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Critical effect of Pol escape mutations associated with detrimental allele HLA-C*15:05 on clinical outcome in HIV-1 subtype A/E infection

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Objective: The mechanism explaining the role of detrimental HLA alleles in HIV-1 infections has been investigated in very few studies. HLA-A*29:01-B*07:05-C*15:05 is a detrimental haplotype in HIV-1 subtype A/E-infected Vietnamese individuals. The accumulation of mutations at Pol 653/657 is associated with a poor clinical outcome in these individuals. However, the detrimental HLA allele and the mechanism responsible for its detrimental effect remains unknown. Therefore, in this current study we identified the detrimental HLA allele and investigated the mechanism responsible for the detrimental effect.

Design and methods: A T-cell epitope including Pol 653/657 and its HLA restriction were identified by using overlapping HIV-1 peptides and cell lines expressing a single HLA. The effect of the mutations on the T-cell recognition of HIV-1-infected cells was investigated by using target cells infected with the mutant viruses. The effect of these mutations on the clinical outcome was analyzed in 74 HLA-C*15:05$^+$ Vietnamese infected with the subtype A/E virus.

Results: We identified HLA-C*15:05-restricted SL9 epitope including Pol 653/657. PolS653A/T/L mutations within this epitope critically impaired the T-cell recognition of HIV-1-infected cells, indicating that these mutations had escaped from the T cells. T-cell responders infected with these mutants showed significantly lower CD4$^+$ T-cell counts than those with the wild-type virus or Pol S653K/Q mutants, which are not associated with HLA-C*15:05.

Conclusion: The accumulation of Pol S653A/T/L escape mutants critically affected the control of HIV-1 by SL9-specific T cells and led to a poor clinical outcome in the subtype A/E-infected individuals having the detrimental HLA-C*15:05 allele.

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Introduction

Previous studies using longitudinal analysis of HLA class I alleles associated with the progression to AIDS showed particular HLA alleles or haplotypes associated with AIDS progression [1–7], while cross-sectional analyses also demonstrated that several HLA class I alleles are strongly associated with clinical parameters such as plasma viral load (pVL) and CD4$^+$ T-cell count (CD4$^+$ cell count) in HIV-1-infected individuals [8–18]. HLA-B*57, HLA-B*27,

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HLA-B*67:01, and HLA-B*52:01-C*12:02 haplotypes have a protective effect on disease progression in whites, Africans, and/or Japanese [6,7,10–13,19], whereas HLA-B*35, HLA-B*07, HLA-B*58:02, HLA-B*08, HLA-B*18, HLA-C*07, and HLA-A*02:07 have a detrimental effect on it [1–4,9,10,13,17–20].

Previous studies suggested possible mechanisms for the effect of protective alleles on the progression to AIDS, such as strong abilities of protective epitope-specific cytotoxic T lymphocytes [21–26] and low fitness of mutant virus selected by these T cells [27,28]. In contrast, little is known about the mechanism underlying the effects of detrimental alleles on disease outcome. A few studies proposed possible mechanisms for the detrimental effect of HLA-B*35 [29–31]. Huang *et al.* [29] demonstrated that strong binding of HLA-B*35:03 molecules to immunoglobulin-like transcript 4 expressed on dendritic cells downregulated the expression of CD86 and HLA-DR and the secretion of IL-6, resulting in impaired dendritic cell function. Matthews *et al.* [30] showed that HLA-B*35:01-restricted T cells specific for the Gag p24 NY10 epitope, which had the ability to suppress HIV-1 replication *in vivo*, were elicited among HIV-1 subtype C-infected HLA-B*35:01$^+$ individuals in Africa, where B*35:01 is a neutral allele, whereas they were not found among the subtype B-infected HLA-B*35:01$^+$ patients in Mexico and Japan, where this allele is a detrimental one. In addition, a recent study demonstrated that the accumulation of the Y to F mutation at Nef135, which is selected by HLA-A*24:02-restricted NefRF10-specific T cells, critically impairs the suppression of HIV-1 replication by HLA-B*35:01-restricted NefYF9-specific T cells in HIV-infected Japanese individuals, suggesting that the accumulation of a single escape mutation is a key factor for the detrimental effect of HLA-B*35:01 on clinical outcome in Japanese [31].

We recently showed that HLA-A*29:01-B*07:05-C*15:05 is a detrimental HLA haplotype in Vietnamese individuals infected with HIV-1 subtype A/E [14]. However, it remained unclear which HLA was the detrimental allele and how HIV-1 mutations were associated with the detrimental effect. In the current study, we sought to identify the detrimental HLA allele in the HLA-A*29:01-B*07:05-C*15:05 haplotype and further to clarify the mechanism responsible for the detrimental effect of this HLA allele on the clinical outcome in HIV-1-subtype A/E-infected Vietnamese.

Methods

Patients

We recruited 74 HLA-C*15:05$^+$ and 312 C*15:05$^-$ ART-naive, Vietnamese individuals chronically infected with HIV-1 subtype A/E from the National Hospital of Tropical Disease in Hanoi during the period of October 2012–December 2017. All participants were adults with an HIV-1 infection, 56% male and 46% female. HIV-1 infection was confirmed by ELISA within 12 months before recruitment (mostly within 3 months). This study was approved by the ethics committees of Kumamoto University and by the Ethics Committee of the Vietnamese Ministry of Health. Informed consent was obtained from all individuals according to the Declaration of Helsinki. HLA types of HIV-infected individuals were determined by standard sequence-based genotyping.

Peptides

HIV-1 peptides with more than 90% purity were synthesized and used in this study.

Cell lines

*HLA-A*29:01*, *HLA-B*07:05*, and *HLA-C*15:05* genes were clones from the RNA of HLA-positive donors. Each *HLA* gene was ligated into the pcDNA3.1/Neo(+) expression plasmid (Invitrogen, Carlsbad, California, USA). 721.221-CD4$^+$ cells expressing HLA-A*29:01, HLA-B*07:05, or HLA-C*15:05 were generated by transfecting 721.221-CD4$^+$ cells with each of these *HLA* genes, as previously described [32,33]. RMA-S cells expressing HLA-C*15:05 (RMA-S-C1505) were generated by transfecting RMA-S cells with the *HLA-C*15:05* gene.

