



Research paper

Impact of a single HLA-A*24:02-associated escape mutation on the detrimental effect of HLA-B*35:01 in HIV-1 control



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ABSTRACT

Background: HLA-B*35 is an HLA allele associated with rapid progression to AIDS. However, a mechanism underlying the detrimental effect of HLA-B*35 on disease outcome remains unknown. Recent studies demonstrated that most prevalent subtype HLA-B*35:01 is a detrimental allele in HIV-1 clade B-infected individuals. We here investigated the effect of mutations within the epitopes on HLA-B*35:01-restricted CD8⁺ T cells having abilities to suppress HIV-1 replication.

Methods: We analyzed 16 HLA-B*35:01-restricted epitope-specific T cells in 63 HIV-1 clade B-infected Japanese B*35:01⁺ individuals and identified HLA-B*35:01-restricted CD8⁺ T cells having abilities to suppress HIV-1 replication. We further analyzed the effect of HLA-associated mutations on the ability of these T cells.

Findings: The breadth of T cell responses to 4 epitopes was inversely associated with plasma viral load (pVL). However, the accumulation of an Y135F mutation in NefYF9 out of the 4 epitopes, which is selected by HLA-A*24:02-restricted T cells, affected the ability of YF9-specific T cells to suppress HIV-1 replication. HLA-B*35:01⁺ individuals harboring this mutation had much higher pVL than those without it. YF9-specific T cells failed to suppress replication of the Y135F mutant *in vitro*. These results indicate that this mutation impairs suppression of HIV-1 replication by YF9-specific T cells.

Interpretation: These findings indicate that the Y135F mutation is a key factor underlying the detrimental effect of HLA-B*35:01 on disease outcomes in HIV-1 clade B-infected individuals.

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1. Introduction

It is well known that HIV-1 replication is controlled at the level of individual cells and the overall host by several host cellular proteins called restriction factors [1] as well as host immunity [2]. Studies of host genetic variation showed that homozygosity for the *CCR5* deletion mutation *CCR5Δ32* (*CCR5 delta 32*) showed significant protection against HIV-1 infection [3–8]. Although several variants of restriction factors and other genes have been proposed to confer similar protection [9–11], the results have not been replicated in other cohorts. Genome-wide association studies of protection against HIV-1 infection have consistently demonstrated the major impact of variation in HLA class I genes on

HIV-1 outcome [12,13], suggesting that CTLs and NK cells may predominantly contribute to the control of HIV-1 replication.

Previous longitudinal analyses of HLA alleles associated with progression to AIDS showed that the presence of particular HLA class I alleles or haplotypes is associated with disease progression [14–20], and cross-sectional analyses have demonstrated associations of several HLA class I alleles with clinical parameters such as plasma viral load (pVL) and CD4⁺ T cell count [12,13,21–28]. HLA-B alleles more strongly influence clinical outcome than HLA-A and HLA-C [26]. HLA-B*57 and HLA-B*27 are consistently associated with control of HIV-1 or slow progression to disease in Caucasians and Africans [19–23,29], whereas HLA-B*35, -B*07, -B*58:02, -B*08, -B*18 and HLA-A*29-B*07-C*15 haplotype are associated with rapid progression to AIDS [2,13,14–17,21,26,27–30]. HLA-B35 subtypes such as HLA-B*35:02 and B*35:03 (with the peptide binding motif of Proline at P2 and a non-Tyrosine residue at the C terminus, referred to as HLA-B*35-P_X) had a significant association with rapid

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Research in Context

Evidence before this study

Several HLA alleles are strongly associated with progression to AIDS. Previous longitudinal studies demonstrated that HLA-B*35 was significantly associated with rapid progression to AIDS. Recent cross-sectional analyses of HLA alleles and clinical parameters in the large cohorts infected with HIV-1 clade B showed an association of most prevalent HLA-B*35 subtype HLA-B*35:01 with higher plasma viral load (pVL) in Caucasian, Mexican, and Japanese. These findings indicate that HLA-B*35:01 is a detrimental allele in HIV-1 clade B infection. However, a mechanism underlying the detrimental effect of this allele on disease outcome in HIV-1 clade B-infected individuals remains unclear. A previous study demonstrated that there was a strong T-cell response to an HLA-B*35:01-restricted GagNY10 epitope in HIV-1 clade C-infected African cohort which has a neutral effect on pVL, while this response was absent in the clade B infected subjects, suggesting a possibility that a lack of the T cells specific for this epitope may result in the detrimental effect of HLA-B*35:01 on clinical outcome in the clade B-infected individuals. However, there is no direct evidence for the mechanism underlying the detrimental effect of HLA-B*35:01 in HLA-B*35:01⁺ individuals infected with the clade B virus.

Added value of this study

We here demonstrated T cells specific for 4 HLA-B*35:01-restricted epitopes having abilities to suppress HIV-1 replication in HIV-1 clade B-infected individuals having this allele. Only Y135F mutation within NefYF9 out of the 4 epitopes, which selected by HLA-A*24:02-restricted T cells, impaired an ability of YF9-specific T cells to suppress HIV-1 replication *in vivo* and *in vitro*. This study indicates that this mutation is a critical factor for the detrimental effect of HLA-B*35:01 on disease outcome in the clade B-infected individuals.

Implications of all the available evidence

This study for the first time clarified the mechanism of detrimental effect of HLA-B*35:01 on disease outcome in HIV-1 infection. Therefore, this study could contribute to understanding of pathogenesis of HIV-1 infection.

progression in Caucasians, whereas other subtypes with the motif Proline at P2 and Tyrosine at the C-terminus (HLA-B*35-P_Y) did not [15,16]. On the other hand, a recent large scale cross-sectional study in a European cohort infected with HIV-1 clade B demonstrated that amongst HLA-B*35:01⁺ individuals the frequency of HIV-1 controllers was significantly lower than that of progressors [13]. A study of Japanese individuals infected with HIV-1 clade B also showed that HLA-B*35:01⁺ individuals had significantly higher pVL and lower CD4 count than those without HLA-B*35:01 [24]. These findings together suggest that HLA-B*35:01 remains a detrimental allele in HIV-1 clade B-infected individuals. However, the mechanisms underlying the detrimental impact of these HLA-B*35 alleles on the clinical outcome of HIV-1 clade B-infected individuals remains unclear.

A previous study revealed that the HLA-B*35:01 allele had a neutral effect on the control of HIV-1 in an HIV-1 clade C-infected African cohort from Botswana, whereas this allele was associated with poor outcome

in HIV-1 clade B-infected Mexican and Japanese cohorts [31]. This study further showed that, while in the clade C-infected cohort there was a strong CD8⁺ T cell response to Gag p24 NY10 epitope, this response was largely absent in the clade B infected subjects. These findings suggest that T cells specific for this epitope possesses the ability to suppress HIV-1 replication. However, there is no direct evidence for the detrimental effect of HLA-B*35:01 on disease outcome in clade B infection and the role of other HLA-B*35:01-restricted CD8⁺ T cells in poor clinical outcome remains unclear.

