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

Running title: HLA down-regulation ability of HIV Nef mutants

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Author contributions

Conceived the study: JKM and TN. Constructed mutant clones: ER, QM and PM under the supervision of JKM. Performed HLA down-regulation 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.

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 down-regulation 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 down-regulate HLA-I was measured by flow cytometry in a CEM-derived T cell line. A substantial negative effect of 199Y on HLA-I down-regulation and Nef expression was observed, while 102H and 105R displayed negative effects on HLA-I down-regulation ability and Nef expression to a lesser extent. The total magnitude of CTL responses in individuals harbouring 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 down-regulation ability and total magnitude of CTL responses was observed, suggesting that there is a higher requirement for HLA-I down-regulation with increased CTL pressure. These results highlight a region of Nef that could be targeted by vaccine-induced CTL to reduce HLA-I down-regulation and maximise CTL efficacy.

Key words: HIV-1 Nef, Nef-mediated HLA-I down-regulation, CTL responses, mutations

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 down-regulation 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 a 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 down-regulation ability was observed [21]. In addition, several HLA-I associated Nef polymorphisms (likely escape mutations as described in [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 down-regulation ability, were identified [21]. HLA-I down-regulation 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 down-regulation [23], maintenance of HLA-I down-regulation activity in chronic infection [24, 25], and correlation of HLA-I down-regulation 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 down-regulate HLA-I. The effects of these mutations on Nef expression and magnitude of CTL responses were also explored.

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Methods

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 133V was statistically associated with increased HLA-I down-regulation [26], indirectly suggesting that escape at this codon compromises HLA-I down-regulation 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 down-regulation ability [26]. Based on the previous statistical analysis of patient-derived sequences, H199Y was expected to have a greater impact on HLA-I down-regulation 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 down-regulation 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, USA). 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, USA).

CD4 and HLA-I down-regulation 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 down-regulation 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 normalised 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 down-regulation activity and a value of 100% indicated down-regulation activity equivalent to SF2 Nef, as previously published [26-28]. Experiments were performed at least in triplicate and results averaged.

HLA-I down-regulation assays in peripheral blood mononuclear cells (PBMCs)

PBMCs from two HIV-negative donors expressing HLA-A*02 were stimulated with phytohaemmaglutinin (5 μg/ml) and IL-2 (20 U/ml) for three days prior to 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 Nef harbouring 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 down-regulation in PBMCs was calculated using the following equation: (PE median fluorescence intensity $[MFI]_{Gag- cells} - PE\ MFI_{Gag+ cells})/PE\ MFI_{Gag- cells}$ X 100.

Western blot

Western blots were performed as previously described to measure 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.

Data analysis

ANOVA with Tukey post-hoc tests was performed to test for significant differences between the CD4/HLA-I down-regulation 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's T test was used. Nef expression levels and magnitude of CTL responses were correlated with HLA-I down-regulation ability using Pearson's or Spearman's correlation, depending on whether the data was normally distributed or not. Fisher's exact test was used to compare the frequency of Nef clones grouped according to high/low magnitude of CTL response and high/low magnitude of HLA-I down-regulation ability. The p value cut-off was 0.05.

Results

Mutations 102H, 105R and 199Y decrease HLA-I down-regulation

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 down-regulation 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 down-regulation, to 81%, 69% and 63% of wild-type levels, respectively (ANOVA with Tukey post-hoc tests; all $p<0.001$) (Figure 2B). The 133T mutation only slightly decreased HLA-I down-regulation, to 94% of wild-type levels, and this was not statistically significant. As expected, the mutation 93D did not affect HLA-I down-regulation ability and displayed the same activity as the wild-type Nef (100%). Although the mutation 108D was previously statistically associated with decreased HLA-I down-regulation 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

194 in the SK446 Nef sequence background were consistent with those obtained in the SK93 Nef
195 sequence background in several respects: 102H and 199Y significantly decreased HLA-I
196 down-regulation ability (ANOVA with Tukey post-hoc tests; both $p<0.001$), and 133T, 93D
197 and 108D did not significantly alter HLA-I down-regulation ability (96%, 98% and 100% of
198 wild-type, respectively) (data not shown). None of the Nef mutants tested in this study
199 compromised CD4 down-regulation ability (all were within the range of 98-100% relative to
200 the respective wild-type sequences) (Figure 2C). In summary, mutants 102H, 105R and
201 199Y, were confirmed to negatively affect HLA-I down-regulation ability, but the effects of
202 102H and 105R were milder and less consistent than 199Y in different sequence
203 backgrounds.