IFN-γ ELISPOT assays

IFN-γ ELISPOT assays were performed as previously described [23,34]. The mean + 3SD of the spot number of samples from 13 HIV-1 naive individuals for overlapping HIV-1 peptides were 162 spots/10^6 CD8$^+$ T cells [23]. Therefore, we defined more than 200 spots/10^6 CD8$^+$ T cells as positive responses.

HIV-1 mutant strains

The Pol S653A, S653T, S653L, S653K, or S653Q mutation was inserted into the 93JP-NH1 plasmid containing a Pol region between ApaI and Af1II sites [35]. The plasmids were digested with ApaI and AflII. The ApaI–AflII 2.7-kb fragment was purified and then ligated into the same site of ApaI–AflII digested 93JP-NH1 plasmids. To obtain mutant viruses, we transfected 293T cells with the 93JP-NH1 plasmids including each mutation by using Lipofectamine 2000 (Invitrogen).

Intracellular cytokine staining assay

Peripheral blood mononuclear cells (PBMCs) from HLA-C*15:05$^+$ individuals were stimulated with a 1 μmol/l concentration of each epitope peptide and cultured for 14 days to induce epitope-specific bulk T cells. Responses of bulk T cells to 721.221 cells prepulsed with each peptide or infected with each virus were analyzed by performing IFN-γ-intracellular cytokine staining (ICS) assays, as previously described [34]. A list of SL9 and its mutant

peptides is shown in Fig. S1, http://links.lww.com/QAD/B854. Data were analyzed with a FACS Canto II (BD Biosciences, San Jose, California, USA). To normalize recognition of HIV-1-infected cells by T cells, we calculated the relative percentage of IFN-γ-producing cells among $CD8^+$ T cells as follows: absolute percentage of IFN-γ-producing cells among $CD8^+$ T cells/(infection rate with wild-type or mutant viruses/100) [28].

HLA class I stabilization assay

The affinity of peptide binding to HLA-C*15:05 was examined by using RMA-S-C1505 cells as previously described [36]. Relative peptide binding affinity was calculated as follows: [MFI (mean fluorescence intensity) of RMA-S cells prepulsed with peptide − MFI of RMA-S cells without peptide]/(MFI of RMA-S cells kept at 26 °C indefinitely − MFI of RMA-S cells without peptide) × 100 [36,37].

Bulk sequence of autologous virus

Bulk sequencing of autologous plasma viral RNA from HIV-1-infected patients was performed as described previously [38].

Statistical analysis

For comparison of two groups in this study, two-tailed Mann–Whitney's test, Wilcoxon rank test, and unpaired *t* test were performed. *P* values less than 0.05 were considered to be statistically significant.

Results

Identification of a novel HLA-C*15:05-restricted Pol epitope

Previous studies showed that 9 Pol and 3 Nef mutations are associated with at least one of the HLA alleles in HLA-A*29:01-B*07:05-C*15:05 haplotype [38] and that two mutations, at Pol 653 and Pol 657, are associated with a poor clinical outcome [14], implying that T cells recognizing an epitope including these positions are involved in the detrimental effect of this haplotype. We therefore sought to identify the T-cell epitope since there is no reports of HLA-A*29:01-restricted, HLA-B*07:05-restricted, or HLA-C*15:05-restricted T-cell epitopes including Pol 653 and Pol 657. We first identified five individuals having this haplotype who had T-cell responses to one 17-mer overlapping peptide cocktail (Pol cocktail 15) including these positions by using the ELISPOT assay (data not shown) and then selected patient VI-479, who was HLA homozygous for this haplotype. We further analyzed T-cell responses to eight 17-mer single peptides included in the Pol cocktail 15 and found that the response to the Pol 17–118 peptide was the strongest among those to these peptides (Fig. 1a), suggesting that this 17-mer peptide contained a T-cell epitope restricted by at least one of the three HLA alleles.

We next sought to define the HLA restriction for the T-cell response to the Pol 17–118 peptide. PBMCs from VI-479 were cultured with the Pol 17–118 peptide for 14 days, and then the bulk T cells were analyzed by performing an ICS assay using the peptide-pulsed 721.221 cells expressing each one of the HLA alleles. The T-cell response to the Pol 17–118 peptide was found only when 721.221-C1505 cells were used as the target cells (Fig. 1b), indicating that this T-cell response was restricted by HLA-C*15:05. We next sought to identify the optimal T-cell epitope by using four overlapping 11-mer peptides covering the Pol 17–118 17-mer peptide. The T cells recognized both Pol11–354 and Pol11–355 peptides (Fig. 1c). Further analysis using truncated peptides of these two peptides demonstrated that the optimal T-cell epitope peptide was Pol SL9: SGIRKVLFL (Fig. 1d). Finally, we investigated whether SL9-specific T cells could recognize HIV-1-infected cells. SL9-specific T-cell line effectively recognized HIV-1-infected 721.221-C1505 cells but neither HIV-1-uninfected ones nor HIV-1-infected 721.221 cells (Fig. 1e), indicating that SL9 was presented as a T-cell epitope in HIV-1-infected HLA-C*15:05$^+$ cells.

Effect of HLA-C*15:05-associated mutations in SL9 epitope on clinical outcome in HIV-1-infected HLA-C*15:05$^+$ individuals

Five HLA-C*15:05-associated mutations (Pol S653A, Pol S653T, Pol S653L, Pol I655V, and Pol K657R) were found within SL9 epitope in Vietnamese individuals infected with the HIV-1 subtype A/E virus [38]. To investigate the effect of these mutations on clinical outcome, we analyzed sequences of the SL9 epitope in 74 HLA-C*15:05$^+$ Vietnamese individuals chronically infected with the subtype A/E virus and then analyzed differences in clinical parameters between the HLA-C*15:05$^+$ individuals infected with the wild-type virus and those with mutant viruses at Pol 653, Pol 655, or Pol 657. The individuals infected with the wild-type virus had a significantly higher $CD4^+$ cell count than those infected with the Pol S653A or Pol S653T mutant virus and higher $CD4^+$ cell count, but not significantly so, than the individuals infected with Pol S653L (Fig. 2a). They also had a significantly higher $CD4^+$ cell count than those infected with one of the mutant viruses (Fig. 2b). The individuals infected with the Pol S653A, Pol S653T, or Pol S653L virus had a higher pVL than those infected with the wild-type one, but no significant difference was found between wild-type and mutant groups (Fig. S2, http://links.lww.com/QAD/B854). On the other hand, no significant difference in $CD4^+$ cell count (Fig. 2a) or in pVL (Fig. S2, http://links.lww.com/QAD/B854) was found between HLA-C*15:05$^+$ individuals infected with the wild-type virus and those with the Pol I655V or Pol K657R virus. No significant difference in $CD4^+$ cell

