Nef-mediated inhibition of NFAT following TCR stimulation differs between HIV-1 subtypes

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ABSTRACT

Functional characterisation of different HIV-1 subtypes may improve understanding of viral pathogenesis and spread. Here, we evaluated the ability of 345 unique HIV-1 Nef clones representing subtypes A, B, C and D to inhibit NFAT signalling following TCR stimulation. The contribution of this Nef function to disease progression was also assessed in 211 additional Nef clones isolated from unique subtype C infected individuals in early or chronic infection. On average, subtype A and C Nef clones exhibited significantly lower ability to inhibit TCR-mediated NFAT signalling compared to subtype B and D Nef clones. While this observation corroborates accumulating evidence supporting relative attenuation of subtypes A and C that may paradoxically contribute to their increased global prevalence and spread, no significant correlations between Nef-mediated NFAT inhibition activity and clinical markers of HIV-1 infection were observed, indicating that the relationship between Nef function and pathogenesis is complex.

1. Introduction

The HIV-1 accessory protein Nef plays an important role in enhancing HIV-1 pathogenesis (Deacon et al., 1995; Kestler et al., 1991) through facilitating viral immune evasion (Swigut et al., 2004) and increasing viral infectivity and replication (Iafrate et al., 2000) (reviewed in (Foster and Garcia, 2008)). Major activities of Nef include CD4 downregulation to enhance viral budding (Ross et al., 1999) and facilitate evasion of antibody-dependent cell-mediated cytotoxicity (Veillette et al., 2014), HLA-I downregulation to evade CD8+ T cells (Ali et al., 2005; Schwartz et al., 1996), and CD4-independent enhancement of virion infectivity (an important mechanism includes inhibition of SERINC5 incorporation into virions (Rosa et al., 2015; Usami et al., 2015b)). In addition, Nef tailors TCR signalling to modulate the activation state of infected cells and this has been recognised as a key function of Nef (Abraham and Fackler, 2012; Fackler et al., 2007), however there is a lack of studies defining the role of this function in HIV-1 pathogenesis.

Nef has been shown to have both activating (Fenard et al., 2005; Manninen et al., 2000) and inhibitory (Haller et al., 2007; Thoulouze et al., 2006) effects on different aspects of TCR signalling in infected cells. Following TCR stimulation, lymphocyte-specific protein tyrosine kinase (Lck) is the most proximal protein to be activated, leading to multiple downstream events that include phosphorylation of signalling mediators zeta-chain-associated protein kinase 70 (ZAP-70), linker for activation of T cells (LAT) and phospholipase C-gamma 1 (PLC-gamma1). This is followed by activation of transcription factors, such as nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1), that then result in optimal production of IL-2. In quiescent primary T cells, through distal aspects of the TCR signalling pathway Nef enhances NFAT and IL-2 production thereby increasing viral replication, while in pre-activated primary T cells Nef inhibits NFAT and IL-2 production.
through disrupting proximal events following TCR stimulation (Neri et al., 2011). A model has emerged to reconcile apparently contradictory findings, whereby Nef reduces the availability of Lck and LAT at the plasma membrane and redirects these to an intracellular compartment, resulting in inhibition of signalling from the plasma membrane yet stimulation of a relocalised narrow signalling response from the intracellular compartment (Abraham and Fackler, 2012). Thus, Nef disrupts proximal TCR signalling events and activates selected downstream events, and which effect predominates is determined by the activation state of the infected cell. Overall, Nef fine tunes activation to achieve an intermediate activation state where HIV replication is promoted and activation-induced cell death (which would limit production of progeny viruses) is avoided (Abraham and Fackler, 2012).

The relative significance of each Nef function for HIV-1 disease progression remains incompletely understood, although there is evidence that Nef-mediated CD4 down-regulation and enhancement of infectivity are likely the major contributors to Nef’s effect of enhancing pathogenicity (Jafar et al., 2000; Stoddart et al., 2003; Watkins et al., 2013). Few studies have linked Nef activities with clinical outcome using patient-derived sequences and the majority of these have focused on subtype B infection, although subtype C is predominant world-wide. Nef clones derived from elite controllers with subtype B infection did not display major genetic defects however they showed impairments in HLA-I/CD4 down-regulation, CD74 upregulation, enhancement of infectivity and stimulation of replication in peripheral blood mononuclear cells (PBMCs), in comparison to normal progressors, suggesting that these Nef activities may affect clinical outcome in subtype B infection (Mwimanzì et al., 2013a). Furthermore, a study of multiple Nef activities in subtype B infection supported biological importance of Nef-mediated enhancement of virion infectivity for HIV pathogenesis (Mwimanzì et al., 2013b). Nef-mediated CD4 and HLA-I down-regulation have been associated with viral set point and rate of CD4+ T cell decline in early subtype C infection (Mann et al., 2014), however it remains unknown whether natural variation in other Nef functions, such as alteration of TCR signalling, influences HIV-1 subtype C disease progression.