In the present study, we analyzed HLA-B*35:01-restricted CD8⁺ T cell responses in 63 treatment-naïve, HLA-B*35:01⁺ Japanese individuals chronically infected with HIV-1 clade B to clarify the underlying mechanism by which HLA-B*35:01 affects disease outcome. To identify HLA-B*35:01-restricted T cell responses capable of suppressing HIV-1 replication *in vivo*, we first studied CD8⁺ T cell responses to 16 known HLA-B*35:01-restricted epitopes in HLA-B*35:01⁺ individuals and then analyzed the differences in pVL between responders and non-responders. We next investigated the effects of viral mutations associated with HLA-B*35:01 and other HLA alleles within each protective epitope on pVL and the epitope-specific T-cell responses. In the present study, we sought to identify the mechanism underlying the detrimental effect of HLA-B*35:01 in HIV-1 clade B infection in Japanese subjects.

2. Materials and methods

2.1. Subjects

We recruited 63 chronically HIV-1 clade B-infected, antiretroviral therapy naïve, HLA-B*35:01⁺ Japanese individuals from the AIDS Clinical Center, National Center for Global Health and Medicine during January 2009–January 2013. This study was approved by the Ethical Committee of the National Center for Global Health and Medicine and Kumamoto University. Informed consent was obtained from all individuals according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping.

2.2. Measurement of pVL

We measured pVL of all of the 63 patients by using a Cobas TaqMan HIV-1 real-time PCR version 2.0 assay (Roche Diagnostics, NJ, USA). Measurements of pVL by using this kit is based on a nucleic acid amplification. The concentration of HIV-1 RNA in plasma that can be detected with a positive rate of >95% by using this kit is about 200 copies/mL. This kit showed a high reproducibility of measurements (standard deviation: 1.1–1.2 copies/mL) [32]. The data were from a single measurement of pVL of each individual.

2.3. Cells

C1R and RMA-S cells expressing HLA-B*35:01 were previously generated by transfecting C1R and RMA-S cells with the HLA-B*35:01 gene [33,34] and they were maintained in RPMI medium containing 10% fetal calf serum (FCS, R10) and 0.15 mg/mL hygromycin.

2.4. IFN- γ ELISPOT assays

IFN- γ ELISPOT assays were performed as previously described [35]. Briefly, to standardize the number of spots to spots/10⁶ CD8⁺ T cells, we stained PBMCs from each subject with phycoerythrin (PE)-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark) and measured a frequency of CD8⁺ T cells amongst PBMCs using a flow cytometry. Next, 100,000 PBMCs from HLA-B*35:01⁺ HIV-1-infected individuals were plated in

each well in the ELISPOT plate along with a concentration of 100 nM of each of 16 known HLA-B*35:01-restricted epitope peptides [31,33,36–42]. We calculated the number of CD8⁺ T cells in each well containing 100,000 PBMCs by using the frequency of CD8⁺ T cells amongst PBMCs and determined spots/10⁶ CD8⁺ T cells in each well [35,43–45]. The number of spots for each peptide-specific T cell response was finally calculated by subtracting the number of spots in wells without peptides. A mean + 4 SD of the spots of samples from 9 HIV-1-naïve individuals for the epitope peptides was 170 spots/10⁶ CD8⁺ T cells. Therefore, we defined a positive ELISPOT response as >200 spots/10⁶ CD8⁺ T cells.

2.5. Intracellular cytokine staining (ICS) assay

The CTL clones were cocultured with C1R-B3501 pulsed with YF9 or YF9-1F peptide (0.1–10 nM) for 2 h at 37 °C. Brefeldin A (10 µg/mL) was then added, and the cells were incubated for an additional 4 h. Cells were stained with allophycocyanin (APC)-labeled anti-CD8 mAb (DAKO, Glostrup, Denmark) and subsequently fixed (4% paraformaldehyde), permeabilized (0.1% saponin and 5% FCS), and intracellularly stained with fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ mAb (BD Bioscience, CA). Data were analyzed on a FACS Canto II.

2.6. HLA class I stabilization assay

The binding of peptides to HLA-B*35:01 molecules was tested as previously described [34,46]. RMA-S-B3501 cells transfected with HLA-B*35:01 and human β₂-microglobulin were cultured at 26 °C for 16 h. The cells were incubated at 26 °C for 1 h with Nef YF9 or YF9-1F peptide at various concentrations and then at 37 °C for 3 h. After 2 washes with PBS containing 5% FCS (PBS-5% FCS), they were subsequently incubated with mAb SFR8-B6 for 30 min. After 2 washes with PBS-5% FCS, the cells were incubated for 30 min on ice with FITC-conjugated sheep IgG with anti-mouse Ig specificity (Silenus Laboratories, Hawthorn, Australia). Finally, the fluorescence intensity was measured using a flow cytometer.

2.7. Tetramer binding assay

HLA-class I-peptide tetrameric complexes (tetramers) were synthesized as previously described [47]. Nef YF9 or YF9-1F peptides were added to the refolding solution containing the biotinylation sequence-tagged extracellular domain of the HLA-B*35:01 molecule and β₂-microglobulin. The purified monomer complexes were mixed with PE-labeled streptavidin (molecular Probes) at a molar ratio of 4:1. PBMCs were stained with YF9 or YF9-1F tetramers, respectively, at 37 °C for 30 min. The cells were then washed twice with R10, followed by staining with FITC-anti-CD8 mAb (DAKO, Glostrup, Denmark), and 7-AAD (BD Pharmingen) at 4 °C for 30 min. The cells were washed twice with R10. Data were analyzed on a FACS Canto II instrument.

2.8. HIV-1 mutant clones

NL4-3 mutants (NL4-3YF9-1F) were previously generated by introducing the Nef YF9-1F mutation into NL4-3 using a site-directed mutagenesis system (Invitrogen, USA) [48,49].

2.9. HIV-1 replication suppression assay

The ability of epitope-specific CTLs to suppress HIV-1 replication was examined as previously described [50,51]. CD4⁺ T cells isolated from PBMCs of HLA-B*35:01⁺ healthy donors were infected with NL4-3 or NL4-3YF9-1F and then these cells were co-cultured with epitope-specific CTL clones at an E:T ratio of 1:1. On day 5–6 post infection, the

concentration of p24 Ag in the culture supernatant was measured by using an enzyme-linked immunosorbent assay.

2.10. Bulk DNA sequencing

Bulk sequencing of autologous plasma viral RNA was performed as described previously [52].

