Received: 8 October 2019 | Accepted: 7 January 2020

DOI: 10.1002/jmv.25676

JOURNAL OF MEDICAL VIROLOGY WILEY

RESEARCH ARTICLE

Consequences of HLA-associated mutations in HIV-1 subtype C Nef on HLA-I downregulation ability

Jaclyn K. Mann[1] | Erasha Rajkoomar[1] | Steven W. Jin[2] | Qiniso Mkhize[1] | Omolara Baiyegunhi[3] | Pholisiwe Mbona[1] | Mark A. Brockman[2,4,5] | Thumbi Ndung'u[1,3,6,7,8]

[1]HIV Pathogenesis Programme, University of KwaZulu-Natal, Durban, South Africa

[2]Faculty of Health Sciences, Simon Fraser University, Burnaby, Burnaby, BC, Canada

[3]Africa Health Research Institute, Durban, South Africa

[4]Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

[5]British Columbia Centre for Excellence in HIV/AIDS, Vancouver, BC, Canada

[6]Ragon Institute of MGH, MIT and Harvard University, Cambridge, Maryland

[7]Max Planck Institute for Infection Biology, Berlin, Germany

[8]Division of Infection and Immunity, University College London, London, United Kingdom

Correspondence
Thumbi Ndung'u, PhD, HIV Pathogenesis Programme, University of KwaZulu-Natal, 719 Umbilo Road, Durban 4013, South Africa.
Email: ndungu@ukzn.ac.za

Funding information
Sullivan Family Foundation; Howard Hughes Medical Institute; Victor Daitz Foundation; DELTAS Africa Initiative,
Grant/Award Number: DEL-15-006; South African Department of Science and Technology

Abstract

Identification of CD8+ T lymphocyte (CTL) escape mutations that compromise the pathogenic functions of the Nef protein may be relevant for an HIV-1 attenuation-based vaccine. Previously, HLA-associated mutations 102H, 105R, 108D, and 199Y were individually statistically associated with decreased Nef-mediated HLA-I downregulation ability in a cohort of 298 HIV-1 subtype C infected individuals. In the present study, these mutations were introduced by site-directed mutagenesis into different patient-derived Nef sequence backgrounds of high similarity to the consensus C Nef sequence, and their ability to downregulate HLA-I was measured by flow cytometry in a CEM-derived T cell line. A substantial negative effect of 199Y on HLA-I downregulation and Nef expression was observed, while 102H and 105R displayed negative effects on HLA-I downregulation ability and Nef expression to a lesser extent. The total magnitude of CTL responses in individuals harboring the 199Y mutation was lower than those without the mutation, although this was not statistically significant. Overall, a modest positive relationship between Nef-mediated HLA-I downregulation ability and total magnitude of CTL responses was observed, suggesting that there is a higher requirement for HLA-I downregulation with increased CTL pressure. These results highlight a region of Nef that could be targeted by vaccine-induced CTL to reduce HLA-I downregulation and maximize CTL efficacy.

KEYWORDS

CTL responses, HIV-1 Nef, mutations, Nef-mediated HLA-I downregulation

1 | INTRODUCTION

A major barrier to the development of an effective vaccine against human immunodeficiency virus 1 (belonging to the genus *Lentivirus* and the family Retroviridae) is the high mutability of the virus which promotes escape from immune responses.[1] Although escape is overall advantageous to the virus, certain CD8+ T lymphocyte (CTL) escape mutations, particularly those in conserved regions, result in diminished HIV-1 replication ex vivo.[2] One proposed vaccine strategy involves directing immune responses to multiple regions of the virus where escape mutations would substantially compromise replication, with the aim of preventing viable escape or driving the virus to an attenuated form should partial escape occur.[3,4] In support of this concept, elite controllers tend to make CTL responses to a structurally and functionally constrained region of Gag where multiple mutations are unlikely due to the overall replication cost to the virus.[3]

Several attenuating immune-driven mutations have been identified in the Gag protein.[5–10] However, the Nef protein is a critical virulence factor in HIV infection,[11,12] and highly immunogenic,[13] and is, therefore, an attractive vaccine target. Although somewhat limited, there is growing evidence that certain immune-driven escape mutations in Nef could result in replicative costs.[14–18] Specifically, some combinations of escape mutations in Nef have been reported to reduce HLA-I downregulation activity (a Nef activity that allows evasion of CTL responses),[15–17] and several HLA-associated mutations in Nef were linked to reversion, indirectly suggesting that they compromise viral replication.[14] Furthermore, CTL responses to certain Nef epitopes have been linked to low viremia.[14,19,20]

Through functional analysis of a large population of patient-derived HIV-1 subtype C Nef sequences, a significant relationship between increasing numbers of reversion-associated HLA-associated polymorphisms in Nef and decreased Nef-mediated HLA-I downregulation ability was observed.[21] In addition, several HLA-I associated Nef polymorphisms (likely escape mutations as described in Carlson et al[22]), namely 102H (HLA-B*44), 105R (C*07:01), 108D (B*44 and B*18), and 199Y (C*16), that were individually statistically associated with decreased Nef-mediated HLA-I downregulation ability, were identified.[21] HLA-I downregulation is an important activity of Nef as indicated by restoration of this Nef function in macaques infected with SIV that was mutated in Nef to selectively impair HLA-I downregulation,[23] maintenance of HLA-I downregulation activity in chronic infection,[24,25] and correlation of HLA-I downregulation ability of Nef sequences obtained in acute HIV-1 subtype C infection with subsequent rate of CD4+ T cell decline.[21] Therefore, in the current study the aim was to directly test the effect of the HLA-associated mutations 102H, 105R, 108D, and 199Y, by site-directed mutagenesis, on the ability of HIV-1 Nef to downregulate HLA-I. The effects of these mutations on Nef expression and magnitude of CTL responses were also explored.