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205 **199Y consistently decreases HLA-I down-regulation in different sequence backgrounds**

206 In the SK93 and SK446 Nef sequences, the 199Y mutation had the most impact on HLA-I
207 down-regulation ability (Figure 2B). In patient-derived sequences, the presence of this HLA-
208 associated mutation was associated with 28% lower HLA-I down-regulation ability on
209 average when compared with 102H, 105R and 108D which were associated with 6-8% lower
210 HLA-I down-regulation ability [21]. Furthermore, 199Y was naturally present in only 7 out of
211 298 patient-derived Nef sequences, while 102H, 105R and 108D were present in 45, 46 and
212 138 sequences, respectively [21]. The negative effect of 199Y on HLA-I down-regulation
213 ability was confirmed in a further two different Nef sequence backgrounds (Figure 2D). In
214 the SK141 Nef sequence, the presence of 199Y reduced HLA-I down-regulation ability to
215 83% of wild-type levels (Student's T test; $p=0.0004$) (Figure 2D). The 199Y mutation was
216 naturally present in the SK73 Nef sequence (which had a Nef-mediated HLA-I down-
217 regulation 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 down-regulation ability to 123% of wild-type levels (Student's T test; p=0.002) (Figure 2D).

Mutant 199Y Nef decreases HLA-I down-regulation in PBMCs

To further confirm the effect of 199Y on HLA-I down-regulation, 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 down-regulation. The mutation 199Y decreased HLA-I down-regulation ability to 66% and 12% of the wild-type Nef in donor 1 and 2, respectively (Student's T test; p=0.03), confirming the negative effect of this mutation on HLA-I down-regulation ability (Figure 3).

Nef expression levels of mutants directly correlate with HLA-I down-regulation ability

Previous reports indicate that mutants with decreased Nef expression have a decreased ability to down-regulate 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 down-regulation 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 down-regulation ability (Pearson's correlation; r=0.79 and p=0.02) (Figure 4B).

HLA down-regulation ability is positively correlated with magnitude of CTL responses

Anmole *et al.* (2015) showed that HLA-A*02 down-regulation 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 [31]. Furthermore, in another study, the ability of different virus constructs to down-regulate 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 Nef-mediated HLA-I down-regulation, would result in 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 harbour this Nef mutation (3401 vs. 6379 spot-forming units/million cells; Mann-Whitney, $p = 0.17$) (Figure 4A). Surprisingly, an analysis of the correlation between the HLA-I down-regulation 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, $r = 0{,}13$ and $p = 0.08$) (Figure 4B), suggesting that increased Nef-mediated HLA-I down-regulation 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-down-regulation ability is rarely impaired (upper left quadrant), while Nef clones with low HLA down-regulation activity more frequently correspond with a low magnitude CTL response (lower left quadrant) (Fisher's exact, $p = 0.046$). 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 down-regulation 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 down-

regulation, high magnitude CTL) appears to overall influence the correlation in a positive direction.

Discussion

The Nef protein has diverse functions that aid virus replication *in vivo* [34]. Nef-mediated down-regulation 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 down-regulation 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 down-regulation also correlated strongly with protein expression level, suggesting that the effect of 102H, 105R and 199Y on HLA-I down-regulation was mediated through decreased Nef expression or stability. The decrease in Nef protein levels mediated by these mutations did not however affect CD4 down-regulation ability, which is consistent with previous reports that higher intracellular concentrations of Nef are required for HLA-I down-regulation when compared with that required for CD4 down-regulation [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 down-regulation 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 down-regulation 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 down-regulation, 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 down-regulation 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 stability of the 3-way interaction

between Nef, HLA-I and AP-1, in addition to affecting stability of the Nef protein, thereby affecting Nef-mediated HLA-I down-regulation.

