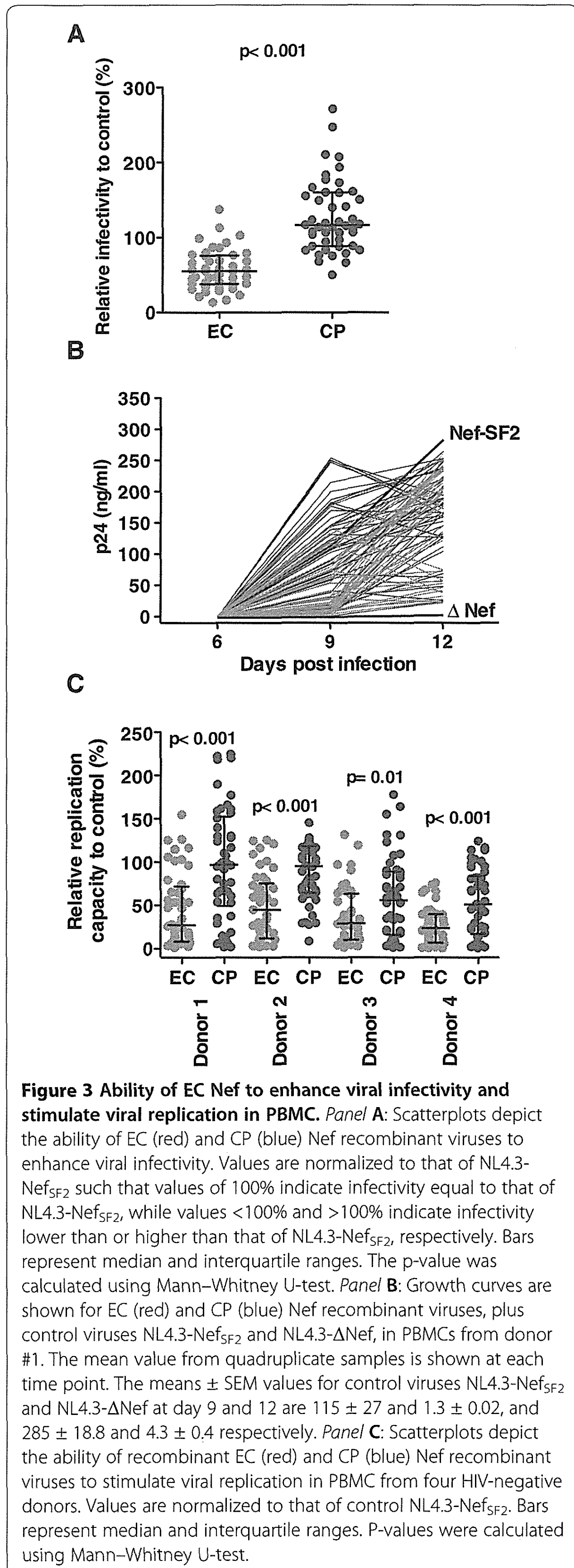

Figure 3 Ability of EC Nef to enhance viral infectivity and stimulate viral replication in PBMC. *Panel* **A**: Scatterplots depict the ability of EC (red) and CP (blue) Nef recombinant viruses to enhance viral infectivity. Values are normalized to that of NL4.3-Nef_{SF2} such that values of 100% indicate infectivity equal to that of NL4.3-Nef_{SF2}, while values <100% and >100% indicate infectivity lower than or higher than that of NL4.3-Nef_{SF2}, respectively. Bars represent median and interquartile ranges. The p-value was calculated using Mann–Whitney U-test. *Panel* **B**: Growth curves are shown for EC (red) and CP (blue) Nef recombinant viruses, plus control viruses NL4.3-Nef_{SF2} and NL4.3-ΔNef, in PBMCs from donor #1. The mean value from quadruplicate samples is shown at each time point. The means ± SEM values for control viruses NL4.3-Nef_{SF2} and NL4.3-ΔNef at day 9 and 12 are 115 ± 27 and 1.3 ± 0.02, and 285 ± 18.8 and 4.3 ± 0.4 respectively. *Panel* **C**: Scatterplots depict the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to stimulate viral replication in PBMC from four HIV-negative donors. Values are normalized to that of control NL4.3-Nef_{SF2}. Bars represent median and interquartile ranges. P-values were calculated using Mann–Whitney U-test.

associations were observed at $p < 0.05$ ($q < 0.4$) in B*57$^+$ EC (Figure 6A). With the exception of V85L, these B*57-associated polymorphisms were distinct from those previously identified in large population-level analyses of chronically subtype B infected individuals (N >1500) [20,29], suggesting that they may be largely unique to EC. In contrast, a search for B*57-associated polymorphisms in our cohort of 46 CP revealed several expected Nef polymorphisms at $p < 0.05$, including V85L and H116N [20,29,30] (not shown), supporting our ability to identify HLA-associated polymorphisms in cohorts of the present size. Therefore, we reasoned that the unconventional B*57-associated polymorphisms observed in EC merited further attention.

Among B*57-expressing EC (N = 17), we observed significant inverse relationships between the number of EC-specific B*57-associated polymorphisms and Nef-mediated replication (Spearman R = −0.5, p = 0.04), HLA-I down-regulation (R = −0.57, p = 0.02) and CD74 up-regulation (R = −0.6, p = 0.01); and a modest, albeit not statistically significant, negative correlation with CD4 down-regulation (R = −0.37, p = 0.1) (Figure 6). No similar correlations were observed between EC-specific polymorphisms associated with other HLA-I alleles observed at comparable frequencies in our cohort (i.e. A*01, A*02, A*03, A*30, C*06, C*07) and Nef function in EC expressing these alleles (not shown).