2 | METHODS

2.1 | Nef mutants

Mutations Y102H, K105R, E108D, and H199Y were introduced into patient-derived subtype C Nef sequences SK93 (GenBank accession KC906748) and SK446 (GenBank accession KM263139), since these Nef sequences had the highest similarity to the HIV-1 Nef consensus C sequence (93.2% and 92.7% amino acid similarity, respectively) in a large cohort of subtype C infected individuals.[26] In addition, V133T (HLA-B*35- associated), the most common mutation at this codon, was tested in these Nef backgrounds as the consensus 133 V was statistically associated with increased HLA-I downregulation,[26] indirectly suggesting that escape at this codon compromises HLA-I downregulation function. Furthermore, E93D (B*44:03-associated) was included as control since it is an HLA-associated mutation that was not significantly associated with altered HLA-I downregulation ability.[26] Based on the previous statistical analysis of patient-derived sequences, H199Y was expected to have a greater impact on HLA-I downregulation than Y102H, K105R, and E108D, therefore the effect of the 199Y mutation was tested in two additional patient-derived subtype C sequence backgrounds (SK73, GenBank accession KC906739; and SK141, GenBank accession KC906760). Both SK73 and SK141 had 91.7% amino acid similarity to the consensus C sequence, while SK73 was a patient-derived sequence in which H199Y was naturally present. The 199Y mutation was reverted to consensus 199H in the SK73 Nef sequence and the 199Y mutation was introduced into the SK141 Nef sequence. None of the tested mutations were previously associated with Nef-mediated CD4 downregulation ability in patient-derived sequences. These patient-derived Nef sequences, in relation to the consensus C Nef sequence, and the mutations tested are highlighted in Figure 1.

The patient-derived Nef sequences were cloned into a TOPO vector using the TOPO TA Cloning kit (Invitrogen, San Diego). The relevant mutations were then introduced into the Nef-TOPO plasmids by site-directed mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene).

2.2 | CD4 and HLA-I downregulation assays in a CEM-derived T cell line

The resulting mutated Nef sequences were re-cloned into a pSELECT green fluorescent protein (GFP) reporter expression plasmid, and subjected to an assay simultaneously measuring Nef-mediated HLA-I and CD4 downregulation abilities, as previously described.[27] Briefly, the mutated Nef-pSELECT plasmids were electroporated into an HLA-A*02-expressing CEM-derived CD4 T cell line followed by antibody staining for HLA-A*02 and CD4 and flow cytometry measurements. GFP expression was a marker of transfected cells. The median fluorescent intensity (MFI) of CD4 or HLA-A*02 in GFP-expressing cells was normalized to the MFI of the SF2 Nef-pSELECT plasmid positive control and the empty pSELECT plasmid negative control such that a value of 0% indicated no downregulation activity and a value of 100% indicated downregulation activity equivalent to SF2 Nef, as previously published.[26–28] Experiments were performed at least in triplicate and results averaged.

2.3 | HLA-I downregulation assays in peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from two HIV-negative donors expressing HLA-A*02 were stimulated with phytohemagglutinin (5 μg/mL) and IL-2 (20 U/mL) for 3 days before infection, and thereafter cultured in R10 with IL-2 only. One million stimulated PBMCs were infected in triplicate with 500 ng p24 of NL4-3 recombinant viruses encoding either wild-type SK93 Nef or SK93

```
CONSENSUS_C NNADCAWLEA QEEEEVGFPV RPQVPLRPMT YKAAFDLSFF LKEKGGLEGL 100
SK93        NNADCAWLQA QEEEEVGFPV RPQVPLRPMT YKAAVDLSFF LKEKGGLEGL
SK446       TNADCAWLEA QEEEEVGFPV RPQVPLRPMT FKGAFDLSFF LKEKGGLDGL
SK73        NNAACAWLEA QEEEEVGFPV RPQVPVRPMT YKAAFDLSFF LKEKGGLEGL
SK141       NNAECAWLQA QEEEEVGFPV RPQVPLRPMT YKAAVDLSFF LKEKGGLEGL

CONSENSUS_C IYSKKRQEIL DLWVYHTQGY FPDWQNYTPG PGVRYPLTFG WCFKLVPVDP 150
SK93        IYSKKRQEIL DLWVYHTQGF FPDWQNYTPG PGVRYPLTFG WCFKLVPVDP
SK446       IYSKKRQEIL DLWVYNTQGF FPDWQNYTPG PGVRYPLTFG WCYKLVPVDP
SK73        IYSKKRQEIL DLWVYNTQGF FPDWQNYTPG PGTRFPLTFG WCFKLVPVDP
SK141       IYSKRRQDIL DLWVYNTQGY FPDWQNYTPG PGVRYPLTFG WCFKLVPVDP

CONSENSUS_C REVEEANEGE NNCLLHPMSQ HGMEDEDREV LKWKFDSHLA RRHMARELHP 200
SK93        REVEEANEGE NNCLLHPMSQ HGIEDEEREV LRWKFDSSLA RRHLARELHP
SK446       REVEEANKGE NNCLLHPMSQ HGMEDENREV LKWQFDSSLA RRHMARELHP
SK73        REVEEENEGE NNSLLHPMSL HGMEDEHREV LKWKFDSQLG RRHMARELYP
SK141       REVEEANTGE NNCLLHPMSL HGIEDEEREV LKWQFDSSLA RRHMARELHP
```

FIGURE 1 Patient-derived Nef sequences into which mutations were introduced. Codons 50 to 200 of the patient-derived Nef sequences are shown relative to the consensus C Nef sequence. Sequences were aligned to HXB2. The additional subtype C specific residue in the $_{62}$EEEE$_{65}$ motif with respect to HXB2 was stripped out. Codons at which mutations were introduced are highlighted in red

Nef harboring the 199Y mutation, and incubated overnight. The culture was then pelleted, resuspended in fresh R10 medium with IL-2 and incubated for a further 48 hours. Thereafter, cells were stained using the LIVE/DEAD Fixable Aqua kit (Thermo Fisher Scientific) to discriminate between live and dead cells, and PE-labelled anti-HLA-A*02 antibody (BD Biosciences), followed by fixing and permeabilization using the BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were then stained with fluorescein isothiocyanate-labelled anti-HIV-1 Gag p24 antibody (clone kc57; Beckman Coulter) to detect infected cells. Data was acquired on the BD-LSRII (BD Biosciences). The percentage of HLA-I downregulation in PBMCs was calculated using the following equation: (PE median fluorescence intensity [MFI]$_{\text{Gag}-\text{cells}}$ − PE MFI$_{\text{Gag}+\text{cells}}$)/PE MFI$_{\text{Gag}-\text{cells}}$ × 100.

