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Virology

Nef-mediated down-regulation of CD4 and HLA class I in HIV-1 subtype C infection: Association with disease progression and influence of immune pressure

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ABSTRACT

Nef plays a major role in HIV-1 pathogenicity. We studied HIV-1 subtype C infected individuals in acute/early ($n=120$) or chronic ($n=207$) infection to investigate the relationship between Nef-mediated CD4/HLA-I down-regulation activities and disease progression, and the influence of immune-driven sequence variation on these Nef functions. A single Nef sequence per individual was cloned into an expression plasmid, followed by transfection of a T cell line and measurement of CD4 and HLA-I expression. In early infection, a trend of higher CD4 down-regulation ability correlating with higher viral load set point was observed ($r=0.19$, $p=0.05$), and higher HLA-I down-regulation activity was significantly associated with faster rate of CD4 decline ($p=0.02$). HLA-I down-regulation function correlated inversely with the number HLA-associated polymorphisms previously associated with reversion in the absence of the selecting HLA allele ($r=-0.21$, $p=0.0002$). These data support consideration of certain Nef regions in HIV-1 vaccine strategies designed to attenuate the infection course.

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Introduction

HIV-1 Nef is a small accessory protein that plays a major role in viral replication and pathogenesis (Foster et al., 2011). Nef displays multiple functions *in vitro*, including down-regulation of cell-surface CD4 (to enhance viral spread and possibly evade antibody-dependent cell-mediated cytotoxicity of infected cells), down-regulation of HLA

class I (HLA-I) A and B molecules (to evade CD8+ T cell responses), activation of T cells and CD4-independent enhancement of virion infectivity (Foster and Garcia, 2008; Veillette et al., 2014). The relative importance of these Nef activities *in vivo* remain incompletely understood, although some studies suggest that Nef-mediated CD4 down-regulation, enhancement of infectivity, and one or more unknown Nef functions represent major pathogenic contributors (Iafrate et al., 2000; Watkins et al., 2013).

Nef's influence on HIV-1 pathogenesis is clearly demonstrated by an attenuated disease course in individuals infected with HIV-1 harbouring gross genetic Nef defects (Deacon et al., 1995; Kestler et al., 1991; Kirchhoff et al., 1995). However, more subtle, naturally-occurring variation in Nef may also influence disease outcomes. For example, Nef proteins derived from HIV-1 subtype B infected elite controllers displayed relative functional impairments compared to those from progressors in the absence of major genetic defects (Mwimanzi et al., 2011c, 2013). Studies linking Nef function and disease outcomes in HIV-1 subtype C, the predominant subtype of the HIV pandemic, are however lacking.

Nef is also a highly immunogenic protein (Lichterfeld et al., 2004). Cross-sectional studies have shown that the overall breadth of Nef-specific CD8+ T cell responses does not correlate with viral control (Kiepiela et al., 2007) and in some studies a higher magnitude of these responses correlated with higher viral loads (Novitsky et al., 2003; Radebe et al., 2011). Nevertheless, the observed associations between lower viral loads and CD8+ T cell targeting of certain Nef epitopes in humans (Adland et al., 2013) and non-human primate models (Budde et al., 2012; Mudd et al., 2012), suggests that CD8+ T cell responses to specific Nef regions may be beneficial.

There is also somewhat limited evidence that immune-driven escape mutations can impair Nef function. Two HLA-B*35-associated CD8+ T cell escape mutations in a conserved proline-rich region of Nef impaired Nef-mediated HLA class I down-regulation and virus replication when occurring in combination (Mwimanzi et al., 2011a; Ueno et al., 2008). Accumulation of novel HLA-B*57-associated polymorphisms largely unique to elite controllers was associated with reduced Nef function in these individuals (Mwimanzi et al., 2013). Furthermore, a recent study predicted that over 50% of HLA-associated Nef polymorphisms would revert on transmission to an HLA-mismatched recipient, supporting biologically-relevant fitness costs of certain immune-driven mutations in Nef (Adland et al., 2013). Identification of immune-driven Nef mutations influencing its pathogenic functions could therefore reveal regions of potential relevance for inclusion in an HIV-1 vaccine designed to attenuate the infection course (Allen and Altfeld, 2008). Thus, studies to investigate this are warranted.

Also important to vaccine design is the identification of specific features of viruses that are transmitted successfully (*i.e.* understanding the transmission bottleneck). Transmitted/early viruses tend to exhibit particular Env characteristics, including shorter variable loops with fewer glycosylation sites (Derdeyn et al., 2004; Sagar, 2010) and near-exclusive CCR5 co-receptor usage (Sagar, 2010); however, there is little knowledge regarding whether Nef characteristics of early viruses may differ from those from later infection stages (Noviello et al., 2007). Similarly, envelope length and number of glycosylation sites may increase during the infection course (Sagar et al., 2006) (though this remains somewhat controversial (Novitsky et al., 2009a)); however, few studies have examined early sequence and/or functional changes in Nef in large numbers of patients, particularly in a subtype C context (Brumme et al., 2008; Kuang et al., 2014; Noviello et al., 2007).

To address these knowledge gaps, we assessed two Nef functions implicated in pathogenesis – down-regulation of CD4 and HLA-I – in clonal plasma HIV RNA-derived Nef sequences isolated from untreated HIV-1 subtype C infected individuals from different disease stages. We studied a single representative Nef clone from each patient, including 120 individuals in early infection (of whom a subset of 68 individuals were additionally characterised for Nef function one year later), and an independent group of 207 individuals in the chronic phase of infection. We investigated (i) the relationship between Nef function in early infection and subsequent rate of HIV-1 disease progression, and (ii) the relationship between Nef function and sequence, in particular immune-driven HLA-associated Nef mutations. In addition, as an exploratory analysis we assessed differences in the genetic characteristics of Nef in early and chronic infection as well as changes in Nef function and sequence over the first year of infection.

Results

Nef clones: function and expression

Nef-mediated CD4 and HLA-I down-regulation were measured for 395 Nef clones from 327 individuals (120 with acute/early infection, for whom 68 also had a follow-up clone at 1 year post-infection, as well as 207 with chronic infection) in a CEM T cell line expressing HLA-A*02:01. It should be noted that we previously observed an excellent correlation between the relative ability of Nef clones to down-regulate HLA-A*02 and HLA-B*07, justifying that HLA-A*02 down-regulation is in general representative of HLA-I down-regulation (Mann et al., 2013). The functions of patient-derived Nef sequences were expressed relative to that of the subtype B reference Nef sequence SF2, which represented 100% activity (Supplementary Table 2, representative flow plots in Fig. 1). Overall, patient-derived Nef clones showed a median CD4 down-regulation ability of 96% (IQR, 77.6 to 99.7%) and a median HLA-I down-regulation ability of 76% (IQR, 53.4 to 87%) compared to the control subtype B SF2 strain. A minority of Nef clones ($n=47$, 12%) was poorly functional ($<45\%$ for both Nef functions). To confirm their Nef protein expression, Western blots were performed on this subset as well a randomly-selected set of 78 Nef clones that displayed $>50\%$ CD4 or HLA-I down-regulation ability relative to SF2 (indicating that Nef was expressed). Of the 47 poorly functional clones, 41 were not readily detectable by Western blot using polyclonal anti-Nef sera from rabbit or sheep (defined as $<30\%$ of SF2 control, Supplementary Table 2), while 8 of the 78 functional clones were below this threshold of detection. Since we cannot exclude the possibility that Nef clones with poor expression and function are attributable to cloning or other *in vitro* artifact, we excluded these 41 isolates from all further analyses. This left a total of 354 Nef clones for analysis: 107 from acute/early infection, 61 from 1 year post-infection (including 56 paired clones at baseline and 1 year post-infection), and 186 from chronic infection. The Western blot band intensities of the remaining Nef clones did not correlate significantly with CD4 down-regulation (Spearman's, $r=0.16$ and $p=0.15$) or HLA-I down-regulation (Spearman's, $r=0.18$ and $p=0.1$) (Supplementary Fig. 1).

