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Modulation of TCR-dependent NFAT signaling is impaired in HIV-1 Nef isolates from elite controllers

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ABSTRACT

HIV-1 Nef modulates the activation state of $CD4^+$ T cells by altering signaling events elicited by the T cell receptor (TCR). Primary *nef* sequences exhibit extensive inter-individual diversity that influences their ability to downregulate CD4 and HLA class I; however, the impact of *nef* variation on modulation of T cell signaling is poorly characterized. Here, we measured TCR-mediated activation of NFAT transcription factor in the presence of *nef* alleles isolated from 45 elite controllers (EC) and 46 chronic progressors (CP). EC Nef clones displayed lower ability to inhibit NFAT signaling (median 87 [IQR 75–93]% relative to SF2 Nef) compared to CP clones (94 [IQR 89–98]%) ($p < 0.001$). Polymorphisms in Nef's N-terminal domain impaired its ability to inhibit NFAT signaling. Results indicate that primary *nef* alleles exhibit a range of abilities to modulate TCR-dependent NFAT signaling, implicating natural variation in this function as a potential contributor to differential HIV-1 pathogenesis.

1. Introduction

HIV-1 Nef is a 27 kDa myristoylated accessory protein that is critical for viral pathogenesis (Dyer et al., 1999; Kestler et al., 1991; Zaunders et al., 2011). Nef displays multiple functions *in vitro*, including the ability to downregulate CD4 (Garcia and Miller, 1991), HLA class I (Collins et al., 1998; Le Gall et al., 1998; Schwartz et al., 1996), chemokine coreceptors (Michel et al., 2005; Toyoda et al., 2015; Venzke et al., 2006), and the restriction factor SERINC5 from the surface of infected cells (Rosa et al., 2015; Usami et al., 2015), which are thought to enhance viral infectivity and replication (Miller et al., 1994; Trautz et al., 2016; Vermeire et al., 2011). At least some of these activities facilitate viral evasion of host immune responses and promote viral persistence *in vivo* (Alsahafi et al., 2015; Arhel and Kirchhoff, 2009; Chen et al., 2012a; Veillette et al., 2014; Yang et al., 2002). HIV-1 *nef* sequences exhibit substantial genetic and functional diversity (Brumme et al., 2008; Corro et al., 2012; Gray et al., 2011; Kirchhoff et al., 1999; Kuang et al., 2014; Mann et al., 2013; Meribe et al., 2015; Mwimanzi et al., 2013a, 2013b; Zuo et al., 2012), which may have important implications for disease progression.

One line of evidence supporting Nef genotype/phenotype diversity as a correlate of HIV-1 pathogenesis comes from studies of elite controllers (EC), rare infected individuals (< 1%) who spontaneously maintain plasma viral loads below 50 RNA copies/mL without antiretroviral treatment (Blankson and Siliciano, 2008; Walker and Yu, 2013). It is well established that the EC phenotype is due at least in part to favorable host genetics (Carrington et al., 1999; Carrington and O'Brien, 2003; Carrington and Walker, 2012; International-HIV-Controllers-Study et al., 2010), namely possession of protective HLA class I alleles such as B*57:01 or B*27:05 that restrict effective antiviral $CD8^+$ T cell responses (Betts et al., 2006; Chen et al., 2012b, 2009; Ladell et al., 2013; Migueles et al., 2003, 2000). However, viral genetics can also play a major role (Casado et al., 2013; Chen et al., 2015; Kikuchi et al., 2015; Lassen et al., 2009; Lobritz et al., 2011), for example through acquisition of an attenuated HIV-1 strain or within-host selection of fitness-reducing viral mutations under strong immune pressure (Alsahafi et al., 2015; Brumme et al., 2011; Kuang et al., 2014; Miura et al., 2009a, 2009b, 2010; Mwimanzi et al., 2013a; Salgado et al., 2014; Troyer et al., 2009). We and others have demonstrated that while *nef* sequences isolated from EC are generally intact (Miura et al., 2008; Salgado et al., 2010), they nevertheless displayed lower function for a range of *in vitro* activities that contribute to viral pathogenesis (Alsahafi et al., 2015; Kuang et al., 2014; Mwimanzi et al., 2013a; Toyoda et al., 2015), supporting impaired Nef function as a feature of

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spontaneous HIV-1 control. Further characterization of Nef genotype/phenotype relationships, particularly in EC populations, could inform the discovery of new therapeutic or vaccine strategies.

Nef is reported to inhibit T cell receptor (TCR)-mediated signaling events in $CD4^+$ T cells by re-localizing the crucial Src family kinase $p56^{Lck}$ to an intracellular compartment (Collette et al., 1996; Greenway et al., 1996; Iafrate et al., 1997) and by disrupting actin cytoskeleton remodeling that is crucial to generate a stable immunological synapse with antigen presenting cells (Rudolph et al., 2009; Thoulouze et al., 2006). Lck is the most proximal protein to be activated following TCR stimulation and it directs multiple downstream events, including phosphorylation of signaling mediators Zeta-chain-associated protein kinase 70 (ZAP-70), Linker of Activated T cells (LAT) and Phospholipase C-gamma 1 (PLC-gamma1) that lead to calcium flux and activation of various transcription factors (Denny et al., 2000; Lo et al., 2018; Lovatt et al., 2006). Actin cytoskeletal remodeling mediates cell polarization and establishment of cell–cell contacts that are required to form a stable immunological synapse (Yu et al., 2013). By dampening antigen-specific TCR signaling, Nef may reduce activation-induced cell death and thereby increase progeny virion production (Abraham et al., 2012; Abraham and Fackler, 2012; Fackler et al., 2007). Residues in Nef's N-terminal domain (Baur et al., 1997; Wolf et al., 2008) and Proline-rich motif ($P_{72}XXP_{75}$) (Fackler et al., 2001; Haller et al., 2007; Trible et al., 2006) contribute to its ability to alter T cell signaling; however, past studies were conducted mainly using Nef isolates derived from reference strains and relatively few reports have examined primary *nef* alleles. Therefore, the degree to which natural sequence variation influences Nef's ability to modulate TCR signaling has not been fully addressed.

Based on our observations that primary *nef* alleles isolated from EC displayed relative functional impairments compared to those from chronic progressors (CP) (Kuang et al., 2014; Mwimanzi et al., 2013a; Toyoda et al., 2015), we hypothesized that EC-derived Nef clones would also display a reduced ability to modulate TCR-mediated signaling events. To assess this, we used a nuclear factor of activated T cells (NFAT)-dependent luciferase reporter assay in Jurkat T cells to compare the *in vitro* function of 45 Nef clones isolated from EC and 46 clones isolated from untreated CP. Consistent with some prior studies (Ahmad and Venkatesan, 1988; Niederman et al., 1992, 1993, 1989), we observed that most primary *nef* alleles were able to dampen TCR-mediated NFAT signaling. Notably, the median activity of EC-derived clones was lower than that of CP-derived clones. Analyses of three poorly functional EC Nef clones identified polymorphisms in the N-terminal domain that contributed to their impairment. Our results highlight the impact of natural sequence variation on Nef's ability to modulate T cell signaling and suggest that differences in this Nef function may contribute to differential clinical outcomes following HIV-1 infection in some individuals.

2. Materials and methods

2.1. Study participants

HIV-1 subtype B-infected elite controllers (EC) and chronic progressors (CP) have been described previously (Mwimanzi et al., 2013a). Briefly, the EC cohort comprised 45 untreated individuals with a median plasma viral load (pVL) of 2 RNA copies/mL (interquartile range [IQR] 0.2 – 14) and a median CD4 count of 811 cells/mm^3 (IQR 612 – 1022). The CP cohort comprised 46 untreated individuals with a median pVL of 80,500 RNA copies/mL (IQR 25,121 – 221,250) and a median CD4 count of 293 cells/mm^3 (IQR 73 – 440). All participants were recruited from the Boston area, provided written informed consent, and were not receiving antiretroviral therapy at the time of sample collection. This study was approved by the Research Ethics Board at Simon Fraser University (Burnaby, BC Canada) and the Massachusetts General Hospital (Boston, MA USA).