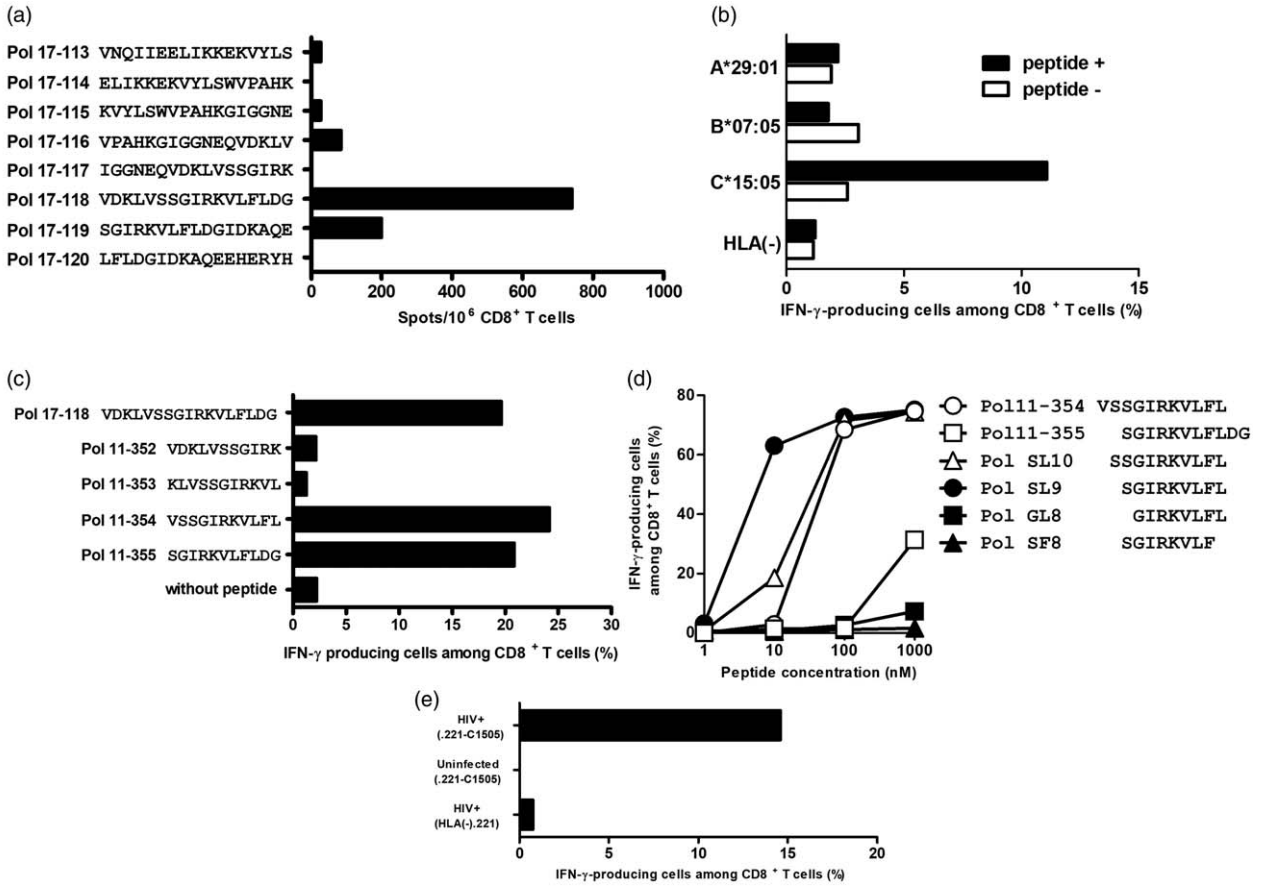

Fig. 1. Identification of SL9 epitope restricted by HLA-C*15:05. (a) T-cell responses to eight 17-mer overlapping Pol peptides in the Pol cocktail 15. T-cell responses by peripheral blood mononuclear cells from an HLA-A*29:01-B*07:05-C*15:05 haplotype-positive individual (VI-479) to each 17-mer overlapping peptide at a concentration of 1 μmol/l were analyzed by using the ELISPOT assay. (b) HLA restriction of the T-cell responses to the Pol 17–118 17-mer peptide. T-cell responses of bulk T cells stimulated with the Pol 17–118 peptide to 721.221-A2901, 721.221-B0705, 721.221-C1505, or HLA-negative 721.221 cells, prepulsed with the Pol 17–118 peptide, were analyzed by use of the intracellular cytokine staining assay. (c) Identification of overlapping 11-mer peptides recognized by the specific T cells restricted by HLA-C*15:05. T-cell responses of the bulk T cells to the 721.221-C1505 prepulsed with 11-mer overlapping peptides covering the Pol 17–118 17-mer one were analyzed by performing the intracellular cytokine staining assay. (d) Identification of the optimal epitope restricted by HLA-C*15:05. Responses of the bulk T cells to 721.221-C1505 prepulsed with individual truncated peptides were analyzed by use of the intracellular cytokine staining assay. (e) Recognition of clade A/E virus-infected cells by T cells specific for SL9. T-cell responses of the bulk T cells to 721.221-C1505 cells infected with HIV-1 93JP-NH1 were analyzed by performing the intracellular cytokine staining assay. Frequencies of p24 antigen-positive cells among stimulator cells were as follow: 721.221-C1505 and HLA-negative 721.221 infected with HIV-1 were 9.3 and 35%, respectively.

count or pVL was found between the individuals infected with wild-type virus and those with Pol S653A/T/L mutant viruses among a total of 312 HLA-C*15:05-negative individuals (data not shown). These results taken together suggest that three Pol653 mutations affected HIV-1 suppression by SL9-specific T cells.

Effect of Pol S653A, S653T, and S653L mutations on SL9-specific T-cell recognition

To investigate the effects of Pol S653A/T/L mutations on SL9-specific T-cell recognition, we analyzed the recognition of the mutant epitope peptides by SL9-specific bulk T cells. We generated the bulk T cells by stimulating PBMCs from two wild-type virus-infected individuals (VI-479 and VI-278) with the wild-type peptide. The specific T cells from both individuals showed significantly weaker recognition of the 1A peptides than that of the wild-type one and almost failed to recognize the 1T and 1L ones (Fig. 2c). These bulk T cells recognized the cells infected with the Pol S653A mutant virus much more weakly than those with the wild-type virus, whereas they failed to recognize the cells infected with the Pol S653T or Pol S653L mutant virus (Fig. 2d), indicating that these three mutations were escape ones from SL9-specific T cells.