Similar Nef function and sequence in different cohorts and laboratories

Our Nef sequences from acute/early infection were derived from various cohort and clinical studies in Botswana (Tshedimoso [$n=27$]) and South Africa (HPP acute infection [$n=33$] and TRAPS [$n=47$] cohorts). In addition, samples from the HPP acute infection cohort and Tshedimoso cohort were assayed in the HIV Pathogenesis Programme Laboratory at the University of KwaZulu-Natal while those from the TRAPS cohort were assayed in the HIV/AIDS
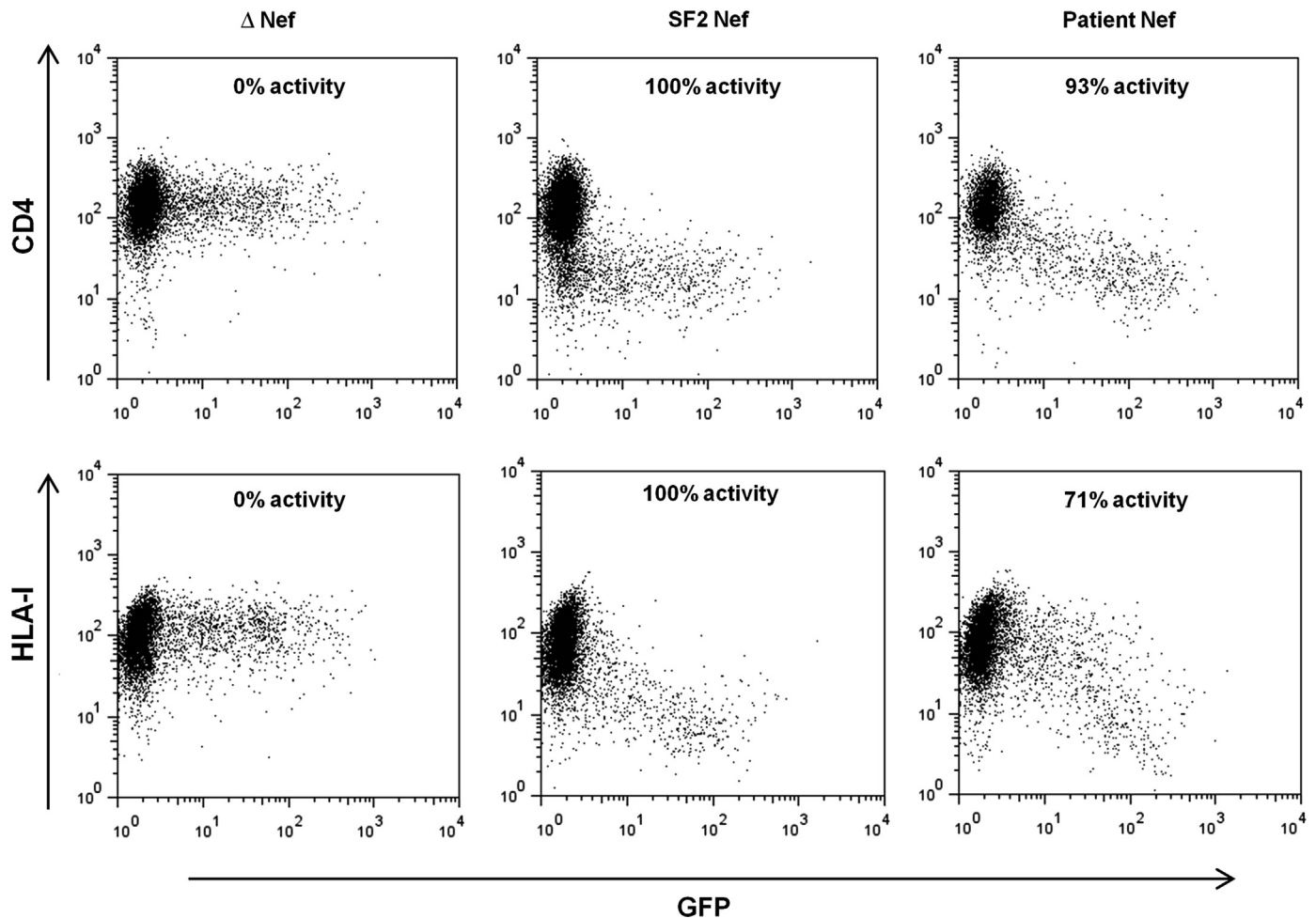
Fig. 1. Nef-mediated down-regulation of cell-surface CD4 and HLA-I. Representative flow cytometry plots show cell-surface expression of CD4 or HLA-I (*y* axis) in cells transfected with empty plasmid (Δ Nef, negative control), wild-type Nef plasmid (SF2 Nef, positive control), and plasmid expressing a patient-derived Nef. Green fluorescent protein (GFP) expression (*x* axis) was used as a marker of cells transfected with plasmids.

Molecular Epidemiology Laboratory at Simon Fraser University. As such, we confirmed that there were no significant cohort- or laboratory-specific biases in Nef function before pooling data for analysis. Both Nef-mediated CD4 down-regulation and HLA-I down-regulation activities of Nef clones from recent infection were comparable across cohorts (Kruskal–Wallis, $p > 0.2$) and laboratories (Mann–Whitney $p > 0.2$) (Fig. 2A and B). In addition, no gross clustering of Nef clones by site was observed in a combined phylogeny (Fig. 2C), and no significant differences in the average pairwise distance between patient Nef clone and published Nef consensus C sequence were observed by cohort (Kruskal–Wallis, $p = 0.84$). Thus, all Nef sequences from acute/early infection were combined in subsequent analyses.

Association of Nef-mediated CD4 and HLA-I down-regulation function in acute/early infection with subsequent disease progression

To assess the relationship between Nef function in acute/early infection and subsequent disease progression, CD4 and HLA-I down-regulation abilities of Nef clones from the earliest time-point post-infection were correlated with subsequent viral load set point and rate of CD4 decline. Viral load set point (median of 4.4 $\log_{10}$ copies/ml; IQR, 3.7–5 $\log_{10}$ copies/ml) and rate of CD4 decline (median of -4 cells/mm^3 per month; IQR, -10 to -1 cells/mm^3 per month) were calculated for 102 and 107 individuals, respectively. Rate of CD4 decline was calculated over a median treatment-free follow-up of 672 days; IQR, 346–1304 days.

A trend of increased Nef-mediated CD4 down-regulation function at baseline correlating with higher viral load set point was observed (Spearman's, $r = 0.19$ and $p = 0.05$), but no relationship was observed between HLA-I down-regulation and viral load set point (Spearman's, $r = 0.14$ and $p = 0.18$) (Fig. 3A and B). Furthermore, both CD4 and HLA-I down-regulation functions were associated inversely with rate of CD4 decline, the latter significantly so (Spearman's, $r = -0.18$, $p = 0.07$ and $r = -0.24$, $p = 0.01$ respectively). However, baseline CD4 count and length of follow-up time also significantly correlated with CD4 decline in our cohorts (Spearman's, $r = -0.25$, $p = 0.01$ and $r = 0.31$, $p = 0.001$ respectively); therefore, a multiple linear regression was performed to assess the relationship between Nef function and rate of CD4 decline while controlling for these potential confounders (see *Methods* and Table 1). After accounting for these and other baseline parameters such as viral load, the association between higher Nef-mediated HLA-I down-regulation and faster rate of CD4 decline remained statistically significant ($p = 0.02$) (Table 1). In a multivariate analysis additionally adjusting for cohort site, HLA-I down-regulation still remained significantly associated with rate of CD4 decline ($p = 0.048$; data not shown).