Amino acids associated with EC Nef function

To investigate the relationship between Nef sequence and function in EC, we performed an exploratory analysis to correlate amino acids with each of the five functions tested, regardless of host HLA. A total of 23 polymorphisms occurring at 14 sites were associated with Nef-mediated modulation of HLA-I, CD74 and CD4 ($p < 0.05$, $q < 0.4$; Table 1). No Nef polymorphisms associated with infectivity or replication were observed at this threshold.

Discussion

We assessed five *in vitro* Nef functions using clonal plasma HIV RNA sequences from 45 EC and 46 CP. We observed that EC Nef clones were generally functional, especially for Nef's most characteristic activities, CD4 and HLA-I down-regulation. Nevertheless, median EC Nef activities were significantly lower for all five functions when compared to those from CP. Median CP Nef activities were consistent with that of HIV-1 strain SF2 used as a normalization control for all assays, indicating that our selection of chronic Nef clones is representative of chronic Nef isolates examined previously. The range in Nef activities observed here may help to resolve discrepancies between previous studies of HIV long-term non-progressors or controllers, which have reported

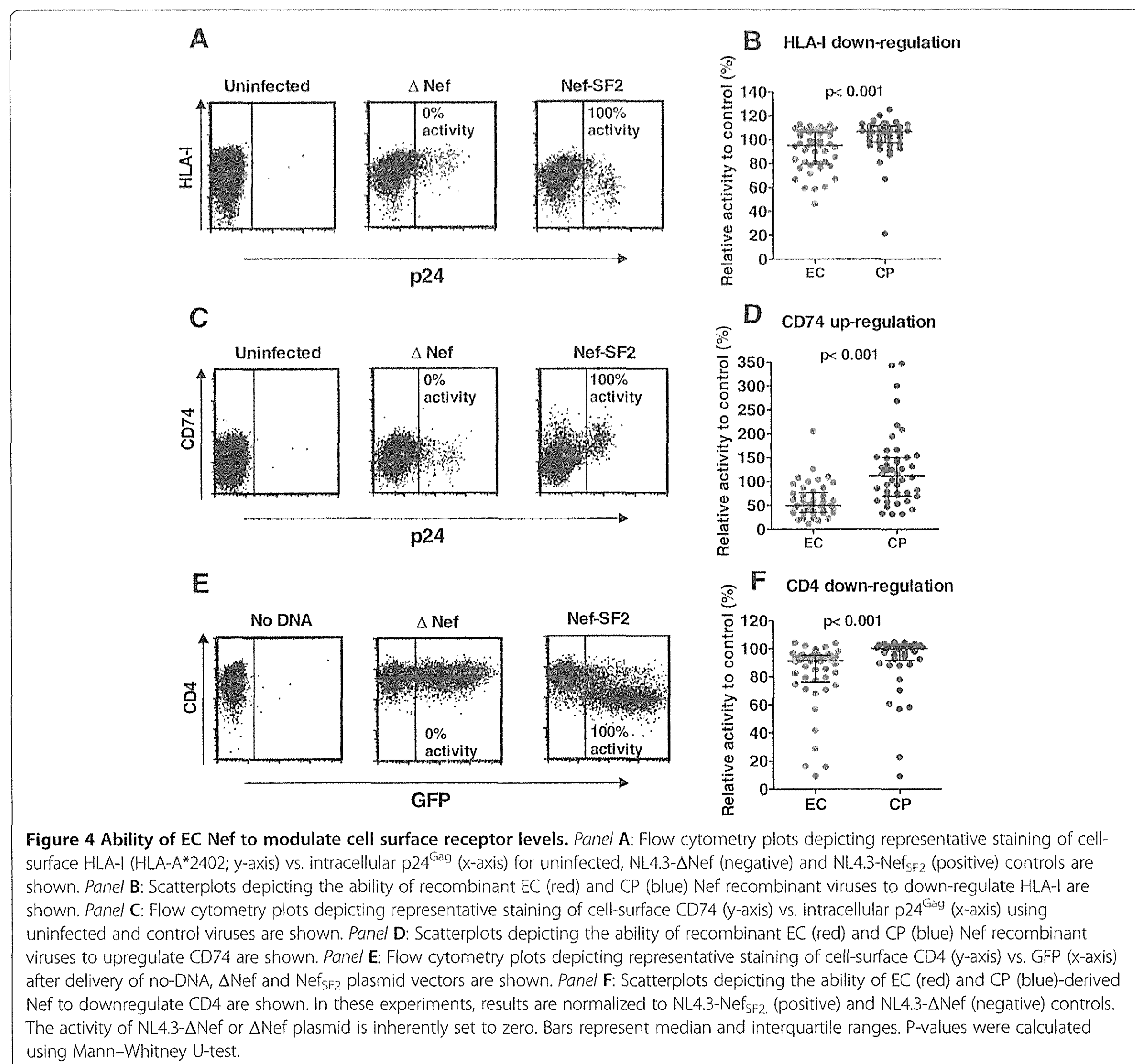

Figure 4 Ability of EC Nef to modulate cell surface receptor levels. *Panel* **A**: Flow cytometry plots depicting representative staining of cell-surface HLA-I (HLA-A*2402; y-axis) vs. intracellular $p24^{Gag}$ (x-axis) for uninfected, NL4.3-ΔNef (negative) and NL4.3-Nef_{SF2} (positive) controls are shown. *Panel* **B**: Scatterplots depicting the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to down-regulate HLA-I are shown. *Panel* **C**: Flow cytometry plots depicting representative staining of cell-surface CD74 (y-axis) vs. intracellular $p24^{Gag}$ (x-axis) using uninfected and control viruses are shown. *Panel* **D**: Scatterplots depicting the ability of recombinant EC (red) and CP (blue) Nef recombinant viruses to upregulate CD74 are shown. *Panel* **E**: Flow cytometry plots depicting representative staining of cell-surface CD4 (y-axis) vs. GFP (x-axis) after delivery of no-DNA, ΔNef and Nef_{SF2} plasmid vectors are shown. *Panel* **F**: Scatterplots depicting the ability of EC (red) and CP (blue)-derived Nef to downregulate CD4 are shown. In these experiments, results are normalized to NL4.3-Nef_{SF2} (positive) and NL4.3-ΔNef (negative) controls. The activity of NL4.3-ΔNef or ΔNef plasmid is inherently set to zero. Bars represent median and interquartile ranges. P-values were calculated using Mann–Whitney U-test.