We next analyzed peptide binding affinities to HLA-C*15:05 by performing an HLA stabilization assay using RMA-S-C1505 cells. The wild-type peptide and all three of the mutant peptides evenly bound to HLA-C*15:05 at low peptide concentrations, though the binding affinities

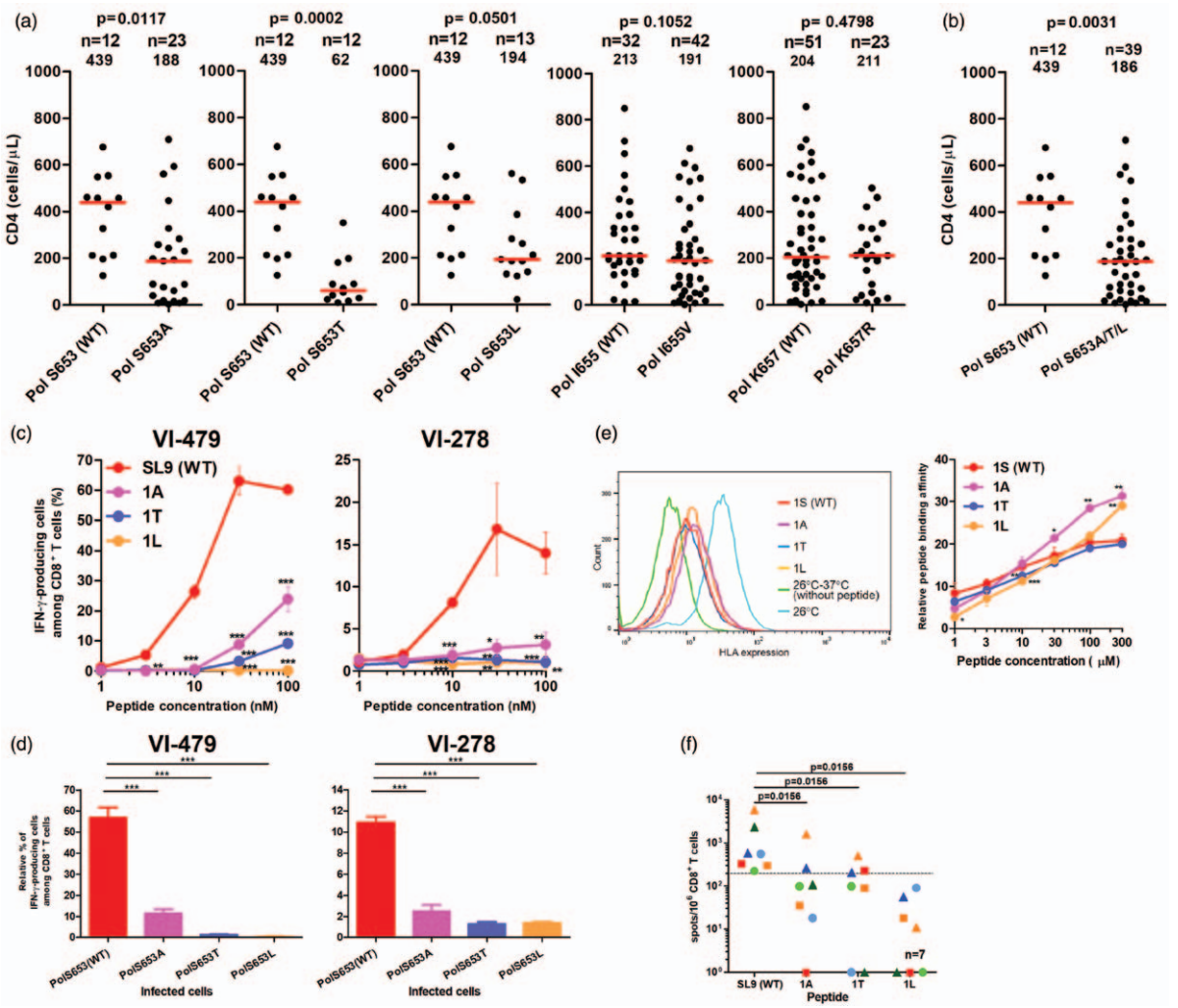

Fig. 2. Effects of Pol S653A, Pol S653T, and Pol S653L on CD4$^+$ cell count and T-cell recognition. Comparison of CD4$^+$ cell count between HLA-C*15:05-positive individuals infected with Pol S653 (wild-type) virus and those with each of Pol S653A, Pol S653T, Pol S653L, Pol I655V, and Pol K657R (a) or with at least one of the Pol S653A/T/L mutant viruses (b). The red lines and the values in each graph represent medians of CD4$^+$ cell count. Statistical analysis was performed by using the Mann–Whitney test. (c) Recognition of 1A, 1T, and 1L mutant epitope peptides by bulk T cells specific for the SL9 epitope. T-cell responses of bulk T cells specific for SL9 epitope, established from two wild-type virus-infected individuals (VI-479 and VI-278), to 1S (wild-type), 1A, 1T, and 1L peptide-prepulsed 721.221-C1505 cells were analyzed by using the intracellular cytokine staining assay. The results are shown as the means and SDs of triplicate assays. Statistical analyses were performed by using the unpaired *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (d) Recognition of mutant-infected cells by SL9-specific T cells. Bulk T cells were stimulated with CD4-expressing 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653}}$ (wild-type), 93JP-NH1$_{\text{Pol S653A}}$, 93JP-NH1$_{\text{Pol S653T}}$, or 93JP-NH1$_{\text{Pol S653L}}$, and IFN-γ production from the T cells was measured by performing the intracellular cytokine staining assay. Proportions of 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653}}$ (wild-type), 93JP-NH1$_{\text{Pol S653A}}$, 93JP-NH1$_{\text{Pol S653T}}$, and 93JP-NH1$_{\text{Pol S653L}}$ were 93.2, 94.9, 93.6, and 88.7%, respectively. To normalize recognition of HIV-1-infected cells by T cells, we calculated the relative percentage of IFN-γ-producing cells among CD8$^+$ T cells as follows: absolute percentage of IFN-γ-producing cells among CD8$^+$ T cells/(infection rate with wild-type or mutant viruses/100). The results are shown as means and SDs of triplicate assays. Statistical analyses were performed by using the unpaired *t* test. $^{***}P < 0.001$. (e) Binding affinity of wild-type, 1A, 1T, and 1L peptides to HLA-C*15:05. Binding affinity of the peptides to HLA-C*15:05 was measured by performing HLA class I stabilization assays using the RMA-S-C1505 cell line. The results are shown as the means and SDs of triplicate assays (right). Relative peptide binding affinity was calculated to evaluate the binding of each peptide to HLA-C*15:05 (right, see Methods). Representative data from flow cytometry analysis are shown at the left. Peptide concentration = 300 μmol/l; 26 °C, incubated at 26 °C throughout; 26–37 °C (without peptide), incubated at 26 °C overnight and then at 37 °C for 3 h. Statistical analyses were performed by using the unpaired *t* test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (f) T-cell responses to the wild-type and three mutant peptides in HIV-1-infected C*15:05$^+$ individuals. T-cell responses to the wild-type and three mutant peptides in 12 wild-type virus-infected individuals were analyzed by using the ex-vivo ELISPOT assay. The data for seven individuals with T-cell responses to the wild-type peptide are shown in the figure. Each symbol indicates one individual. The dotted line at 200 spots/10^6 CD8$^+$ T cells represents the threshold for a positive response. Statistical analyses were performed by using the Wilcoxon rank test.