The ability of Nef to down-regulate HLA-I was previously shown to correspond with 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 down-regulation ability was observed. Consistent with this, a positive correlation between Nef-mediated HLA-I down-regulation 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 down-regulation 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 down-regulation ability and CTL response in the acute phase may differ however, as suggested by a higher CTL response at 4-16 weeks post-infection in macaques infected with SIV defective for Nef-mediated HLA-I down-regulation when compared with those infected by wild-type SIV [23]. Taken together, not only does HLA-I down-regulation ability shape the CTL response but the CTL response also influences HLA-I down-regulation ability: a likely explanation for the overall positive correlation between the CTL response and HLA-I down-regulation following the acute phase is that Nef adapts to its environment over time – greater HLA-I down-regulation 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 down-regulation ability over the course of infection, and longitudinal studies will be required to confirm this hypothesis. Overall, targeting HLA-I down-regulation 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 down-regulation 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 105-114 is targeted by protective HLA alleles (B*27:05 in humans and Mamu-B*08 in macaques) and CTL responses to an overlapping peptide 88-105 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 102-108. In Mauritian cynomolgus macaques, targeting of Nef codons 196-203 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 down-regulation. 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 down-regulation [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 down-regulation [15]. Similarly, in an elite controller harbouring a Nef sequence encoding several mutations associated with their HLA alleles, HLA-I down-regulation 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 down-regulation 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 down-regulation 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 down-regulated less efficiently than HLA-A alleles [42-44]. While the magnitude of down-regulation differs between HLA-A and HLA-B alleles, the down-regulation of these alleles by different Nef clones are very strongly correlated ($r=0.89$ and $p<0.0001$) [27]. Furthermore, although polymorphisms at two Nef codons, 9 and 202, were reported to differentially affect HLA-A and HLA-B down-regulation 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 down-regulation 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 down-regulation.

In summary, these results highlight regions of Nef where HLA-driven mutations may affect its ability to down-regulate 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.

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Figure legends

Figure 1. Patient-derived Nef sequences into which mutations were introduced.

Codons 50-200 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.

Figure 2. HLA-I and CD4 down-regulation 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 which 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 down-regulation activity (HLA/CD4d), as well as calculations to normalise activity to the controls (Δ Nef and SF2 Nef), are in panel A. The HLA-I and CD4 down-regulation activities of the SK93 mutants are shown in panels B and C, respectively, while HLA-I down-regulation activities of the SK141 and SK73 mutants are shown in panel D. The HLA-I down-regulation ability expressed relative to SF2 was 91%, 75%, and 52% for SK93, SK73 and SK141, respectively. In panels B-D, down-regulation 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. ANOVA with Tukey post-hoc tests was performed to assess which SK93 mutants differed significantly from the wild-type, and the Student's T test was used to assess whether the mutation at codon 199 in the SK141 and SK73 sequences significantly affected HLA-I down-regulation ability (indicated by asterisks; all $p<0.01$).

Figure 3. HLA-I down-regulation activity of the 199Y mutant in peripheral blood mononuclear cells (PBMCs)

HLA-A*02 down-regulation 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 harbouring 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 down-regulation. In panel D, down-regulation activity is expressed relative to the WT, which represents 100% activity. Bars represent the mean of three replicates, and error bars represent standard

563 deviations from the means. The 199Y mutation significantly decreased HLA-I down-
564 regulation activity when compared to the wild-type (Student's T test; p=0.03).