Our previous observation of inter-subtype differences in the ability of Nef isolates to downregulate CD4 and HLA-I (Mann et al., 2013), which are Nef activities shown to correlate with markers of disease progression (Mann et al., 2014), raised the hypothesis that inter-subtype differences in Nef activities may contribute to differing prevalences and spread of subtypes through affecting pathogenicity. Inter-subtype functional differences have also been reported for Gag and Pol, and these have been linked to inter-subtype differences in HIV-1 disease progression and prevalence (Kigoya et al., 2017; Ng et al., 2014). HIV-1 subtypes differ greatly in prevalence and they are expanding unevenly: the most prevalent HIV-1 genetic subtypes include subtypes A, B and C, with subtype C being the most prevalent (Hemelaar, 2012; Tebit and Arts, 2011), and subtypes A and C may be expanding more rapidly than other subtypes (Conroy et al., 2010; Gräfl and Pinto, 2013; Tebit and Arts, 2011). Studies have also shown that the transmissibility and the rate of disease progression may differ between HIV-1 subtypes (Kanki et al., 1999; Kiwanuka et al., 2009; Palm et al., 2014; Renjifo et al., 2004; Silveira et al., 2012). Identifying inter-subtype differences is of interest to better understand the global distribution and prevalence of subtypes, and uncovering biological factors contributing to these differences may have influence on strategies for therapeutic or vaccine development. However, it remains unknown whether the ability of Nef to modulate TCR signalling, which may be an important function of Nef, differs between HIV-1 subtypes.

In the present study, we investigated whether there are differences in Nef-mediated alteration of TCR signalling between HIV-1 subtypes using previously published patient-derived Nef clones from subtypes A, B, C and D (Mann et al., 2013). We used a high throughput NFAT-based luciferase reporter T cell assay to measure the ability of each Nef clone to inhibit NFAT, a downstream molecule of TCR signalling, following TCR stimulation. We further analysed the impact of Nef-mediated inhibition of NFAT signalling on HIV-1 disease progression and assessed which amino acid variants may affect this Nef function by studying a large number of previously published Nef clones derived from individuals with recent and chronic HIV subtype C infection (Mann et al., 2014).

2. Methods

2.1. Patient-derived Nef clones

Patient-derived Nef sequences assessed in the present study were previously cloned into a modified pSELECT-GFPzeo plasmid (that expresses Nef and green fluorescent protein [GFP] from separate promoters) and assessed for their CD4 and HLA down-regulation abilities (Mann et al., 2013, 2014). Nef clones were derived from individuals chronically infected with subtypes A (n = 94), B (n = 92), C (n = 74), and D (n = 85) from cohorts in Uganda, Canada and South Africa (Mann et al., 2013) and from recently (n = 101) and chronically (n = 110) HIV-1 subtype C infected individuals from observational cohorts and clinical sites in South Africa and Botswana (Mann et al., 2014). A single clone per infected individual was analysed and all individuals were antiretroviral naive. Viral load, CD4 count and HLA class I data were available. Nef protein expression was confirmed on a subset by Western blot (Mann et al., 2013, 2014). Nef sequences are available under Genbank accession numbers KC906733-KC907077, KF208819, KF208821, KF208823, KF208825-KF208828, KF208831-KF208834, KF208836, KF208838-KF208839, KF208842-KF208843, KF208845, KF208847-KF208853, KF208855, KF208857-KF208861, KF208863-KF208865, KF208867, KF208870, KF208872-KF208873, KF208878-KF208879, KF208886, KF208889, KF208893-KF208895, KM262907-KM262921, KM262925-KM262926, KM262928-KM262994, KM262956-KM262985, KM263030-KM263118, and KM263120-KM263141.