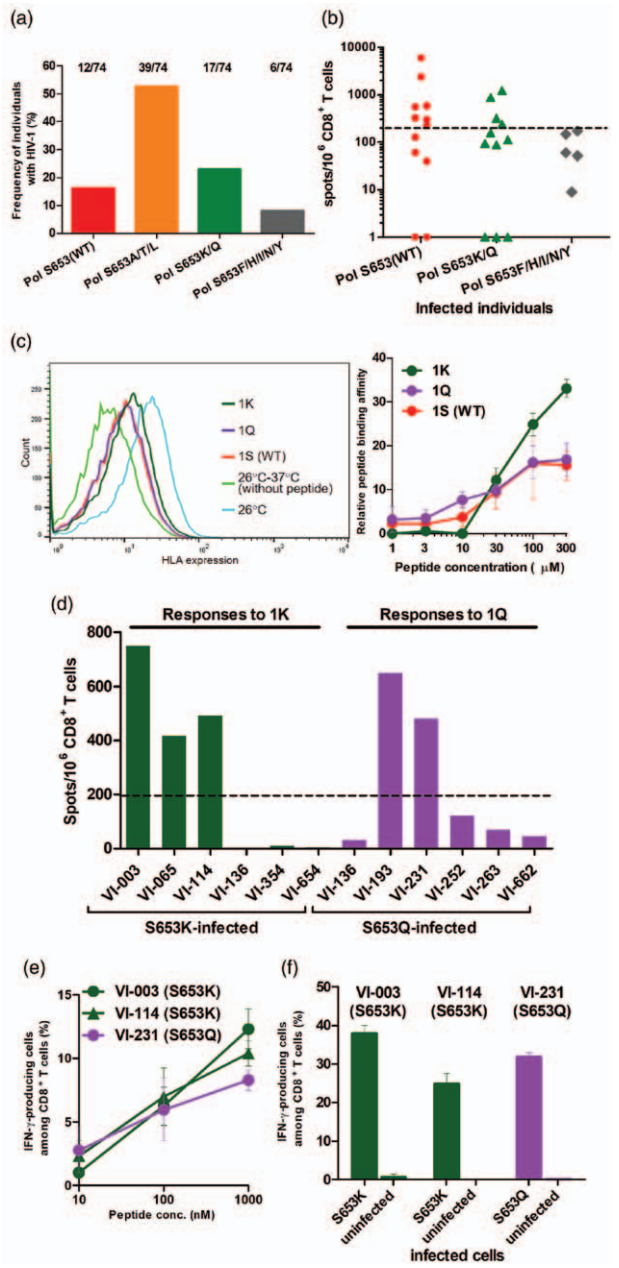

Fig. 3. Detection of T cells recognizing 1K and 1Q mutant epitopes. (a) Frequency of individuals infected with mutant viruses at Pol 653. Frequencies of individuals infected with wild-type, Pol S653A/T/L, S653K/Q, or S653F/H/I/N/Y virus in 74 HLA-C*15:05$^+$ individuals are shown. (b) T-cell responses to the SL9 epitope in individuals infected with mutants at Pol 653 other than Pol S653A/T/L. T-cell responses to wild-type peptide were analyzed by use of the ELISPOT assay. The dotted line at 200 spots/10^6 CD8$^+$ T cells represents the threshold for a positive response. (c) Binding affinity of wild-type, 1K, and 1Q peptides to HLA-C*15:05. Binding affinity of the peptides to HLA-C*15:05 was measured by performing the HLA class I stabilization assay using the RMA-S-C1505 cell line. The results are shown as the means and SDs of triplicate assays (right). Relative peptide binding affinity was calculated as shown in the legend of Fig. 3c (right). Representative data from flow cytometry analysis are shown at the left. Peptide concentration = 300 μmol/l; 26 °C, incubated at 26 °C throughout; 26–37 °C (without peptide), incubated at 26 °C overnight and then at 37 °C for 3 h. (d) T-cell responses to SL9-1K and SL9-1Q epitope peptides in Pol S653K/Q-infected individuals. T-cell responses to SL9-1K and SL9-1Q peptides were analyzed in PolS653K-infected and S653Q-infected individuals, respectively. For patient VI-136 infected with a mixture of S653K/Q viruses, T-cell responses to both 1K and 1Q peptides were analyzed. (e) Recognition of 1K or 1Q epitope peptides by bulk T cells specific for 1K/1Q epitopes. T-cell responses of the bulk T cells, established from two Pol S653K-infected individuals (VI-003 and VI-114) or one S653Q-infected individual (VI-231), to 1K or 1Q peptide-prepulsed 721.221-C1505 cells were analyzed by performing the intracellular cytokine staining assay. The results are shown as the means and SDs of triplicate assays. (f) Recognition of cells infected with S653K or S653Q viruses by 1K/1Q-specific T cells. The bulk T cells were stimulated with CD4-expressing 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653K}}$, or 93JP-NH1$_{\text{Pol S653Q}}$; and IFN-γ production from the T cells was measured by using the intracellular cytokine staining assay. The results are shown as the means and SDs of triplicate assays. Proportions of 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653K}}$ and 93JP-NH1$_{\text{Pol S653Q}}$ were 90.0 and 95.7%, respectively. The results are shown as means and SDs of triplicate assays.

of 1A and 1L peptides to HLA-C*15:05 were significantly higher than that affinity of the wild-type peptide at high peptide concentrations (Fig. 2e), suggesting that these mutations minimally affected peptide binding to HLA-C*15:05. These results taken together suggest that SL9-specific T cells had much lower T-cell receptor (TCR) affinities for 1A/1T/1L mutant epitopes than for the wild-type one.