Sequence-function analysis part 1: lack of genetic differences between acute/early and chronic Nef sequences

We next wished to identify sequence determinants of Nef function using a maximally-powered dataset. To do this, we first
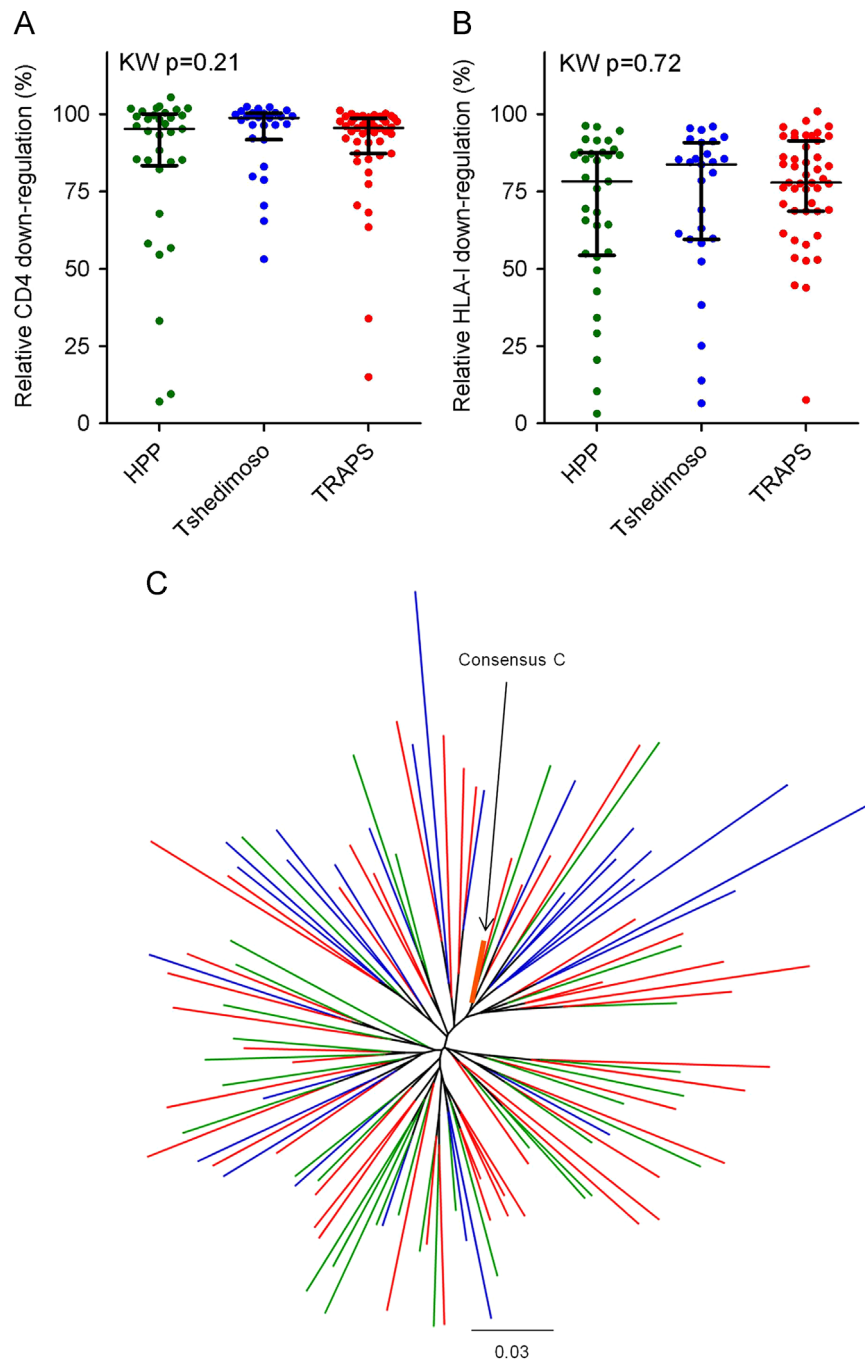
Fig. 2. Functional and sequence comparison of acute/early infection Nef clones derived from different cohorts and assayed in different laboratories. Panel A and B: graphs show no significant differences in CD4 (A) and HLA-I (B) down-regulation function between Nef clones derived from the HPP (green), Tshedimoso (blue) and TRAPS (red) cohorts, as tested by Kruskal–Wallis (KW). No significant differences in either Nef function were found between Nef clones constructed and assayed in different laboratories (HPP and Tshedimoso versus TRAPS) using the Mann–Whitney *U* test ($p > 0.2$). Bars indicate the median and whiskers represent the inter-quartile range. Panel C: the maximum-likelihood phylogenetic tree shows good intermixing of Nef clonal sequences from HPP (green), Tshedimoso (blue) and TRAPS (red) cohorts. The reference 2004 consensus C Nef sequence from the Los Alamos HIV sequence database is highlighted in orange.

compared Nef sequences from acute/early ($n=107$) and chronic infection ($n=186$) to investigate whether the former possess features that distinguish them from the latter and whether combining Nef sequence data across different disease stages for our sequence-function analysis would introduce major biases. No significant differences in Nef sequence length (Mann–Whitney, $p=0.35$) and no significant differences in the frequency of the predominant (consensus) amino acids were observed at any Nef codon between clones collected during acute/early and chronic infection (Fisher's exact test, $p > 0.09$; data not shown). Moreover, acute/early and chronic sequences were interspersed in a

phylogenetic tree (Fig. 4). There were, however, marginally greater pairwise distances from Nef consensus C sequence in early infection clones (median 11.9% [IQR, 10.6–13.9%]) compared to 11.1% for chronic infection clones [IQR, 10–12.6%], Mann–Whitney, $p=0.007$). In summary, although we cannot exclude the possibility that there is some transmission bottleneck in the Nef gene resulting in increased transmission of non-consensus sequences, we found overall that there were no major differences between Nef sequences from acute/early and chronic infection. Given the lack of major differences between Nef sequences from acute/early and later infection and phylogenetic inter-mixing of these groups
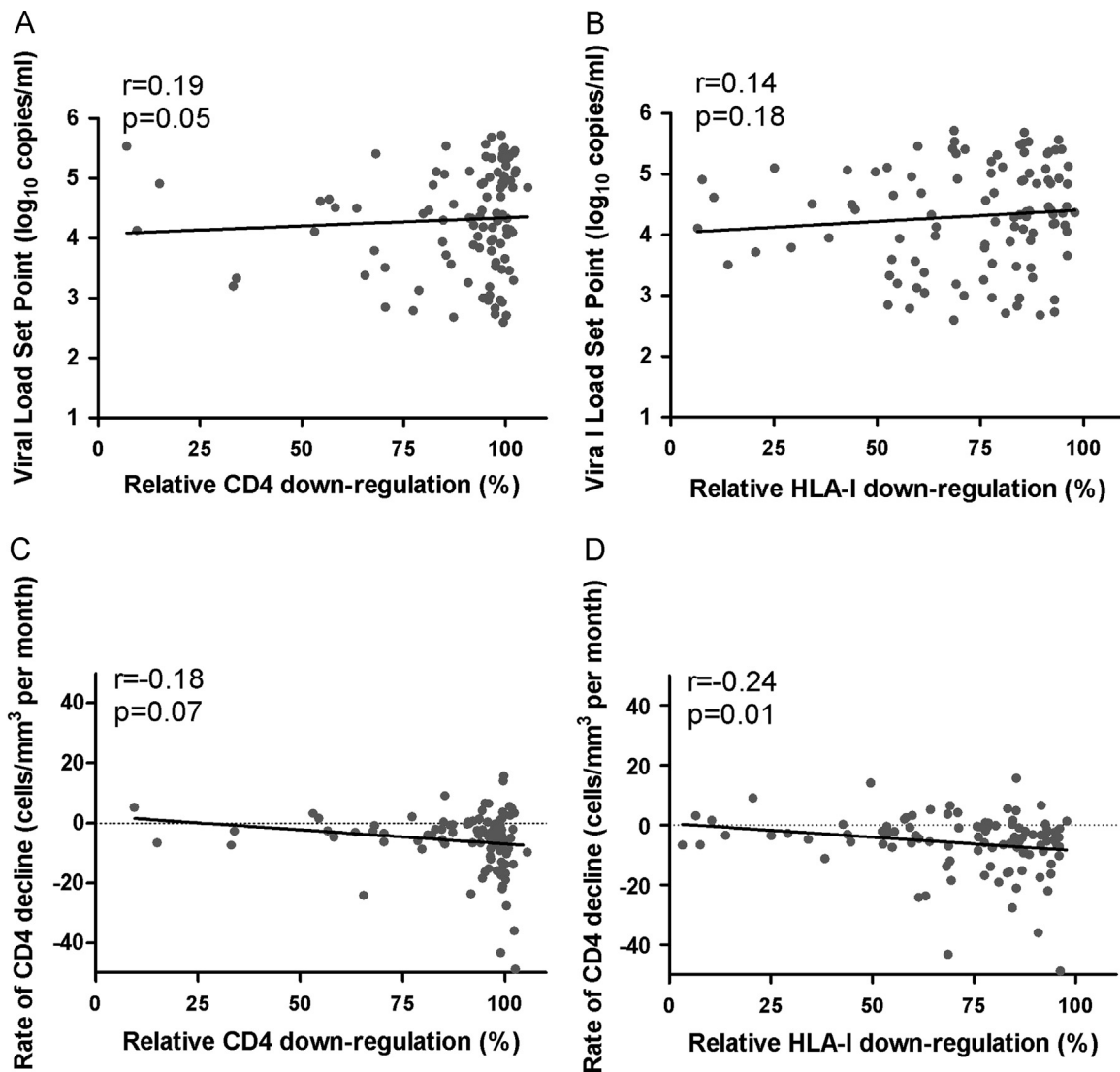
Fig. 3. Relationship between Nef functions in acute/early infection and markers of disease progression. Panel A and B: A trend for a positive correlation between higher CD4 down-regulation in acute/early infection and subsequently higher viral load set point is shown (A), while no significant relationship was observed between HLA-I down-regulation and viral load set point (B) ($n=102$). Panel C and D: associations between faster rate of CD4 decline and higher Nef-mediated CD4 (C) or HLA-I (D) down-regulation in acute/early infection are shown, but this was statistically significant for HLA-I down-regulation only ($n=107$). Data in graphs C and D are restricted to -50 to $+50$ cells/mm^3 per month, and thus do not include 5 outlier data points. Spearman's correlation was used to assess significance of relationships.