2.2. NFAT-based luciferase reporter T cell assay

The ability of each Nef clone to alter TCR-mediated signalling was assessed using a transfection-based assay that measures luciferase production driven by nuclear factor of activated T cells (NFAT) following stimulation of Jurkat T cells (Jin et al., 2019). Briefly, 5 million Jurkat T cells, 10 µg Nef clone and 5 µg pNFAT-luciferase were combined in 400 µl of OptiMEM without phenol red (Gibco) and electro-porated at 250 V and 950 µF using a Gene Pulser Xcell electroporator (Biorad). Transfected Jurkat cells were mixed with 600 µl of RPMI-1640 medium without phenol red (Sigma) supplemented with 10% foetal bovine serum (Gibco), 2 mM-L-glutamine (Sigma), 10 mM Hepes (Gibco) and 50/µl penicillin-streptomycin (Gibco), and incubated for 18 h. Transfection efficiency (measured as the percentage of green fluorescent protein [GFP] positive cells) and the percentage live cells were assessed after the 18 h incubation by flow cytometry to confirm that these parameters were similar across different samples within the same experiment. Simultaneously, 100 µl of transfected Jurkat cells was transferred in triplicate to a plate coated with 0.1 µg/ml anti-CD3 antibody (eBioscience) and incubated for 6 h to stimulate TCR signalling (Smith et al., 1997). Following the 6 h incubation, 50 µl of stimulated cells were transferred to a half-well white plate (Greiner) and mixed with 50 µl of Steady-Glo luciferase substrate (Promega). The luminescence signal was measured following a 10 min incubation using a GloMax-Multi Microplate Multimode Reader (Promega) with an integration time of 2000 ms and settling time of 500 ms. The 3 luminescence readings per Nef clone were averaged and the ability of each Nef clone to inhibit NFAT, a downstream molecule of TCR signalling, following
luminescence) / (G2A Nef luminescence − SF2 Nef luminescence). For each Nef clone, normalised activity from two independent experiments was averaged.

2.3. Generation and testing of Nef mutants

Selected mutations were introduced into patient-derived subtype C Nef sequence SK329 (GenBank accession KC906797), which displayed high similarity to the HIV-1 Nef consensus C sequence (91.7% amino acid similarity), using overlap extension PCR. Wild-type SK329 Nef and SK329 Nef mutants were assessed in the context of the pSELECT backbone in triplicate independent experiments for the ability to inhibit TCR-mediated NFAT signalling as well as ability to down-regulate CD4, HLA-I and SERINC5. CD4 and HLA-I down-regulation activities were measured in a CEM-derived cell line expressing high levels of HLA-A*02 using flow cytometry as previously described (Mann et al., 2013). SERINC5 down-regulation was also assessed in the CEM-A*02 cell line using flow cytometry by co-transfection of 1 µg Nef and 5 µg pSELECT-SERINC5-iHA-ΔGFP, encoding an internal HA-tagged variant of SERINC5 (Usami et al., 2015a; Jin et al., 2019), followed by antibody staining the next day. Expression levels of the mutant Nef proteins were assessed by Western blot using rabbit polyclonal anti-HIV-1 Nef serum, as previously described (Mann et al., 2013), following transfection of 2.5 million Jurkat cells with 10 µg Nef.

2.4. Data analysis

The capacity of Nef to inhibit TCR-mediated NFAT signalling was compared between subtypes, as well as between mutant Nefs and the wild-type, using ANOVA with the Tukey post-hoc test since the data was normally distributed. The comparison between subtypes was done while controlling for socio-demographic and clinical factors, by performing multivariable linear regression using capacity of Nef to inhibit TCR-mediated NFAT signalling as the dependent variable and the following as independent variables: gender, log10 plasma viral load, log CD4 count, and HIV-1 subtype. For log transformed variables, model coefficients quantify the change in outcome per one log unit increase in the dependent variable of interest. The reference category for gender was female and subtype B was the reference category for HIV-1 subtypes.

The relationships between Nef-mediated inhibition of NFAT signalling in HIV-1 subtype C acute infection and subsequent viral load set point as well as the rate of CD4+ T cell decline were determined. Viral load set point was calculated as the average viral load of an infected individual between 3 and 12 months after infection, and Spearman’s correlation was used to correlate Nef-mediated inhibition of NFAT signalling function with viral load set point. The rate of CD4+ T cell decline was computed, as previously described (Wright et al., 2010), for each infected individual over a treatment-free follow-up period. A multiple linear regression was performed, taking into account follow-up time and baseline CD4+ T cell count, to determine the relationship between Nef-mediated inhibition of NFAT signalling function and the rate of CD4+ T cell decline. Since the CD4+ T cell decline data was a mixture of positive and negative values, it could not be easily transformed to normality without compromising the meaningful interpretation of coefficients. Therefore, the CD4+ T cell decline range was
limited to ~35 to 50 cells/mm$^3$ per month to approximate a normal distribution and ensure that the final model met the assumption of normally distributed residuals as assessed by the Shapiro Wilk test. This resulted in 7 observations (7%) being omitted from the analysis. Distinct Nef amino acids associated with altered ability of subtype C Nef to inhibit TCR-mediated NFAT signalling were assessed using codon-by-codon Mann–Whitney U tests. Q values were used to account for multiple tests (Storey and Tibshirani, 2003). All analyses were performed using GraphPad Prism 5 and Stata 14.