2.4 | Western blot analysis

Western blots analysis was performed as previously described to measure the expression levels of the Nef mutants.[27] Briefly, Nef was detected using rabbit polyclonal anti-HIV-1 Nef serum following transfection of 1 million HLA-A*02-expressing CEM-derived T cells with 10 μg Nef clone. Nef band intensity was calculated using ImageJ.[29] Actin was simultaneously detected and quantified, and Nef band intensity was normalized to that of actin. Western blot experiments were performed in duplicate and the results were averaged.

2.5 | Data analysis

Analysis of variance (ANOVA) with Tukey post hoc tests was performed to test for significant differences between the CD4/HLA-I downregulation function of the mutants and the wild-type in each Nef sequence background, where more than one mutant was evaluated. Where only two groups were compared, the Student *T* test was used. Nef expression levels and magnitude of CTL responses were correlated with HLA-I downregulation ability using Pearson's or Spearman's correlation, depending on whether the data were normally distributed or not. Fisher's exact test was used to compare the frequency of Nef clones grouped according to the high/low magnitude of CTL response and high/low magnitude of HLA-I downregulation ability. The *P* value cut-off was .05.

3 | RESULTS

3.1 | Mutations 102H, 105R, and 199Y decrease HLA-I downregulation

Mutations E93D, Y102H, K105R, E108D, V133T, and H199Y were introduced into patient-derived subtype C Nef sequences (as shown in Figure 1) and HLA-I as well as CD4 downregulation ability of these Nef mutant sequences was measured. Representative flow plots are shown in Figure 2A. In the SK93 Nef sequence background, the mutations 102H, 105R, and 199Y significantly impaired HLA-I downregulation, to 81%, 69% and 63% of wild-type levels, respectively (ANOVA with Tukey post hoc tests; all $P < .001$) (Figure 2B). The 133T mutation only slightly decreased HLA-I downregulation, to 94% of wild-type levels, and this was not statistically significant. As expected, the mutation 93D did not affect HLA-I downregulation ability and displayed the same activity as the wild-type Nef (100%). Although the mutation 108D was previously statistically associated with decreased HLA-I downregulation ability in patient-derived sequences,[26] it displayed 103% activity relative to the wild-type in the SK93 Nef sequence background. The Nef mutations were also introduced into the SK446 Nef sequence background. The effects of mutations 102H, 105R and 199Y were much less pronounced in the SK446 Nef sequence background, where these mutations displayed 91%, 96%, and 85% activity relative to the wild-type, respectively. However, the results obtained in the SK446 Nef sequence background were consistent with those