We next analyzed T-cell responses to wild-type and three mutant epitope peptides by PBMCs from 12 wild-type virus-infected HLA-C*15:05$^+$ individuals and found that seven of them showed positive responses to the wild-type peptide. T-cell responses to the three mutant peptides were significantly much weaker than that response to the wild-type one in these seven individuals (Fig. 2f), confirming these three mutations to be escape ones.

We next analyzed replication capacities of PolS653A/S653T/S653L mutant viruses *in vitro*. The replication capacity of the Pol S653A mutant virus was higher than that of the wild-type virus, whereas that of the Pol S653T one was lower. However, we found only a small difference in replication capacity between S653A/T and wild-type virus (Fig. S3, http://links.lww.com/QAD/B854), suggesting that the effects of these mutations on replication capacity *in vivo* were minimal.

Recognition of Pol S653K/Q virus-infected cells by SL9-1K/1Q-specific T cells

We detected Pol S653K/S653Q mutations in 17 of 23 HLA-C*15:05$^+$ individuals infected with mutants at Pol653 other than Pol S653A/T/L (Fig. 3a). These mutations are not HLA-C*15:05-asssociated ones [38],

suggesting that they were not escape mutations. We therefore speculated that HLA-C*15:05^{+} individuals infected with these mutant viruses would be able to elicit T cells recognizing SL9 wild-type or these mutant peptides. To clarify this possibility, we first analyzed ex-vivo T-cell responses to the wild-type peptide in 16 individuals whose PBMCs were available for the analysis. We detected T-cell responses to SL9 wild-type peptide in four of 11 Pol S653K/Q-infected individuals but in none of five Pol S653F/H/N/Y/I-infected ones (Fig. 3b), suggesting that cross-reactive T cells may have been elicited in the Pol S653K/Q-infected individuals.

We next analyzed the binding affinities of 1K and 1Q mutant peptides to HLA-C*15:05 (Fig. 3c, right). Binding affinities of 1Q and 1S (wild-type) peptides to HLA-C*15:05 were similar, whereas the binding affinity of 1K peptide was higher than that of the 1S one at a high concentration of the peptides such as 300 μmol/l (Fig. 3c, left), indicating that 1K/1Q mutations did not affect the peptide binding to HLA-C*15:05. These results support the idea that HLA-C*15:05^{+} individuals infected with PolS653K or PolS653Q virus could elicit SL9-1K-specific or SL9-1Q-specific T cells. Indeed, the T-cell responses to the mutant peptides were detected in five of the 11 individuals infected with Pol S653K/Q viruses: responses to the SL9-1K peptide were detected in three of the six Pol S653K-infected individuals, while those to SL9-1Q were found in two of the six Pol S653Q-infected individuals (Fig. 3d). Thus, T cells recognizing 1K/1Q mutant epitopes were elicited in individuals infected with HIV-1 harboring the same mutation. We finally generated bulk T cells specific for SL9-1K and SL9-1Q epitopes by stimulating PBMCs from patients VI-003, VI-114, and VI-231 with SL9-1K or SL9-1Q peptides (Fig. 3e). These T cells effectively recognized PolS653K or PolS653Q virus-infected target cells (Fig. 3f).

Longitudinal analysis on selection of Pol S653T/L viruses by SL9-1K-specific T cells in an HLA-C*15:05^{+} individual

We performed a longitudinal analysis of a sequence at Pol 653 and T-cell responses to SL9 epitope in an HLA-C*15:05^{+} individual, VI-065, and found a change in the Pol 653 sequence from K (November 2012) to L (April 2013) and then to S/T/L (October 2013; Fig. 4a). We next analyzed T-cell responses in this individual. Ex-vivo ELISPOT analysis revealed T-cell responses to SL9-1K and SL9-1S but not those to SL9-1T in November 2012 and April 2013, but did not show those to SL9-1L peptide at any of the three time points (Fig. 4b), supporting the idea that SL9-1K-specific T cells selected Pol S653L and S653T mutant viruses. Indeed, SL9-1K-specific T cells, which were generated by stimulating PBMCs isolated in November 2012 or April 2013 with the 1K peptide, failed to recognize 1A/1T/1L mutant and 1S peptides (Fig. 4c) or the cells infected with these three mutant and wild-