relative preservation of CD4 and/or HLA-down-regulation function [32,33], inefficient Nef-mediated CD4 and/or HLA-down-regulation [34-36] and reduced infectivity [35] compared to CP. Our data suggest that there is *in vivo* pressure on Nef in EC to maintain CD4 and HLA-I down-regulation functions.

Relative functional impairments between EC and CP clones are not likely to be due to differences in Nef protein stability or expression levels, nor to recent descent from a defective common ancestor. Similarly, while enrichment of protective HLA alleles in EC may contribute to Nef sequence, it is not likely to be the only explanation for relative functional attenuation observed here, since differences between groups persisted after persons who expressed protective HLA alleles were excluded from analysis. Indeed, although significantly lower CD4 down-regulation activity was observed in B*57 compared to non-B*57 EC (Figure 5E), this was not true for other Nef functions, indicating that B*57 expression alone does not guarantee Nef attenuation in this group.

Rather, our results are consistent with functional variability of naturally occurring Nef sequences from EC, which may be attributable in part to non-canonical HLA-associated escape mutations selected in this rare group. Previously, in order to investigate the influence of HLA-associated viral polymorphisms on HIV-1 protein function in EC, we have made use of reference lists of common HLA-associated polymorphisms derived from population-level studies of chronically infected individuals [20,29]. However, such lists may not capture rare

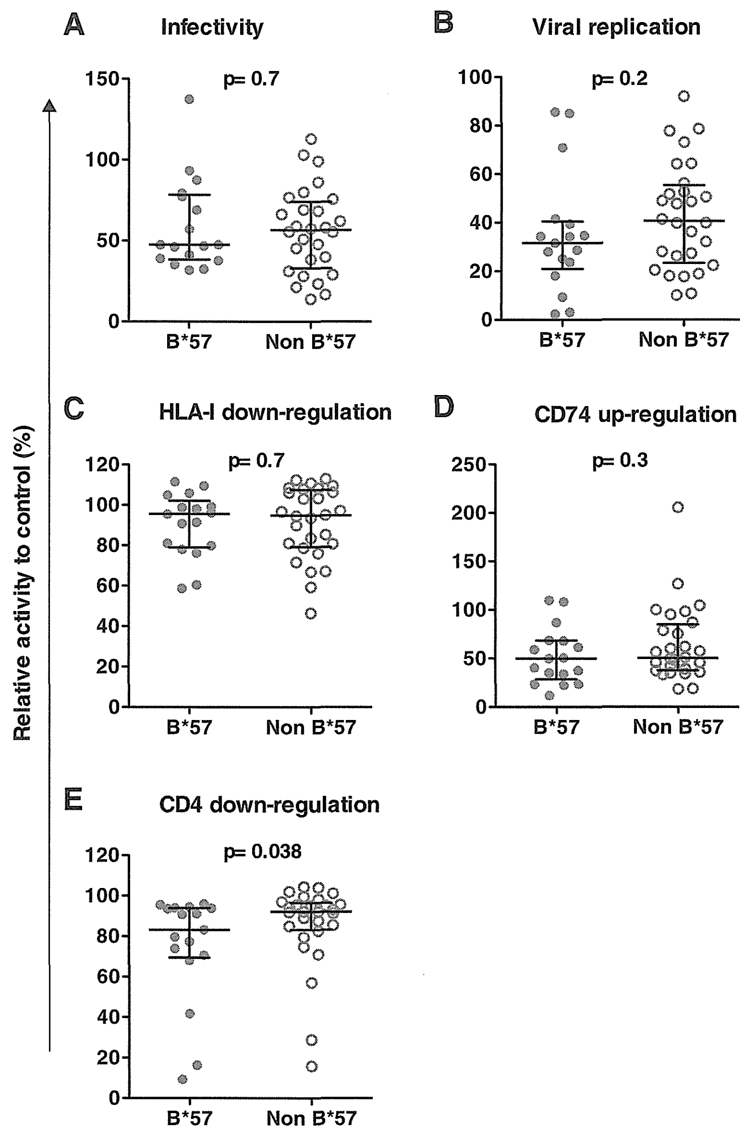

Figure 5 Relationship between host HLA-B*57 expression and Nef activities in EC. Scatterplots are shown depicting the ability of EC-derived Nefs from B*57-expressing (closed red circles) and non-B*57 expressing (open red circles) to enhance virion infectivity (*panel* **A**), enhance viral replication in PBMC (*panel* **B**; data depict means for all four PBMC donors), down-regulate HLA-I (*panel* **C**), up-regulate CD74 (*panel* **D**) and down-regulate CD4 (*panel* **E**). All results are normalized to Nef_{SF2}. Bars represent median and interquartile ranges. P-values were calculated using Mann–Whitney U-test.