2.11. Statistical analysis

For comparison of two groups in this study, a two-tailed Mann-Whitney's test or an unpaired *t*-test were performed. Correlations between the breadths or the magnitudes and pVL were statistically analyzed using Spearman rank test. *P* values < 0.05 were considered to be statistically significant. Multiple tests were accounted for using *q*-values, a measure in terms of the false-discovery rate, a significance threshold of *q* < 0.2 was employed [52].

3. Results

3.1. HLA-B*35:01-restricted CD8⁺ T cells have the capacity to suppress HIV-1 replication *in vivo*

To clarify the role of HLA-B*35:01-restricted HIV-1-specific CD8⁺ T cells in the control of HIV-1, we investigated CD8⁺ T cell responses to 16 known HLA-B*35:01-restricted epitopes [31,33,36–42] in 63 chronically HIV-1-infected ART-free HLA-B*35:01⁺ Japanese individuals using ELISPOT assays. These HLA-B*35:01⁺ individuals were selected by HLA genotyping from 502 HIV-1-infected ART-free individuals in our cohort. The frequency of B*35:01⁺ individuals was 12.5%, which is a little lower than that (16.5%) in Japanese population [53]. The clinical characteristics of these B*35:01⁺ individuals are shown in Supplementary Table 1. We analyzed the T cell responses in the PBMCs collected at each sampling date (the first visiting or within 4 weeks after the first visiting) in each individual and then statistically analyzed correlation between the T cell responses and pVL that were measured at same date as sampling of the PBMCs in the 63 individuals. We found a relatively weak correlation between the breadth ($r = -0.39, p = 0.0013$) and magnitude ($r = -0.39, p = 0.0015$) of responses to these epitopes and pVL in these subjects (Fig. 1a and b). These results suggest that some but not all of these T cells may contribute to suppression of HIV-1 replication *in vivo*. On the other hand, it is speculated that known protective alleles may influence these results. To determine whether or not this might be due to the presence of protective HLA alleles in HLA-B*35:01⁺ individuals, we excluded 10 individuals with the protective alleles HLA-B*52:01/C*12:02 or HLA-B*67:01 and repeated the analysis. The analysis in the remaining 53 individuals showed similar or a little weaker correlation between the magnitude and breadth of CD8⁺ T-cell responses to B*35:01 epitopes and pVL (Fig. 1a and b). Indeed, pVL did not differ between individuals with and without these protective alleles (Fig. 1c). These results indicate that neither the B*52:01/C*12:02 haplotype or the HLA-B*67:01 allele influenced pVL in these HLA-B*35:01⁺ individuals. We therefore evaluated the influence of these T cell responses on pVL using the data from all 63 individuals. Responders to four epitope peptides, p17NY9, RTVY10, NefRY11, or NefYF9, had significantly lower pVL than non-responders, while those with responses to two epitope peptides, IntY11 or gp120TVW9, showed a trend to lower pVL than the non-responders (Table 1). These results suggest that CD8⁺ T cells specific for the 4 epitopes have ability to suppress HIV-1 replication *in vivo*. To investigate whether there was an additive or synergistic effect of these HLA-B*35:01-restricted CD8⁺ T cell responses on pVL, we looked for a correlation between the breadth or total magnitude of these T cell responses and pVL in HLA-B*35:01⁺ individuals. Both the breadth and total

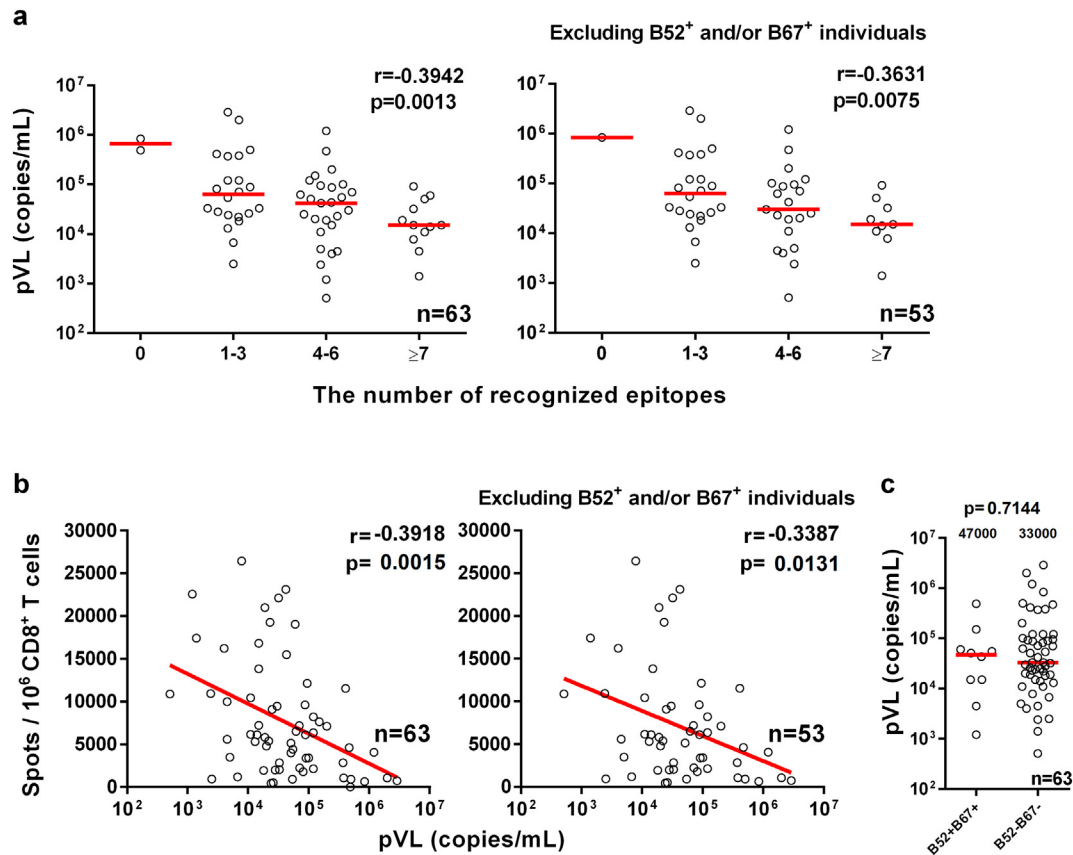


Fig. 1. Correlation between multiple CD8⁺ T cell responses to 16 HLA-B*35:01-restricted epitopes and pVL. Epitope-specific CD8⁺ T cell responses were analyzed using ELISPOT assays at a peptide concentration of 100 nM. Correlation between the breadth (a) and total magnitude (b) of CD8⁺ T cell responses to the 16 HLA-B*35:01-restricted epitopes and pVL in 63 HLA-B*35:01⁺ HIV-1-infected Japanese individuals (left) or a subset of 53 individuals, excluding those with protective alleles HLA-B*52:01 or HLA-B*67:01 (right), were statistically analyzed using the Spearman rank correlation test. (c) Comparison of pVL between HLA-B*52:01/C*12:02 or B*67:01-positive and -negative individuals. Statistical analyses were performed using the Mann-Whitney test.