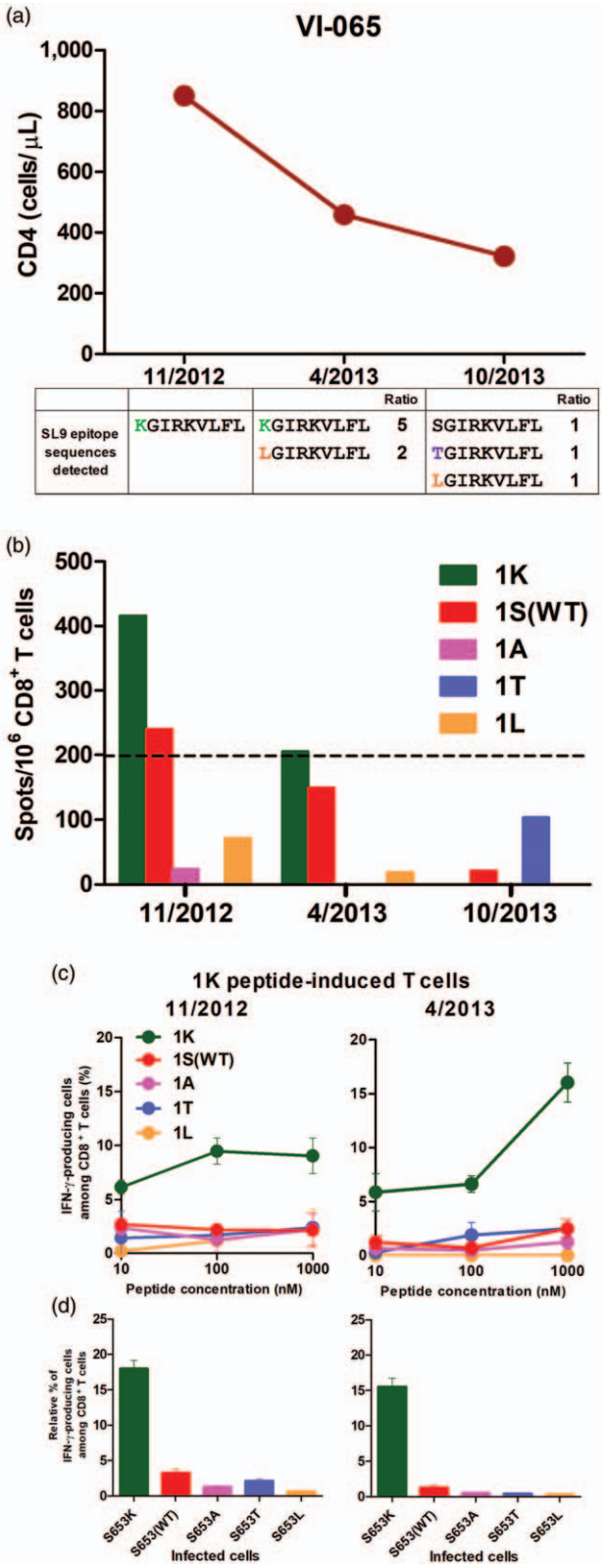

Fig. 4. Longitudinal changes in epitope sequence and T-cell responses in a Pol S653K-infected individual. (a) Longitudinal changes in epitope sequence and CD4^{+} cell count in a Pol S653K-infected individual (VI-065). To evaluate the frequency of amino acids at Pol 653, we performed sequencings

type viruses (Fig. 4d), suggesting that SL9-1K-specific T cells could select the Pol S653T/L mutant virus and the wild-type S virus.

Over all, these results demonstrated that SL9-1K-specific T cells could select the Pol S653L/T mutant virus in this individual.

Critical effect of S653A/T/L mutations on disease outcome via loss of HIV-1 suppression by T cells

We finally clarified the effect of Pol S653A/T/L mutations on HIV-1 suppression by SL9-specific T cells *in vivo*. We first investigated the ability of T cells recognizing 1K/1Q epitopes to suppress HIV-1 replication *in vivo*, since they had abilities to recognize cells infected with Pol S653K/Q mutant viruses (Fig. 3f). Among 11 HLA-C*15:05$^+$ individuals infected with Pol S653K/Q, the responders to SL9-1K or SL9-1Q peptide showed trends toward a higher CD4$^+$ cell count than the nonresponders (Fig. 5a), suggesting that T cells recognizing 1K/1Q epitopes may have had the ability to suppress replication of these mutant viruses *in vivo*. We therefore combined wild-type-infected responders to the wild-type peptide and Pol S653K/Q-infected responders to 1K/1Q ones and then compared the CD4$^+$ cell count between these responders and the Pol S653A/T/L virus-infected responders to the wild-type peptide. The former responders had significantly higher CD4$^+$ cell counts than the latter ones (Fig. 5b), indicating that Pol S653A/T/L mutations impaired the ability of SL9-specific T cells to suppress HIV-1 replication *in vivo*.

Discussion

In the current study, we demonstrated that the accumulation of Pol S653A/T/L mutations within the HLA-C*15:05-restricted Pol SL9 epitope led to a poor clinical outcome in 74 HIV-1 subtype A/E-infected Vietnamese individuals having HLA-C*15:05. A longitudinal analysis of patient VI-065 also showed that the disease progression was associated with the accumulation of the L and T mutations and loss of SL9-1K-specific T cells. A similar mechanism was reported based on the study of the subtype B virus-infected Japanese individuals having a detrimental allele HLA-B*35:01, which study showed that the Nef Y135F mutation selected by HLA-A*24:02-restricted T cells impaired the TCR recognition and viral suppression ability of YF9-specific T cells restricted by HLA-B*35:01 [31]. Both studies showed that the accumulation of escape mutations in the epitopes is a cause of a poor clinical outcome, whereas a different mechanism for the selection of escape mutations was found between the individuals having HLA-C*15:05 and HLA-B*35:01 detrimental alleles.

Since Pol S653K and Pol S653Q mutations are not associated with any HLA alleles [38], it is difficult to clarify the origin of these mutants. These mutations were found in 22% of HLA-C*15:05$^+$ Vietnamese individuals analyzed in the current study. These results suggest that they were natural variations among circulating viruses. We detected T-cell responses to SL9-1K and SL9-1Q mutant epitopes in approximately 45% of individuals infected with Pol S653K/Q viruses. In addition, 1K/1Q-specific T cells effectively recognized cells infected with Pol S653K/Q viruses. These results suggest that the 1K/1Q mutant epitopes were highly immunogenic. A longitudinal analysis of one individual infected with the Pol S653K virus showed that SL9-1K-specific T cells selected Pol S653L and S653T mutant viruses. In addition, responders to SL9-1K or SL9-1Q peptide tended to have a higher CD4$^+$ cell count than nonresponders among HLA-C*15:05$^+$ Vietnamese individuals. These findings support the idea that SL9-1K/1Q-specific T cells can suppress the replication of these mutant viruses as SL9-specifc T cells can do that of the wild-type virus.