565

566 **Figure 4. Expression of Nef mutants.**

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

577

578 **Figure 5. Relationship between the total magnitude of CD8+ T cell (CTL) responses and**
579 **Nef-mediated HLA-I down-regulation ability.**

580 The total magnitude of HIV-specific CTL responses was measured by ELISPOT assays in
581 spot-forming units (SFU) per million cells. The difference in the magnitude of CTL
582 responses made by patients harbouring viruses with and without the Nef 199Y mutation is
583 shown in panel A. Bars indicate the mean, error bars indicate standard deviation from the
584 mean, and the Mann-Whitney U test p value is shown. A weak positive correlation between
585 the total magnitude of CTL responses and the ability of Nef to down-regulate HLA-I is
586 shown in panel B (Spearman's correlation). Grey lines indicate four quadrants on the graph

587 in panel B corresponding to: low HLA-I down-regulation and high magnitude CTL response
588 (upper left quadrant), low HLA-I down-regulation and low magnitude CTL response (lower
589 left quadrant), high HLA-I down-regulation and high magnitude CTL response (upper right
590 quadrant), and high HLA-I down-regulation and low magnitude CTL response (lower right
591 quadrant). The frequency of Nef clones is significantly different between the four groups
592 (Fisher's exact).
593

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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
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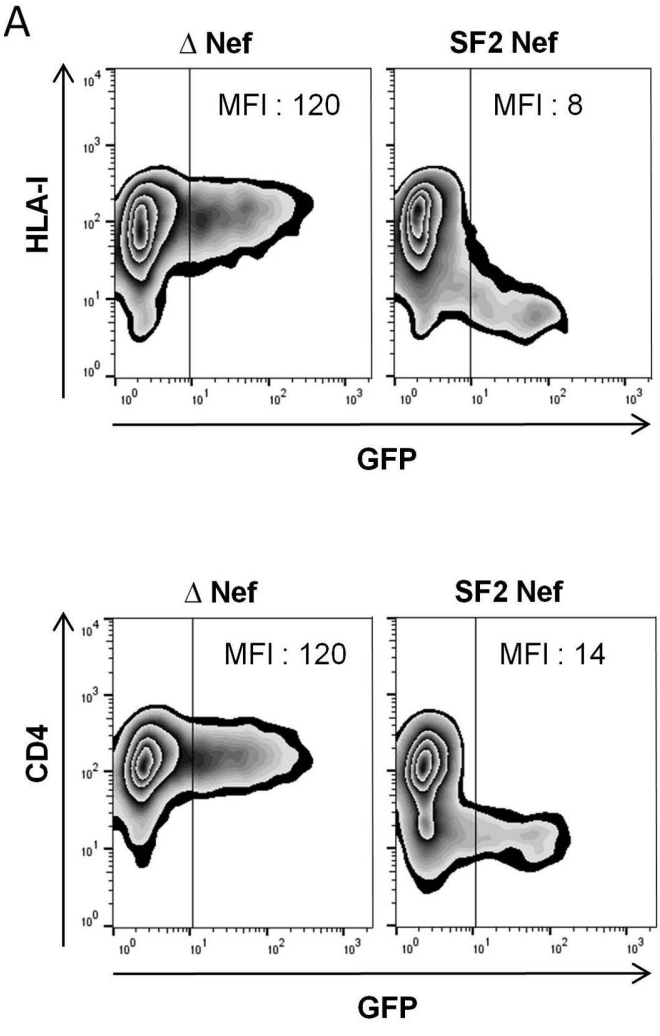
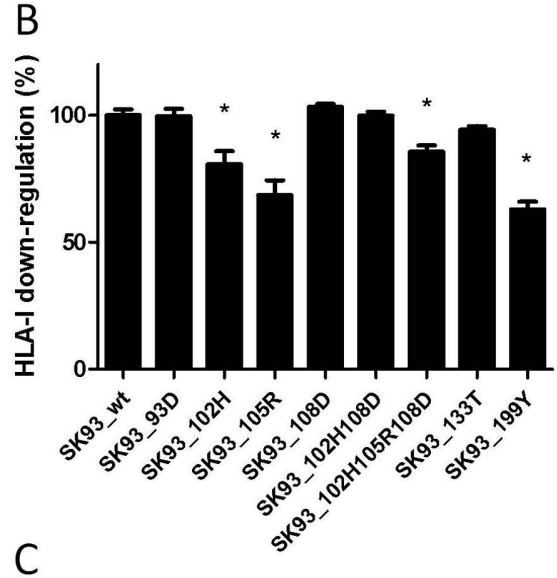
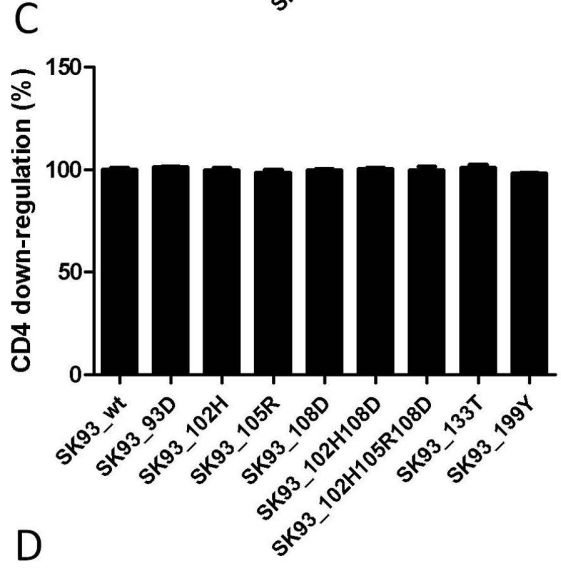
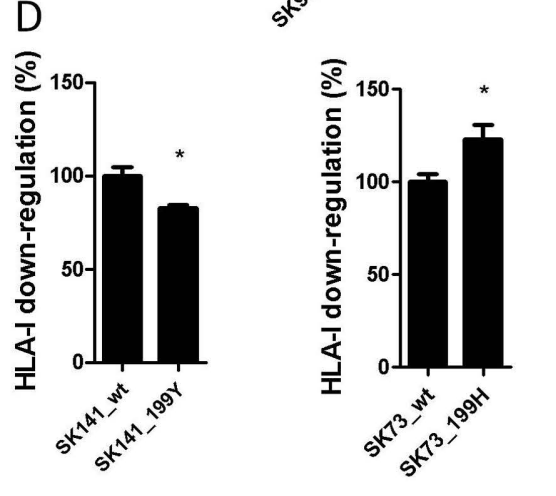