2.2. Generation of HIV-1 nef constructs

The HIV-1 *nef* gene was amplified from plasma viral RNA and a single phylogenetically representative *nef* sequence was cloned into pIRES2-EGFP (Clontech) for a previous study (Mwimanzi et al., 2013a). The same Nef clones were evaluated here; however, because modulation of TCR signaling requires relatively higher protein levels (Liu et al., 2001), all sequences were re-cloned into pSELECT-GFPzeo (InvivoGen), which features a composite hEF1-HTLV promoter driving *nef* and an independent CMV promoter driving expression of a GFP: zeocin reporter protein. To do this, we modified the multiple cloning site in pSELECT-GFPzeo to incorporate *Asc*I and *Sac*II restriction sites. Each *nef* gene was amplified by PCR using degenerate primers incorporating these restriction sites (Fwd: 5′-AGAGCACC**GG CGCGCC**TCCA CATAC-CTASA AGAATMAGAC ARG-3′, HXB2 nt 8746–8772 underlined, *Asc*I site in bold; Rev: 5′-GCCT**CCGCGG** ATCGATCAGG CCACRCCTCC CTGGAAASKC CC-3′, HXB2 nt 9474–9449 underlined, *Sac*II site in bold), cloned into the modified pSELECT-GFPzeo vector, and validated by sequencing. *Nef* sequences were deposited into Genbank previously; accession numbers are JX171199-JX171243 (EC) and JX440926-JX440971 (CP).

The same strategy was used to clone *nef* from the HIV-1 subtype B reference strain SF2, which served as the positive control in all experiments. A glycine (G) to alanine (A) substitution was introduced into SF2 Nef at residue 2 (G2A) by overlap extension PCR, which served as a negative control. The G2A substitution prevents myristoylation of the Nef protein, thus rendering it defective for most functions, including modulation of T cell signaling (Djordjevic et al., 2004). Overlap extension PCR was also used to generate other mutations in SF2 or primary *nef* sequences and to construct chimeric constructs encoding various regions of primary *nef* and SF2 *nef*, as described in the text. All mutations and chimeric genes were validated by sequencing.

Nef polymorphisms are reported using the HXB2 numbering convention. Sequences were pairwise-aligned to the reference strain HXB2 (GenBank accession number K03055) and insertions with respect to HXB2 were removed using an in-house alignment algorithm based on the HyPhy platform (Kosakovsky Pond et al., 2005).

2.3. NFAT-luciferase signaling assay

To assess TCR signaling, 10 μg of pSELECT-GFPzeo encoding each *nef* allele plus 5 μg of pNFAT-luciferase (Agilent Biosciences) were used to co-transfect 5×10^6 Jurkat T cells resuspended in 200 μL Opti-MEM medium, without phenol red (Thermo Fisher Scientific). Cells were electroporated in 96-well plates using a BioRad GenePulser MXCell™ instrument (square wave protocol: 250 V, 2000 μF, infinite Ω, 25 ms, single pulse). Transfected cultures were recovered for 18 h in 800 μL of RPMI 1640 medium without phenol red, supplemented with 2 mM L-glutamine, 1000 U/mL Penicillin and 1 mg/mL Streptomycin (R10 +) (all from Sigma-Aldrich) at 37 °C plus 5% CO_2 to allow for Nef expression. Following this, 50 μL of culture (2.5×10^5 cells) was assessed by flow cytometry for transfection efficiency (GFP expression) and viability using annexin V-APC antibody and 7-AAD (both from BioLegend). In parallel, 100 μL of the culture (5.0×10^5 cells) was stimulated for 6 h in triplicate on flat-bottomed 96-well culture plates (Greiner CELLSTAR) pre-coated with 0.01–1 μg/mL of anti-CD3 antibody (OKT3 clone; eBioscience), allowing mobilization of cytoplasmic Ca^{2+} and activation of NFAT-mediated transcription of the luciferase reporter gene (Ledbetter et al., 1987a, 1987b). Luciferase activity was detected using the Steady-Glo® Luciferase Assay system (Promega) by mixing substrate/lysis solution (50 μL) with stimulated cells (50 μL) on a flat-bottom, white polystyrene, half-area microplate (Corning or Grenier). Luminescence was measured as absolute light units (ALUs) using a Tecan Infinite M200 PRO plate reader (2000 ms integration time; 100 ms settle time). Luminescence values for each Nef clone were normalized to positive (pSELECT-nef_{SF2}-GFPzeo) and negative