3. Results

3.1. Measurement of Nef-mediated inhibition of NFAT signalling in a transfection-based assay

Nef-mediated inhibition of NFAT signalling activity was assessed by transfecting the Nef clones into a Jurkat T cell line, stimulating the TCR with anti-CD3 antibody, and then measuring the effect on NFAT-driven luciferase production through luminescence detection (Jin et al., 2019). Following transfection, flow cytometry was used to confirm similarity of live cell percentages and transfection efficiency within an experiment (representative plots shown in Fig. 1A and B). Representative luminescence values are shown for the controls and patient-derived Nef clones, as well as the normalisation of these values for the patient-derived Nef clones to that of the controls (Fig. 1C and D). Reproducibility of the assay was excellent with independent duplicate measurements correlating strongly (Pearson’s correlation; $r = 0.86$ and $p < 0.0001$).

3.2. Inter-subtype comparison of Nef-mediated inhibition of NFAT signalling

Differences in NFAT inhibition activity were investigated between Nef clones of subtypes A ($n = 94$), B ($n = 92$), C ($n = 74$) and D ($n = 85$) derived from chronically infected individuals (Mann et al., 2013). A maximum likelihood phylogenetic tree showing distinct clustering of subtypes is shown in Fig. 2A. Nef’s capacity to inhibit TCR-mediated NFAT signalling was compared between subtypes using one-way ANOVA. Significant differences were observed between subtypes (mean [standard deviation], subtype A, 73% [28]; B, 82% [26]; C, 66% [28]; and D, 86% [26]) ($p < 0.0001$; Fig. 2B). Tukey post-hoc tests indicated that subtype C was significantly less functional than subtypes B and D ($p < 0.001$ for both) and that subtype A was significantly less functional than subtype D ($p < 0.01$). Therefore, the hierarchy of Nef-mediated inhibition of NFAT signalling activity was assessed by transfecting the Nef clones into a Jurkat T cell line, stimulating the TCR with anti-CD3 antibody, and then measuring the effect on NFAT-driven luciferase production through luminescence detection (Jin et al., 2019). Following transfection, flow cytometry was used to confirm similarity of live cell percentages and transfection efficiency within an experiment (representative plots shown in Fig. 1A and B). Representative luminescence values are shown for the controls and patient-derived Nef clones, as well as the normalisation of these values for the patient-derived Nef clones to that of the controls (Fig. 1C and D). Reproducibility of the assay was excellent with independent duplicate measurements correlating strongly (Pearson’s correlation; $r = 0.86$ and $p < 0.0001$).

CD4 counts and viral load estimates are expressed per log10 increment. Age is expressed per year increment. Female was the reference group for gender. Subtype B was the reference group for HIV-1 subtypes.

Sociodemographic (gender and age) and clinical (viral load and CD4 count) variables differ significantly between the cohorts described here (Mann et al., 2013) and could potentially confound the inter-subtype comparison of Nef-mediated inhibition of NFAT signalling (Addo and Altfield, 2014; Antonio et al., 2016; Corró et al., 2012; Huang et al., 1995; Miura et al., 2008; Mwimanzi et al., 2011b, 2013a). To adjust for these potential confounders when comparing Nef function between subtypes, a multivariate analysis was performed. In univariable analysis, where the outcome variable of interest was Nef-mediated inhibition of NFAT signalling, we considered subtype, viral load, CD4 count, gender and age as potential predictors (Table 1). Subtype and log CD4 count were identified as significant predictors of NFAT inhibition, and subsequently considered in the multivariable analysis. Consistent with the ANOVA analysis, in the multivariable analysis subtype remained significantly associated with NFAT inhibition, after controlling for CD4 count ($p = 0.01$). We observed 15% lower NFAT inhibition ability in subtype C compared to subtype B ($p < 0.001$). There was no significant difference observed between NFAT inhibition ability in subtypes B and D ($p = 0.49$).

### Table 1

<table>
<thead>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>D</td>
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<td>Log viral load</td>
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<td>Log CD4</td>
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</tr>
<tr>
<td>Age</td>
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<td>0.5</td>
</tr>
</tbody>
</table>

Function was C $< A < B < D$, where C and A were not significantly different from one another and B and D were not significantly different from one another.