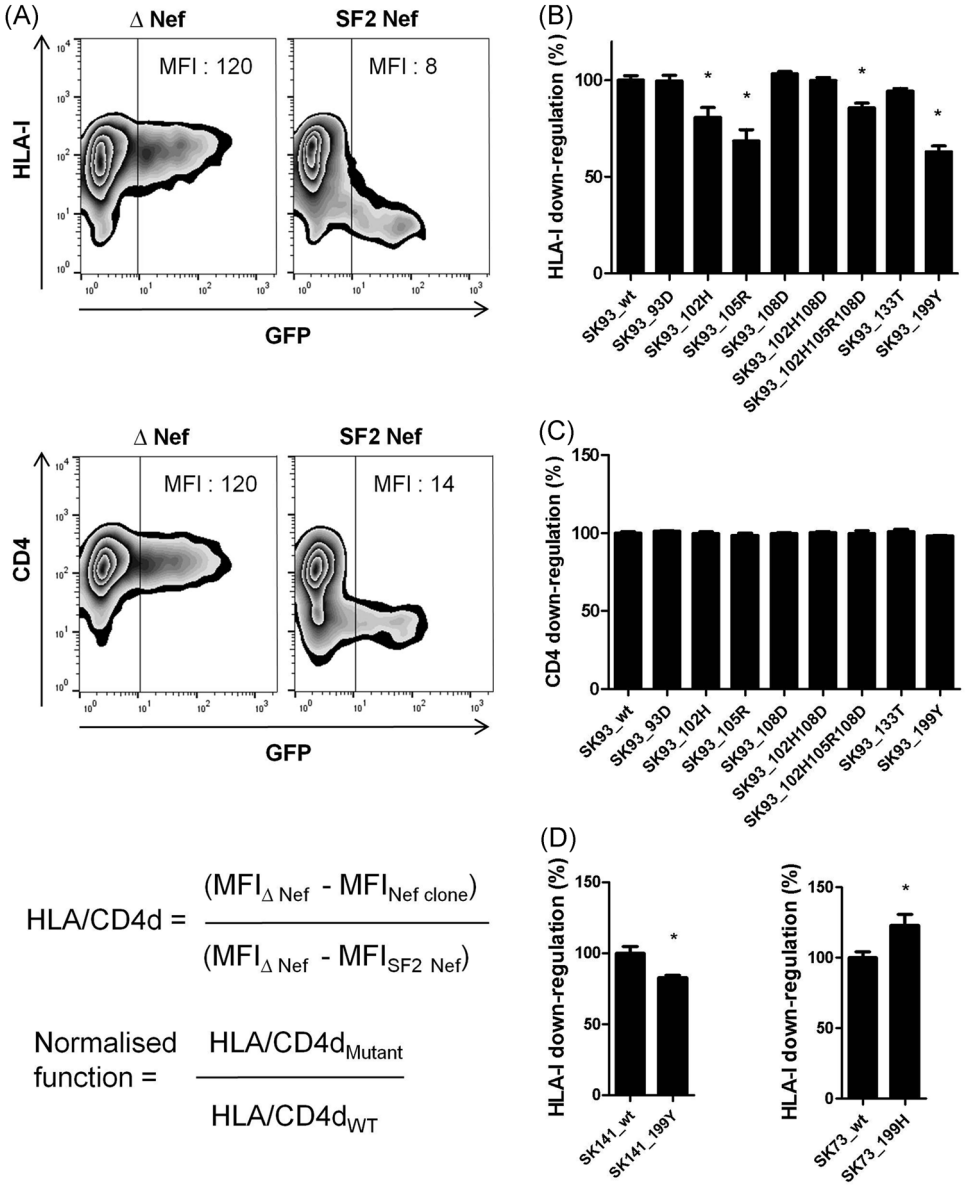

FIGURE 2 HLA-I and CD4 downregulation activities of Nef sequences into which HLA-associated mutations were introduced. A panel of HLA-associated mutations were introduced into a subtype C patient-derived Nef sequence (SK93) of high similarity to the consensus C Nef sequence. In addition, the 199Y mutation was introduced into SK141, and a patient-derived sequence that naturally encoded 199Y (SK73) was mutated to 199H. Representative flow cytometry plots showing median fluorescence intensities (MFI) of HLA-A*02/CD4 in cells expressing green fluorescent protein (GFP; Nef-transfected cells) for measurement of HLA-I/CD4 downregulation activity (HLA/CD4d), as well as calculations to normalize activity to the controls (Δ Nef and SF2 Nef), are in panel A. The HLA-I and CD4 downregulation activities of the SK93 mutants are shown in panels B and C, respectively, while HLA-I downregulation activities of the SK141 and SK73 mutants are shown in panel D. The HLA-I downregulation ability expressed relative to SF2 was 91%, 75%, and 52% for SK93, SK73, and SK141, respectively. In panels B-D, downregulation activity is expressed relative to the respective wild-type (WT) protein, which represents 100% activity. Bars represent the mean of at least three replicates, and error bars represent standard deviations from the means. Analysis of variance (ANOVA) with Tukey post hoc tests was performed to assess which SK93 mutants differed significantly from the WT and the Student *T* test was used to assess whether the mutation at codon 199 in the SK141 and SK73 sequences significantly affected HLA-I downregulation ability (indicated by asterisks; all $P < .01$)

obtained in the SK93 Nef sequence background in several respects: 102H and 199Y significantly decreased HLA-I downregulation ability (ANOVA with Tukey post hoc tests; both $P < .001$), and 133T, 93D and 108D did not significantly alter HLA-I downregulation ability (96%, 98%, and 100% of wild-type, respectively) (data not shown). None of the Nef mutants tested in this study compromised CD4 downregulation ability (all were within the range of 98% to 100% relative to the respective wild-type sequences) (Figure 2C). In summary, mutants 102H, 105R, and 199Y were confirmed to negatively affect HLA-I downregulation ability, but the effects of 102H and 105R were milder and less consistent than 199Y in different sequence backgrounds.

3.2 | 199Y consistently decreases HLA-I downregulation in different sequence backgrounds

In the SK93 and SK446 Nef sequences, the 199Y mutation had the most impact on HLA-I downregulation ability (Figure 2B). In patient-derived sequences, the presence of this HLA-associated mutation was associated with 28% lower HLA-I downregulation ability on average when compared with 102H, 105R and 108D which were associated with 6% to 8% lower HLA-I downregulation ability.[21] Furthermore, 199Y was naturally present in only 7 out of 298 patient-derived Nef sequences, while 102H, 105R and 108D were present in 45, 46 and 138 sequences, respectively.[21] The negative effect of 199Y on HLA-I downregulation ability was confirmed in a further two different Nef sequence backgrounds (Figure 2D). In the SK141 Nef sequence, the presence of 199Y reduced HLA-I downregulation ability to 83% of wild-type levels (Student *T* test; $P = .0004$) (Figure 2D). The 199Y mutation was naturally present in the SK73 Nef sequence (which had a Nef-mediated HLA-I downregulation ability of 52% relative to SF2 Nef), and, consistent with the negative effect of 199Y, the reversion of 199Y to the subtype C consensus 199H increased HLA-I downregulation ability to 123% of wild-type levels (Student *T* test; $P = .002$) (Figure 2D).

3.3 | Mutant 199Y Nef decreases HLA-I downregulation in PBMCs

To further confirm the effect of 199Y on HLA-I downregulation, NL4-3 recombinant viruses encoding SK93 Nef with and without the 199Y mutation were constructed and used to infect PBMCs from two different HIV-negative donors expressing HLA-A*02, followed by measurement of HLA-A*02 downregulation. The mutation 199Y decreased HLA-I downregulation ability to 66% and 12% of the wild-type Nef in donor 1 and 2, respectively (Student *T* test; $P = .03$), confirming the negative effect of this mutation on HLA-I downregulation ability (Figure 3).

3.4 | Nef expression levels of mutants directly correlate with HLA-I downregulation ability

Previous reports indicate that mutants with decreased Nef expression have a decreased ability to downregulate HLA-I.[28,30] Therefore, the effect of the Nef mutations studied on protein expression was investigated. Expression of all Nef mutant proteins was detected (Figure 4A), however expression of Nef mutants 102H and 199Y, which also significantly decreased HLA downregulation ability, were less than 70% of wild-type levels (63% and 33%, respectively). Furthermore, overall there was a significant correlation between protein expression and HLA-I downregulation ability (Pearson's correlation; $r = .79$ and $P = .02$) (Figure 4B).

3.5 | HLA downregulation ability is positively correlated with magnitude of CTL responses

Anmole et al[31] showed that HLA-A*02 downregulation ability of different Nef alleles, as measured in the same cell line and by the same methods described here, correlates strongly with effector T cell recognition of A*02-restricted FK10 peptide-pulsed cells expressing these different Nef alleles. Furthermore, in another study, the ability of different virus constructs to downregulate HLA-A*02 correlated negatively with HIV-specific CTL-mediated suppression in vitro.[32] This led to the idea that the Nef 199Y mutation, through impairing

