Mini Elute Virus spin kit (Qiagen). cDNA was synthesized from the RNA with Superscript II and random primer (Invitrogen). We amplified HIV RT and integrase sequences by nested PCR with RT-specific primers 5′-CCAAAAGTTAAGCAATGGCC-3′ and 5′-CCCATCCAAAGGAATGG AGG-3′ or 5′-CCTTGCCCCTGCTTCTGTAT-3′ for the first-round PCR and 5′-AGTTAGGAATACCACACCCC-3′ and 5′-GTAAATCCCC ACCTCAACAG-3′ or 5′-AATCCCCACCTCAACAGAAG-3′ for the second-round PCR and integrase-specific primers 5′-ATCTAGCTTTGCAG GATTCGGG-3′ and 5′-CCTTAACCGTAGTACTGGTG-3′ or 5′-CCTG ATCTCTTACCTGTCC-3′ for the first-round PCR and 5′-AAAGGTCT ACCTGGCATGGG-3′ or 5′-TTGGAGAGCAATGGCTAGTG-3′ and 5′-AGTCTACTTGTCCATGCATGGC-3′ for the second-round PCR. PCR products were sequenced directly or cloned with a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator v1.1. cycle sequencing kit (Applied Biosystems) and analyzed by an ABI PRISM 310 Genetic Analyzer.

Statistical analysis with phylogenetically corrected odds ratios. Strength of selection was measured by using a phylogenetically corrected odds ratio as previously described (19). Briefly, the odds of observing a given amino acid (e.g., 135V) was modeled as $P/(1 - P) = (a \times X) + (b \times T)$, where P is the probability of observing 135V in a randomly selected individual, X is a binary (0/1) variable representing whether or not an individual expresses the HLA allele in question (e.g., B*52:01), and T equals 1 if the transmitted/founder virus for that individual carried 135V and −1 otherwise. Because the transmitted/founder virus is unknown, we averaged over all possibilities by using weights informed by a phylogeny that was constructed from the RT sequences of all of the individuals in the study. The parameters a and b were determined by using iterative maximum-likelihood methods. The maximum-likelihood estimate of a is an estimate of the natural logarithm of the odds ratio of observing 135V in individuals expressing X versus individuals not expressing X, conditioned on the individuals' (unobserved) transmitted/founder virus. P values are estimated by using a likelihood ratio test that compares the above model to a null model in which a equals 0.

To compare the odds of selection between two cohorts, we modified the phylogenetically corrected logistic regression model to include a cohort term, $Z = X \times Y$, where X is the HLA allele, and Y is a 0/1 variable that indicates cohort membership, yielding $P/(1 - P) = (a \times X) + (b \times T) + (c \times Z)$, as previously described (19, 34). A P value testing if the odds of escape are different in the two cohorts was estimated by using a likelihood ratio test that compared this model to a null model where c equals 0.

Generation of HLA class I tetramers. HLA class I-peptide tetrameric complexes (tetramer) were synthesized as described previously (31, 35). The Pol283-8 peptide was used for the refolding of HLA-B*51:01 or HLA-B*52:01 molecules. Phycoerythrin (PE)-labeled streptavidin (Molecular Probes) was used for generation of the tetramers.

Tetramer binding assay. HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones were stained at 37°C for 30 min with PE-conjugated HLA-B*51:01-tetramer and HLA-B*52:01-tetramer, respectively, at concentrations of 5 to 1,000 nM. After two washes with RPMI 1640 medium supplemented with 10% FCS (RPMI 1640–10% FCS), the cells were stained with FITC-conjugated anti-CD8 MAb at 4°C for 30 min, followed by 7-amino-actinomycin D at room temperature for 10 min. After two more washes with RPMI 1640–10% FCS, the cells were analyzed by the FACS-Cant II flow cytometer. The tetramer concentration that yielded the half-maximal MFI (the EC_{50}) was calculated by probit analysis.

Crystallization, data collection, and structure determination. Soluble HLA-B*52:01 (with beta-2 microglobulin and peptide TAFTIPSI) was prepared as described above. Prior to crystallization trials, HLA-B*52:01 was concentrated to a final concentration of 20 mg ml^{-1} in 20 mM Tris-HCl (pH 8.0) buffer containing 250 mM NaCl. This was done with a Millipore centrifugal filter device (Amicon Ultra-4, 10-kDa cutoff; Millipore). Screening for crystallization was performed with commercially available polyethylene glycol (PEG)-based screening kits, PEGs and PEGs II suites (Qiagen). Thin needle crystals were observed from PEGs II suite 23 (0.2 M sodium acetate, 0.1 M HEPES [pH 7.5], and 20% PEG 3000). Several conditions were further screened by the hanging-drop method with 24-well VDX plates (Hampton Research) by mixing 1.5 μl protein solution and 1.5 μl reservoir to be equilibrated against reservoir solution (0.5 ml) at 293 K. Best crystals were grown from macro seeding with the initial crystals obtained with 0.2 M sodium acetate, 0.1 M Bis Tris propane [pH 7.5], and 20% PEG 3350.

The data set was collected at beamline BL41XU of SPring-8 with Rayonix charge-coupled device detector MX225HE. Prior to diffraction data collection, crystals were cryoprotected by transfer to a solution containing 25% (vol/vol) glycerol and incubation in it for a few seconds, followed by flash cooling. The data sets were integrated with XDS (36) and then merged and scaled by using Scala (37). HLA-B*52:01 crystals belonged to space group $P2_12_12_1$, with unit cell parameters $a = 69.0$ Å, $b = 83.3$ Å, and $c = 170.3$ Å. Based on the values of the Matthews coefficient (V_M) (38), we estimated that there were two protomers in the asymmetric unit with a V_M value of 1.37 A^3/Da (Vsolv = 10.5%). For details of the data collection and processing statistics, see Table S1 in the supplemental material.