Table 1

Association of CD8⁺ T cell responses to HLA-B*35:01-restricted epitopes with pVL in 63 HIV-1-infected Japanese individuals carrying HLA-B*35:01.

Epitope				# of subjects		pVL		p value ^b	q value ^c
Protein	Designation	HXB2 position	Sequence	Response ^a	Response ^a	Median (copies/mL)	Median (copies/mL)		
				+	-	+	-		
p17 Gag	NY9	Gag 124–132	NSSQVSQNY	6	57	9400	43000	<i>0.0186</i>	0.138
p24 Gag	HA9	Gag 216–224	HPVHAGPIA	15	48	22000	47000	0.1939	0.348
p24 Gag	NY10	Gag 253–262	NPPIPVGEIY	6	57	66500	33000	0.9907	0.991
RT	TY9	Pol 262–270	TVLDVGDAY	27	36	25000	37500	0.2087	0.348
	VY10	Pol 273–282	VPLDKDFRKY	20	43	20000	54000	<i>0.0367</i>	0.138
	NQY9	Pol 330–338	NPDIVIYQY	28	35	37500	33000	0.3326	0.454
	EY10	Pol 587–596	EPVGAETFY	19	44	33000	42000	0.5692	0.610
Int	IY11	Pol 804–814	IPAETGQETAY	8	55	11400	42000	0.0885	0.266
Rev	KY10	Rev 14–23	KTVRLIKFLY	4	59	17000	42000	0.2478	0.372
gp120	TVW9	Env 37–45	TVVYGVVW	8	55	16500	43000	0.1096	0.274
	VL11	Env 42–52	VPVWKEATITL	5	58	15000	42500	0.3666	0.458
	DL9	Env 78–86	DPNPQEVVL	18	45	26500	43000	0.4332	0.500
gp41	TW9	Env 606–614	TAVPWNASW	1	62	–	–	–	–
Nef	VY8	Nef 74–81	VPLRPMTY	23	40	30000	48500	0.175	0.348
	RY11	Nef 71–81	RPQVLRPMTY	32	31	26500	62000	<i>0.0309</i>	0.138
	YF9	Nef 135–143	YPLTFGWCF	49	14	30000	245000	<i>0.016</i>	0.138

Italics indicate that differences were statistically significant.

^a A positive ELISPOT response was defined as >200 spots/10⁶ CD8⁺ T cells.

^b Statistical analysis was performed using the Mann-Whitney test.

^c Multiple tests were accounted for using q-values, a measure in terms of the false-discovery rate, a significance threshold of $q < 0.2$ was employed.⁵²

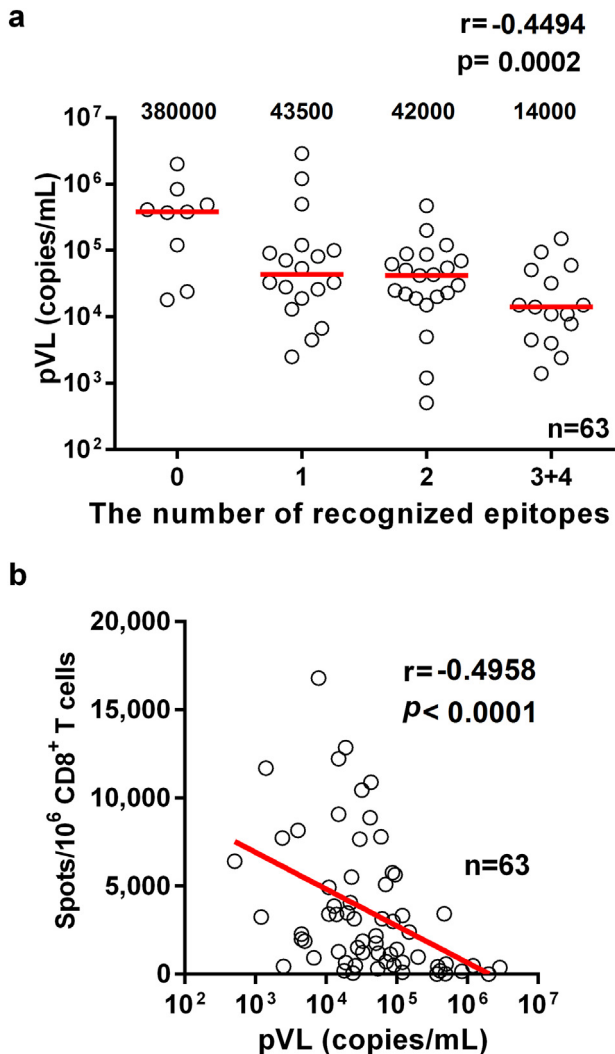


Fig. 2. Correlation between T cell responses to 4 HLA-B*35:01-restricted epitopes and pVL. Epitope-specific CD8⁺ T cell responses at a peptide concentration of 100 nM were analyzed using ELISPOT assays. Correlation between breadth (a) and total magnitude (b) of CD8⁺ T cell responses to the 4 epitopes (p17NY9, RTVY10, NefRY11, and NefYF9) and pVL in the 63 HLA-B*35:01⁺ HIV-1-infected Japanese individuals were statistically analyzed using the Spearman rank correlation test.

magnitude of responses revealed an inverse correlation with pVL (breadth: $r = -0.4494$, $p = .0002$, magnitude: $r = -0.4958$ $p < 0.0001$, Fig. 2a and b). Thus, CD8⁺ T cell responses to these 4 epitopes showed an additive or synergistic effect on the control of HIV-1 in HLA-B*35:01⁺ individuals.

3.2. Impact of Nef Y135F mutation on pVL in HIV-1-infected HLA-B*35:01⁺ individuals

To determine whether the emergence of escape mutations within the epitopes might affect the control of HIV-1 by HLA-B*35:01-restricted CD8⁺ T cells, we searched for HLA-B*35:01-associated mutations within each epitope. We previously identified one HLA-B*35:01-associated polymorphism, Nef Y81F, which is in the NefRY11 epitope, amongst HIV-1-infected Japanese individuals [52]. We investigated the effect of this mutation on pVL, but did not find any significant difference in pVL between those individuals carrying wild-type (WT) virus and those harboring the Nef Y81F mutant (Supplementary Fig. 1), indicating that this mutation has little impact on the suppression

of HIV-1 by RY11-specific CD8⁺ T cells. We next investigated the possibility that mutations selected by CTLs restricted by other HLA alleles could influence the control of HIV-1 by CD8⁺ T cells specific for the 4 HLA-B*35:01-restricted epitopes. A previous study had described 2 HLA-A*11:01-associated polymorphisms; Gag S126G and Nef T80D, an HLA-C*07:02-associated polymorphism Nef R71K, and an HLA-A*24:02-associated polymorphism Nef Y135F in Japanese donors (Supplementary Table 2) [52]. We investigated the frequencies of HIV variants containing Gag S126G, Nef T80D, Nef R71K, or Nef Y135F mutations in the HLA-B*35:01⁺ individuals. Gag S126G, Nef T80D, and Nef R71K mutations did not accumulate in HLA-B*35:01⁺ Japanese individuals, whereas Nef Y135F was found in 61% of the cohort (Fig. 3a). We also found a Nef Y135L mutation in approximately 8% of the HLA-B*35:01⁺ individuals (data not shown) although this mutation did not appear to be associated with specific HLA alleles. However, the 1 L epitope peptide was recognized equally well as the WT YF9 peptide by an YF9-specific HLA-B*35:01-restricted T cell clone (Fig. 3b), indicating that the Y135L mutation does not impair recognition by YF9-specific T cells.