Table 1
Multiple linear regression models investigating the relationship between rate of CD4 decline and Nef functions.

Variables	CD4 decline, model 1[a]		Variables	CD4 decline, model 2[b]	
	Co-efficient	*p*-value		Co-efficient	*p*-value
CD4 down-regulation	−7.7	0.12	HLA-I down-regulation	−9.07	0.02
Square-root baseline CD4	−1.02	< 0.0001	Square-root baseline CD4	−0.99	< 0.0001
Follow-up time	0.01	< 0.0001	Follow-up time	0.01	< 0.0001
Log baseline viral load	−0.87	0.32	Log baseline viral load	−1.04	0.22

[a] Model for assessing relationship between CD4 decline and CD4 down-regulation: adjusted $r^2=0.25$, $p=<0.0001$ and $n=102$.
[b] Model for assessing relationship between CD4 decline and HLA-I down-regulation: adjusted $r^2=0.28$, $p=<0.0001$ and $n=102$.

of sequences, we combined these into a single dataset to increase power. Therefore, subsequent sequence-function analyses were performed on a set of $n=298$ Nef clones from unique patients as follows: 107 from acute/early infection, 5 from 1 year post-infection and 186 from chronic infection (the acute/early sequence was used in the event where both acute/early and 1 year post-infection sequences were available for the same individual).

Sequence-function analysis part 2: Nef amino acids associated with increased or decreased HLA-I down-regulation function

A Nef sequence-function analysis for CD4 down-regulation identified no Nef amino acids significantly associated with variations in this Nef function. However, a similar analysis for HLA-I down-regulation identified 31 amino acid variants at 22 different
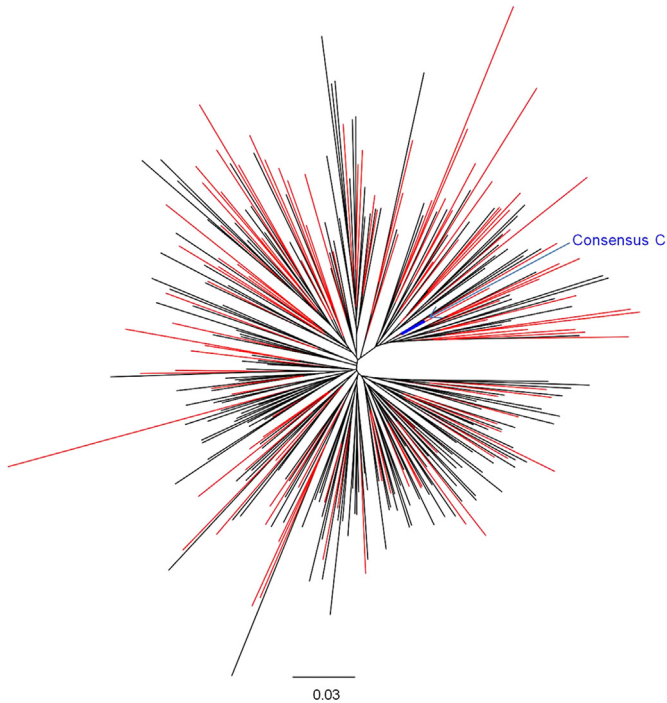
Fig. 4. Phylogenetic tree of HIV-1 subtype C Nef sequences from acute/early and chronic infection. The maximum-likelihood phylogenetic tree shows good inter-mixing of Nef clonal sequences derived from acute/early (red) and chronic (black) infection. The reference 2004 consensus C Nef sequence from the Los Alamos HIV sequence database is shown in blue.

Table 2

Amino acids ($n \geq 5$) associated with increased or decreased Nef HLA-I down-regulation function.

Codon[a]	AA[b]	Cons.[c]	Relative Nef function (%)[d]		No. of samples[e]		p-value	q-value
			With AA	Without AA	With AA	Without AA		
3	N	G	85.0	77.8	32	266	0.03	0.4
8	S	S	77.8	84.7	256	35	0.02	0.3
9	S	S	76.2	90.1	250	20	< 0.0001	0.01
9	R	S	85.5	77.1	11	259	0.007	0.2
12	G	G	77.0	84.0	262	14	0.01	0.2
15	E	A	84.9	77.1	47	251	0.03	0.3
20	M	I	83.0	77.6	88	210	0.03	0.4
20	I	I	76.9	81.1	179	119	0.04	0.4
23	T	T	76.3	83.9	186	112	0.009	0.2
23	A	T	83.9	76.8	106	192	0.01	0.2
40	Y	H	74.8	82.0	126	172	0.002	0.1
40	H	H	82.1	74.9	165	133	0.003	0.1
51	S	N	64.7	79.3	9	289	0.02	0.3
62	E	E	79.1	37.7	293	5	0.005	0.2
64	E	E	80.2	71.1	253	45	0.01	0.3
64	D	E	71.1	79.9	27	271	0.02	0.3
79	M	M	79.1	44.6	293	5	0.02	0.3
102	H	Y	71.8	79.9	45	253	0.01	0.2
105	R	K	74.1	80.2	46	252	0.03	0.4
108	E	E	82.7	74.2	159	139	0.001	0.1
108	D	E	74.3	82.7	138	160	0.002	0.1
133	V	V	80.2	68.6	265	33	0.005	0.2
146	V	V	79.1	53.1	293	5	0.02	0.3
156	N	A	89.1	78.1	6	292	0.02	0.3
161	D	N	84.7	77.0	53	244	0.007	0.2
161	N	N	76.9	83.2	235	62	0.02	0.3
178	K	R	61.1	79.2	14	284	0.02	0.3
178	G	R	84.9	77.9	40	258	0.04	0.4
188	N	S	21.1	79.1	5	293	0.03	0.3
199	H	H	79.4	47.8	290	8	0.002	0.1
199	Y	H	51.8	79.3	7	291	0.005	0.2