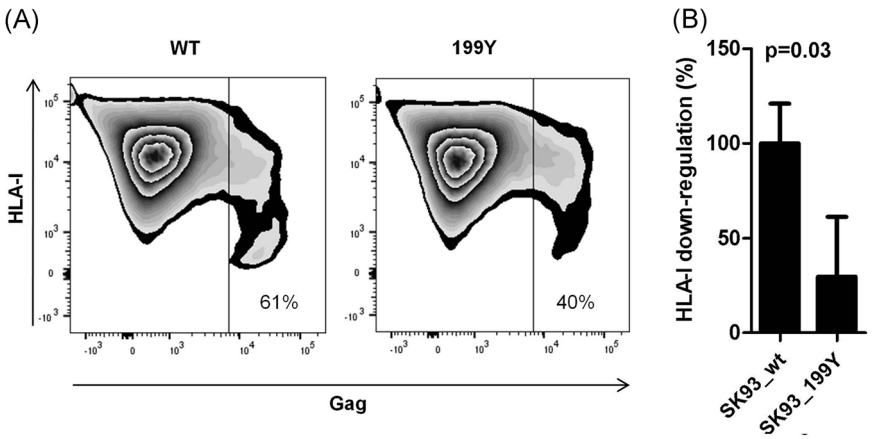

FIGURE 3 HLA-I downregulation activity of the 199Y mutant in peripheral blood mononuclear cells (PBMCs). HLA-A*02 downregulation activity was measured in PBMCs, from two different donors, that were infected with NL4-3 viruses encoding either the wild-type (WT) subtype C patient-derived Nef sequence (SK93) or SK93 Nef harboring the 199Y mutation. Flow cytometry plots in panel A show the HLA-A*02 expression levels in infected cells (cells positive for Gag) from donor 1, and values denote the percentage of HLA-A*02 downregulation. In panel D, downregulation activity is expressed relative to the WT, which represents 100% activity. Bars represent the mean of three replicates, and error bars represent standard deviations from the means. The 199Y mutation significantly decreased HLA-I downregulation activity when compared to the wild-type (Student *T* test; $P = .03$)

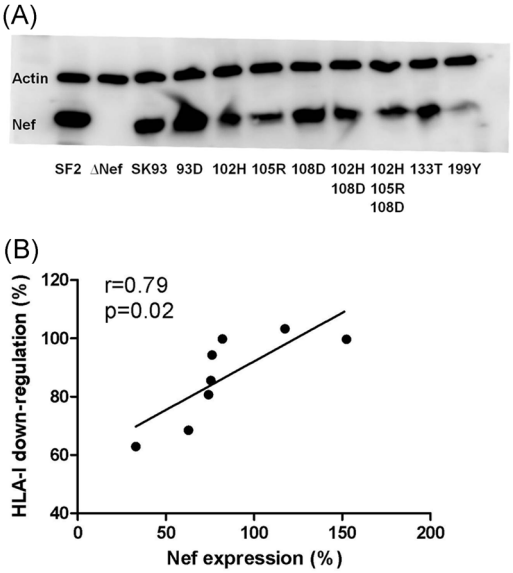

FIGURE 4 Expression of Nef mutants. The steady-state protein expression of Nef mutants by Western blot was measured in duplicate and a representative image is shown in panel A. SF2 Nef and empty vector (ΔNef) were included as positive and negative controls, respectively, while beta-actin protein was included as a cellular loading control. Band intensity, calculated using ImageJ, was used as the measure of Nef expression, which was normalized to that of beta-actin loading control. In panel B, a direct relationship between Nef expression level and HLA-I downregulation activity as assessed by Pearson's correlation test is shown. Nef expression and downregulation activity are expressed relative to the respective wild-type protein (SK93), which represents 100% expression/activity. The expression level and HLA-I downregulation ability of wild-type SK93 Nef expressed relative to SF2 Nef was 76% and 91%, respectively

Nef-mediated HLA-I downregulation, would result in an increased magnitude of CTL responses in vivo. However, in patients from whom Nef clones expressing the 199Y mutation were derived, the average magnitude of CTL responses, as previously measured by ELISPOT assays,[13,33] was lower than in those patients who did not harbor this Nef mutation (3401 vs. 6379 spot-forming units/million cells; Mann-Whitney, $P = .17$) (Figure 4A). Surprisingly, an analysis of the correlation between the HLA-I downregulation ability of all patient-derived Nef clones previously studied[21] and magnitude of CTL responses similarly showed a trend of an overall positive relationship between these two parameters (Spearman's correlation, $r = .13$ and $P = .08$) (Figure 4B), suggesting that increased Nef-mediated HLA-I downregulation ability may be required in response to increased CTL pressure. Accordingly, further analysis of Figure 4B by quadrants indicates that when CTL magnitude is high, HLA-downregulation ability is rarely impaired (upper left quadrant), while Nef clones with low HLA downregulation activity more frequently correspond with a low magnitude CTL response (lower left quadrant) (Fisher's exact, $P = .046$). The initially expected association of high HLA-I down-regulation ability and low magnitude of CTL response nevertheless appears to play a role, as Nef clones with high HLA-I downregulation ability less frequently correspond with a high magnitude CTL response (upper right quadrant) when compared with a low magnitude CTL response (lower right quadrant). Despite the two opposing drivers, the lack of data points in the upper left quadrant (low HLA downregulation, high magnitude CTL) appears to overall influence the correlation in a positive direction (Figure 5).

4 | DISCUSSION

The Nef protein has diverse functions that aid virus replication in vivo.[34] Nef-mediated downregulation of HLA-I from the surface of the infected cell is an important Nef function that allows HIV to avoid recognition and elimination of infected cells by CTL.[23] Previously it was shown that this activity of Nef is associated with disease progression rate in HIV-1 subtype C infection, and several HLA-associated mutations (likely CTL-driven escape mutations) were individually statistically associated with decreased Nef-mediated HLA-I downregulation ability in patient-derived Nef sequences.[21] CTL escape mutations that compromise the function of the pathogenic Nef protein may be relevant for an HIV-1 attenuation-based vaccine, particularly since Nef is a highly immunogenic protein[13] that has been included in most vaccine candidates that have undergone human clinical trials.[35] An attenuation-based vaccine seeks to exploit the natural escape routes of the virus that diminish its replication ability.[2] Therefore, the current study sought to confirm whether or not naturally-occurring HLA-associated mutations identified by statistics in a previous study[21] affected HLA-I down-regulation ability of the Nef protein.