Finally, we investigated the effect of the Nef Y135F mutation on pVL. Since the Y135L mutation did not affect recognition by YF9-specific T cells, we assigned this mutation into the WT group. When pVL was compared between Y135-infected or Y135L-infected (Y135/Y135L-infected) and Y135F-infected HLA-B*35:01⁺ individuals, those infected with the Y135F mutant had significantly higher pVL than those with Y135/Y135L (Fig. 3c). A previous study demonstrated that this mutation did not affect replication capacity [48]. These findings together suggest that the Y135F mutation may impair the suppression of HIV-1 replication by YF9-specific T cells, even though the mutation is not driven by HLA-B*35:01. To determine the effect of this mutation on the suppressive capacity of YF9-specific T cells, we compared pVL between individuals with YF9 responses infected with either Y135/Y135L or Y135F virus. Y135/Y135L virus-infected responders had much lower pVL than Y135F virus-infected responders (Fig. 3d), indicating that the emergence of the Y135F mutant impairs the ability of YF9-specific CD8⁺ T cells to suppress the mutant virus in the HLA-B*35:01⁺ individuals. In addition, the Y135F-infected responders had significantly lower pVL than the Y135F-infected non-responders, although both had relatively high pVL (Fig. 3d). This result raises the possibility that mutant-specific T cells can to some extent suppress replication of the Y135F virus. In contrast, there was no difference in pVL between responders and non-responders infected with Y135/Y135L virus (Fig. 3d), implying that T cells restricted by other HLA alleles may compensate for the lack of immune pressure exerted by YF9-specific T cells in the non-responders.

3.3. Effect of CD8⁺ T cell responses to the 3 epitopes on pVL in Y135F-infected individuals

To determine the role of HLA-B*35:01-restricted CD8⁺ T cells specific for the other 3 epitopes in Y135F-infected individuals, we compared pVL between those with and without responses to p17NY9, RTVY10, or NefRY11 epitope. Responders to RY11 or VY10 had lower pVL than non-responders amongst Y135F-infected individuals although this was only statistically significant for RY11 and VY10 (Fig. 4a). The breadth of the T cell responses to these 3 peptides and YF9 showed a strong correlation with lower pVL in Y135F-infected individuals (Fig. 4b). These results suggest that T cells specific for these epitopes can suppress replication of the Y135F mutant virus. In contrast, the effect of these T cell responses was minimal in WT or Y135L-infected individuals, although responders to NY9 had a slightly lower pVL than non-responders (Supplementary Fig. 2). Overall, these results suggest that these HLA-B*35:01-restricted T cells can suppress HIV-1 to a moderate level of pVL in these individuals.

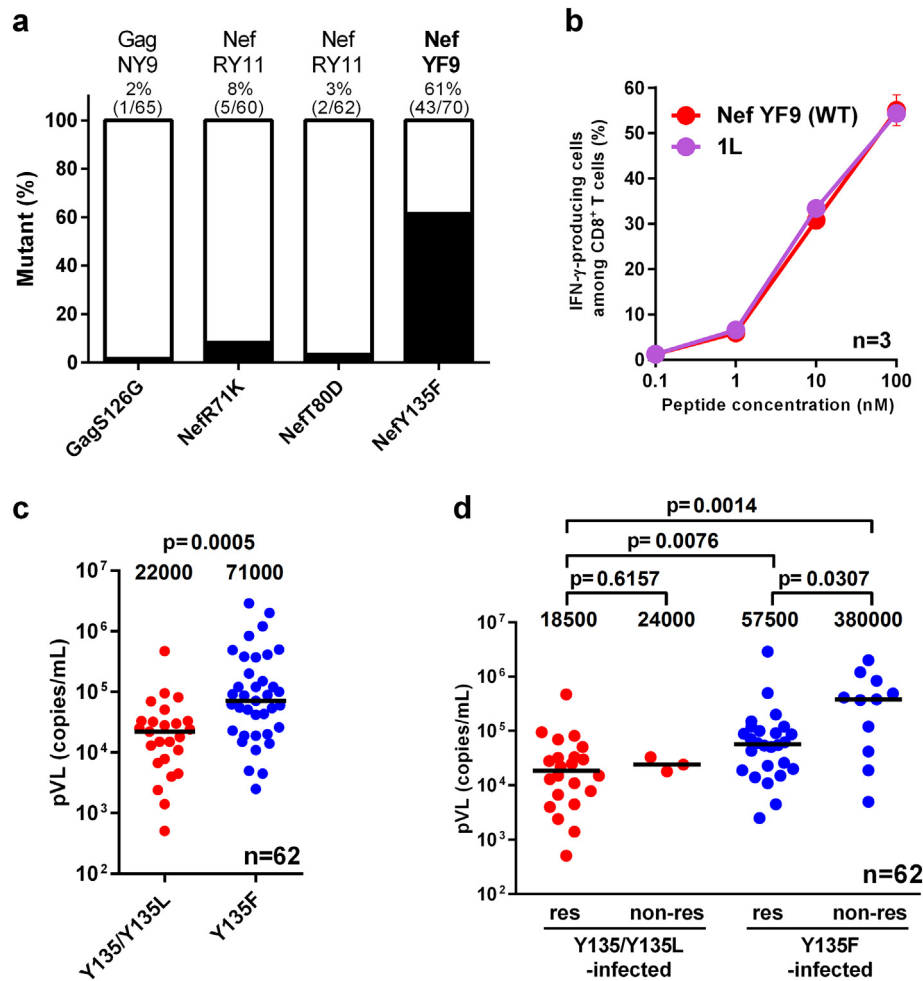


Fig. 3. Effect of NefY135F mutant on pVL in the Japanese individuals carrying HLA-B*35:01. (a) Accumulation of HLA-associated mutations in Japanese individuals carrying HLA-B*35:01. (b) Cross-recognition of 1 L mutant peptide by YF9-specific CD8⁺ T cells. A YF9-specific CTL clone (clone B7) was established from KI-642 infected with WT virus. IFN- γ production from the CTL clone was detected by performing an ICS assay. (c) Comparison of pVL between Y135-infected+Y135L-infected and Y135F-infected HLA-B*35:01-positive individuals. ($n = 62$). Statistical analyses were performed using the Mann-Whitney test. (d) Comparison of pVL between responders to YF9 and non-responders in Y135/Y135L-infected and Y135F-infected individuals ($n = 62$). Statistical analyses were performed by using the Mann-Whitney test.