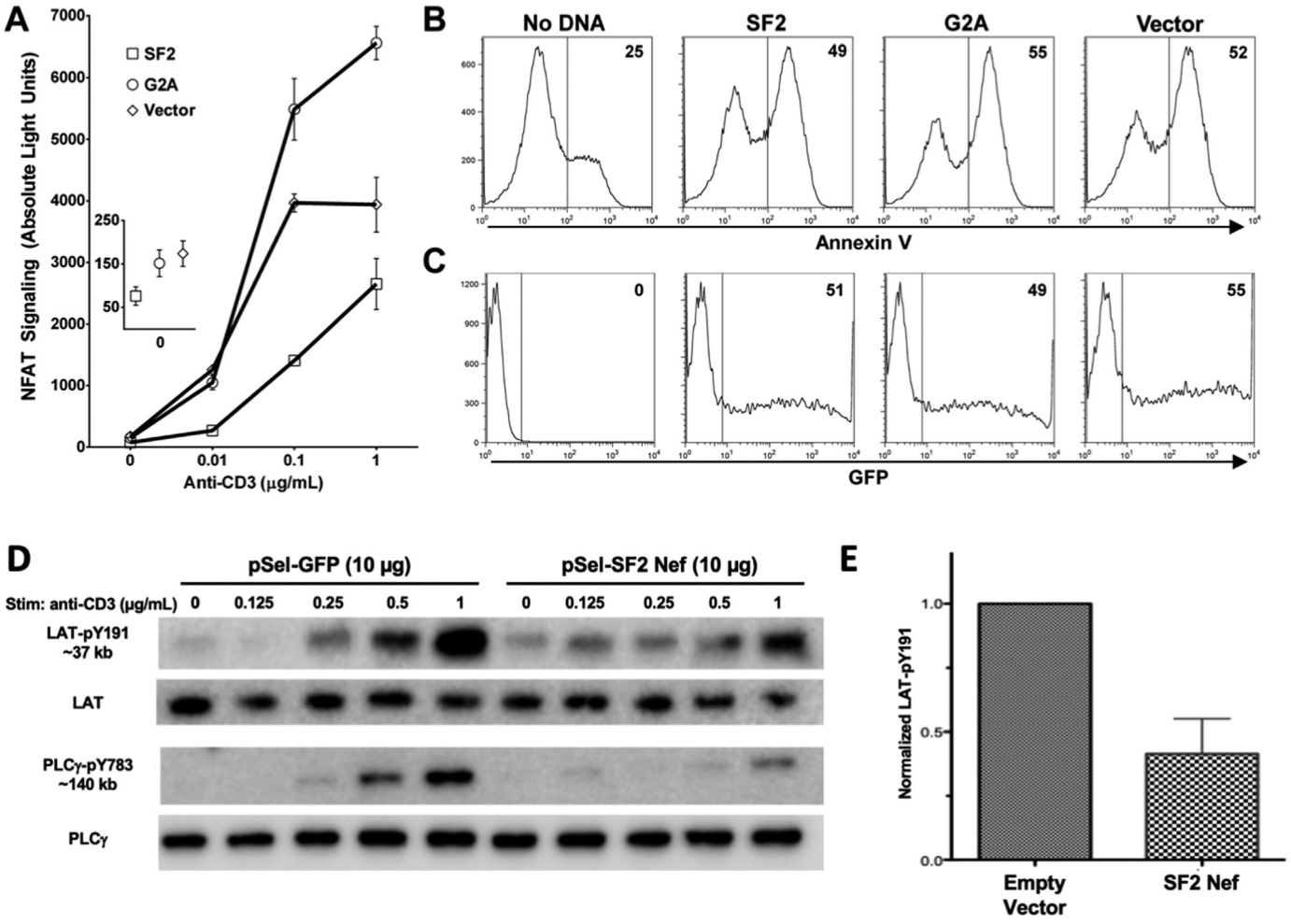

Fig. 1. ***In vitro*** **assay to measure Nef-mediated inhibition of NFAT signaling.** (***A***) NFAT-driven luciferase activity was quantified using luminescence (y-axis) in Nef-transfected Jurkat T cells following stimulation with anti-CD3 antibody at various concentrations (x-axis). Absolute light units detected from cells expressing either wild type SF2 Nef, myristoylation-defective Nef G2A mutant, or empty vector are shown at 6 h post-stimulation. The inset figure displays basal NFAT-driven luciferase activity in Jurkat cells in the absence of stimulation. (***B***) Cellular toxicity was assessed by flow cytometry. Histograms show the proportion of Jurkat cells that were positive for the early apoptosis marker Annexin V (x-axis) following electroporation with no DNA or with pNFAT-luciferase plasmid plus SF2 Nef, Nef G2A, or empty vector. (***C***) DNA transfection efficiency was measured using flow cytometry to detect the proportion of Jurkat cells producing GFP, which is expressed from an independent promoter present in the pSELECT-GFPzeo plasmid. Histograms show the proportion of annexin-negative cells (from panel *B*) that expressed GFP (x-axis) following electroporation with no DNA or with pNFAT-luciferase plasmid plus SF2 Nef, Nef G2A, or empty vector. (***D***) Nef-mediated inhibition of proximal TCR signaling events was confirmed using Western blot to detect phosphorylation of LAT (pY191) and PLCgamma (pY783). Jurkat cells were transfected with empty pSELECT-GFPzeo or vector encoding SF2 Nef. GFP + (transfected) cells were isolated by FACS, stimulated with anti-CD3 antibody at various concentrations, and phosphorylation measured at 2 min post-stimulation. (***E***) Nef-mediated inhibition of LAT phosphorylation (pY191) was quantified based on results from three independent experiments following stimulation with anti-CD3 antibody at 0.5 μg/mL.

(pSELECT-*nef*$_{G2A}$-GFPzeo) controls using the formula: (ALU_{G2A} – $ALU_{patient}$)/(ALU_{G2A} – ALU_{SF2}) × 100%, such that function less than or greater than the positive control SF2 Nef is represented by values of < 100% or > 100%, respectively. The inhibition activity of each Nef clone is reported as the mean ± standard deviation (SD) based on at least three independent transfection experiments.

2.4. Downregulation of CD4 and HLA class I

Selected Nef mutants and chimeras were evaluated for their ability to downregulate CD4 and HLA-A* 02 (as a representative HLA class I molecule), as described previously (Kuang et al., 2014; Mwimanzi et al., 2013a), with minor modifications. Briefly, 1 × 10^6 CEM-A* 02^+ T cells were transfected with 4 μg of pSELECT-GFPzeo encoding each *nef* allele by electroporation (as described above) and stained 20 h later with anti-human CD4-APC and anti-human HLA-A* 02-PE antibodies (BD Biosciences). The median fluorescence intensities (MFI) of surface CD4 and HLA-A* 02 expression in GFP-negative and GFP-positive cell subsets were determined by flow cytometry.

2.5. Western blot

To assess TCR-mediated phosphorylation, 5 × 10^6 Jurkat T cells were transfected with 10 μg of empty pSELECT-GFPzeo or vector encoding Nef SF2 by electroporation (as described above). After 20 h, GFP-positive cells were isolated by FACS and 1 × 10^6 cells were stimulated with anti-CD3 antibody (OKT3 clone) at the indicated concentration in R10 + medium for 2 min. Cells were immediately chilled on ice, lysed and prepared for Western blot as described previously (Mwimanzi et al., 2011, 2013a). Linker for Activation of T cells (LAT) was detected using mouse clone 1111 (total protein; BioLegend) (1:2000) and rabbit clone 3584 (pY191; Cell Signaling Technology) (1:500). Phospholipase-C gamma1 (PLC-gamma1) was detected using rabbit clone 2822 (total; Cell Signaling Technology) (1:2000) and

rabbit clone 2821 (pY783; Cell Signaling Technology) (1:1000). To assess steady-state Nef expression, 5×10^6 Jurkat T cells were transfected with 10 μg of pSELECT-GFPzeo encoding a selected *nef* allele or chimera by electroporation as described above. After 18 h, cells were pelleted, lysed and prepared as described. Nef was detected using polyclonal rabbit serum (ARP444; NIBSC Center for AIDS Reagents) (1:2000). Secondary staining was conducted using donkey anti-mouse or donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare) (1:30,000). Proteins were detected using Clarity Western ECL substrate (Bio-Rad) and visualized on an ImageQuant LAS 4000 imager (GE healthcare). Band intensities were quantified using Image Lab™ software (Bio-Rad).

2.6. Statistical analysis

Statistical analyses were performed using Prism v.7 (Graphpad). Mann-Whitney *U* test was used to compare differences in median Nef function between EC and CP cohorts. One-sample *t*-test was used to compare the function of individual Nef clones, chimeras or mutants to that of the positive control SF2 Nef (whose function was set to 100%). Spearman test was used to assess correlations between study results. Students T-test was used to compare differences in mean PLA fluorescence intensity between Nef mutants in microscopy studies. For all analyses, tests were two-tailed and significance was defined as $p < 0.05$. The Mann-Whitney *U* test was also used in pair-wise genotype/phenotype analyses to identify natural Nef polymorphisms associated with differential *in vitro* activity. Briefly, for all Nef polymorphisms observed at least five times in the dataset, the function of all Nef clones encoding the specific polymorphism was compared to that of clones lacking it. Here, multiple comparisons were addressed using q-values, the p-value analogue of the false discovery rate (FDR). The FDR is the expected proportion of false positives among results deemed significant at a given p-value threshold (e.g. at $q < 0.2$, we expect less than 20% of identified associations to be false positives).

3. Results

3.1. Inhibition of TCR-mediated NFAT signaling by HIV-1 Nef

To examine TCR-mediated signaling in the presence of HIV-1 Nef, we employed an NFAT luciferase reporter assay in Jurkat T cells stimulated with anti-CD3 antibody. NFAT transcription factor activity is induced by calcium flux following TCR engagement and contributes to expression of key cytokines and other activation-induced proteins (Muller and Rao, 2010). In control experiments, cells were transfected with wild type SF2 Nef, myristoylation-defective Nef G2A mutant, or empty vector and then stimulated with anti-CD3 at concentrations ranging from 0 to 1 μg/mL. We observed that NFAT-dependent luminescence was reduced approximately 4-fold in the presence of SF2 Nef compared to Nef G2A under most stimulation condition tested (Fig. 1**A**). Higher luminescence values were consistently seen for cells transfected with Nef G2A compared to empty vector, particularly following stimulation with 1 μg/mL anti-CD3. The reason for this is unclear; however, we speculate that it may be due to Nef's ability to inhibit cell death following antigen stimulation by interfering with the pro-apoptotic protein Bad (Wolf et al., 2001), which does not necessarily depend on its localization to the plasma membrane. Also, while NFAT signaling was minimal in the absence of stimulation, luminescence values were consistently lower in the presence of SF2 Nef compared to Nef G2A or empty vector controls (Fig. 1**A**, inset), indicating that wild type Nef dampened basal NFAT signaling in Jurkat cells.

To further characterize our *in vitro* system, we examined cytotoxicity and transfection efficiency by flow cytometry. Representative data are shown in Fig. 1**B** and **C**. We assessed toxicity by measuring induction of the apoptotic marker annexin V. Following mock electroporation (no DNA), 25% of Jurkat cells displayed annexin V surface staining (Fig. 1**B**). While higher proportions of cells were annexin V-positive following co-transfection with Nef and NFAT-luciferase reporter plasmids, this staining was comparable between SF2 Nef (49%), Nef G2A (55%), and empty vector (52%). Similar results were observed using 7-AAD labeling as an indicator of cell death, which indicated 12% positive cells in mock treated cells compared to 28–31% positive cells following transfection (*data not shown*). Together, these results suggest that cytotoxicity was due to electroporation with plasmid DNA, rather than expression of Nef protein itself. Next, we assessed transfection efficiency by measuring the proportion of viable (annexin-V low) cells that were positive for GFP, which is expressed from an independent promoter on pSELECT-GFPzeo. A similar high percentage of cells were observed to express GFP following transfection with SF2 Nef (51%), Nef G2A (49%), or empty vector (55%) (Fig. 1**C**), indicating that DNA transfection was efficient and comparable between constructs. Based on these results, we concluded that reductions in NFAT signaling observed in cells transfected with SF2 Nef were not likely to be due to differences in toxicity or transfection efficiency. Despite overall toxicity being relatively high following electroporation, this was similar among Nef clones and was not considered to be a major confounder to interpreting assay results. We did not examine cytotoxicity following stimulation with anti-CD3, which might contribute to observed differences in luminescence values between Nef G2A and the empty vector control, as discussed above. For subsequent experiments, we stimulated cells using an intermediate concentration of anti-CD3 antibody (0.1 μg/mL) and selected SF2 Nef and the Nef G2A mutant as positive and negative controls, respectively, since they provided the broadest range to assess relative *in vitro* function.