escape mutations that are unique to EC [26,27]. Therefore, we used our EC dataset to identify HLA-associated polymorphisms specific to this population, in a novel analysis of this type. It is important to note that this analysis was aimed at identifying HLA-B*57-associated polymorphisms in the EC dataset itself (and is therefore distinct from the comparative analyses described in [21] that sought to identify polymorphisms enriched among EC compared to CP). Statistical power is a major limitation of this analysis, but HLA-associated polymorphisms can be identified in modestly sized datasets (e.g. [37]), especially if analyses are limited to specific alleles. At $p < 0.05$, we identified nine B*57-associated polymorphic sites specific to EC (Figure 6A), most of which differed from B*57-associated polymorphisms commonly identified in population-level analyses [20,29]. In contrast, B*57-associated polymorphisms identified in our CP dataset using the same approach were largely as expected [20,29,30]. ELISpot reactivity to overlapping peptides spanning codons 28, 55, 85, 178 and 198 has been documented in B*57+ EC [27]; and V85L has been described to function as an escape mutation in the B*57/58-KF9

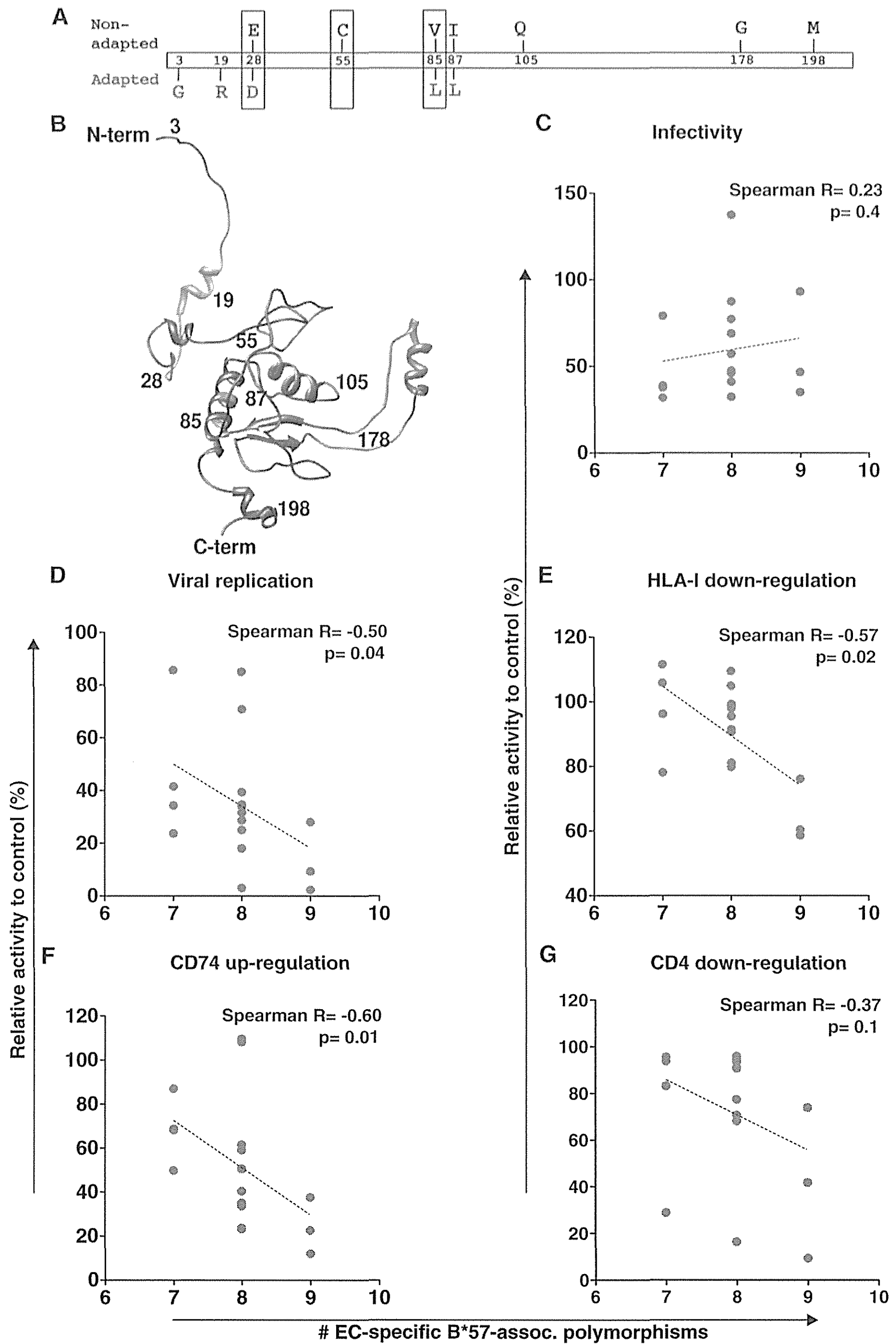

Figure 6 HLA-B*57-associated polymorphisms in EC, and their relationship with Nef function. *Panel* **A**: Two-dimensional map of B*57-associated polymorphisms identified in an exploratory analysis of the EC cohort using phylogenetically-corrected approaches with p<0.05 [20]. "Nonadapted" forms (those less likely to be observed in the presence of B*57 at a specific location) are shown above the Nef protein in blue; "adapted" forms (those enriched among B*57-expressing persons) are shown below the Nef protein in red. Due to limited statistical power and other reasons, both nonadapted and adapted forms are not always identified at a given position. Boxed codons indicate those where the amino acid varied in B*57 EC; the remainder were either expressed in 100% of B*57 EC (adapted forms 3G and 19F) or 0% of B*57 EC (nonadapted forms 105Q, 178G, 198M). In subsequent analyses, Nef sequences from B*57-expressing persons were counted as harboring a B*57-associated polymorphism at a given site if that site expressed anything other than the nonadapted form, or the adapted form if no nonadapted form was identified. *Panel* **B**: The locations of the nine EC-specific B*57-associated polymorphisms are indicated in green on the structure model of the Nef protein (composite crystal structure kindly provided by Art F. Y. Poon, [31]) *Panels* **C-G**: Correlations between the number of B*57-associated polymorphisms in Nef sequences from B*57-expressing EC, and five Nef functions evaluated. Statistical analyses were done using Spearman's correlation.

epitope [38], further suggesting that these polymorphism may be due to CTL selection pressure in B*57 EC. In addition, paired Nef sequences from PBMC and plasma were available for two B*57 EC in the present study. The first exhibited identical amino acids in both compartments at all 7 of the residues putatively associated with HLA-B*57, while the second exhibited different amino acids at three of the seven B*57-associated sites (codons 85, 105 and 198). Although it is not possible to make conclusions based on only two patients, these data support evolution of these sites *in vivo*.