Site-directed mutagenesis experiments using representative subtype C Nef alleles showed that 102H, 105R, and 199Y mutations have a significant negative effect on the HLA-I down-regulation ability of Nef, although only 199Y had a substantial negative effect in all Nef backgrounds tested. Supporting that 102H and 199Y have a viral fitness cost, in a previous analysis of >700 subtype C Nef sequences these HLA-associated mutations were statistically associated with reversion in the absence of the selecting HLA allele.[14]

The impact of the Nef mutations studied here on HLA-I downregulation also correlated strongly with protein expression level, suggesting that the effect of 102H, 105R, and 199Y on HLA-I downregulation was mediated through decreased Nef expression or stability. The decrease in Nef protein levels mediated by these mutations did not however affect CD4 downregulation ability, which is consistent with previous reports that higher intracellular concentrations of Nef are required for HLA-I downregulation when compared with that required for CD4 downregulation.[28,36]

Nef residue 105 is one of the residues that was previously reported to contribute to Nef dimerization, which is essential for Nef-mediated CD4 downregulation and enhancement of viral replication.[37] Previously it was shown that 105E but not 105R/K affected dimerization and that all Nef mutants partially or completely affecting dimerization had substantial negative effects on CD4

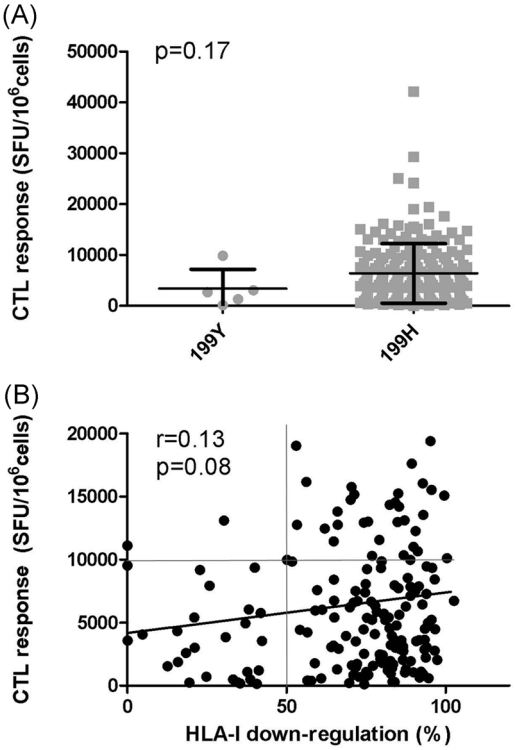

FIGURE 5 Relationship between the total magnitude of CD8+ T cell (CTL) responses and Nef-mediated HLA-I downregulation ability. The total magnitude of HIV-specific CTL responses was measured by ELISPOT assays in spot-forming units (SFU) per million cells. The difference in the magnitude of CTL responses made by patients harboring viruses with and without the Nef 199Y mutation is shown in panel A. Bars indicate the mean, error bars indicate standard deviation from the mean, and the Mann-Whitney U test p value is shown. A weak positive correlation between the total magnitude of CTL responses and the ability of Nef to downregulate HLA-I is shown in panel B (Spearman's correlation). Gray lines indicate four quadrants on the graph in panel B corresponding to: low HLA-I downregulation and high magnitude CTL response (upper left quadrant), low HLA-I downregulation and low magnitude CTL response (lower left quadrant), high HLA-I downregulation and high magnitude CTL response (upper right quadrant), and high HLA-I downregulation and low magnitude CTL response (lower right quadrant). The frequency of Nef clones is significantly different between the four groups (Fisher's exact)

downregulation ability.[37] Since the naturally-occurring K105R mutation in subtype C Nef (105R is the consensus amino acid in subtype B) did not affect CD4 downregulation, it is unlikely to have had an impact on Nef dimerization.

Nef residue 199 is at the carboxy-terminus of Nef, which plays an important role in stabilizing the Nef, HLA-I and AP-1 complex that is formed during Nef-mediated downregulation of HLA-I.[38] Mutation of both the 202 and 203 Nef residues to alanine abrogates formation of the complex.[38] Given the close proximity of Nef residue 199 to residues 202 and 203, it is possible that the 199Y mutation partly affects the stability of the 3-way interaction between Nef, HLA-I, and AP-1, in addition to affecting the stability of the Nef protein, thereby affecting Nef-mediated HLA-I downregulation.

The ability of Nef to downregulate HLA-I was previously shown to correspond with the ability to evade CTL-based elimination of infected cells.[23,31,32] In the current study a trend, albeit modest, of a lower magnitude of CTL responses in patients who harbor Nef alleles with decreased HLA-I downregulation ability was observed. Consistent with this, a positive correlation between Nef-mediated HLA-I downregulation and breadth of CTL response in chronic infection has been observed,[32] and it was demonstrated that CTL pressure in vitro selects Nef sequences with high HLA-I downregulation function from the in vivo quasispecies.[39] Similarly, in another study, preservation of HLA-downregulation ability from acute infection to establishment of viral set point was associated with a greater breadth of CTL response.[40] The relationship between HLA-I downregulation ability and CTL response in the acute phase may differ, however, as suggested by a higher CTL response at 4 to 16 weeks postinfection in macaques infected with SIV defective for Nef-mediated HLA-I downregulation when compared with those infected by wild-type SIV.[23] Taken together, not only does HLA-I downregulation ability shape the CTL response but the CTL response also influences HLA-I downregulation ability: a likely explanation for the overall positive correlation between the CTL response and HLA-I downregulation following the acute phase is that Nef adapts to its environment over time—greater HLA-I downregulation ability is selected for when there is strong CTL pressure.[32,39] Due to the cross-sectional nature of the current study we were unable to fully explore the relationship between the CTL response and HLA-I downregulation ability over the course of infection, and longitudinal studies will be required to confirm this hypothesis. Overall, targeting HLA-I downregulation through vaccination could improve CTL activity against infected cells and thereby improve virus control. Considering the HLA-associated mutations studied here that affect HLA-I downregulation ability of Nef, 102H and 105R occur in an epitope-rich region of Nef which is targeted by several different HLA alleles.[41] Interestingly, the region 105 to 114 is targeted by protective HLA alleles (B*27:05 in humans and Mamu-B*08 in macaques) and CTL responses to an overlapping peptide 88 to 105 were associated with significantly lower viral loads.[14] In contrast, very few epitopes that span codon 199 have been reported and the HLA restriction is narrow,[41] thus this region may be more challenging to target with a CTL-based vaccine than codons 102 to 108. In Mauritian cynomolgus macaques, targeting of Nef codons 196 to 203 correlated with virus control,[19] supporting that this is a beneficial region of Nef to target with a CTL-based vaccine.

Following the sequence-function analysis of 298 patient-derived Nef sequences[21] and mutagenesis confirmation described here, 199Y was the only HLA-driven mutation found to notably and consistently affect HLA-I downregulation. This is consistent with previous studies showing that single immune-driven mutations infrequently have much effect on the function of the Nef protein. For example, in the PxxP motif, CTL escape mutations at codons 75 and 85 in combination, but not individually, affected HLA-I downregulation.[16] HLA-B*13-associated Nef mutations did not significantly affect virus replication or Nef function, however one combination of these mutations (E24Q-Q107R) resulted in substantially reduced HLA-I downregulation.[15] Similarly, in an elite controller harboring a Nef sequence encoding several mutations associated with their HLA

alleles, HLA-I downregulation ability was only impaired when all mutations were present.[17] Thus, with few exceptions noted,[28,30] Nef mutations that occur naturally seldom significantly affect its function when occurring individually.