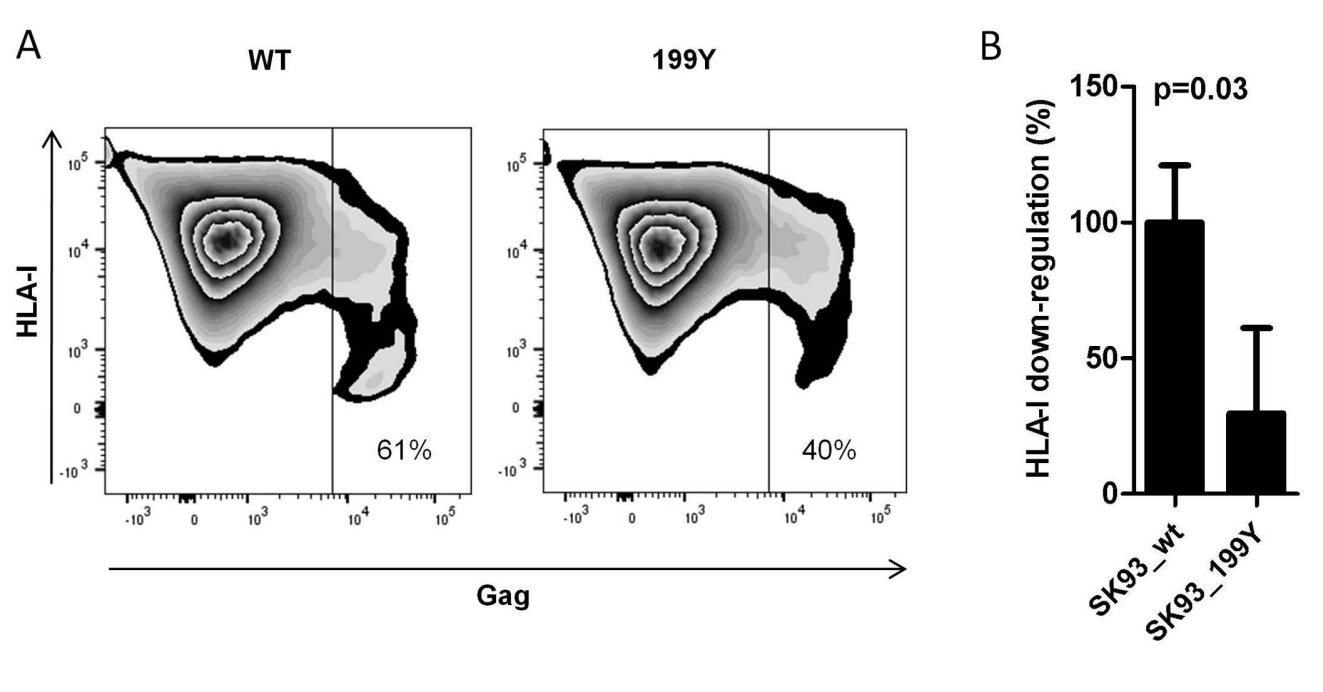




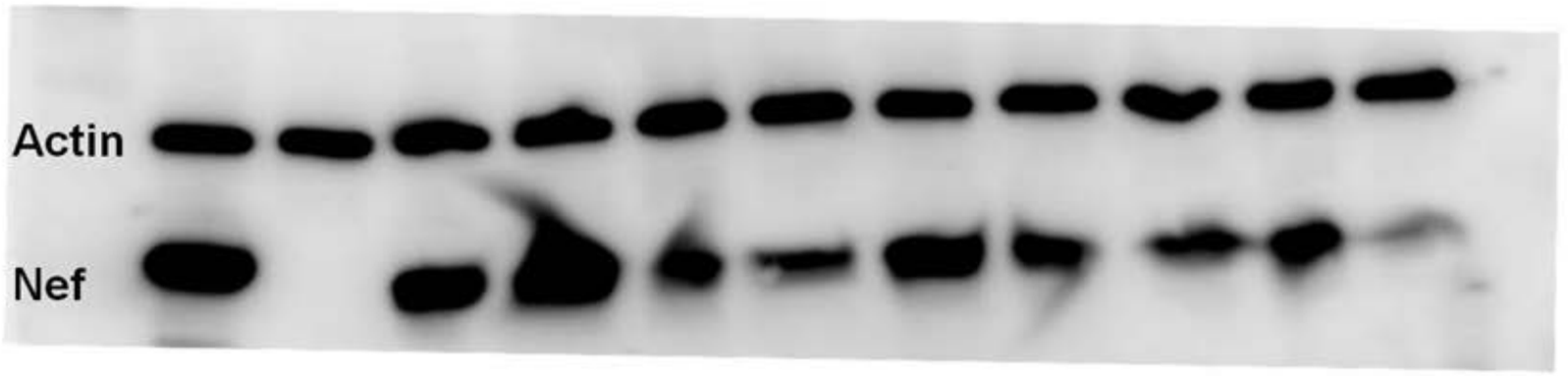
$$\text{HLA/CD4d} = \frac{(\text{MFI}_{\Delta\ \text{Nef}} - \text{MFI}_{\text{Nef clone}})}{(\text{MFI}_{\Delta\ \text{Nef}} - \text{MFI}_{\text{SF2 Nef}})}$$

$$\text{Normalised function} = \frac{\text{HLA/CD4d}_{\text{Mutant}}}{\text{HLA/CD4d}_{\text{WT}}}$$