Notably, we observed dramatic inverse associations between the number of EC-specific B*57-associated polymorphisms and Nef-mediated replication, HLA-I down-regulation and CD74 up-regulation. CD4 down-regulation, the only function in which host expression of B*57 was in itself significantly associated with poorer function in EC, also displayed a modest, albeit not significant, negative relationship between the burden of B*57-associated escape mutations and function (Figure 6). These remarkable inverse relationships were particular to B*57 and not observed for other HLA alleles with similar frequency in our EC cohort. Taken together, results suggest that HLA-B*57-associated CTL pressures select for non-canonical polymorphisms in EC, which contribute additively to multiple functional impairments in EC Nef. Building upon previous studies of recombinant viruses encoding *gag* and *pol* sequences from the same EC cohort [3,4], our results support a complex relationship between B*57-associated immune pressures and Nef function.

In an exploratory analysis of Nef amino acid sequences, we identified 23 polymorphisms, located at 14 residues, associated with Nef function in EC. None overlapped with mutations previously identified in HIV non-progressors [39] or with sites reported to affect HLA-I down-regulation

Table 1 Analysis of Nef residues associated with EC Nef functions (all N>5 and q<0.4)

Nef activity	Position on HXB2	AA[a]	No. of subjects[b]		Relative Nef activity (%)		p-value	q-value
			With AA	Without AA	With AA	Without AA		
HLA-I down-regulation	8	S	17	27	83.6	97.2	0.01	0.3
	8	R	14	30	106.1	91.1	0.02	0.3
	11	V	20	21	87.2	98.9	0.01	0.3
	14	P	34	11	91.1	108	0.004	0.3
	46	S	36	8	96.7	77.1	0.01	0.3
	108	D	34	11	91.1	105.8	0.02	0.3
	108	E	11	34	105.8	91.1	0.02	0.3
	138	C	5	40	79.8	96.5	0.02	0.3
	138	T	40	5	96.5	79.8	0.02	0.3
	192	H	36	9	97	78.1	0.01	0.3
	192	R	6	39	84	96.8	0.04	0.4
	205	N	22	23	101	83.6	0.02	0.3
	205	D	22	23	85.9	98.9	0.03	0.3
CD74 up-regulation	8	S	17	27	37.7	60	0.004	0.3
	28	E	14	31	73.7	42.4	0.008	0.3
	28	D	29	16	40.4	65.3	0.009	0.3
	135	F	10	35	35	57.5	0.003	0.3
	135	Y	34	11	57.1	35.1	0.01	0.3
CD4 down-regulation	8	S	17	27	84.8	92.6	0.007	0.3
	14	P	34	11	86.1	97.9	0.003	0.1
	14	S	5	40	102.9	87.2	0.02	0.4
	15	A	11	34	76.9	91.3	0.02	0.4
	21	K	11	34	70.7	91.6	0.002	0.1
	21	R	28	17	91.6	76.9	0.02	0.4
	105	R	12	33	76.6	91.9	0.003	0.1
	163	C	9	36	98.1	86.6	0.02	0.4

[a] AA, amino acid.
[b] Row totals vary depending on the codon position because sequences with gaps in the alignment are considered missing data.

activity in chronic infection [40], a discrepancy that might be due to non-canonical polymorphisms observed in EC. Of interest, 8S was associated with Nef-mediated modulation of cell-surface CD4, HLA-I and CD74, an observation consistent with codon 8's involvement in myristylation [41]. Also, 28D and 105X (in this case R), associated with modulation of CD74 and CD4, respectively, are EC-specific B*57-associated polymorphisms identified in the present study.