![Fig. 2. Inter-subtype comparison of HIV-1 Nef-mediated inhibition of NFAT signalling.](image)

(A) A maximum likelihood phylogenetic tree of Nef sequences was constructed using Phyml (Guindon and Gascuel, 2003). Subtypes form distinct clusters, with subtypes A, B, C, and D shown in red, green, blue, and purple, respectively. The SF2 strain is shown in black. (B) ANOVA with the Tukey post-hoc tests was used to compare Nef’s ability to inhibit TCR-mediated NFAT signalling (measured by NFAT-driven luciferase activity through luminescence detection) between subtypes A, B, C and D. Luminescence values for the patient-derived Nef clones are normalised to SF2 Nef, which represents 100% activity. Each dot represents the average of two independent experiments (each with triplicate measurements) for one patient-derived Nef clone. The ANOVA p value is shown. The asterisks above the bar indicate significant differences between subtypes, with the bar indicating the two subtypes being compared. The level of significance is indicated by the number of asterisks: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)

Means and standard deviations are indicated by bars and whiskers, respectively.
Previously, Nef-mediated CD4 down-regulation and HLA down-regulation activities were measured for the same Nef clones (Mann et al., 2013, 2014). Although these Nef activities are to a large extent genetically separable (Foster et al., 2011), CD4 down-regulation and HLA down-regulation activities of primary Nef clones nevertheless correlate to some extent (e.g. as previously reported, CD4 and HLA functions of the Nef clones studied here correlated weakly [Spearman’s correlation; \( r \geq 0.3 \) and \( p < 0.01 \]) (Mann et al., 2013). Therefore, we investigated the relationship between Nef-mediated inhibition of NFAT signalling and Nef-mediated HLA down-regulation as well as CD4 down-regulation. A significant relationship between Nef-mediated inhibition of NFAT signalling and Nef-mediated HLA down-regulation activity within each subtype with use of Spearman’s correlation (panel A). However Nef-mediated inhibition of NFAT signalling was not correlated with CD4 down-regulation ability (panel B), log\(_{10}\) plasma viral load (panel C), or CD4+ T cell count (panel D). Values are normalised to SF2, which represents 100% activity. Subtypes A, B, C, and D are represented in red, green, blue, and purple, respectively. Significance is indicated by asterisks: \( p < 0.05 \) (*), \( p < 0.01 \) (**), and \( p < 0.001 \) (***)

No significant correlations were observed between Nef’s function of inhibiting TCR-mediated NFAT signalling and patient CD4 count or viral load within each subtype (Spearman’s correlation; \( r \geq -0.18 \) and \( \leq 0.1 \) and \( p \geq 0.13 \)) (Fig. 3C and D). Although it should be noted that these cohorts largely comprised individuals with late-stage infection (e.g. average CD4 counts were < 200 cells/mm\(^3\) for subtype A and D cohorts) when associations with clinical parameters may no longer be strongly detectable.

3.3. Lack of association between Nef-mediated inhibition of NFAT signalling in early infection and subsequent disease progression

To further assess whether Nef-mediated inhibition of NFAT signalling significantly influences disease progression, we employed 101 previously constructed Nef clones of the same subtype (C) that were derived from recently infected individuals, as well as an additional 110 previously constructed subtype C Nef clones from chronic infection
and observed that function was significantly lower in TRAPs versus HPP, however the functional difference was not statistically significant (estimate = −0.03 and p = 0.79). Due to the functional differences observed between the acute cohorts, a linear regression analysis was performed to check for an effect of the interaction of cohorts and this Nef function on viral load set point. No significant effect was found (Table 2), indicating that the impact of this Nef function on viral load set point was similar between cohorts.

Next, a multiple linear regression was performed to determine the relationship between Nef-mediated inhibition of NFAT signalling and the rate of CD4+ T cell decline while controlling for baseline log viral load and confounding factors, namely baseline CD4+ T cell count (transformed by square root) and follow-up time, which were both known to affect the rate of CD4+ T cell decline (Mann et al., 2014). Cohort was not included as a variable in the final model since there was no significant difference in the effect of Nef-mediated inhibition of NFAT signalling on CD4 decline in the TRAPS and Tshedimoso cohorts compared to the HPP cohort (p = 0.64 and p = 0.67, respectively). In both univariate and multivariate analyses there was no significant relationship between Nef-mediated inhibition of NFAT signalling and rate of CD4 decline (Table 3).

Furthermore, in agreement with the recent infection analysis, in the chronic infection cohort there was no significant correlation between Nef-mediated inhibition of NFAT signalling and log viral load (Spearman's correlation; r = 0.1 and p = 0.29) or CD4+ T cell count (Spearman's correlation; r = −0.05 and p = 0.62).

3.4. Sequence-function analysis: Amino acids associated with increased or decreased ability of subtype C Nef to inhibit TCR-mediated NFAT signalling

A secondary aim of this study was to identify amino acid variants that significantly affect Nef-mediated inhibition of NFAT signalling

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<th>Multivariable(^a)</th>
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CD4+ counts are expressed per one square root unit increase and viral load estimates are expressed per log10 increment. Follow-up time is expressed per day increment.