A possible limitation of the methods in the present study is the measurement of Nef-mediated HLA-I downregulation in a CEM-derived cell line engineered to express HLA-A*02 only. However, the results for the 199Y mutation were validated in PBMCs. Furthermore, previous studies have shown that Nef-mediated HLA-I downregulation results are highly concordant between different cell lines as well as primary cells and between different HLA alleles within the HLA-A and HLA-B groups respectively.[42,43] HLA-B alleles are however consistently downregulated less efficiently than HLA-A alleles.[42–44] While the magnitude of downregulation differs between HLA-A and HLA-B alleles, the downregulation of these alleles by different Nef clones are very strongly correlated ($r = .89$ and $P < .0001$).[27] Furthermore, although polymorphisms at two Nef codons, 9 and 202, were reported to differentially affect HLA-A and HLA-B downregulation with more pronounced effects on HLA-B alleles,[42,43] these polymorphisms significantly affect both groups of alleles in the same direction.[27,42,43] Taken together, HLA-A and HLA-B downregulation abilities of Nef clones are closely linked and the results obtained in this study are likely to be overall reflective of Nef-mediated HLA-I downregulation.

In summary, these results highlight regions of Nef where HLA-driven mutations may affect its ability to downregulate HLA-I and consequently evade CTL responses. These regions may be useful as vaccine targets to maximize the effectiveness of CTL responses through diminishing Nef's ability to evade them.

ACKNOWLEDGMENTS

This study was supported by a Sullivan Early Career Scientist Award from the Sullivan Family Foundation to JM. JM received additional funding from the National Research Foundation. TN received additional funding from the South African Department of Science and Technology through the National Research Foundation (South African Research Chairs Initiative), the Victor Daitz Foundation and the Howard Hughes Medical Institute. Additional funding was provided by the Sub-Saharan African Network for TB/HIV Research Excellence (SANTHE), a DELTAS Africa Initiative (grant # DEL-15-006). The DELTAS Africa Initiative is an independent funding scheme of the African Academy of Sciences (AAS)'s Alliance for Accelerating Excellence in Science in Africa (AESA) and supported by the New Partnership for Africa's Development Planning and Coordinating Agency (NEPAD Agency) with funding from the Wellcome Trust (grant #107752/Z/15/Z) and the UK government. The views expressed in this publication are those of the author(s) and not necessarily those of AAS, NEPAD Agency, Wellcome Trust or the UK government.

AUTHOR CONTRIBUTIONS

Conceived the study: JKM and TN. Constructed mutant clones: ER, QM, and PM under the supervision of JKM. Performed HLA downregulation analysis: JKM, ER, QM, and OB. Performed Western blot: SWJ under the supervision of MAB. Analyzed the data and wrote the paper: JKM. Critically reviewed and edited the paper: SWJ, OB, MAB, and TN.

ORCID

Jaclyn K. Mann http://orcid.org/0000-0002-2398-1273

REFERENCES

1. Johnston MI, Fauci AS, An HIV. Vaccine—challenges and prospects. *N Engl J Med*. 2008;359(9):888-890.
2. Chopera DR, Wright JK, Brockman MA, Brumme ZL. Immune-mediated attenuation of HIV-1. *Future Virol*. 2011;6(8):917-928.
3. Dahirel V, Shekhar K, Pereyra F, etal. Coordinate linkage of HIV evolution reveals regions of immunological vulnerability. *Proc Natl Acad Sci USA*. 2011;108(28):11530-11535.
4. Ferguson AL, Mann JK, Omarjee S, Ndung'u T, Walker BD, Chakraborty AK. Translating HIV sequences into quantitative fitness landscapes predicts viral vulnerabilities for rational immunogen design. *Immunity*. 2013;38:606-617.
5. Brockman MA, Schneidewind A, Lahaie M, etal. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *J Virol*. 2007;81(22):12608-12618.
6. Crawford H, Lumm W, Leslie A, etal. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *J Exp Med*. 2009;206:909-919.
7. Schneidewind A, Brockman MA, Yang R, etal. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *J Virol*. 2007;81:12382-12393.
8. Wright JK, Naidoo VL, Brumme ZL, etal. Impact of HLA-B*81-associated mutations in HIV-1 Gag on viral replication capacity. *J Virol*. 2012;86(6):3193-3199.
9. Troyer RM, McNevin J, Liu Y, etal. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLOS Pathog*. 2009;5(4):1-13.
10. Rolland M, Manocheewa S, Swain JV, etal. HIV-1 conserved-element vaccines: relationship between sequence conservation and replicative capacity. *J Virol*. 2013;87(10):5461-5467.
11. Kestier HW, Ringler DJ, Mori K, etal. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. *Cell*. 1991;65(4):651-662.
12. Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N Engl J Med*. 1995;332:228-232.
13. Radebe M, Nair K, Chonco F, etal. Limited immunogenicity of HIV CD8+ T-cell epitopes in acute clade C virus infection. *J Infect Dis*. 2011;204(5):768-776.
14. Adland E, Carlson JM, Paioni P, et al. Nef-specific CD8+ T cell responses contribute to HIV-1 immune control. *PLOS One*. 2013;8(9):e73117. https://doi.org/10.1371/journal.pone.0073117
15. Shahid A, Olvera A, Anmole G, etal. Consequences of HLA-B*13-associated escape mutations on HIV-1 replication and Nef function. *J Virol*. 2015;89(22):11557-11571. https://doi.org/10.1128/JVI.01955-15
16. Ueno T, Motozono C, Dohki S, etal. CTL-Mediated selective pressure influences dynamic evolution and pathogenic functions of HIV-1 Nef. *J Immunol*. 2008;180:1107-1116.
17. Kuang XT, Li X, Anmole G, etal. Impaired Nef function is associated with early control of HIV-1 viremia. *J Virol*. 2014;88(17):10200-10213. https://doi.org/10.1128/JVI.01334-14