Viral genetic studies of EC feature numerous challenges and limitations. Although care was taken to choose a Nef clone that reflected each patient's original bulk HIV RNA sequence, and to rule out proviral DNA contamination, potential biases associated with PCR amplification from extremely low copy-number templates must be acknowledged. On the other hand, use of a single sequence per patient alleviates potential biases associated with quasispecies approaches to compare samples with low vs. high genetic diversity. As our goal was to specifically investigate the function of Nef in EC, we employed recombinant virus (and single-protein expression) approaches to eliminate potential confounding effects of other HIV-1 proteins; however, such approaches may not reflect the characteristics of infectious molecular clones or whole-virus isolates recovered from PBMC, procedures that are rarely successful in EC [21,42]. Recombinant virus approaches are also inherently limited by potential incompatibilities between insert and backbone; our choice of a recombinant control strain (NL4.3-Nef_{SF2}) alleviates this to a minor extent. Although we assessed Nef activity using primary PBMCs and immortalized cell lines, Nef's multiple functions [16-18,43] may vary in different cell types [43-45]. Furthermore, the *in vivo* relevance of our observations - in particular, the extent to which these functional differences contribute to the viremia control in EC - remains unclear. Although our results are consistent with the transmission of partially attenuated Nef sequences in at least some EC, and/or further immune-mediated attenuation in others [3,4,46], it is not possible to disentangle cause and effect in cross-sectional studies. Furthermore, Nef function may change over the infection course [47], therefore longitudinal analysis of Nef function in controllers, beginning in the acute/early phase of infection, is warranted. Finally, although our results suggest that non-canonical polymorphisms in EC may contribute to attenuated Nef function, it will be important to validate these findings in larger EC cohorts. Despite these limitations, our study represents the largest linked analysis of multiple *in vitro* Nef functions in EC to date, and to our knowledge the only study assessing CD74 up-regulation and replication capacity in this group.

Conclusions

EC Nef clones were generally functional; however, all five activities assessed were significantly impaired compared to CP Nef clones. HLA-I-restricted immune pressure, most notably by B*57, may contribute to the differences observed. Taken together with previous studies of HIV Gag, Pol, and Env function in EC [3,4,7], our results support decreased viral protein function as a hallmark of the EC phenotype and underscore the potential role of immune pressures in modulating viral protein function in this rare group.

Methods

Study subjects

45 EC (median [interquartile range, IQR] pVL 2 [0.2-14] RNA copies/ml [25]; median [IQR] CD4 count 811 [612–1022] cells/mm^3) and 46 CP (median [IQR] pVL 80500 [25121–221250] RNA copies/ml); median [IQR] CD4 count 292.5 [72.5-440] cells/mm^3) were studied as described previously [3,4,21,25]. All EC and CP were HIV-1 subtype B-infected, untreated at the time of sample collection, recruited from the Boston area, and comparable with respect to ethnicity and date of HIV diagnosis (1985–2006 for EC vs. 1981–2003 for CP). This study was approved by the institutional review board of Massachusetts General Hospital, Boston USA; all participants provided written informed consent.

Cloning and analysis of nef genes

For EC, HIV RNA was extracted from a starting volume of 4.5 to 35.0 ml of plasma and amplified using nested RT-PCR, as described [21]. Given that the median pVL in our EC cohort was 2 RNA copies/ml [IQR 0.2-14] [25], we estimate that on average, 40 viral RNA templates were extracted for each EC patient. To rule out proviral DNA contamination, all extractions included a DNAse treatment step; controls lacking RT enzyme were also performed [21]. For CP, HIV RNA was extracted from 0.5ml of plasma and amplified in the same manner. Nef amplicons were cloned into pIRES2-EGFP expression vector (Clontech). A minimum of three Nef clones were sequenced per patient, and a single clone with an intact Nef reading frame that closely resembled the original bulk plasma RNA sequence was selected. Genbank accession numbers for clonal Nef sequences are JX171199-JX171243 (EC) and JX440926-JX440971 (CP).

Recombinant virus construction and verification of Nef expression

Nef clones were transferred into a pNL4.3ΔNef plasmid as described [23] and confirmed by DNA sequencing. Recombinant viruses harboring *nef* from HIV strain SF2 (NL4.3-Nef_{SF2}), and lacking *nef* (NL4.3ΔNef) were used as positive and negative controls, respectively. Infectious viruses were generated as described [48]. Briefly, HEK-293T cells were transfected with each proviral clone. Virus-containing culture supernatants were harvested 48 hr following transfection, titered by $p24^{Gag}$ ELISA

(ZeptoMetrix Corp.) and aliquots stored at −80°C until use.

HEK-293T cell pellets collected at this time point were used to prepare total cell lysates as described [45] that were subjected to SDS-PAGE in duplicate and transferred to nitrocellulose membranes. Nef genetic diversity poses a challenge to antibody-based detection as differences in reactivity may reflect suboptimal antibody binding rather than variation in protein levels. To ensure detection of patient-derived Nef, duplicate blots were probed using unique anti-Nef polyclonal antisera, developed from sheep (ARP 444; provided by O.T. Fackler, Heidelberg University, Germany) or rabbit (NIH AIDS Research and Reference Reagent Program). Band intensities were quantified using ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Virion infectivity and replication assays

Recombinant virus infectivity was determined by exposing 10^4 TZM-bl cells (NIH AIDS Research and Reference Reagent Program) to 3 ng $p24^{Gag}$ recombinant virus followed by chemiluminescence detection 48 hr later as described [49]. Infectivity values represented the mean of triplicate experiments, normalized to control strain NL4.3-Nef_{SF2}, such that values > 100% and < 100% indicated increased or decreased infectivity, respectively. Recombinant virus replication was assessed by exposing 10^6 freshly isolated PBMC from four HIV-negative donors to 10 ng $p24^{Gag}$ recombinant virus for 8 hr, washing twice, and then resuspending cells in a culture medium (RPMI 1640, 10% FCS) as described [22,23]. Three days later, PBMCs were stimulated with phytohemagglutinin at 5 μg/ml. Culture supernatants were collected and replaced with fresh medium supplemented with human rIL-2 every 3 days. Viral replication was monitored by measuring $p24^{Gag}$ in the culture supernatant using ELISA over 12 days. ELISA values during the initial burst of viral replication (on day 9) were used as our measure of replication capacity. Results were expressed as the mean of quadruplicate assessments in each donor, normalized to control strain NL4.3-Nef_{SF2}.