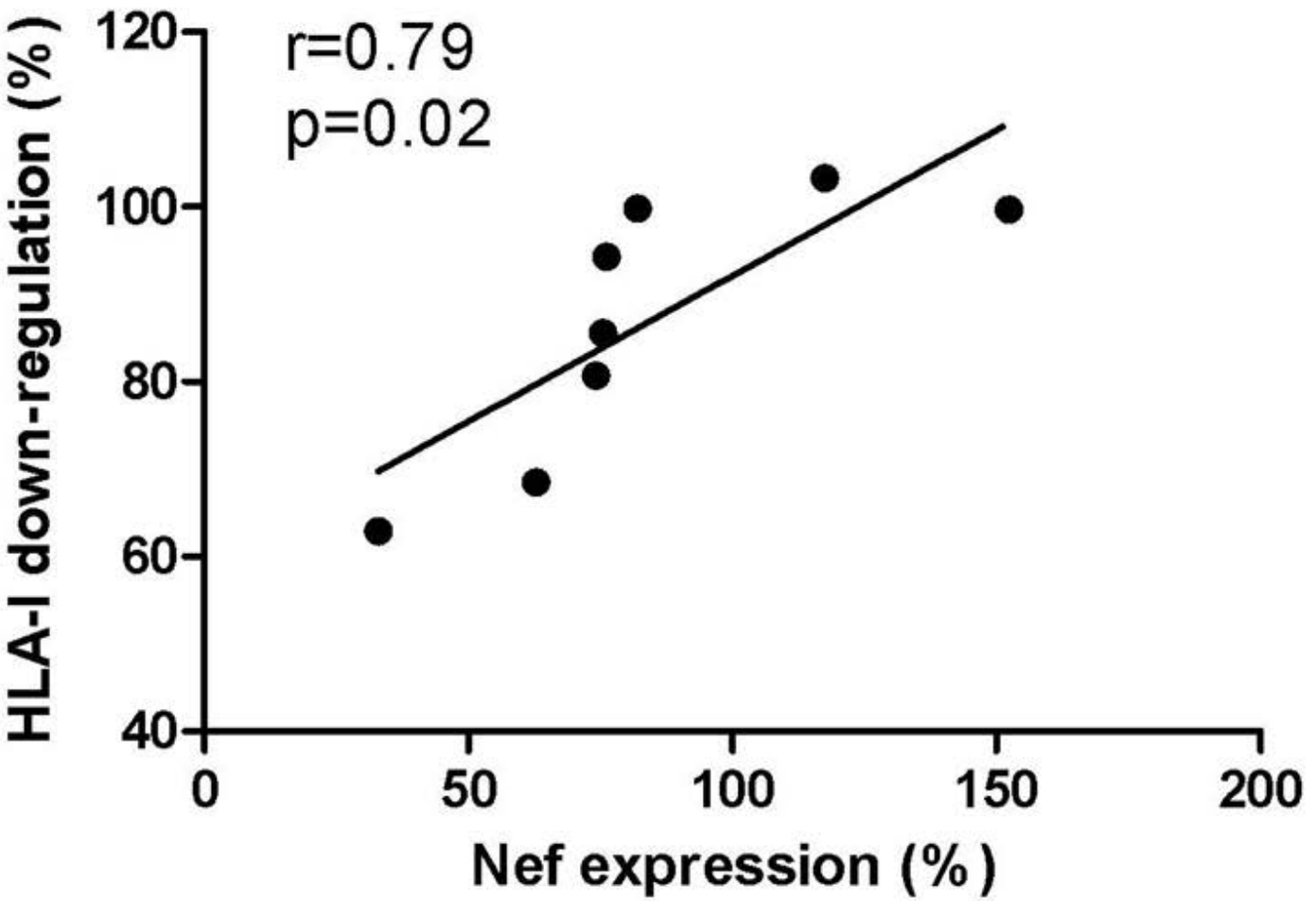

A
WT
199Y
HLA-I
Gag
61%
40%
B
HLA-I down-regulation (%)
p=0.03
150
100
50
0
SK93_wt
SK93_199Y