3.4. Effect of HLA-A*24:02-restricted CD8⁺ T cells on pVL in HLA-A*24:02⁺B*35:01⁺ individuals

To determine whether HLA-A*24:02-restricted CD8⁺ T cells can affect HIV-1 control in B*35:01⁺ individuals, we compared pVL between Y135/Y135L-infected and Y135F-infected HLA-A*24:02⁺ or A*24:02⁻ individuals but found no significant differences (Fig. 5a). This was also the case for HLA-A*24:02⁺HLA-B*35:01⁻ individuals (Supplementary Fig. 3). These findings suggest that HLA-A*24:02-restricted CTLs confer a minimal effect on pVL in our cohort. It is well known that the Y135F mutation is selected by RF10-specific T cells restricted by HLA-A*24:02 [48,49]. We therefore investigated T cell responses to the RF10 peptide in Y135/Y135L-infected HLA-A*24:02⁺ individuals and responses to RF10 and RF10-2F peptides in subject infected with Y135F virus. All five Y135/Y135L-infected HLA-A*24:02⁺ individuals were responders to RF10 peptide (data not shown), whereas 21 of 27 Y135F-infected individuals showed responses to RF10 and/or RF10-2F (Fig. 5b). When responses to these peptides were analyzed in relation to pVL, no significant differences were seen between responders and non-responders or between responders and HLA-A*24:02⁻ individuals amongst those infected with Y135F virus (Fig. 5b). These results together indicate that RF10 and RF10-2F-specific T cells have only minimal impact on pVL in B*35:01⁺ individuals.

3.5. The abilities of YF9-specific T cells to suppress HIV-1 replication *in vitro*

We analyzed the ability of YF9-specific T cells to suppress YF9 (WT) or 1F mutant viruses *in vitro*. To perform this analysis, we established an YF9-specific CTL clone (clone H3) from a WT virus-infected HLA-B*35:01⁺ individual (KI-642). The YF9-specific clone showed significantly stronger recognition of the YF9 peptide than the 1F variant (Fig. 6a). HLA class I stabilization assays using RMA-S-B3501 cells demonstrated that the YF9 and 1F peptides showed very similar binding affinities for HLA-B*35:01 (Fig. 6b), indicating that the peptide binding affinity does not influence T cell recognition of these peptides. We next investigated the contribution of the T-cell receptor (TCR) to recognition of these epitope variants using YF9- and 1F-tetramers. The T-cell clone showed greater binding affinity for the YF9-tetramer than the 1F-tetramer (Fig. 6c), indicating that YF9-specific T cells have a low affinity for the HLA-B*35:01/YF9-1F peptide complex. We finally analyzed the ability of the T-cell clone to suppress replication of either the YF9 or 1F viruses *in vitro*. The T-cell clone suppressed the replication of WT virus much more potently than that of 1F virus (Fig. 6d), suggesting that YF9-specific CD8⁺ T cells will effectively suppress replication of YF9 virus but not the mutant 1F virus *in vivo*. These findings confirm the hypothesis that the emergence of this HLA-A*24:02-associated mutation impairs the control of HIV-1 by YF9-specific CD8⁺ T cells in individuals with HLA-B*35:01.

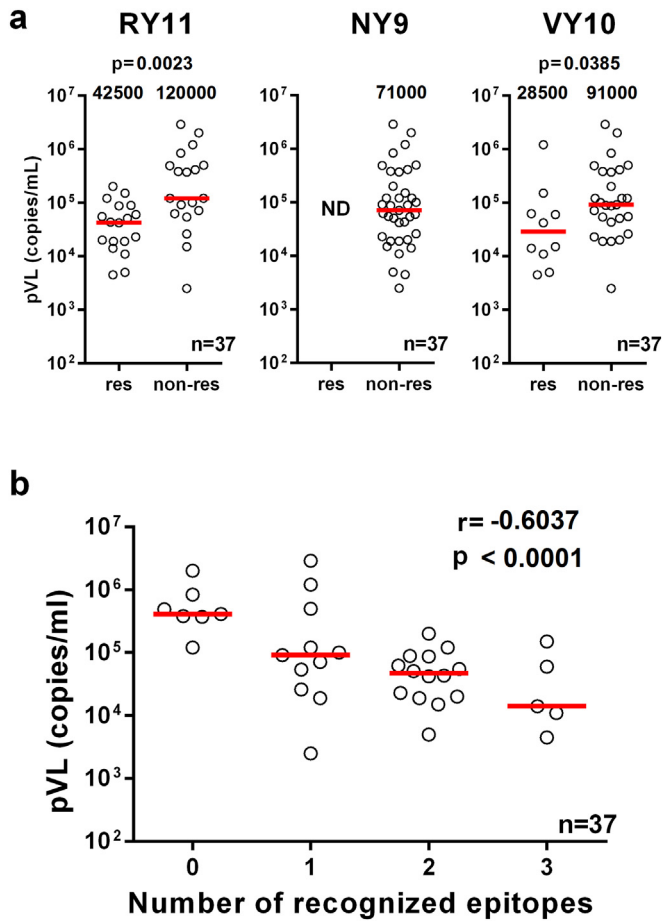


Fig. 4. Effect of the T cell responses to 3 HLA-B*35:01-restricted epitopes on pVL in Y135F virus-infected individuals. (a) The T cell responses to the 3 epitope peptides (p17NY9, RTVY10, or NefRY11) were analyzed using IFN- γ ELISPOT assays. The differences in pVL between responders and non-responders to each epitope peptide in individuals infected with Y135F virus were statistically analyzed using the Mann-Whitney test. (b) Correlation between the breadth of T cell responses to the 3 epitope peptides and to the NefYF9 epitope and pVL in individuals infected with the Y135F virus was statistically analyzed using Spearman rank test.