[a] Numbered according to HXB2.
[b] Amino acid associated with increased or decreased HLA-I down-regulation function.
[c] Cohort consensus amino acid at that particular codon.
[d] Median percentage HLA-I down-regulation function (expressed relative to SF2 control) of Nef clones with and without the amino acid.
[e] Number of sequences with and without the amino acid. Amino acid totals vary, as gaps in the alignment are considered missing data.

codons significantly associated with this function (Table 2). Several of these associations corroborated those identified in previous studies: amino acid associations with differential HLA-I down-regulation were identified at codons 3, 8, 9, 40, 102, 105, and 108 in a study of Nef subtypes A, B, C and D that included 74 subtype C Nef clonal sequences analysed in the present study (Mann et al., 2013) and similarly at codons 8 and 108 in HIV subtype B Nef sequences (Mwimanzi et al., 2013). Of note, 3 codons identified in this analysis, *viz.* 20, 62 and 64, are codons previously identified by mutational analysis to play a role in HLA-I down-regulation (Akari et al., 2000; Foster et al., 2011), while non-consensus variants 102H, 105R, 108D and 199Y, associated with reduced HLA-I down-regulation (Table 2), are HLA*B44, C*07:01, B*44 and B*18, and C*16-associated polymorphisms respectively (Supplementary Table 1). In contrast, the consensus 108E, which is enriched in individuals with HLA-B*57:02 (Supplementary Table 1), is linked to higher HLA-I down-regulation (Table 2). Together, these results suggest that HLA-I-restricted CD8+ T cell responses could select polymorphisms which impact Nef-mediated HLA-I down-regulation.

Sequence-function analysis part 3: increased numbers of HLA-associated polymorphisms correlate with reduced HLA-I down-regulation

To further explore the effect of HLA-associated polymorphisms on Nef function, the number of such polymorphisms present in each Nef sequence (defined according to an independently-derived reference list of HLA-associated polymorphisms in HIV subtype C; Supplementary Table 1) was correlated with Nef function (see Methods). No significant relationship between CD4 down-regulation and number of polymorphisms was observed (Spearman's, $r=0.008$ $p=0.89$). However, a weak trend of inverse correlation between number of HLA-associated Nef polymorphisms and HLA-I down-regulation function was observed (Spearman's, $r=-0.11$ and $p=0.06$) (Fig. 5A).

Several HLA-associated mutations in Nef have been predicted to revert upon transmission to a host lacking the original restricting HLA, suggesting they may have fitness costs (Adland et al., 2013). To investigate the relationship between such "reverting" polymorphisms and Nef HLA-I down-regulation function ($n=21$ at 15 sites previously associated with reversion, (Adland et al., 2013) and Supplementary Table 1), we repeated our analysis considering only these polymorphisms. Of note, we observed a stronger inverse correlation with HLA-I down-regulation function (Spearman's, $r=-0.21$ and $p=0.0002$) (Fig. 5B) compared to the original analysis undertaken on all HLA-associated polymorphisms (Fig. 5A). Collectively, these results suggest that HLA alleles can drive the selection of certain polymorphisms in Nef that impair its function.

*Sequence-function analysis part 4: polymorphisms associated with HLA-B*44, but not protective alleles B*57, B*58:01 and B*81, correlate with reduced HLA-I down-regulation*

Previous studies have shown correlations between presence of polymorphisms associated with protective HLA alleles and reduced Gag-protease function (Brockman et al., 2007; Crawford et al., 2009; Schneidewind et al., 2007; Wright et al., 2011), and between increasing numbers of B*57-associated Nef polymorphisms largely unique to
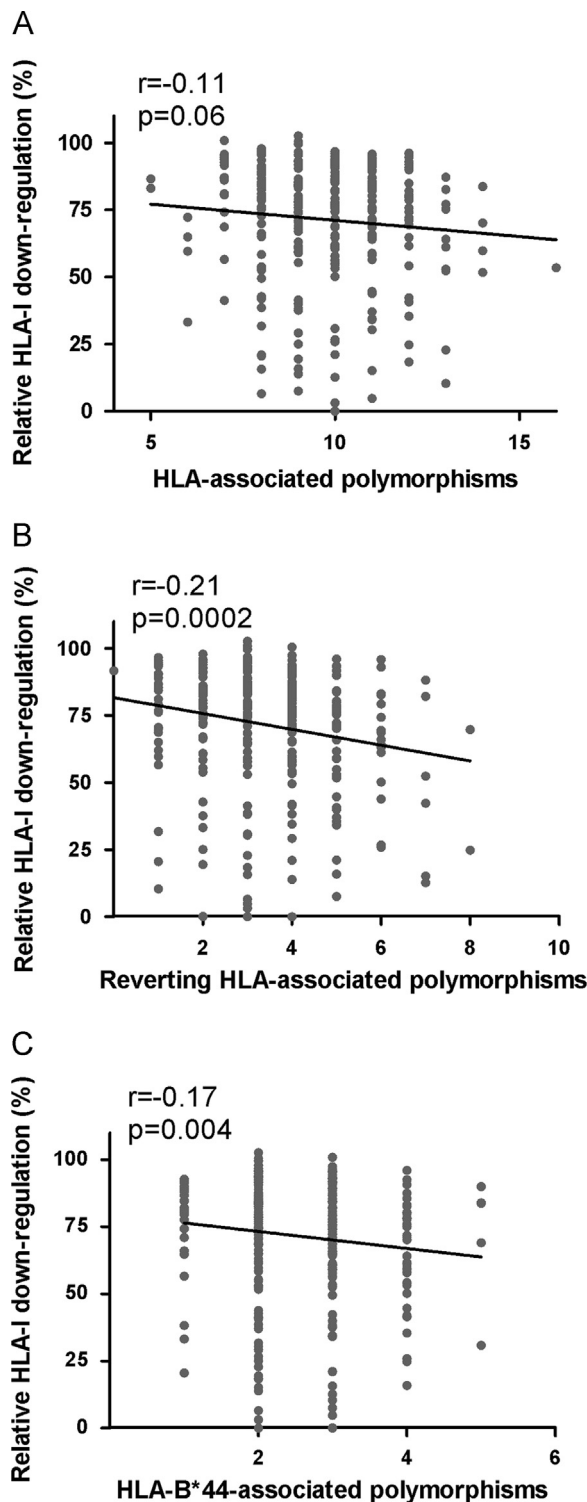
Fig. 5. Relationship between HLA-associated Nef polymorphisms and Nef-mediated HLA-I down-regulation activity. Panel A: A trend for lower HLA-I down-regulation activity with increasing numbers of HLA-associated polymorphisms is shown. Panel B: A significant negative correlation between the number of non-consensus polymorphisms at HLA-associated codons previously associated with reversion (Adland et al., 2013) and HLA-I down-regulation ability is shown. Panel C: the number of HLA-B*44-associated polymorphisms present correlates negatively with HLA-I down-regulation function. Spearman's correlation was used to assess significance of relationships ($n = 298$ for all analyses).

C-specific protective HLA alleles B*57, B*58:01 and B*81 (Kiepiela et al., 2004) exhibited decreased Nef function. No relationship between the numbers of these polymorphisms and either Nef function was observed (Spearman's, $p > 0.17$; data not shown). In fact, the number of polymorphisms specifically associated with HLA-B*57 (83G, 83E, 108E, and 116N) was positively associated with HLA-I down-regulation function (Spearman's, $r = 0.12$ and $p = 0.04$) (data not shown). This result appeared to be driven by 108E, the consensus amino acid at codon 108, which alone was associated with higher HLA-I down-regulation (Mann–Whitney, $p = 0.001$).