To confirm the impact of Nef on proximal TCR-mediated signaling events, we also examined the phosphorylation of two cellular proteins that are crucial to the induction of calcium flux following TCR engagement, namely Linker for Activation of T cells (LAT) and Phospholipase-C gamma 1 (PLC-γ1). Jurkat cells were electroporated with empty vector (expressing GFP only) or vector expressing SF2 Nef. Transfected (GFP^+) cells were isolated by FACS at 20 h and subsequently stimulated for 2 min with anti-CD3 antibody at concentrations ranging from 0 to 1 μg/mL. In the absence of Nef, phosphorylation of LAT (at Y191) and PLC-γ1 (at Y783) was rapid and increased with stimulation dose (Fig. 1**D**). In the presence of Nef, phosphorylation of LAT and PLC-γ1 was inhibited by 59% and 61%, respectively (Fig. 1**E** and *data not shown*). A modest increase in LAT phosphorylation was seen at lower concentrations of anti-CD3 antibody in some experiments (Fig. 1**D**), but this observation was inconsistent and not assessed further in our studies. Overall, these results agree with prior studies indicating that Nef modulates TCR-mediated signaling by trafficking $p56^{Lck}$, which acts upstream of LAT and PLC-γ1, away from the plasma membrane. Phosphorylation of PLC-γ1 is crucial for induction of calcium flux and subsequent activation of NFAT, providing a plausible mechanism to explain Nef's impact on NFAT-mediated signaling.

3.2. Modulation of TCR-mediated NFAT signaling is attenuated in EC Nef clones

We next used this *in vitro* assay to examine the ability of 91 primary Nef clones to modulate TCR-mediated NFAT signaling. Of these, 45 clones were obtained from untreated elite controllers (EC) and 46 clones were obtained from untreated chronic proregressors (CP). Representative luminescence data for three EC- and three CP-derived Nef clones, plus controls, are shown in Fig. 2**A**. Raw luminescence values were subsequently normalized to those of the positive (SF2) and negative (G2A) controls such that Nef inhibition activity less than, equal to or greater than SF2 Nef was represented as < 100%, 100% or > 100%, respectively; and the activity of G2A Nef was represented as 0% (Fig. 2**B**). For each EC- and CP-derived Nef clone, we then calculated an average normalized value based on triplicate data from at

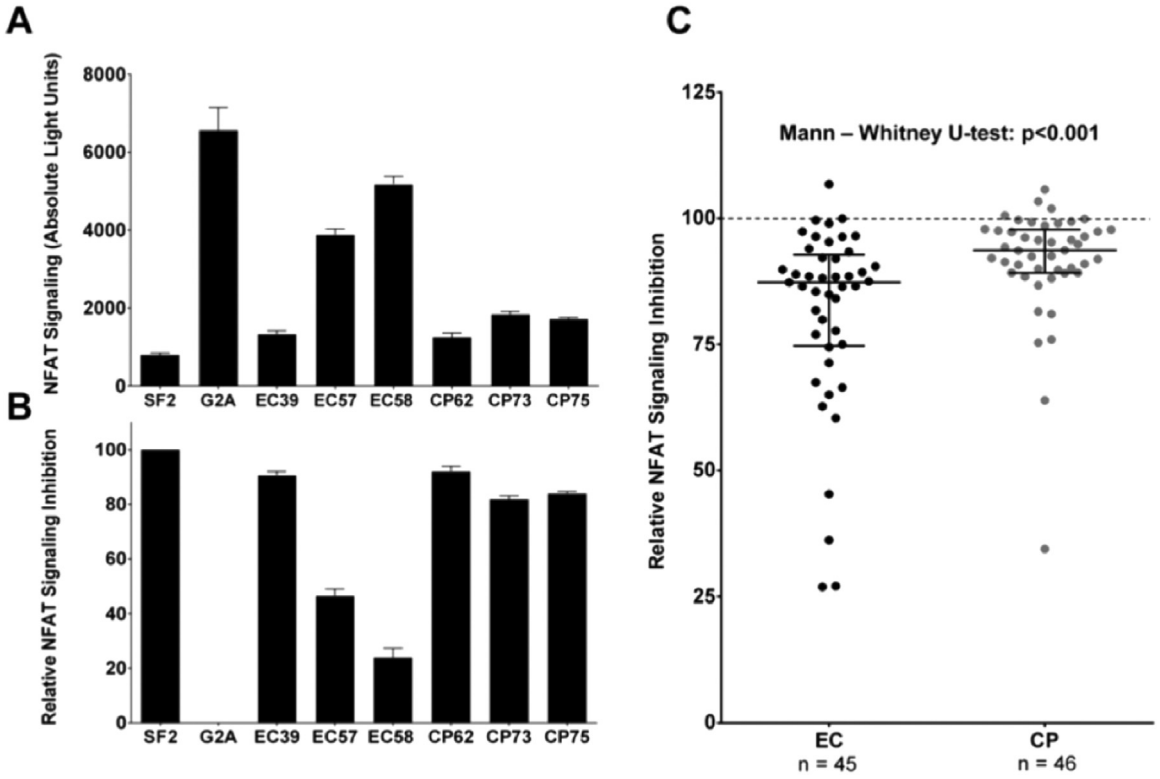

Fig. 2. Nef-mediated inhibition of TCR signaling is attenuated in HIV-1 elite controllers (*A*) Representative NFAT-luciferase results (absolute light units, y-axis) are shown for three elite controller (EC)- and three chronic progress (CP)-derived Nef clones, compared to wild type SF2 Nef (positive control) and myristoylation-defective G2A Nef mutant (negative control). (***B***) Results shown in panel ***A*** were normalized to the positive control (set to 100%) and the negative control (set to 0%), as described in the Methods. Values greater than or less than SF2 Nef are represented as > 100% or < 100%, respectively. (***C***) The relative ability of 45 EC-derived (black) and 46 CP-derived (gray) Nef clones to inhibit NFAT signaling is shown. Individual circles represent the mean value for each patient Nef clone, based on triplicate values obtained from at least three independent experiments. Bars represent the median and interquartile ranges for each population. The p-value was calculated using the Mann-Whitney *U*-test.

least three independent transfection experiments. All EC and CP Nef clones displayed at least partial function in this assay, in that their ability to inhibit TCR-mediated NFAT signaling exceeded that of the negative control, Nef G2A (Fig. 2**C**). Nevertheless, as a group, the EC-derived Nef clones displayed a significantly lower ability to inhibit signaling (median 87 [IQR 75–93] %) than the CP-derived Nef clones (median 94 [IQR 89–98] %) ($p < 0.001$). These results demonstrate that primary Nef clones display a range of abilities to modulate TCR-mediated NFAT signaling, with EC-derived *nef* alleles exhibiting relatively lower function.

We next assessed whether *in vitro* inhibition of NFAT signaling correlated with other Nef functions. The 91 Nef clones used in this study were characterized previously for their ability to downregulate CD4 and HLA class I (Mwimanzi et al., 2013a). While we observed statistically significant associations between Nef-mediated NFAT inhibition and downregulation of CD4 (Spearman $R = 0.31$, $p = 0.003$) and HLA ($R = 0.44$, $p < 0.001$) (Fig. 3**A and B**), we noted that all three activities also correlated with steady-state Nef expression levels as determined by Western blot (NFAT: Spearman $R = 0.30$, $p = 0.003$; CD4: $R = 0.31$, $p = 0.003$; HLA: $R = 0.18$, $p = 0.09$) (Fig. 3**C** and *data not shown*). These results are consistent with prior work indicating that Nef's ability to modulate TCR signaling is distinct from its other activities (Iafrate et al., 1997), but also suggest that intrinsic differences in the expression or stability of natural isolates are also likely to affect this function, which requires a relatively high intracellular concentration of Nef (Liu et al., 2001).