\(^a\) Multivariable model for rate of CD4+ T cell decline: multiple \(r^2 = 0.27\), p < 0.00001.
through performing sequence-function analyses. We used the larger dataset of the additional 211 subtype C clones to identify these variants. Taking into account each amino acid variant at each codon present at least 3 times or more in the combined dataset, we identified 11 amino acids variants at 6 different codons significantly associated with increased or decreased Nef-mediated inhibition of NFAT signalling (Table 4). Several of these amino acid associations (3N, 3G, and 71K) corresponded with codons previously described in the literature to be involved in modulation of TCR signalling (Arolf et al., 1997; Foster et al., 2011; Geyer et al., 2001; Lee et al., 1996), while others were adjacent to such previously described residues (9S, 9R, 108D and 108E) (Table 4). In addition, 57W, 71K, 71R, 108D and 108E were previously reported as HLA-associated polymorphisms (Carlson et al., 2014) and 71K was confirmed to confer escape from CDB+ T cell responses to the 68FPVRPQVPLR77 epitope (Du et al., 2016), indicating that immune-driven polymorphisms can impact the ability of Nef to modulate TCR signalling.

To further explore the contribution of amino acid variants to Nef's ability to modulate TCR signalling, the same codon-by-codon analysis was performed on the independent panel of chronic subtype C Nef clones (n = 74) that were used in the cross-clade comparison. Although, no amino acid variants were identified that met the q value cutoff for significance in this smaller sample subset (q < 0.2), three variants that were identified in the previous analysis were associated with altered NFAT inhibition function at p ≤ 0.07, namely 57W (p = 0.06), 108D (p = 0.07) and 108E (p = 0.07). Furthermore, 8S (p = 0.002), which was associated with decreased Nef activity, was the most significant association in the smaller dataset and was present at p = 0.03 and q = 0.28 in the larger analysis. We therefore introduced non-consensus mutations at codons 8, 57 and 108 into a patient-derived sequence, SK329, of high similarity to the consensus C sequence to further assess their functional consequences. In addition, since 71K was associated with the most pronounced decrease in ability to inhibit TCR-mediated NFAT signalling in the larger dataset, this mutation was also included in the mutant panel for functional assessment. As expected, mutants 71K and 108D displayed significantly lower ability to inhibit NFAT signalling (46% and 79% relative to the wildtype, respectively) (ANOVA; p < 0.001 and p < 0.05, respectively) (Fig. 5A). The negative consequence of 108D was specific to NFAT inhibition activity as this mutation did not influence Nef's ability to down-regulate CD4, HLA or SERINC5. In contrast, 71K significantly impaired Nef-mediated HLA downregulation ability (p < 0.01) but it had no effect on CD4 or SERINC5 down-regulation (Fig. 5B-D). It was predicted that 8R, 57Q and 57R would increase Nef's ability to inhibit TCR-mediated NFAT signalling. However, while 8R displayed similar function as the wildtype (99% of the wildtype), 57Q and 57R significantly decreased this Nef function (67% and 39% of the wildtype, respectively) (p < 0.01 and p < 0.001, respectively; Fig. 5A) as well as Nef's ability to down-regulate CD4 (both p < 0.01; Fig. 5B). Furthermore, WS7R was impaired for HLA-I down-regulation and SERINC5 down-regulation, respectively (p < 0.01 and p < 0.001, respectively; Fig. 5A and D). The expression of all Nef mutants was readily detected by Western blot, although the expression of 71K was modestly reduced when compared to the wildtype SK329 (Fig. 5E). This could partly account for the lower ability of 71K to down-regulate HLA-I and inhibit NFAT as these activities both require high intracellular Nef concentrations relative to that required for CD4 down-regulation (Liu et al., 2001). Consistent with this, in primary Nef isolates both HLA-I down-regulation ability and NFAT inhibition ability correlate positively with Nef protein expression levels by Western blot (Jin et al., 2019). In summary, these results confirm the impact of 71K and 108D on Nef-mediated inhibition of NFAT signalling, but suggest that unidentified secondary polymorphisms may alter the impact of 8R, 57Q, and 71R on this Nef function. Additional studies will be needed to address this question.

4. Discussion

HIV-1 subtypes are uneven in their prevalence and spread globally (Buonaguro et al., 2007; Hemelaar, 2012) and the biological reasons for this are not fully understood. Nef-mediated alteration of TCR signalling may be important for virus survival and persistence, since it optimizes the activation state of infected cells for optimal virus replication. We aimed to compare the Nef-mediated alteration of TCR signalling between subtypes A-D, through measuring the ability of Nef to inhibit NFAT, and to relate this to differences in subtype prevalence and expansion.