HLA-B*44:03, the most common subtype of B*44 in South Africa, is associated with a modest (≈ 0.25 log) reduction in viral load (Kiepiela et al., 2004; Leslie et al., 2010) and restricts a CD8+ T cell epitope (KY11, codons 92–102) within a "vulnerable" Nef region (codons 88–105) to which CD8+ T cell responses have been associated with a protective effect (Adland et al., 2013). There are also more Nef polymorphisms ($n = 7$; 65E, 71R, 93D, 102H, 108D, 176D, and 176V) associated with HLA-B*44 than any other allele (Supplementary Table 1), five of which are predicted to revert in the absence of HLA-B*44 (Adland et al., 2013). Consistent with a cumulative effect of HLA-B*44-driven polymorphisms on Nef function, we observed a significant inverse correlation between number of such polymorphisms present and HLA-I down-regulation function (Spearman's, $r = -0.17$ and $p = 0.0042$) (Fig. 5C).

Nef sequence and function in the first year of infection: adaptation to host environment and lack of consistent functional change

Exploratory analyses of 56 paired acute/early and 1 year post-infection Nef clones were next performed to investigate immune-driven evolution in Nef and functional changes over the first year of infection.

Firstly, sequences of matched clones were analysed to identify common sequence changes away from or towards a particular amino acid, that were observed in ≥ 5 individuals, between baseline and 1 year post-infection. Five such changes were identified: X83A, X102Y, X105K, E108D, and H116N, where X indicates any amino acid (Table 3); all represent codons at which HLA-associated polymorphisms have been identified (Supplementary Table 1). Changes towards consensus (X83A, X102Y, and X105K) may thus represent reversions of escape mutations, suggesting fitness costs for these (Matthews et al., 2008). Indeed, reversions at codons 83 and 102 were predicted in a recent study (Adland et al., 2013) and non-consensus amino acids at codons 83, 102 and 105 have been statistically associated with reduced HLA-I down-regulation previously (Mann et al., 2013; Mwimanzi et al., 2013) and/or in the present study (Table 2). A minority of these reversions to consensus, particularly X102Y, occurred in the presence of the associated HLA allele, however such an occurrence has previously been documented (Allen et al., 2004). In contrast, HLA-associated mutations from consensus (E108D and H116N) may represent HLA-driven escape. Indeed, H116N is an HLA-B*57 escape mutation (Frater et al., 2007; Pillay et al., 2005) that has not been associated with functional costs, and 108D, which associated with decreased HLA-I down-regulation in the present and previous studies (Mann et al., 2013), was selected four out of five times in patients harbouring the associated HLA alleles B*44 and B*18. Thus, common Nef sequence changes in the first year of infection appear to be largely driven by adaptation to host HLA.

Some of these common early sequence changes are expected to increase Nef function and some are expected to decrease Nef function. As such, it is perhaps unsurprising that in a longitudinal analysis, no overall consistent changes in Nef-mediated CD4 or HLA-I down-regulation functions were observed during the first year of infection (Wilcoxon matched-pairs signed-ranks test, $p = 0.54$ and $p = 0.56$, respectively) (Fig. 6A and B).

elite controllers and reduced Nef CD4 down-regulation function (Mwimanzi et al., 2013). Therefore, we investigated whether Nef sequences harbouring polymorphisms associated with HIV subtype

Table 3
Common sequence differences ($n \geq 5$) occurring from acute/early to 1 year post-infection in 56 pairs of Nef clones.

Change	Con.[a]	HLA-association[b]	HLA present[c]	Function association[d]
X83A	A	83G – B*57, B*58:01, A*03:01, A*34 83E – B*57	0/5	83G decreased HLA-I down-regulation Mann et al. (2013)
X102Y	Y	102H – B*44	3/5	102H decreased HLA-I down-regulation Mann et al. (2013)
X105K	K	105R – C*07:01	1/5	105 K increased HLA-I down-regulation Mann et al. (2013) 105 R and Q decreased HLA-I down-regulation Mann et al. (2013) 105 R decreased CD4 down-regulation Mwimanzi et al. (2013)
E108D	E	108D – B*44, B*18 108E –B*57:02	4/5	108D decreased HLA-I down-regulation Mann et al. (2013), Mwimanzi et al. (2013)
H116N	H	116N – B*57:03	0/5	No association

[a] Consensus amino acid.
[b] HLA alleles positively associated with an amino acid at the relevant codon at $p < 0.05$, $q < 0.05$ (Supplementary Table 1).
[c] Indicates the number of individuals who expressed the relevant HLA allele (associated with variants at the codon at which the sequence change occurred).
[d] Statistical associations between Nef amino acid variants and Nef function as identified in previous studies.
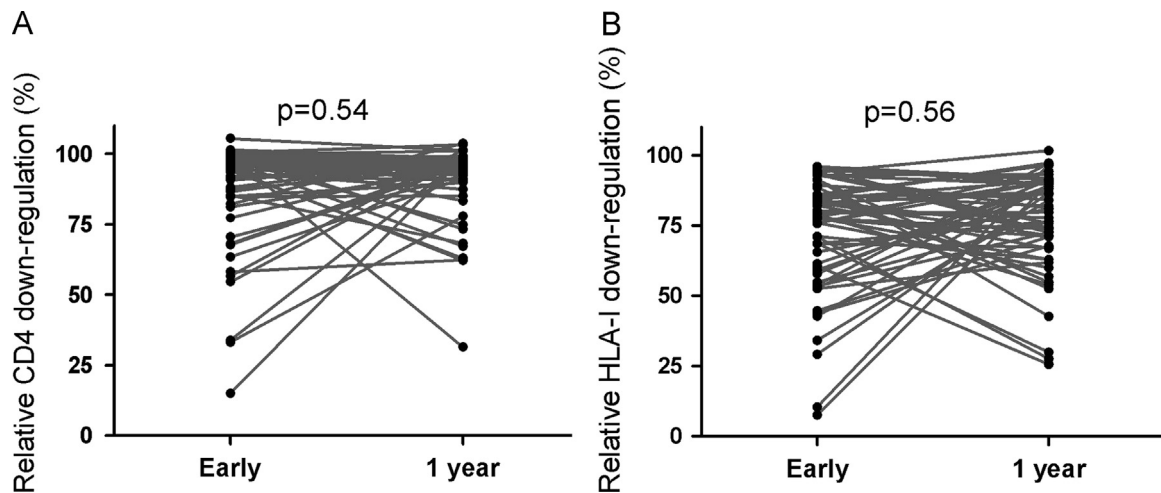
Fig. 6. Nef functions over the first year of infection. Panel A and B: graphs show no consistent increase or decrease in Nef-mediated CD4 (A) and HLA-I (B) down-regulation over the first year of infection. Functions of 56 pairs of matched Nef clones derived from acute/early infection and 1 year post-infection from the same individuals are shown. *P* values from the Wilcoxon matched-pairs test are shown.

Discussion

There is limited direct evidence that CD8+ T cell responses to HIV-1 Nef impose a functional cost on the virus (Adland et al., 2013; Mwimanzi et al., 2013; Ueno et al., 2008) and the contribution of Nef-specific CD8+ T cell responses to viral load control remains debated (Adland et al., 2013; Kiepiela et al., 2007). Furthermore, while gross genetic defects in Nef can contribute to long-term non-progression (Deacon et al., 1995; Kirchhoff et al., 1995) and attenuation of Nef functions in elite controllers have been demonstrated (Mwimanzi et al., 2013), large population-based studies correlating Nef functions with disease progression in HIV-1 subtype C infection are lacking. Therefore, we investigated the relationships between Nef-mediated CD4 and HLA-I down-regulation functions and disease progression, as well as the potential influence of immune-driven Nef sequence variation on these functions, in HIV-1 subtype C infection.