3.3. Natural variation at Nef residue 21 influences modulation of TCR signaling

To investigate potential determinants of Nef's ability to modulate TCR signaling, we conducted genotype/phenotype analyses using data from all 91 primary Nef clones. We identified 12 amino acids located at 9 Nef residues that were significantly associated with this *in vitro* function at $p < 0.05$ and $q < 0.2$ (Table 1). The strongest correlation was seen for lysine at Nef residue 21 (R21K), which was more common in EC clones (24%; 11 of 45) compared to CP clones (7%, 3 of 46) (Fisher's exact test, $p = 0.02$). Nef isolates encoding R21K displayed a median activity of 75 [IQR 60–86] % compared to a median activity of 92 [IQR 88–97] % for those harboring the consensus R at this site ($p < 0.001$). To confirm this observation, we substituted the consensus R_{21} in SF2 Nef with each of the five alternative amino acids observed in at least one EC or CP sequence, namely glutamic acid (E), lysine (K), leucine (L), glutamine (Q) or threonine (T) (Fig. 4). All five mutants were able to downregulate CD4 and HLA-A*02 to similar levels as wild type SF2 Nef (Fig. 4**A**), however all mutants were impaired in their ability to inhibit NFAT signaling, displaying normalized functions of 77%, 81%, 74%, 68%, and 80% relative to SF2 Nef, respectively (all $p < 0.05$) (Fig. 4**B**). Steady-state Nef levels for all mutants were found to be comparable to SF2 by Western blot (Fig. 4**C**), suggesting that these differences were not due to changes in protein expression or stability. These results demonstrate that natural sequence variation at Nef residue R_{21} can impact its ability to modulate cellular signaling without significantly compromising its other major functions.

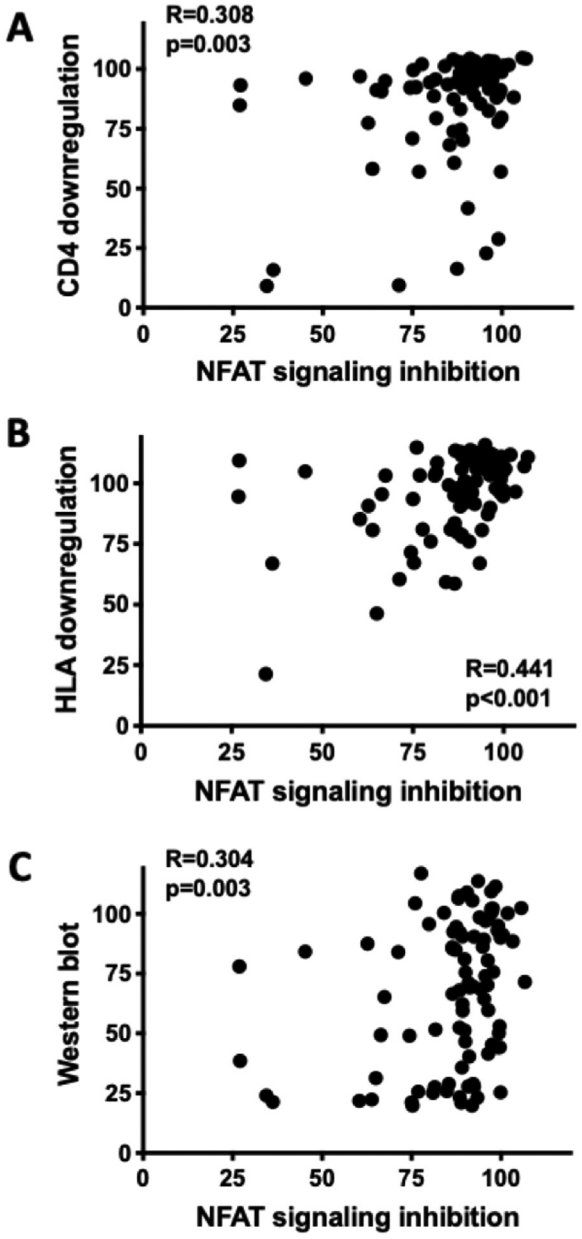

Fig. 3. Associations between *in vitro* Nef functions and protein expression. The ability of each primary Nef clone to inhibit NFAT signaling was correlated with its ability to downregulate CD4 (***A***) or HLA class I (***B***), as well as protein stability as detected by Western blot (***C***) using the Spearman's rank sum test. These data were reported previously in (Mwimanzi et al., 2013a).

3.4. Chimeric Nef constructs highlight a critical role for the N-terminal domain

Variation at residue 21 did not fully account for the range of function observed in patient *nef* sequences. To identify additional genetic determinants of activity, we generated a series of chimeric proteins that combined wild type SF2 Nef with various domains from three Nef clones that displayed impaired TCR signaling inhibition (EC36, EC57 and EC58; exhibiting activities of 27%, 45% and 27%, respectively) (Fig. 5**A**). The least functional CP clone (CP32) and another highly impaired EC clone (EC51) were excluded from this analysis since they also displayed substantially lower CD4 and HLA-A*02 downregulation activities (*data not shown*), suggesting that they encoded mutations that severely affected protein expression or stability.

For EC36, a chimera encoding clone-derived residues 1–103 and SF2-derived residues 104–206 ($EC36_{1–103}$) displayed impaired signaling inhibition function (28%, relative to SF2), while function of the reciprocal chimera ($EC36_{104–206}$) was similar to wild type SF2 Nef (102%). Another chimera encoding clone-derived residues 1–32 ($EC36_{1–32}$) remained significantly impaired (15%), while the function of a chimera encoding only EC36-derived residues 33–103 was comparable to wild type Nef (99%). Together, these data indicated that a major determinant of attenuated function for the EC36 Nef clone was located within the first 32 amino acids. Analogous methods were used to study chimeric Nef products generated using EC57 and EC58 clones. Results demonstrated similarly that polymorphism(s) located within the N-terminal 32 amino acids of Nef were likely to be responsible for the impaired function of these clones.

To examine differences in protein expression or stability as a potential explanation for impaired function, we assessed steady-state Nef levels by Western blot (Fig. 5**B**). Notably, while the rabbit polyclonal antibody used for these assays detected only weak bands for the three native EC-derived clones, all of the functionally impaired EC-SF2 chimeras were readily detected (except $EC57_{1–103}$ and $EC58_{104–206}$, which nevertheless retained ~ 70% and ~ 95% activity respectively). Overall, these results indicate that naturally occurring polymorphisms located in the N-terminal 32 amino acids of Nef significantly alter its ability to modulate NFAT signaling following TCR stimulation.

3.5. Mapping N-terminal determinants of Nef-mediated TCR signaling modulation

We next sought to identify specific polymorphisms responsible for the reduced *in vitro* function of EC36, EC57 and EC58 Nef clones. The N-terminal sequences of these clones were aligned to SF2 Nef (using HXB2 as a numbering reference) and the resulting alignments inspected for differences (Fig. 6**A**). EC36 and EC57 harbored a leucine (L) and lysine (K) respectively at Nef residue 21, which likely contributed in part to their functional impairment (see Fig. 4), while EC58 harbored the consensus arginine at this residue. For all three EC clones, substitutions with respect to SF2 Nef tended to cluster into groups of three residues, suggesting that the predominately alpha-helical structure of Nef's N-terminal domain may constrain amino acid diversity. Introduction of the triple mutations G_8KA_{10} or $H_{14}KV_{16}$ from EC36 Nef into SF2 Nef substantial reduced its *in vitro* function (to 58% or 76%, respectively) (Fig. 6**B**). While this difference was statistically significant for G_8KA_{10} ($p = 0.01$), the residual function of this mutant was still higher than that of native EC36 Nef (27%). Introduction of both G_8KA_{10} and L_{21} into SF2 Nef reduced its function to 16%, whereas combining $H_{14}KV_{16}$ with L_{21} did not result in further impairment. Together, these results indicate that the combination mutation, G_8KA_{10} plus L_{21}, was a major determinant of impaired function for EC36 Nef.

In the case of EC57, introduction of Δ_9VV_{11} into SF2 Nef resulted in no impairment, while introduction of $P_{14}TV_{16}$ reduced its function to 68%. Of interest, while the Δ_9VV_{11} polymorphism improved function of the K_{21} mutant (from 81% to 97%), introduction of $P_{14}TV_{16}$ plus K_{21} reduced SF2 Nef function to 46%, which was similar to that of the native EC57 clone (45%). Together, these results suggest that the combination mutation, $P_{14}TV_{16}$ plus K_{21}, was a major determinant of impaired function for the EC57 Nef clone. Notably, the CD4 downregulation activity of SF2 Nef mutants encoding either G_8KA_{10} plus L_{21} or $P_{14}TV_{16}$ plus K_{21} remained largely intact (76% and 99%, respectively, compared to wild type SF2 Nef) (Fig. 6B), suggesting that these polymorphisms did not have major effects on protein stability or tertiary structure.