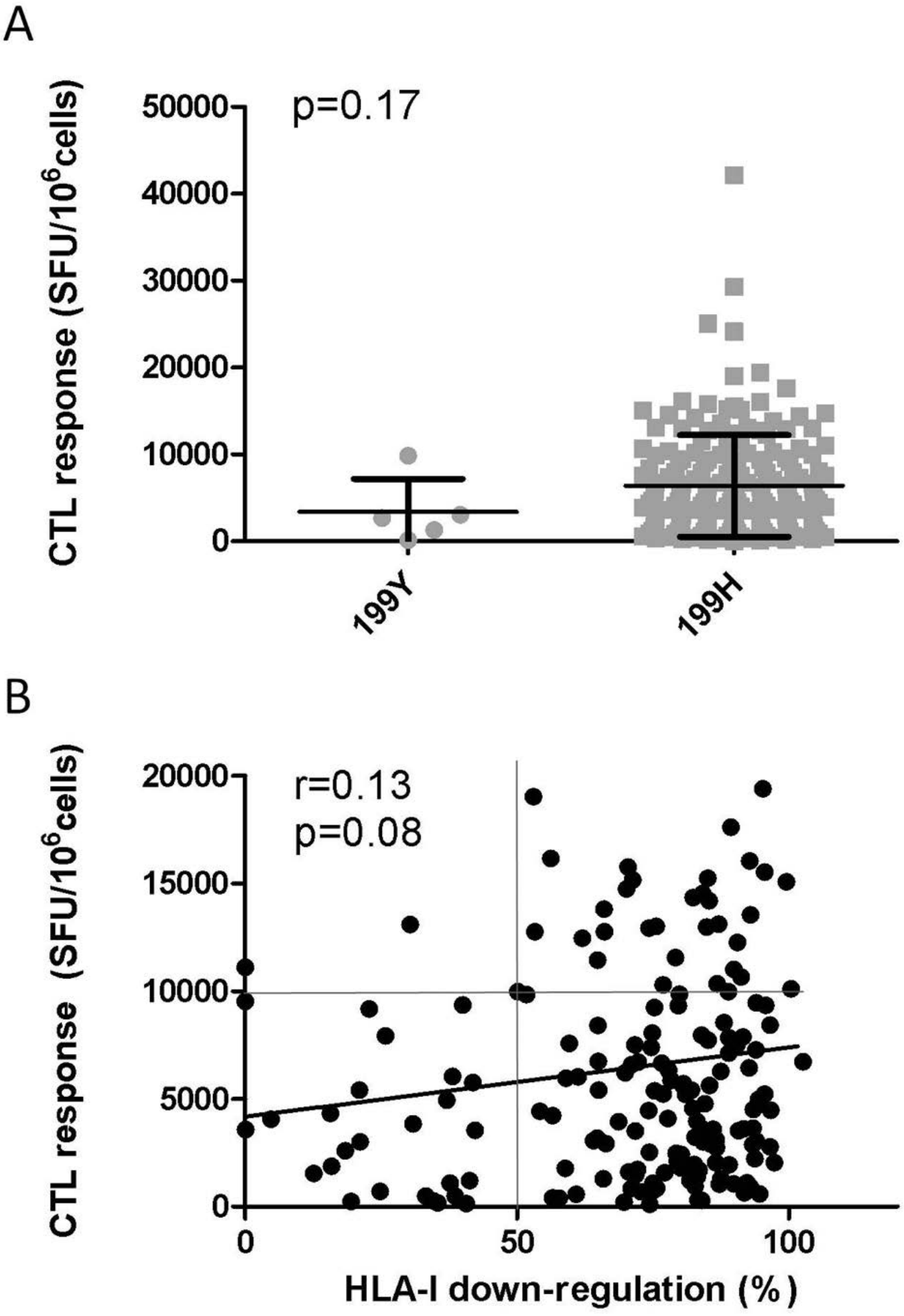
A



SF2 ΔNef SK93 93D 102H 105R 108D 102H 108D 102H 105R 108D 133T 199Y

B




A
p=0.17
CTL response (SFU/10^6cells)
50000
40000
30000
20000
10000
0
199Y
199H
B
r=0.13
p=0.08
CTL response (SFU/10^6cells)
20000
15000
10000
5000
0
0
50
100
HLA-I down-regulation (%)