As previously observed by our group (Mann et al., 2013), Nef-mediated CD4 down-regulation function was considerably more well-conserved than HLA-I down-regulation function in our study, suggesting the former function as particularly important for the virus to conserve. This is consistent with the critical role ascribed to Nef-mediated CD4 down-regulation in mediating viral replication and pathogenesis in humanised mouse and macaque models in early infection, while Nef-mediated HLA-I down-regulation is neither sufficient nor required for these pathogenic effects (Iafrate et al., 2000; Watkins et al., 2013). Despite the fairly

narrow spread of observed CD4 down-regulation values in patient sequences (that could reduce our ability to identify significant correlations between this Nef function and clinical/sequence parameters), we observed a modest positive association ($p=0.05$) between Nef-mediated CD4 down-regulation function in early infection and viral load set point. This result is consistent with reports that CD4 down-regulation directly enhances viral replication through promoting budding of infectious virions (Argañaraz et al., 2003; Lundquist et al., 2002).

On the other hand, we observed no evidence linking Nef-mediated HLA-I down-regulation function in early infection and viral load set point. This is consistent with studies showing a lack of effect on SIV viral loads following knockout of HLA-I down-regulation function in the macaque model (Swigut et al., 2004) and a lack of correlation between this Nef function and viral loads in a small study of HIV-1 infected individuals (Lewis et al., 2008), indicating that this function does not directly affect viral replication. However, we observed that increased Nef-mediated HLA-I down-regulation in early infection was linked with a subsequent faster rate of CD4 decline. This suggests that variability in this Nef function may affect disease progression following the initial CD4 depletion, supporting its relevance *in vivo*. The biological relevance of Nef-mediated HLA-I down-regulation is also supported by strong *in vivo* selection pressure to restore this function following mutational knock-out in an SIV model (Swigut et al., 2004). As such, the relationship between HLA-I down-regulation ability and CD4 decline may be mediated *via* enhanced evasion of CD8+ T cell

responses by HLA-I down-regulation (Lewis et al., 2008; Swigut et al., 2004), though why this is not additionally manifested in higher plasma viral load remains an open question.

In this study, we found evidence that mutations selected by HLA alleles, likely CD8+ T cell escape mutations, may reduce Nef-mediated HLA-I down-regulation ability, but not CD4 down-regulation function (which may be related to the highly conserved nature of that function). The presence of certain HLA-associated polymorphisms (102H, 105R, 108D and 199Y) was individually significantly associated with reduced HLA-I down-regulation. These results extend those of a previous study demonstrating a reduction in HLA-I down-regulation activity of Nef sequences harbouring a combination of escape mutations in the proline-rich region (Ueno et al., 2008). Furthermore, consistent with an inferred fitness cost of Nef HLA-associated polymorphisms associated with reversion (Adland et al., 2013); we observed that increasing numbers of these polymorphisms in our patient Nef sequences correlated strongly with reduced HLA-I down-regulation.

We additionally observed that increasing numbers of mutations associated with HLA-B*44, which has been linked to a modest reduction in viral loads (Kiepiela et al., 2004; Leslie et al., 2010), corresponded with decreased HLA-I down-regulation. Furthermore, we found that the number of polymorphisms within two overlapping peptides (codons 88–105 and 134–148), to which CD8+ T cell responses have been associated with lower viral loads in HIV-1 infection (Adland et al., 2013), correlated significantly and negatively with HLA-I down-regulation function (data not shown). These observations suggest that functional constraints may contribute to the modest benefit associated with HLA-B*44 and the beneficial effects of CD8+ targeting of Nef regions 88–105 and 134–148, either directly (via fitness-costly escape) or indirectly (via sustained CD8+ T cell responses to a mutationally constrained region i.e. delayed escape). In fact, there is much evidence that a balance between these two factors influences viral load (Crawford et al., 2009; Kawada et al., 2006; Wright et al., 2011). Interestingly, we found no evidence that Nef polymorphisms commonly associated with the protective HLA-B*57 allele reduce Nef function, while others have found that the number of non-canonical HLA-B*57-associated Nef polymorphisms particular to elite controllers, but not those commonly associated with B*57, correlated negatively with several Nef functions in elite-controller-derived Nef sequences (Mwimanzi et al., 2013). Nevertheless, it is possible that HLA-B*57 responses to Nef epitopes may contribute to slower progression in B*57 individuals who are not elite controllers, considering that the B*57-restricted Nef epitopes overlap considerably with Nef regions associated with viral control (Adland et al., 2013) and that maintenance of Nef HW9 responses was associated with long-term non-progression in HLA-B*57-expressing individuals (Navis et al., 2008).

Of relevance to HIV vaccine design is whether viruses in early infection differ from those in chronic infection (which might indicate preferential transmission of certain variants). Although Nef sequences from early infection were marginally less similar to the consensus C sequence, we found no features that clearly distinguished early from chronic Nef sequences. This is consistent with a previous report describing similar diversity in donor and acutely-infected recipient Nef sequences as well as a lack of optimisation of specific Nef functions during transmission (Noviello et al., 2007). Our longitudinal analysis of individuals from early to 1 year post-infection revealed no consistent functional changes and that common sequence changes occurring during this period were reversions and selections of HLA-associated mutations. Similarly, it was previously found that the differences between HIV sequences from early and later infection stages largely represented reversion of mutations and escape mutations occurring post-transmission in adaptation to the specific host environment (Treurnicht et al., 2010). Also in line with

these findings, Noviello et al. (2007) observed that CD8+ T cell responses appeared to be a major driver of Nef evolution during early HIV-1 infection (Noviello et al., 2007).

In summary, this study provides evidence that variability in Nef-mediated CD4 and HLA-I down-regulation ability among subtype C viruses in the absence of obvious Nef genetic defects may influence disease progression. Furthermore, immune pressure on certain regions of Nef may result in selection of mutations that reduce the HLA-I down-regulation function of Nef. Together with previous studies showing clinical benefit associated with CD8+ T cell responses to particular epitopes in Nef, results support certain Nef epitopes for consideration as components of an HIV vaccine designed to attenuate the infection course.

Methods

Study subjects

Antiretroviral naïve patients with acute/early and chronic HIV-1 subtype C infection were recruited from established observational cohorts and clinical trial sites in South Africa and Botswana. Study participants with acute/early infection ($n=120$) comprised 39 individuals from the HIV Pathogenesis Programme (HPP) Acute Infection Cohort in Durban, South Africa (Radebe et al., 2011; Wright et al., 2011), 31 from the Tshedimoso Study in Botswana (Novitsky et al., 2009b, 2009c; Wright et al., 2011), and 50 participants of the Tenofovir Gel Research for AIDS Prevention Science (TRAPS) Cohort (in KwaZulu-Natal, South Africa) who seroconverted in the CAPRISA 004 trial and were from the placebo arm (Abdool Karim et al., 2010; Chopera et al., 2013). For acute/early patients, plasma samples used were collected at a median of 49 days post-infection (interquartile range [IQR], 33–66 days post-infection). For 68 of these patients, for whom plasma was available, an additional plasma sample collected at a median of 374 days post-infection [IQR, 363–381 days post-infection] was analysed. Viral load, CD4 count and high resolution HLA class I data were available for all study participants with acute/early infection. Study participants with chronic infection ($n=207$) were from the Sinikithemba Cohort in Durban, South Africa (Kiepiela et al., 2004; Mann et al., 2013; Wright et al., 2010). Viral loads, CD4 counts, and high resolution HLA class I data were available for these chronically infected patients as previously described (Kiepiela et al., 2004).

Written informed consent was obtained from all study participants and ethical approval was obtained from the relevant institutional committees.