Lastly, the EC58 Nef clone harbored a rare proline (P) to threonine (T) substitution at residue 25. Reverting this mutation to consensus proline in the EC58 sequence restored function from 27% to 75% (Fig. 6**C**), demonstrating that this mutation was a major determinant of impaired activity for this clone. Other polymorphisms that are present in the N-terminal sequence of EC58 Nef, including $P_{14}TV_{16}$, may

Table 1
Polymorphisms associated with NFAT inhibition ($p < 0.05$ and $q < 0.2$).

			Relative activity (%)		# Nef clones			
HXB2 residue	Amino acid	Impact	With AA	Without AA	With AA	Without AA	p-value	q-value
11	V	–	86.4	92.5	27	54	0.0002	0.02
21	K	–	75.5	92.0	14	77	0.0001	0.02
26	A	–	89.8	99.7	84	7	0.0073	0.13
28	E	+	95.3	89.2	39	52	0.0118	0.19
157	N	–	89.1	96.4	72	17	0.0013	0.05
157	T	+	96.3	89.2	14	75	0.0062	0.12
163	C	+	95.2	89.1	27	64	0.0051	0.12
170	L	–	88.5	95.1	55	36	0.0007	0.05
170	Q	+	95.1	88.5	34	57	0.0012	0.05
174	D	+	92.5	88.4	53	37	0.0058	0.12
174	E	–	88.4	92.5	37	53	0.0058	0.12
182	Q	+	96.5	89.3	10	81	0.0059	0.12

contribute to the remaining 25% deficit in function.

The impact of these polymorphisms on Nef expression and stability were also assessed by Western blot (Fig. 6**D**). While the G_8KA_{10}–containing mutants displayed lower steady-state protein levels compared to SF2 Nef, detection of the $P_{14}TV_{16}$-containing mutants was similar to wild type Nef. Notably, reversion of T25P in EC58 Nef significantly enhanced detection of this protein, suggesting that improved function was due at least in part to increased protein stability. Overall, we observed a weak correlation between higher protein expression levels and greater ability to inhibit NFAT signaling for polymorphisms identified in EC36 and EC58 Nef clones, but not for EC57 Nef. Additional studies will be necessary to examine this using a larger number of primary Nef isolates and chimeric proteins. Interestingly, we consistently observed lower Western blot band intensity for the Nef G2A

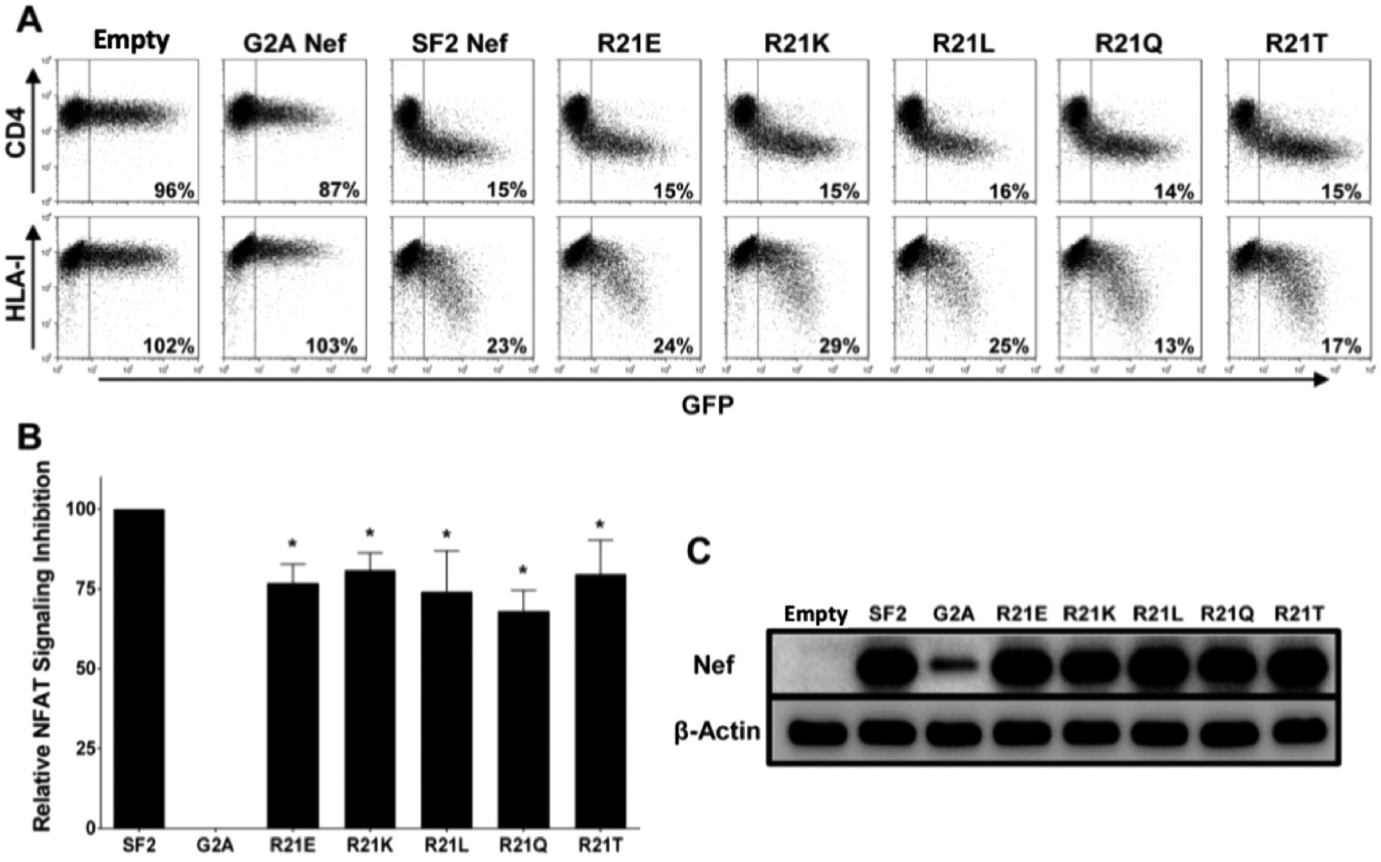

Fig. 4. Polymorphisms at residue R21 impair Nef signaling inhibition function. (***A***) The impact of site-directed mutations at residue 21 of wild type SF2 Nef (R21E, R21K, R21L, R21Q, and R21T) on Nef's ability to downregulate CD4 or HLA class I was assessed by flow cytometry. The proportion of CD4 or HLA-A*02 (y-axis) remaining on the cell surface is indicated in the lower right corner of each panel, which was determined by dividing the median fluorescence intensity (MFI) of GFP-positive cells by that of the GFP-negative subset (x-axis). Empty vector (ΔNef) and myristoylation-defective G2A Nef were included as negative controls. Results are representative of at least three independent experiments. (***B)*** The impact of the R21 mutations shown in panel ***A*** on Nef's ability to inhibit NFAT signaling was assessed in Jurkat cells as described in the Methods. Results were normalized to SF2 Nef (set as 100%) and the myristoylation-defective Nef G2A mutant (set as 0%). Bars represent the mean and standard deviation for each mutant based on at least three independent experiments. Asterisks indicate results that were significantly different compared to SF2 Nef (one sample T-test; $p < 0.05$). (***C***) Western blot analyses were used to assess the steady-state protein expression of R21 mutants (upper blot). Empty vector (ΔNef), SF2 Nef, and Nef G2A mutant were included as controls. Blots were probed for the β-actin protein as a cellular loading control (lower blot).