Inter-subtype differences in Nef-mediated inhibition of NFAT signalling were observed with a hierarchy of Nef functions

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Table 4

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<th>Codon</th>
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<th>Cons1</th>
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<th>q value</th>
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<th>HLA-associated polymorphisms</th>
<th>CTL escape mutations</th>
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* Numbered according to HXB2.
* Amino acid associated with increased or decreased inhibition of NFAT activity.
* Consensus amino acid at a particular codon from the reference 2004 consensus C Nef sequence from the Los Alamos HIV sequence database.
* Median functional difference in percentage inhibition of NFAT activity (expressed relative to SF2 control) between Nef clones with and without the amino acid.
* Number of sequences with (+) and without (-) the amino acid. Amino acid totals vary, as gaps in the alignment are considered missing data.
* Motif/residue previously reported to be involved in Nef-mediated alteration of TCR signalling.
* HLA-associated polymorphisms are polymorphisms positively associated with a specific HLA allele, as previously described (Carlson et al., 2014), and are likely to be immune escape mutations.
* CTL escape mutations listed in HIV Los Alamos immunology database.
C < A < B < D, where A and C, as well as B and D, were not significantly different from one another. Furthermore, this hierarchy was supported by a multivariate analysis controlling for gender, age, viral load and CD4 count, wherein it was estimated that, on average, subtype C Nef clones displayed 15% lower function and subtype A Nef clones displayed 10% lower function relative to subtype B Nef clones. Consistent with the inter-subtype differences observed here, we previously found that subtypes C and A were significantly less functional in Nef-mediated CD4 and HLA-I down-regulation when compared to subtype B (Mann et al., 2013). Similarly, it was demonstrated that subtypes A and C displayed significantly lower Gag-protease-driven replication capacity than subtype D and inter-subtype recombinants, and that subtype C Gag-protease was less functional than that of subtype B (Kiguoya et al., 2017). Furthermore, subtype D Pol isolates were reported to have increased replication capacity compared to subtype A Pol isolates (Ng et al., 2014) and subtype C whole isolates had lower ex vivo replicative fitness compared to isolates from other group M HIV-1 subtypes (Abraha et al., 2009). Taken together, these studies and the present study suggest that subtypes A and C are attenuated compared to other M group subtypes.

The Nef functional hierarchy observed in the present study is also consistent with reported inter-subtype differences in disease progression, prevalence and expansion. In Eastern Africa, subtype A is reported to result in slower disease progression when compared to subtype D (Kanki et al., 1999; Ssemwanga et al., 2013). There are conflicting reports of the rate of disease progression of subtype C relative to other M group subtypes (Ariën et al., 2007; Easterbrook et al., 2010; Neilson et al., 1999; Silveira et al., 2012; Vasan et al., 2006). However, a recent study controlling for clinical factors showed that subtype C infection was associated with slower rate of CD4 decline relative to subtype A or D infection (Venner et al., 2016). Subtypes A and C are the most prevalent subtypes (Buonaguro et al., 2007; Hemelaar, 2012; Tebit and Arts, 2011) which may also be expanding more rapidly than other subtypes, as evidenced by expansion of subtype C in Brazil, India and sub-Saharan Africa (Gräf and Pinto, 2013; Rodriguez et al., 2009; Venner et al., 2016) and increased prevalence of subtype A in Uganda (Conroy et al., 2010). Overall, the most attenuated subtypes – A and C – which were the least functional in the present study of Nef function, display slower disease progression yet are the most prevalent and are expanding rapidly. The increased dominance of subtypes A and C may be due to longer asymptomatic periods (Venner et al., 2016) and/or the low replication capacity of these subtypes may favour transmission due to increased half-life of infected cells in genital fluids, although this hypothesis still requires experimental verification.