Analysis of receptor modulation HIV-infected cell surface

To assess Nef-mediated HLA-I down-regulation and CD74 up-regulation, 721.221 cells stably expressing CD4 and HLA-A*24:02 (provided by Masafumi Takiguchi, Kumamoto University, Japan) were exposed to 300 ng $p24^{Gag}$ recombinant virus for 48 hr, followed by staining with anti-HLA-A24-PE (MBL), anti-CD74-Alexa Fluor-647 (BioLegend), 7-amino-actinomycin D (BioLegend), and anti-p24 Gag-FITC (Beckman Coulter), as described [23]. Mean fluorescence intensity (MFI) of each receptor in live $p24^{Gag}$ positive and negative subsets was determined by flow cytometry (FACS Canto II; BD Biosciences). Results were expressed as the mean of duplicate experiments, normalized to control strain NL4.3-Nef_{SF2}.

Analysis of Nef-mediated CD4 down-regulation

To assess Nef-mediated CD4 down-regulation, 3 × 10^5 CEM-SS cells were transfected with 5 μg plasmid DNA encoding Nef protein and GFP by electroporation (BioRad GenePulser MX) and stained 24 hr later with anti-CD4-APC (BD Biosciences). MFI of GFP-negative and GFP-positive (Nef-expressing) subsets was determined by flow cytometry (Guava easyCyte 8HT, Millipore). Results were normalized to plasmid expressing Nef_{SF2}.

Statistical analyses, including identification of B*57-associated polymorphisms in patient-derived Nef sequences

Phylogenetically-informed methods were used to identify Nef amino acids significantly associated with HLA-B*57allele expression in our EC ([28], implemented at http://research.microsoft.com/en-us/um/redmond/projects/mscompbio/phylododdsratio/default.aspx). Multiplecomparisons were addressed using q-values, the p-value analogue of the false discovery rate (FDR) [50]. The FDR is the expected proportion of false positives among results deemed significant at a given p-value threshold; for example, at a $q \leq 0.2$, we expect 20% of identified associations to be false positives.

Additional file

Additional file 1: Table S1. EC Nef activity with aligned amino acid sequence.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PM, TJM, ZLB, MAB, and TU designed the study; PM, TJM, EM, YO, XTK, MT, and MM performed the experiments; FP, TM and BDW provided access to patient samples and analyzed clinical data; PM, TJM, EM, ZLB, MAB, and TU analyzed data; and PM, ZLB, MAB, and TU wrote the paper. All authors read and approved the final manuscript.

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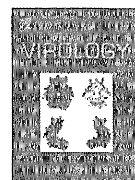

Dynamic range of Nef functions in chronic HIV-1 infection

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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+ 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.

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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

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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