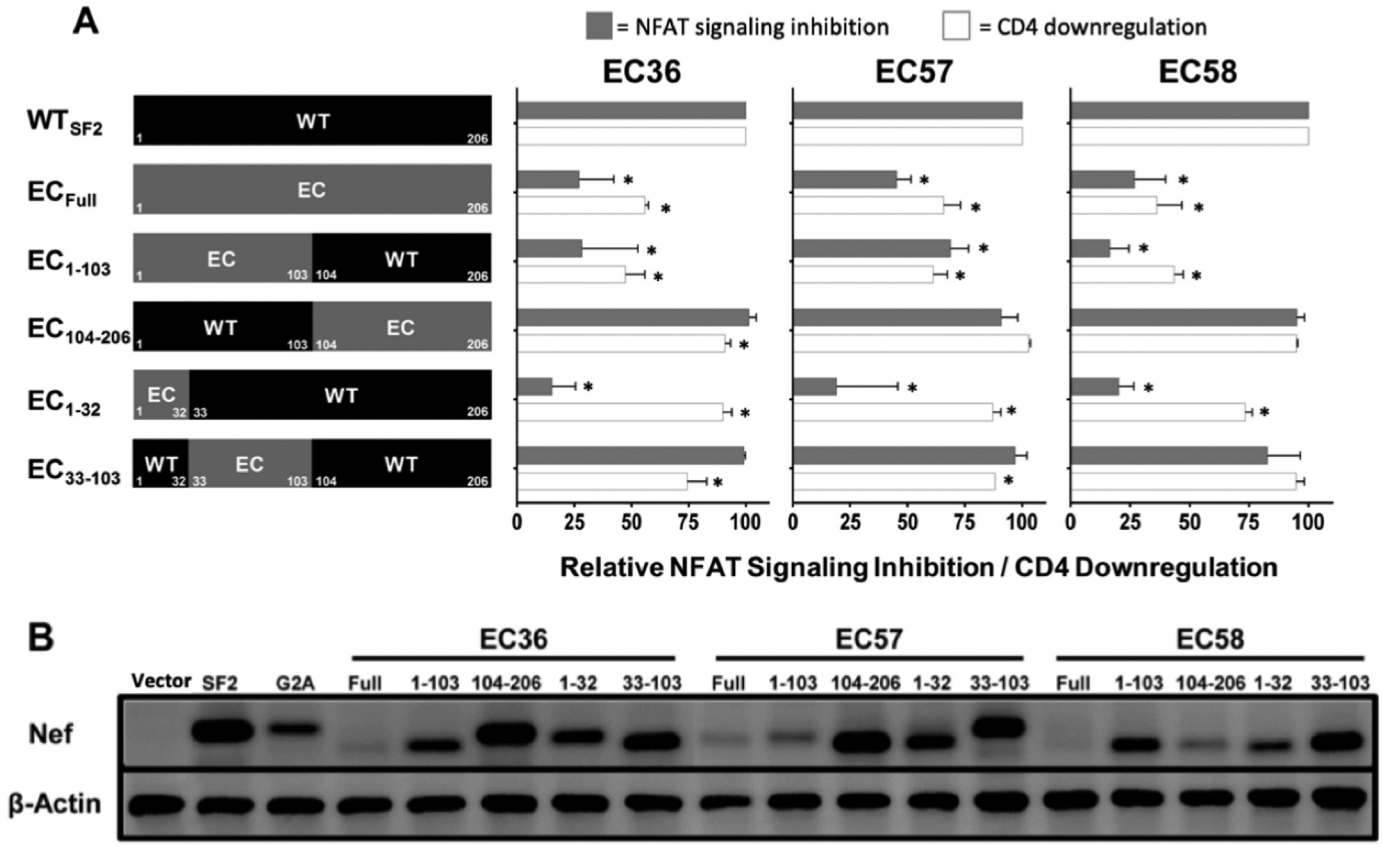

Fig. 5. Determinants of reduced EC Nef function map to the N-terminal domain. (***A***) Chimeric Nef clones were constructed between EC36, EC57 and EC58 isolates and wild type SF2 Nef, and their relative ability to modulate NFAT signaling (shaded bars, x-axis) or CD4 downregulation (open bars, x-axis) was assessed as described in the Methods. Data represent the mean and standard deviation for each construct based on three independent experiments. Asterisks indicate results that were significantly different compared to the SF2 Nef control (one sample T-test; $p < 0.05$). (***B***) Western blot analyses of native EC36, EC57 and EC58 and chimeras were used to assess steady-state protein expression for all constructs (upper blot). Empty vector, SF2 Nef, and Nef G2A mutant were included as controls. Blots were probed for the β-actin protein as a cellular loading control (lower blot).

mutant compared to SF2 Nef (see Figs. 4**C,** 5**B, and** 6**D**), suggesting that myristoylation may help to enhance the stability of wild type Nef. If so, differences in the N-terminal sequence of primary Nef isolates may alter their intracellular localization, thus affecting protein turnover and indirectly reducing Nef function.

4. Discussion

In this report, we examined the ability of HIV-1 Nef to modulate TCR-dependent signaling events by measuring NFAT transcription factor activity following anti-CD3 stimulation. Consistent with prior studies using similar transfection-based methods (Bandres and Ratner, 1994; Iafrate et al., 1997; Markle et al., 2013), we found that reference strain SF2 Nef and 91 primary Nef clones inhibited NFAT signaling in Jurkat cells, while the myristoylation-defective Nef G2A mutant did not. Most primary clones displayed relatively good *in vitro* function (73 of 91 showed > 80% function, relative to SF2 Nef), suggesting that there is selective pressure to retain this activity *in vivo*. Nevertheless, substantial differences were observed among natural isolates, including five Nef clones (4 from EC and 1 from a CP) that displayed < 50% function. Overall, the median NFAT inhibition activity of EC-derived clones (87%) was lower than that of CP-derived clones (94%) ($p < 0.001$). While the difference between these groups was modest, the observed variability among clones suggests that moderate to severe impairment of this Nef function may contribute to the EC phenotype in at least some cases.

We identified 12 Nef polymorphisms located at 9 residues that were associated with differential *in vitro* NFAT inhibition activity. Four sites were located in Nef's N-terminal domain (residues 11, 21, 26, and 28) and five sites were located in Nef's core domain (157, 163, 170, 174 and 182). The strongest association, at residue 21, was confirmed by substituting the consensus arginine in SF2 Nef with five naturally occurring variants at this residue, which resulted in 19–32% reductions in NFAT inhibition activity without appreciably altering Nef's ability to downregulate CD4 or HLA class I. Notably, R_{21} is one of four positively charged arginine residues comprising the $R_{17}xRxRR_{22}$ motif, which is located in an amphipathic helix that is thought to stabilize Nef interaction with the plasma membrane (Gerlach et al., 2010; Welker et al., 1998), suggesting that membrane interaction by this domain is an important factor in Nef's ability to modulate TCR signaling. More modest impairments in Nef function were associated with polymorphisms at residues 11, 26, and 28, which could act similarly to affect the charge or helical structure of Nef's N-terminal domain. Prior studies have also shown that Nef's N-terminal domain can stabilize a multi-protein complex that includes Lck and protein kinase C family kinases (Baur et al., 1997; Wolf et al., 2008), which might be disrupted by polymorphisms in this region. Additional studies will be necessary to explore these potential mechanisms.

A critical role for Nef's N-terminal domain (specifically its first 32 amino acids) was also observed in analyses of chimeric proteins constructed using three Nef clones that were impaired for NFAT inhibition activity. Specific polymorphisms in this region were identified to be major determinants of reduced Nef function in each of these cases. Specifically, incorporation of G_8KA_{10}/L_{21} (analogous to EC36) or $P_{14}TV_{16}/K_{21}$ (analogous to EC57) into SF2 Nef reduced its ability to inhibit NFAT signaling by 84% and 54%, respectively, while having