Generation and sequencing of Nef clones

A single Nef clone per study participant was analysed. A subset of the Nef clones studied here were previously published, including 85 from HIV-1 subtype C chronically infected individuals (Mann et al., 2013) and 48 from individuals in acute/early infection who were in the placebo arm of the CAPRISA 004 trial (Chopera et al., 2013). Nef clones were generated from plasma and sequenced as previously described (Mann et al., 2013) Briefly, nef was amplified from plasma-derived HIV-1 RNA using primers containing AscI and SacII restriction enzyme sites. The nef gene was cloned into a pSELECT-GFPzeo expression plasmid (Invivogen, San Diego, CA, USA), which was engineered with the corresponding restriction enzyme sites and had separate promoters for the nef gene and green fluorescent protein (GFP) expression. Bulk nef polymerase chain reaction (PCR) products as well as nef clonal sequences were sequenced using the ABI Prism Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For each study participant, a single representative Nef clone with an

open reading frame and that clustered with the corresponding bulk sequence in a phylogenetic tree (Guindon and Gascuel, 2003) was selected for CD4 and HLA-I down-regulation analyses. Nef sequences were aligned to HXB2 and stripped of insertions with respect to HXB2 using HyPhy (Kosakovsky Pond et al., 2005). All *nef* clones were confirmed to be HIV-1 subtype C using the recombinant identification program (RIP; http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). Maximum-likelihood phylogenetic trees were constructed using Phyml (Guindon and Gascuel, 2003) and pairwise genetic distances from the 2004 consensus C Nef sequence (available at http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html) were calculated using PATRISTIC (Fourment and Gibbs, 2006). VESPA (http://www.hiv.lanl.gov/content/sequence/VESPA/vespa.html) was used to identify signature differences in consensus amino acids between groups of sequences. Sequences analysed in this study are available under Genbank accession numbers KF208819, KF208821-3, KF208825-8, KF208831-4, KF208836, KF208838-9, KF208842-3, KF208845, KF208847-208853, KF208855, KF208857-208861, KF208863-5, KF208867, KF208870, KF208872-3, KF208878-9, KF208886, KF208889, KF208893-5, KC906734-906805, KC906991-2, and KM262907-263141.

CD4 and HLA-I down-regulation

The CD4 and HLA-I down-regulation activities of Nef clones were measured as previously described using a CEM-derived T cell line expressing CD4 and engineered to stably express HLA-A*02:01 (Mann et al., 2013). Briefly, 300,000 cells were electroporated at 250 V and 950 μF with 4 μg Nef clone and incubated for 20–24 h, followed by staining using APC-labelled anti-CD4 and PE-labelled anti-HLA-A*02 antibodies (BD Biosciences, San Jose, CA, USA). CD4 and HLA-I cell surface expression was measured by flow cytometry in successfully transfected (*i.e* GFP-expressing) cells. The CD4 and HLA-I down-regulation activities (indicated by median fluorescence intensity [MFI] of CD4/HLA-I expression) of Nef clones were expressed relative to that of the positive control (SF2 Nef, set at 100% function) and the negative control (empty pSELECT plasmid, set at 0% function), as follows: $(MFI_{negative\ control} - MFI_{patient\ Nef})/(MFI_{negative\ control} - MFI_{positive\ control})$. For the majority of Nef clones ($n=297$), a single transfection per Nef clone was performed and stained in duplicate, with duplicate measurements in excellent agreement (Spearman's, $r=0.98$ and $p < 0.0001$ for both Nef functions). For a subset of Nef clones ($n=98$), duplicate independent transfections were performed and also correlated well (Spearman's, $r=0.8$ and $p < 0.0001$ for CD4 down-regulation and $r=0.9$ and $p < 0.0001$ for HLA-I down-regulation). Averaged results were analysed.

Nef expression

A minority (47 of 395, 12%) of Nef clones exhibited functions $< 45\%$ of that of the reference strain SF2 for both CD4 and HLA-I down-regulation. For these, as well as a random selection of 78 Nef clones with functions above this threshold, Nef protein levels were measured by Western blot as previously described (Mann et al., 2013; Mwimanzi et al., 2011b). Briefly, following transfection of 1 million CEM cells with 10 μg of Nef clones and a 24 h incubation, cells were lysed. Following SDS-PAGE of cell lysates, proteins were electroblotted onto a polyvinylidene difluoride membrane and the presence of Nef protein was detected using rabbit polyclonal anti-HIV-1 Nef primary antibody (NIH AIDS Research and Reference Reagent Program, USA) and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibody (Amersham Biosciences, Little Chalfont, UK). Samples that failed Nef detection with the rabbit antibody were next probed using sheep polyclonal anti-HIV-1 Nef primary antibody (NIBSC Center for AIDS Reagents,

UK) and HRP donkey anti-sheep IgG secondary antibody (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Actin expression was quantified simultaneously using monoclonal mouse anti-actin primary antibody (Sigma, St. Louis, Missouri, USA) and HRP goat anti-mouse secondary antibody (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). Chemiluminescence of bands was measured using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK). All Nef bands, including that of the SF2 control, were quantified relative to actin expression. These values were then normalised to that of SF2 to obtain percentage expression.

Definition of HLA-associated polymorphisms in HIV-1 subtype C Nef

HLA-associated polymorphisms are HIV amino acid variants that are significantly over- (or under) represented in the presence of a particular HLA allele, as identified in statistical association studies of linked host (HLA) and HIV sequence datasets. A comprehensive list of HLA-associated polymorphisms was previously generated based on 1336 subtype C Nef sequences from individuals from southern Africa using methods correcting for HIV phylogeny, amino acid co-variation, and HLA linkage disequilibrium (Carlson et al., 2014). HIV Nef polymorphisms in this list that were significantly positively associated with the expression of a particular HLA allele at $q < 0.05$ were defined as "HLA-associated polymorphisms" in the present analysis (Supplementary Table 1).

The total number of HLA-associated polymorphisms was counted in each Nef clonal sequence. This was done without taking into account the HLA alleles expressed by the patient, since the aim of this analysis was to investigate the relationship between number of HLA-associated polymorphisms in Nef (irrespective of whether they were transmitted or selected in the present host) and Nef function.

This analysis was repeated by summing only the subset of HLA-associated polymorphisms inferred to be "reverting" (defined as those where the absence of the restricting HLA allele is significantly associated with over-representation of the consensus amino acid or under-representation of the polymorphism in statistical association studies), suggesting that they confer a fitness cost. In this analysis, non-consensus polymorphisms at sites previously defined as "reverting" ((Adland et al., 2013), listed in Supplementary Table 1), were counted in each Nef clonal sequence.

Statistical analysis

Nef function versus disease progression analyses

Nef-mediated CD4 and HLA-I down-regulation functions in acute/early infection were correlated with markers of subsequent disease progression, namely viral load set point and rate of CD4 decline, using Spearman's correlation. Viral load set point was calculated as the average viral load from 3 to 12 months post-infection. Simple linear regression was used to compute the rate of CD4 count decline for each individual over the treatment-free follow up period, where rate of decline was defined as the estimated slope of the fitted regression line (Wright et al., 2010). Multiple linear regression was also performed to assess the relationship between Nef functions and rate of CD4 decline while controlling for baseline CD4 count and length of follow-up. The nature of the CD4 decline data (a mixture of positive and negative values) prevented the use of traditional data transformations (e.g. square root, log, and inverse transformations). We thus limited the analysis to CD4 decline values within the range of -50 to 50 cells/mm^3 per month since this ensured that the assumptions of normality were sufficiently met and included $> 95\%$ of the data. Upon fitting the final model we tested the assumptions of normally distributed residuals using the Shapiro–Wilk test, and

validated that there was no relationship between the residuals and fitted values using Spearman's correlation.

Nef sequence-function analyses

Comparisons of Nef sequence and/or Nef function were assessed using the Mann–Whitney U-test (with the exception of patients for whom paired early versus 1-year Nef clones were compared, in which case the Wilcoxon matched-pairs test was used), and Spearman's correlation as appropriate. Significant differences in cohort consensus amino acids between acute/early and chronic Nef sequences were identified using Fisher's exact test. Unless indicated otherwise, the significance cut-off for all statistical analyses was $p < 0.05$. Specific Nef amino acids (present at a frequency of $n \geq 5$ in our dataset) associated with differences CD4 or HLA-I down-regulation function were assessed using the Mann–Whitney U test. Multiple comparisons were addressed using q-values, the p-value analogue of the false-discovery rate (Storey and Tibshirani, 2003). Here, associations with $p < 0.05$ and $q \leq 0.4$ are reported.

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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.2014.08.009.

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