The current study strongly suggested that HLA-C*15:05 is a detrimental allele though a previous study showed that HLA-A*29:01-B*07:05-C*15:05 is a detrimental HLA haplotype in Vietnam. HLA-C*15:02 is also found in Vietnamese individuals but this allele was not associated with clinical outcome in Vietnam [14]. One substitution

at Pol 653 five times for the 11/2012 sample, seven times for the 4/2013 sample, and three times for the 10/2013 sample. Means of the ratio of each amino acid were calculated and shown in the figure. (b) Longitudinal changes in T-cell responses to wild-type and mutant epitope peptides in VI-065. T-cell responses to the peptides were analyzed by use of the ELISPOT assay. The dotted line at 200 spots/10^6 CD8$^+$ T cells represents the threshold for a positive response. (c) Recognition of 1A, 1T, and 1L mutant epitope peptides by bulk T cells. The bulk T cells were generated by stimulating peripheral blood mononuclear cells from VI-065 at 11/2012 or at 4/2013 with 1K peptide. T-cell responses of the bulk T cells to 1K, wild-type, 1A, 1T, and 1L peptide-prepulsed 721.221-C1505 cells were analyzed by use of the intracellular cytokine staining assay. The results are shown as the means and SDs of triplicate assays. (d) Recognition by 1K/1Q-specific T cells of cells infected with Pol S653K, S653 (wild-type), and S653A/T/L viruses. The bulk T cells were stimulated with CD4-expressing 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653K}}$, 93JP-NH1$_{\text{Pol S653(WT)}}$, 93JP-NH1$_{\text{Pol S653A}}$, 93JP-NH1$_{\text{Pol S653T}}$, or 93JP-NH1$_{\text{Pol S653L}}$; and IFN-γ production from the T cells was measured by using the intracellular cytokine staining assay. Proportions of 721.221-C1505 cells infected with 93JP-NH1$_{\text{Pol S653K}}$, 93JP-NH1$_{\text{Pol S653(WT)}}$, 93JP-NH1$_{\text{Pol S653A}}$, 93JP-NH1$_{\text{Pol S653T}}$, and 93JP-NH1$_{\text{Pol S653L}}$ were 54.6, 29.1, 88.2, 62.4, and 64.8%, respectively. Relative percentage of IFN-γ-producing cells among CD8$^+$ T cells was calculated as shown in the legend of Fig. 2d. The results are shown as the means and SDs of triplicate assays.

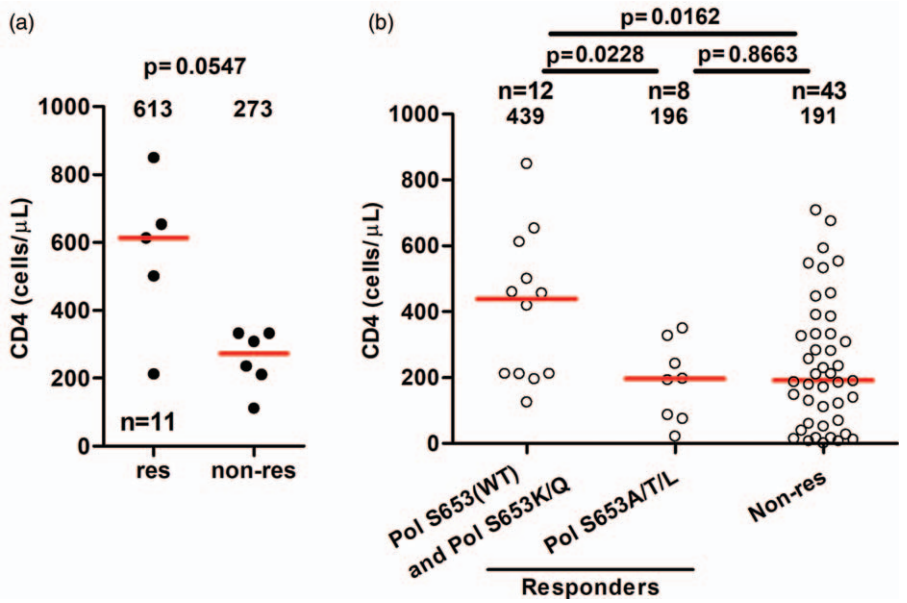

Fig. 5. Impact of Pol S653A/T/L mutations on HIV-1 suppression by T cells specific for the SL9 epitope. (a) Differences in $CD4^+$ cell count between responders and nonresponders to SL9-1K/SL9-1Q epitopes among Pol S653K/Q-infected individuals. (b) Differences in $CD4^+$ cell count between three groups (wild-type-infected responders to SL9 peptide + Pol S653K/Q-infected responders to SL9-1K/SL9-1Q peptides, Pol S653A/T/L-infected responders to SL9 peptide, and nonresponders). T-cell responses to epitope peptides were analyzed by performing the ELISPOT assay. The red lines and the values in each graph represent medians of $CD4^+$ cell count. Statistical analyses were performed by use of the Mann–Whitney test.

is found at position 116 between these two HLA-C*15 subtypes (HLA-C*15:02: Leu and HLA-C*15:05: Phe). Since position 116 is located on the floor of peptide binding groove, this substitution may affect the binding of peptides to these HLA-C*15 molecules. Therefore, it is speculated that HIV-1-specific HLA-C*15:02-restricted T-cell responses are different from the HLA-C*15:05-restricted ones.

The expression level of HLA-C molecules on cells is lower than that of HLA-A or HLA-B ones [39,40]. The lower expression of HLA-C is found even on HIV-1-infected cells in which HLA-A and HLA-B molecules are downregulated [41]. These findings suggest that HLA-C-restricted T cells may be less sufficiently elicited in HIV-1-infected individuals and that they may suppress HIV-1 replication less effectively than HLA-A-restricted and HLA-B-restricted ones. However, a previous study showed that the expression level of HLA-C alleles is negatively correlated with pVL and positively with a frequency of T-cell responses in chronically HIV-1-infected Europeans and African Americans [42], suggesting that some HLA-C-restricted T cells may have the ability to suppress HIV-1 replication *in vivo*. A previous study showed that HIV-1 Vpu-mediated downregulation of HLA-C was found in cells infected with most primary HIV-1 clones [43]. Therefore, we speculated that the HIV-1 Vpu-mediated downregulation of HLA-C*15:05 would critically affect HLA-C*15:05-restricted T-cell recognition for the epitopes and their mutant epitopes.

Together with a previous study on the HLA-B*35:01 detrimental allele in subtype B infections [31], we demonstrated that accumulation of T-cell escape mutations is a key factor for disease progression in HIV-1 infections, especially in individuals having detrimental HLA alleles. We also showed that some HLA-C-restricted T cells had the ability to effectively suppress HIV-1 replication *in vivo* but that the accumulation of escape mutations in the HLA-C-restricted epitope caused a rapid progression of the disease, thus indicating the important role of HLA-C-restricted T cells in the suppression of HIV-1 replication. Further analyses of other HLA-C-restricted T-cell epitopes and their escape mutations will clarify more precisely the mechanism underlying the progression to AIDS and the role of HLA-C-restricted T cells in HIV-1 infections.

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Conflicts of interest

There are no conflicts of interest.

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