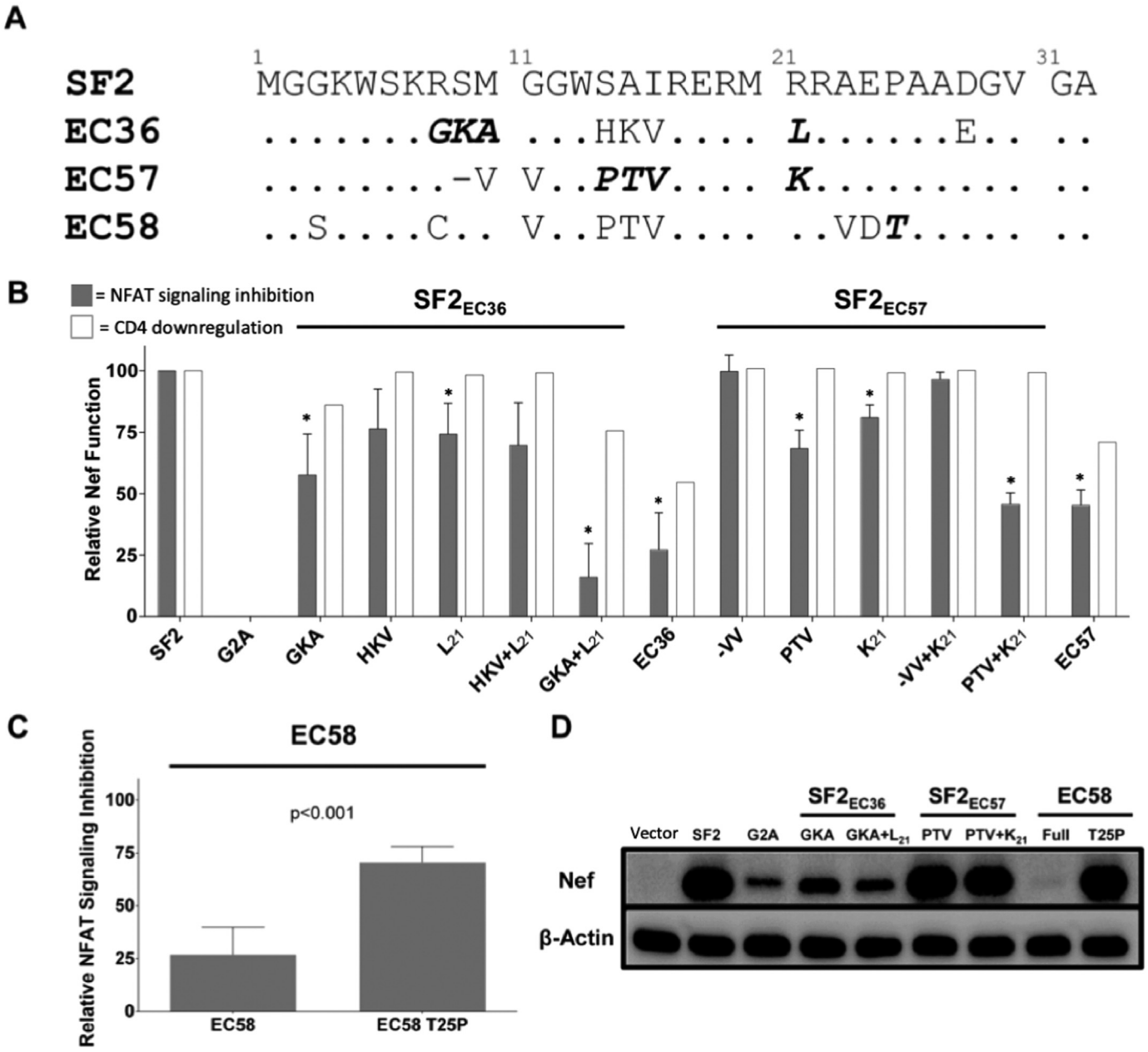

Fig. 6. Identification of polymorphisms responsible for reduced EC Nef function. (***A***) HXB2-aligned sequences for the first 32 amino acid residues of EC36, EC57, EC58 and SF2 Nef are shown. Identities are indicated by a period (.), deletions by a dash (—) and residues that were identified to contribute to Nef function by bold/italicized text. (***B***) Selected polymorphisms and combinations identified in EC36 and EC57 were introduced into SF2 Nef and their relative impact on NFAT signaling inhibition function (shaded bars, y-axis) or CD4 downregulation (open bars, y-axis) was assessed as described in the Methods. Normalized results for SF2 Nef (100%) and Nef G2A (0%) are shown for reference. Data for NFAT signaling inhibition represent the mean and standard deviation for each construct, based on at least three independent experiments. Data for CD4 downregulation represent the mean of two independent experiments, precluding formal statistical analyses. Asterisks indicate results that were significantly different compared to SF2 Nef (one sample T-test; $p < 0.05$). (***C***) A rare polymorphism (T25P) in the EC58 Nef clone was reverted to consensus proline using site-directed mutagenesis and NFAT signaling inhibition function was assessed. Bars represent the mean and standard deviation of each clone, based on triplicate values obtained from at least three independent experiments. The p-value was determined using the student's T-test. (***D***) Western blot analyses were used to assess the steady-state protein expression of native Nef clones and mutants described in this figure (upper blot). Empty vector (ΔNef), SF2 Nef, and Nef G2A were included as controls. Blots were probed for the β-actin protein as a cellular loading control (lower blot).

only a modest effect on its ability to downregulate CD4.

We also identified polymorphisms at five sites located in the core domain of Nef that were associated with NFAT signaling inhibition function. We did not explore these further in this study, so their impact on Nef function remains to be determined, but it is interesting to note that four of these sites (residues 157, 163, 170 and 182) were reported previously to be associated with clinical outcome in an independent cohort of 41 non-progressors and 50 progressors (Kirchhoff et al., 1999). The fifth site (residue 174) is located within a diacidic motif ($D_{174}D_{175}$) that is required for Nef to interact with the AP-2 clathrin adaptor complex and to downregulate CD4 (Janvier et al., 2001; Lindwasser et al., 2008). The importance of this interaction for Nef's ability to modulate T cell signaling is uncertain, since these activities are genetically separable (Iafrate et al., 1997), the polymorphism identified in our study (E_{174}) is frequently observed in natural isolates and E_{174} has been shown to maintain wild type ability to downregulate

CD4 (Lindwasser et al., 2008). Notably, no associations were observed in canonical domains that are required for Nef to interact with cellular kinases, including the proline-rich SH3-binding motif ($P_{72}xxP_{75}$) that allows Nef to re-localize $p56^{Lck}$ to an intracellular compartment (Thoulouze et al., 2006) or the phenylalanine-containing motif (F194 in SF2) that binds Pak2 and disrupts actin cytoskeletal remodeling (Agopian et al., 2006; Stolp et al., 2010). While this was expected since these critical motifs are highly conserved in natural isolates (Renkema and Saksela, 2000; Stolp et al., 2010), variation at one or more of the identified residues may affect the function of these domains through alteration of Nef structure or stability. Additional studies will be necessary to elucidate the impact of these natural Nef polymorphisms.

A limitation of this study is that it relied on overexpression of Nef clones in an immortalize T cell line, which may not fully reflect the role played by Nef in primary HIV-infected T cells. Indeed, several reports have indicated that Nef can enhance NFAT signaling through association with Src-family kinases, protein kinase C-theta, RAS/MAP kinase pathways and inositol trisphosphate receptor (Fortin et al., 2004; Manninen et al., 2001, 2000; Manninen and Saksela, 2002; Neri et al., 2011; Pan et al., 2012). In most of these reports, T cells were stimulated using a combination of anti-CD3 plus anti-CD28 antibodies, which elicits both TCR-dependent and -independent signaling pathways that might alter the impact of Nef on cellular responses. Notably, Neri et al. (2011) demonstrated that Nef displayed a “dual role” that depended on the T cell's activation status and the strength of stimulation: resulting in either superinduction of NFAT in freshly isolated quiescent T cells or suppression of NFAT in sub-optimally activated T cells. The results of our study are consistent with the latter scenario and should be interpreted in this context. Additional research will be necessary to confirm our observations in primary T cell models.

Productive infection of a CD4 + T-cell by HIV-1 is influenced by the cell's activation state. Our data contribute to a model wherein Nef acts to modulate intracellular signaling in virus-infected T cells in order to optimize replication (Abraham and Fackler, 2012). By blocking proximal TCR-dependent signaling events mediated by NFAT and potentially other transcription factors, Nef may prevent activation-induced cell death and thus enhance progeny virion production. This would be particularly advantageous in the context of high antigen load, such as that seen during chronic infection. We speculate that even a modest impairment in Nef's ability to inhibit TCR signaling could result in premature death of infected T cells and a substantial reduction in viral burst size.

In conclusion, this study provides new insight into HIV-1 polymorphisms in primary *nef* sequences that influence its ability to modulate TCR-dependent signaling events. As a group, controller-derived Nef clones displayed modestly lower ability to inhibit NFAT signaling compared to those isolated from progressors, but larger defects were found in a subset of clones. Notably, the data demonstrate that polymorphisms located in the Nef's N-terminal domain, particularly at residue 21 in the arginine-rich ($R_{17}xRxRR_{22}$) motif, contributed to impaired function. Our results highlight the impact of natural sequence variation on Nef's ability to modulate TCR-mediated signaling, which may contribute to clinical outcome in some cases of spontaneous HIV-1 control.

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