Fig. 5. Functional consequences of selected mutations in Nef. Mutations (S8R, W57Q, W57R, R71K and E108D) were introduced into the patient-derived Nef sequence SK329 (47.5% NFAT inhibition activity relative to SF2 Nef) and the Nef-mediated abilities to inhibit NFAT (A), down-regulate CD4 (B), down-regulate HLA-I (C) and down-regulate SERINC5 (D) were measured in triplicate. Activity relative to the wild-type SK329 (representing 100% activity) is shown. The ANOVA p value is shown and asterisks indicate significant differences between the mutants and the wild-type SK329: p < 0.05 (*), p < 0.01 (**), and p < 0.001 (**). (E) Western blot analyses were used to assess the steady-state protein expression of Nef mutants. SF2 Nef and empty vector (ΔNef) were included as controls. Beta-actin protein was included as a cellular loading control.
We then assessed whether any of the variants identified as associated with impaired Nef function in this study were likely CTL escape mutations, as this may have relevance for an HIV-1 attenuation based vaccine, an approach that has been proposed for the highly mutable virus (Allen and Altfeld, 2008; Mann and Ndung’u, 2015). While Nef is a critical virulence factor in HIV infection (Kestler et al., 1991) and highly immunogenic (Radebe et al., 2011), it is also highly variable and largely excluded from the leading conserved T-cell based vaccine immunogens in testing (Borthwick et al., 2014; Mothe et al., 2015). Nevertheless, growing evidence suggests that a limited number of mutations in Nef, particularly specific mutation combinations, can impair the virus (Kuang et al., 2014; Shahid et al., 2015; Ueno et al., 2008), and that CTL targeting of selected Nef epitopes could lead to better clinical outcome (Adland et al., 2013). In the present analysis, two HLA-associated polymorphisms, 71K and 108D, were associated with decreased ability of Nef to inhibit TCR-mediated NefT signalling. 71K was associated with HLA-C*07:02 and HLA-C*17 (Carlson et al., 2014) and confirmed to confer escape from CD8+ T cell responses to the 68FPVRPQVPLR77 epitope (Du et al., 2016), and 108D was associated with HLA-B*44 and HLA-B*18 (Carlson et al., 2014). Therefore, we found limited evidence for immune-driven impairment of Nef-mediated inhibition of NefT signalling. In addition, residue 71 is immediately adjacent to the 2pPxxP72R77 motif, which is important for Nef-mediated HLA-I down-regulation function (Foster and Garcia, 2008), and we confirmed by mutagenesis that 71K also decreases HLA-I down-regulation ability, a Nef function correlated with the rate of CD4+ T cell decline in subtype C infection (Mann et al., 2014). Therefore, residue 71 could represent a vulnerable immune target where escape impairs at least two known functions of Nef, including its immune evasion ability. In support of this, an overlapping peptide (OLP) spanning Nef residue 71 is the only Nef OLP previously identified as a viral target associated with relative control (Mothe et al., 2011) that is included in a human immune data-informed vaccine (Mothe et al., 2015).

Although different Nef functions are reported to be largely genetically separate (Foster et al., 2011), we investigated whether there was any overlap between Nef-mediated inhibition of NefT signalling and HLA down-regulation as well as CD4 down-regulation. It was observed that HLA-I down-regulation activity significantly correlated with NefT inhibition activity. This was not surprising as critical residues for both Nef functions include P72, P75 and R77 of the 68FPVRPQVPLR77 epitope (Du et al., 2016; Geyer et al., 2001). These residues are important in Nef-mediated alteration of TCR signalling because they are the primary binding site of the SH3 domain of signalling kinases (Geyer et al., 2001). In HLA-I down-regulation, these residues play a role through securing the binding of the HLA-I cytoplasmic tail to AP-1 (Collins and Collins, 2014).

It should be noted that when Nef interacts with other viral proteins its function might change and therefore the analysis of Nef in isolation is not a full representation of the microenvironment (Shrivastava et al., 2016). In addition, functions of Nef may differ depending on the infected cell type (Mwimanzi et al., 2011a, 2013a). Another limitation is the assessment of Nef’s ability to alter TCR signalling using Jurkat cells, which have some physiological differences (e.g. defective expression of PTEN) from primary cells that may affect TCR signalling (Abraham and Weiss, 2004). Nevertheless, Jurkat cells have yielded many insights into TCR signalling (Abraham and Weiss, 2004), and have the advantage of uniformity and consistency. Furthermore, the Jurkat-based assay used in the current study and companion report (Jin et al., 2019) was consistent with the effect of Nef to inhibit NefT through impairing early TCR signalling events in pre-activated primary T cells (Neri et al., 2011). Similarly, in Jurkat cells, Nef inhibited NefT production and this corresponded with the ability of Nef to co-localise with Lck, a proximal TCR signalling protein (Jin et al., 2019).

In summary, it was observed that Nef-mediated inhibition of NefT activity differed among HIV-1M group subtypes, with a hierarchy of C < A < B < D. Subtypes A and C were the least functional and this
may correspond with the slower disease progression, increased expansion and high prevalence of these subtypes worldwide. Although we did not find evidence to suggest that Nef-mediated inhibition of NFT signalling has a strong impact on disease progression, other studies indicate that there is selective pressure to maintain multiple Nef functions and that decreased ability to inhibit NFT may contribute to elite control in some cases, supporting that it nevertheless plays a role in disease. Overall, we found limited evidence that CTL escape mutations attenuate this Nef function; however, CTL escape at residue 71 attenuates both Nef-mediated HLA-I down-regulation and inhibition of NFT, and is therefore a potentially useful antiviral target.

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