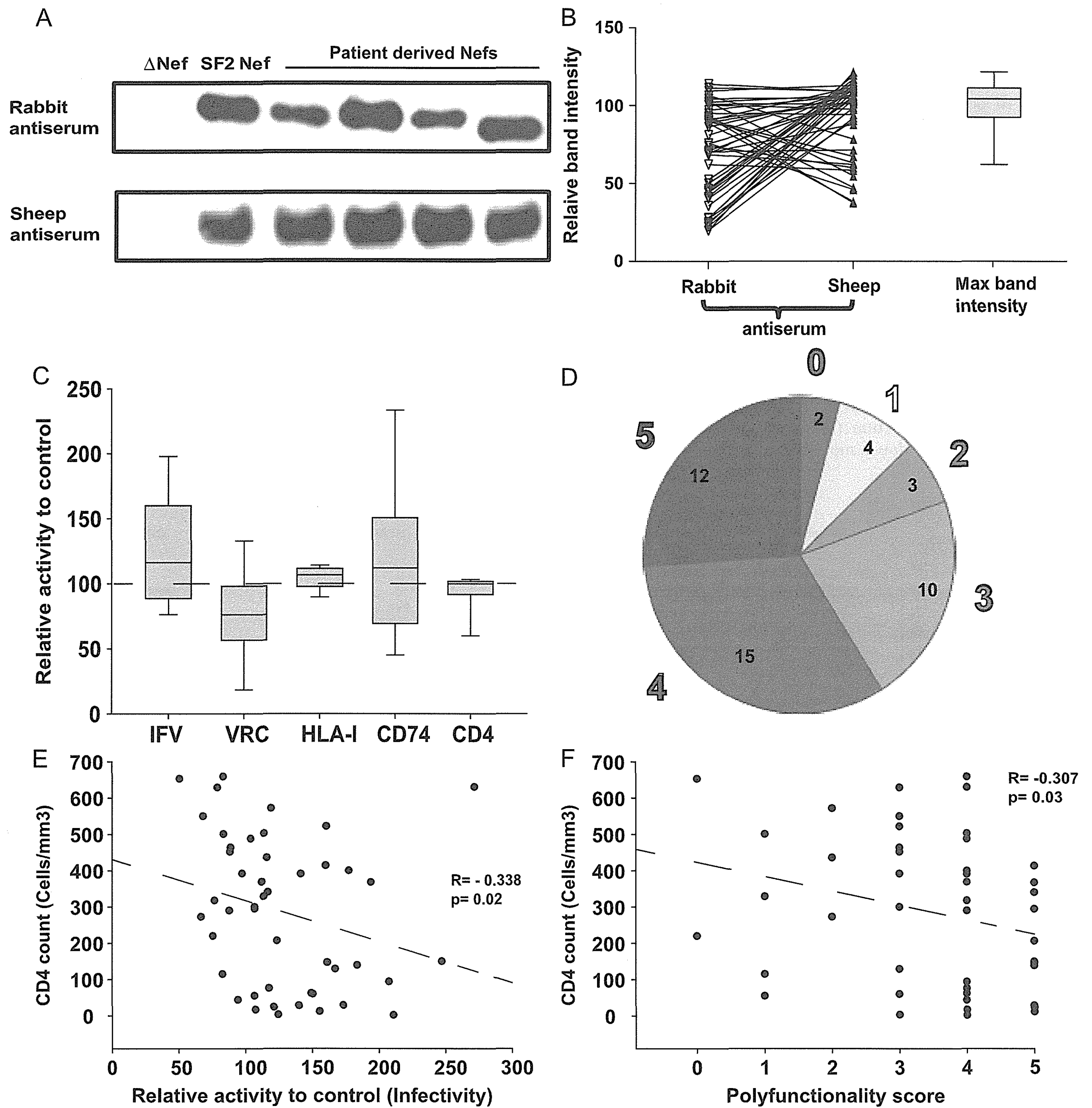

Fig. 1. Western blot and functional profile of 46 patient-derived Nef proteins. (A) Representative blots of ΔNef, SF2 Nef, and four patient-derived Nef clones (B) Band intensities relative to SF2 control, using rabbit (left), sheep (middle), and maximum of rabbit/sheep combined (right), for each patient-derived 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.

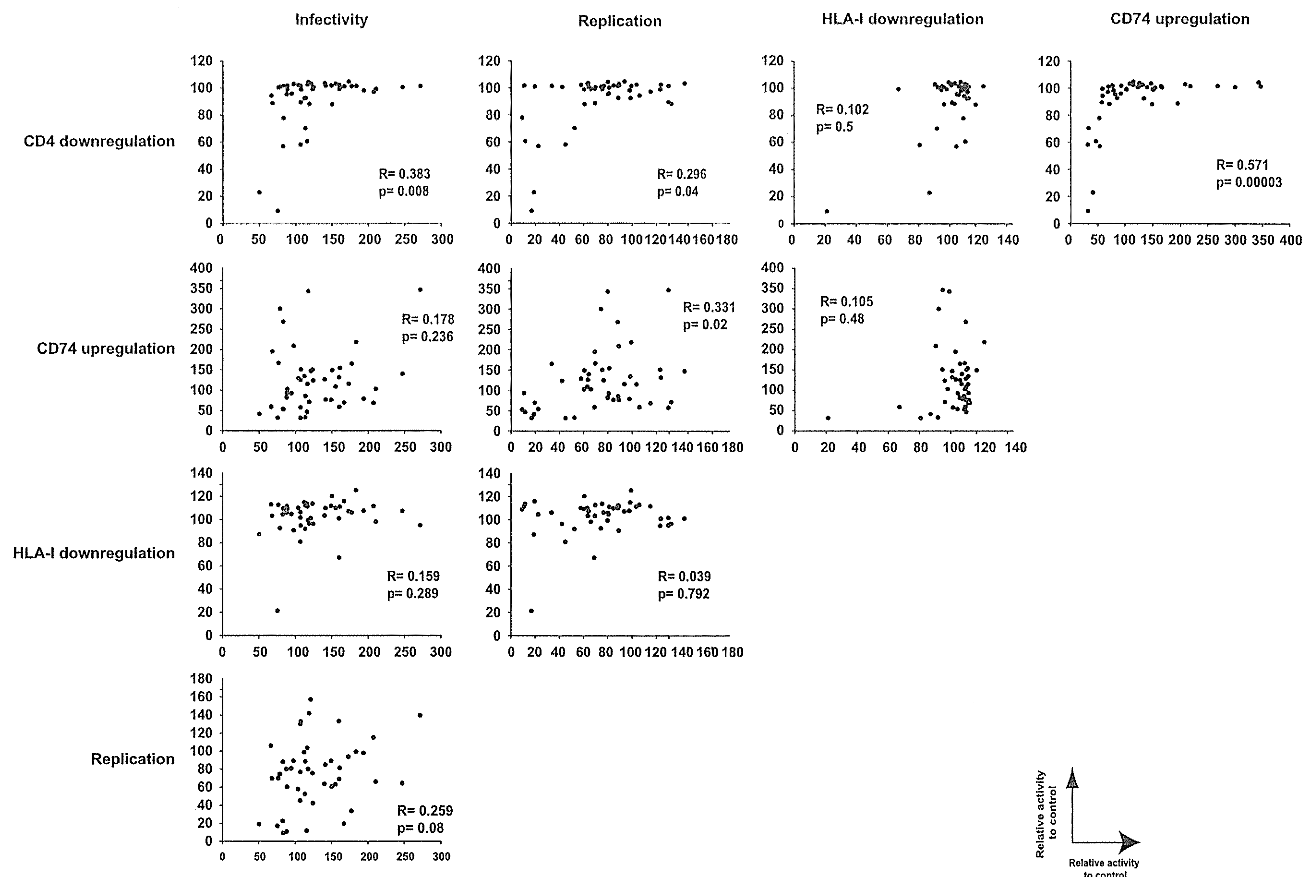

Fig. 2. Co-dependence between *in vitro* Nef activities. Pairwise associations between each of the five *in vitro* functions were examined for the patient-derived Nef clones. Significant correlations were observed between CD4 down-regulation and infectivity, viral replication, and CD74 up-regulation; 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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