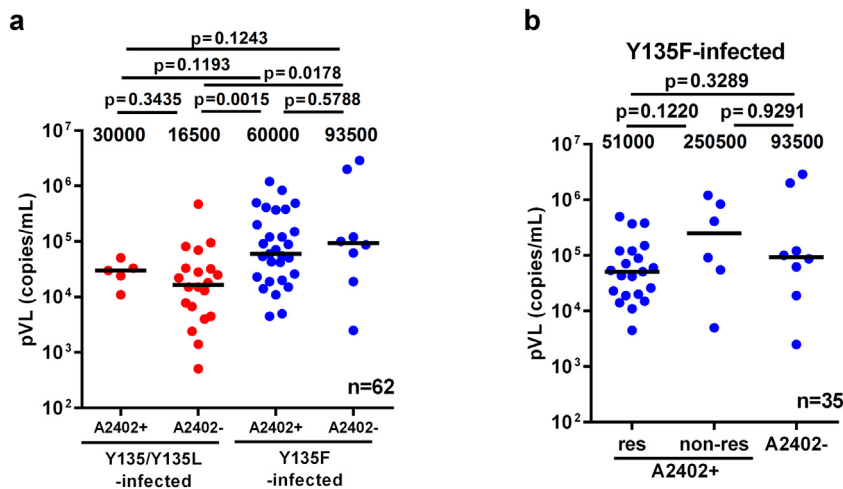


Fig. 5. Effect of HLA-A*24:02-restricted CD8⁺ T cells on pVL in HLA-A*24:02⁺B*35:01⁺ individuals. (a) Comparison of pVL between A*24:02⁺ and A*24:02⁻ in Y135/Y135L-infected or Y135F-infected individuals. (b) Effect of HLA-A*24:02-restricted CD8⁺ T cells specific for RF10 or RF10-2F on pVL in Y135F-infected individuals. The T cell responses to RF10 and RF10-2F epitope peptides were analyzed in Y135F-infected A*24:02⁺ individuals by ELISPOT assays. Statistical analyses were performed using the Mann-Whitney test.

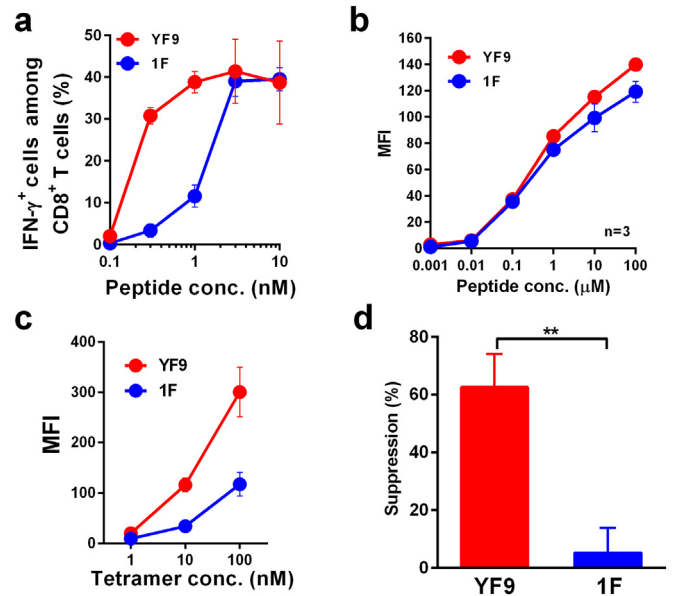


Fig. 6. Effect of the 1F mutation on the function of YF9-specific CD8⁺ T cells. (a) Recognition of YF9 or 1F mutant peptides by a YF9-specific CTL clone. The CTL clone (clone H3) was established from donor KI-642. Recognition of YF9 or 1F mutant peptides by the YF9-specific CTL clone were analyzed by performing ICS assays. (b) Binding affinity of YF9 or 1F peptides to HLA-B*35:01. Binding affinity of the peptides to HLA-B*35:01 was measured by performing HLA class I stabilization assays using the RMA-S-B3501 cell-line. (c) Tetramer binding affinity of the specific CTL clone to YF9- or 1F mutant-tetramers. (d) The abilities of the YF9-specific CTL clone to suppress replication of YF9 or 1F viruses *in vitro*. Primary CD4⁺ T cells from donors carrying HLA-B*35:01 were infected with YF9 or 1F viruses, and then co-cultured with the specific CTL clone at an E:T ratio of 1:1. Suppression % was calculated as follows: (concentration of p24 without the CTLs - concentration of p24 with the CTLs)/concentration of p24 without the CTLs \times 100. The data are presented as the mean and SD (n = 3). ** p < .01.

4. Discussion

The mechanisms underlying the detrimental effect of HLA-B*35:01 on disease outcome in HIV-1 clade B-infected individuals is still unclear. To investigate potential mechanisms, we first sought to identify HLA-

B*35:01-restricted HIV-1-specific CD8⁺ T cells that contribute to HIV-1 suppression in Japanese individuals. We found that HLA-B*35:01-restricted T cells specific for 4 epitopes from a panel of 16 play the major role in suppression of HIV-1 replication in HLA-B*35:01⁺ Japanese individuals. Although together these 4 epitope-specific T cell responses showed an additive or synergistic effect on HIV-1 control, subjects with responses to >3 epitopes had a median pVL of 14,000 copies/mL, which is only 2-fold lower than median pVL in the cohort (29,000 copies/mL) and significantly higher than that of responders to epitopes restricted by protective alleles HLA-B*52:01 or HLA-B*67:01 (<5000 copies/mL) amongst HIV-1-infected Japanese individuals [35]. These findings suggest that the T cell responses to these HLA-B*35:01-restricted epitopes have a relatively modest effect on suppression of HIV-1 replication.

Responders to one of these peptides in whom the Y135F mutant had emerged had a significantly higher pVL than that of WT-infected responders (median: 57,500 copies/mL vs 18,500 copies/mL, Fig. 3d). In addition, YF9-specific T cells failed to suppress replication of the mutant virus *in vitro*. Thus, accumulation of the Nef Y135F mutation, which is strongly associated with HLA-A*24:02, impaired the T-cell response to the YF9 epitope, resulting in a loss of HIV-1 control by YF9-specific CTLs. We have here demonstrated a unique mechanism underlying the detrimental effect of HLA-B*35:01 on disease outcome, that the modest impact of HLA-B*35:01-restricted T cells on HIV-1 control is attenuated by a single mutation selected by HLA-A*24:02-restricted T cells in Japanese individuals. HLA-A*24:02 is found in approximately 70% of Japanese individuals while this mutation was detected in approximately 85% of individuals expressing HLA-A*24:02 [52], suggesting that this mechanism may be more relevant to Japanese cohorts than other ethnicities. Recent cross-sectional studies in a European and Mexican cohort infected with HIV-1 clade B demonstrated that HLA-B*35:01 is also associated with poor disease outcome in these cohorts [13,28]. HLA-A*24:02 is found in 19% and 34% of Caucasians and Mexicans [54,55], respectively, while Y135F was found in >80% of Caucasians expressing this HLA allele [56]. Therefore, this mechanism may be also involved in the detrimental effect of HLA-B*35:01 in these populations.