18. Jin SW, Alsahafi N, Kuang XT, etal. Natural HIV-1 Nef polymorphisms impair SERINC5 downregulation activity. *Cell Rep*. 2019;29(6):1449-1457. https://doi.org/10.016/j.celrep.2019.10.007
19. Budde ML, Greene JM, Chin EN, etal. Specific CD8+ T cell responses correlate with control of simian immunodeficiency virus replication in Mauritian cynomolgus macaques. *J Virol*. 2012;86(14):7596-7604.
20. Mudd PA, Martins MA, Ericsen AJ, etal. Vaccine-induced CD8+ T cells control AIDS virus replication. *Nature*. 2012;491(7422):129-133.
21. Mann JK, Chopera D, Omarjee S, etal. 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. *Virology*. 2014;468-470:214-225. https://doi.org/10.1016/j.virol.2014.08.009
22. Carlson JM, Schaefer M, Monaco DC, etal. HIV transmission. Selection bias at the heterosexual HIV-1 transmission bottleneck. *Science*. 2014;345(6193):1254031. https://doi.org/10.1126/science
23. Swigut T, Alexander L, Morgan J, etal. Impact of Nef-mediated downregulation of major histocompatibility complex class I on immune response to simian immunodeficiency virus. *J Virol*. 2004;78(23):13335-13344.
24. Noviello CM, Pond SLK, Lewis MJ, etal. Maintenance of Nef-mediated modulation of major histocompatibility complex class I and CD4 after sexual transmission of human immunodeficiency virus type 1. *J Virol*. 2007;81(9):4776-4786.
25. Mwimanzi P, Markle TJ, Ogata Y, etal. Dynamic range of Nef functions in chronic HIV-1 infection. *Virology*. 2013;439(2):74-80.
26. Mann JK, Chopera D, Omarjee S, etal. 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. *Virology*. 2014;468:214-225.
27. Mann JK, Byakwaga H, Kuang XT, etal. Ability of HIV-1 Nef to downregulate CD4 and HLA class I differs among viral subtypes. *Retrovirology*. 2013;10(1):100. https://doi.org/10.1186/742-4690-10-100
28. Mann JK, Omarjee S, Khumalo P, Ndung'u T. Genetic determinants of Nef-mediated CD4 and HLA class I down-regulation differences between HIV-1 subtypes B and C. *Virol J*. 2015;12:200. https://doi.org/10.1186/s12985-015-0429-7
29. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. 2012;9:671-675.
30. Johnson AL, Dirk BS, Coutu M, et al. A highly conserved residue in HIV-1 Nef alpha Helix 2 modulates protein expression. *mSphere*. 2016;1(6):e00288-16.
31. Anmole G, Kuang XT, Toyoda M, etal. A robust and scalable TCR-based reporter cell assay to measure HIV-1 Nef-mediated T cell immune evasion. *J Immunol Methods*. 2015;426:104-113. https://doi.org/10.1016/j.jim.2015.08.010
32. Lewis MJ, Balamurugan A, Ohno A, Kilpatrick S, Ng HL, Yang OO. Functional adaptation of Nef to the immune milieu of HIV-1 infection in vivo. *J Immunol*. 2008;180(6):4075-4081.
33. Kiepiela P, Ngumbela K, Thobakgale C, etal. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med*. 2007;13(1):46-53.
34. Foster JL, Denial SJ, Temple BRS, Garcia JV. Mechanisms of HIV-1 Nef function and intracellular signaling. *J Neuroimmune Pharmacol*. 2011;6:230-246.
35. Esparza J. A brief history of the global effort to develop a preventive HIV vaccine. *Vaccine*. 2013;31(35):3502-3518.
36. Liu X, Schrager JA, Lange GD, Marsh JW. HIV Nef-mediated cellular phenotypes are differentially expressed as a function of intracellular Nef concentrations. *J Biol Chem*. 2001;276(35):32763-32770.
37. Poe JA, Smithgall TE. HIV-1 Nef dimerization is required for Nef-mediated receptor downregulation and viral replication. *J Mol Biol*. 2009;394(2):329-342. https://doi.org/10.1016/j.jmb.2009.09.047
38. Jia X, Singh R, Homann S, Yang H, Guatelli J, Xiong Y. Structural basis of evasion of cellular adaptive immunity by HIV-1 Nef. *Nat Struct Mol Biol*. 2012;19(7):701-706.
39. Lewis MJ, Lee P, Ng HL, Yang OO. Immune selection in vitro reveals human immunodeficiency virus type 1 Nef sequence motifs important for its immune evasion function in vivo. *J Virol*. 2012;86(13):7126-735.
40. De La Cruz J, Vollbrecht T, Frohnen P, etal. Ineffectual targeting of HIV-1 Nef by cytotoxic T lymphocytes in acute infection results in no functional impairment or viremia reduction. *J Virol*. 2014;88(14):7881-7892. https://doi.org/10.1128/JVI.00482-14
41. Los Alamos HIV Immunology Database. CTL/CD8+ Epitope summary.
42. Mahiti M, Toyoda M, Jia X, Kuang XT, Mwimanzi F, Mwimanzi P, Walker BD, Xiong Y, Brumme ZL, Brockman MA, Ueno T, et al. Relative resistance of HLA-B to downregulation by naturally occurring HIV-1 Nef sequences. *mBio*. 2016;7(1):e01516-15. https://doi.org/10.1128/mBio.-15
43. Mwimanzi F, Toyoda M, Mahiti M, et al. Resistance of major histocompatibility complex class B (MHC-B) to Nef-mediated down-regulation relative to that of MHC-A is conserved among primate lentiviruses and influences antiviral T cell responses in HIV-1-infected individuals. *J Virol*. 2018;92(1):e01409-17. https://doi.org/10.1128/JVI.01409-17
44. Rajapaksa US, Li D, Peng YC, McMichael AJ, Dong T, Xu XN. HLA-B may be more protective against HIV-1 than HLA-A because it resists negative regulatory factor (Nef) mediated down-regulation. *Proc Natl Acad Sci USA*. 2012;109(33):13353-13358.

How to cite this article: Mann JK, Rajkoomar E, Jin SW, et al. Consequences of HLA-associated mutations in HIV-1 subtype C Nef on HLA-I downregulation ability. *J Med Virol*. 2020;92:1182–1190. https://doi.org/10.1002/jmv.25676