Our previous studies showed that the NefY135F mutation is selected by both HLA-A*24:02-restricted CD8⁺ T cells specific for the NefRF10 (RYPLTFGWCF) epitope and those recognizing a truncated version, RW8 (RYPLTFGW) although HIV-1 suppression was more potent for RF10-specific T cells [48,49,57]. The study also demonstrated that the mutant virus was able to induce RF10-2F-specific CTLs but did not elicit a new T cell repertoire specific for RW8-2F [48]. Thus, the emergence of the Y135F mutant had a distinct impact on T cells specific for the overlapping RW8 and RF10 epitopes. However, the ability of Nef RF10-2F specific CTLs to suppress replication of the mutant virus was much weaker than that of WT-specific T cells to suppress replication of WT virus *in vitro* [49]. In addition, there was no significant difference between A*24:02⁺ and A*24:02⁻ in individuals infected with either the Y135F mutant virus or those with WT virus (Supplementary Fig. 3). In addition, we demonstrated that there was no significant difference in pVL between responders and non-responders in the mutant virus infected individuals. These findings together suggest that HLA-A*24:02-restricted RF10-specific or RF10-2F-specific CTLs have little ability to suppress HIV-1 replication in B*35:01⁺ individuals.

The present study revealed that Y135F-infected responders to YF9 had significantly lower pVL than the Y135F-infected non-responders, although both had relatively high pVL. This finding suggests that the mutant epitope-specific HLA-B*35:01-restricted T cells, which are elicited after the emergence of the mutant virus, may suppress replication of the Y135F virus to some extent. Additional analysis of the breadth of the T cell responses to RTVY10, NefRY11, and NefYF9 epitopes showed a strong correlation with lower pVL in the Y135F-infected individuals, although even responders to 3 epitopes had a relatively high pVL for our cohort (median: 14,000 copies/mL). These findings suggested that these T cells have a modest effect on the control of the mutant virus *in vivo*

even if there are responses to >3 epitopes. Thus, the Y135F mutation, which occurs commonly in Japan, is a critical factor underlying the detrimental effect of HLA-B*35:01 on disease outcome in HIV-1-infected individuals expressing that allele.

A previous study showed that the HLA-B*35:01 allele had a neutral effect on HIV-1 control in HIV-1 clade C-infection where the p24NY10-specific CD8⁺ T-cell response may contribute to HIV-1 control, however, this T cell response is often missing in HLA-B*35:01⁺ individuals infected with HIV-1 clade B [31]. In our present study, we further analyzed the CTL response to the p24NY10 epitope in B*35:01⁺ individuals and found that approximately 10% (6/63) of our cohort responded to the NY10 epitope. T-cell responses to this epitope were associated with low pVL in clade C infection [31], but not with pVL in clade B-infected Japanese individuals. The present study demonstrated that HIV-1 pVL is controlled to a moderate extent by multiple CD8⁺ T cell responses to p17NY9, RTVY10, NefRY11, and NefYF9 epitopes rather than NY10-specific T cells and shows that the control of HIV-1 by NefYF9-specific T cells is impaired by the HLA-A*24:02-associated Y135F mutation within the epitope.

HIV-1-infected individuals with HLA-B*35-P_X progress more rapidly to AIDS than do those with HLA-B*35-P_Y [15]. A previous study demonstrated that B*35-P_X molecule B*35:03 bound to immunoglobulin-like transcript 4 (ILT4) expressed on dendritic cells with greater affinity than B*35-P_Y molecule B*35:01 and showed that dendritic cells matured in the presence of the B*35:03 molecule had lower expressions of CD86 and HLA-DR and secreted a smaller amount of IL-6 as compared to those matured with B*35:01 [58], suggesting that the binding of B*35-P_X to ILT4 may impair dendritic cell function. Thus, there is a possibility that dendritic cell dysfunction may lead to insufficient induction of acquired immunity against HIV-1 in B*35-P_X⁺ individuals. Another study of T cell responses to cells infected with recombinant vaccinia viruses expressing HIV-1 Env, Gag, Pol, or Nef showed that the frequency of IFN- γ -producing cells amongst CD8⁺ T cells specific for Gag but not for other antigens was significantly associated with lower pVL in B*35-P_Y⁺ individuals, but no such association was seen in those with B*35-P_X⁺ [16]. Thus, only Gag epitope-specific CD8⁺ T cells demonstrate the ability to suppress HIV-1 replication in B*35-P_Y⁺ individuals whereas no such ability of HIV-1-specific CD8⁺ T cells is found in those with B*35-P_X. The present study clarified that CTLs specific for 4 epitopes (Gag NY9 but also Pol VY10, and Nef RY11/YF9) have the capacity to suppress HIV-1 in HIV-1-infected B*35:01⁺ individuals. Therefore, further analysis of CTLs specific for these 4 epitopes in B*35-P_X⁺ individuals may help to define the mechanisms for the detrimental effect of B*35-P_X.

Previous studies showed that many factors influence HIV-1 disease progression [3–11] although genome-wide association studies have clearly demonstrated that the primary factor affecting HIV-1 outcome [12] is the impact of variation in HLA class I genes [13], in addition to CCR5 delta 32, which is rarely detected in Caucasian and Africans and not found at all in Japanese population [3]. These findings suggest that CTLs and NK cells predominantly contribute to the control of HIV-1 and clinical outcome. In the present study, we demonstrated that the overall CD8⁺ T cell response to 4 (of a total of 16 defined epitopes) HLA-B*35:01-restricted epitopes show a relatively modest effect in the control of HIV-1. Furthermore, the accumulation of a single common escape mutation in Nef associated with HLA-A*24:02, which is found in approximately 70% of Japanese individuals, significantly impaired the control of HIV-1 by YF9-specific CTLs in HLA-B*35:01⁺ Japanese individuals, although the remaining 3 protective epitope-specific T cell populations (p17NY9, RTVY10, and NefRY11) contribute to suppression of the mutant virus to some extent. These results together indicate that the NefY135F mutation that is almost universally selected by HLA-A*24:02-restricted CTLs is a key factor in the detrimental effect of HLA-B*35:01 on disease outcomes in HIV-1 clade B-infected Japanese individuals. This study provides a novel potential mechanism underlying the detrimental effect of HLA-B*35:01 on HIV-1 outcomes.

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Declaration of interests

The authors have no financial conflicts of interest.

Author contributions

H.M. performed experiments, analyzed data, and wrote the manuscript. T.A. performed experiments on CTL clones. M.K. performed experiments using ELISPOT assays. N.K. generated the mutant virus. T.C. performed experiments on viral sequencing. H.G. and S.O. supplied samples and clinical data from patients. S.R.-J. contributed to writing the manuscript. M.T. designed the study, supervised all experiments, and wrote the manuscript. All authors revised and edited the manuscript.

Appendix A. Supplementary data

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