The structure was solved by the molecular replacement method with Molrep (39). The crystal structure of HLA-B*51:01 (PDB ID: 1E28) was used as a search model. Structure refinement was carried out by using Refmac5 (40) and phenix (41). The final model was refined to an Rfree factor of 34.7% and an R factor of 29.5% with a root mean square deviation of 0.014 Å in bond length and 1.48° in bond angle for all reflections between resolutions of 38.8 and 3.1 Å. Table S1 in the supplemental material also presents a summary of the statistics for structure refinement. The stereochemical properties of the structure were assessed by Procheck (42) and COOT (43) and showed no residues in the disallowed region of the Ramachandran plot.

Protein structure accession number. Atomic coordinates and structure factors for HLA-B*52:01 have been deposited in the Protein Data Bank under accession code 3W39.

RESULTS

Association of I135X variants with HLA-B*52:01. To clarify the possibility that CTLs restricted by other HLA alleles select the I135X mutation, we investigated the association between other HLA alleles and this mutation in 257 Japanese individuals chronically infected with HIV-1. We found an association of HLA-B*52:01 with the I135X variant, though this association was weaker than that with HLA-B*51:01 (phylogenetically corrected ln odds ratio [lnOR] of 11.76 [$P = 8.77 \times 10^{-4}$] for B*52:01 versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] for B*51:01; Table 1). We also analyzed the effects of HLA-B*52:01 and HLA-B*51:01 in chronically HIV-1-infected Japanese individuals, excluding HLA-B*51:01$^+$ and HLA-B*52:01$^+$ individuals, respectively, and found a significant association between HLA-B*52:01 and I135X variants among 200 HLA-B*51:01-negative individuals with chronic HIV-1 infection ($P = 4.7 \times 10^{-4}$; see Fig. S1A in the supplemental material) and that of HLA-B*51:01 with the variants in 202 HLA-B*52:01-negative ones ($P = 5.3 \times 10^{-8}$; see Fig. S1B in the supplemental material). These results together imply that HLA-B*52:01-restricted CTLs selected this mutation.

Identification of HLA-B*52:01-restricted, Pol283-specific CTLs. To identify the HLA-B*52:01-restricted HIV-1 epitope including RT135, we first investigated whether overlapping peptides covering RT135 could elicit CD8$^+$ T cells specific for these peptides in chronically HIV-1-infected individuals. We identified CTLs recognizing the Pol11-142 (TAFTIPSINNE) peptide in a chronically HIV-1-infected HLA-B*52:01$^+$ donor, KI-069 (see Materials and Methods). Since the C terminus of HLA-B*52:01-binding peptides is known to be a hydrophobic residue (30, 44), we speculated that TAFTIPSI (Pol283-8) was the epitope peptide.

TABLE 1 HLA-B*52:01 and HLA-B*51:01 association with variation at RT135 in Japanese and Caucasian cohorts

HLA class I allele	RT135 target variable	PlyloLOR[a]		Within-cohort P value		P value comparing cohorts
		Japanese	IHAC	Japanese	IHAC	
B*51:01	T	13.70	4.53	4.66×10^{-6}	1.70×10^{-35}	0.042
B*52:01	T	−9.77	1.25	0.464	2.04×10^{-3}	0.62
B*51:01	I	−40.00	−5.71	5.78×10^{-12}	1.58×10^{-51}	0.052
B*52:01	I	−11.76	−3.06	8.77×10^{-4}	2.95×10^{-5}	0.52
B*51:01	V	−9.76	8.52	0.884	0.41	0.85
B*52:01	V	12.21	10.15	0.076	1.82×10^{-3}	0.037
B*51:01	R	12.08	13.02	0.038	2.36×10^{-3}	0.42
B*52:01	R	0.26	8.37	0.423	0.469	0.89
B*51:01	L	−0.89	3.21	1	0.038	0.17
B*52:01	L	−0.56	3.61	1	0.231	0.29
B*51:01	K	−0.71	−40.00	1	0.53	0.99
B*52:01	K	−0.69	−40.00	1	0.779	0.99
B*51:01	M	7.76	12.00	0.894	2.10×10^{-4}	0.34
B*52:01	M	11.09	−40.00	0.034	0.517	0.12

[a] PlyloLOR, phylogenetically corrected lnOR.

Indeed, bulk-cultured T cells that had been cultured for 2 weeks after stimulation with Pol17-48 recognized C1R-B*52:01 cells prepulsed with Pol283-8 peptide at a much lower concentration than those incubated with the Pol11-142 peptide (Fig. 1A), strongly suggesting that Pol283-8 is an epitope recognized by HLA-B*52:01-restricted CTLs. These findings were confirmed by ELISPOT assay with PBMCs from two HLA-B*52:01$^+$ individuals chronically infected with HIV-1 (Fig. 1B). To clarify whether this peptide was processed and presented by HLA-B*52:01, we investigated the killing activity of bulk-cultured T cells against HLA-B*52:01$^+$ target cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant. They killed target cells infected with this recombinant but not those infected with wild-type vaccinia virus (Fig. 1C), indicating that the Pol283-8 peptide was presented by

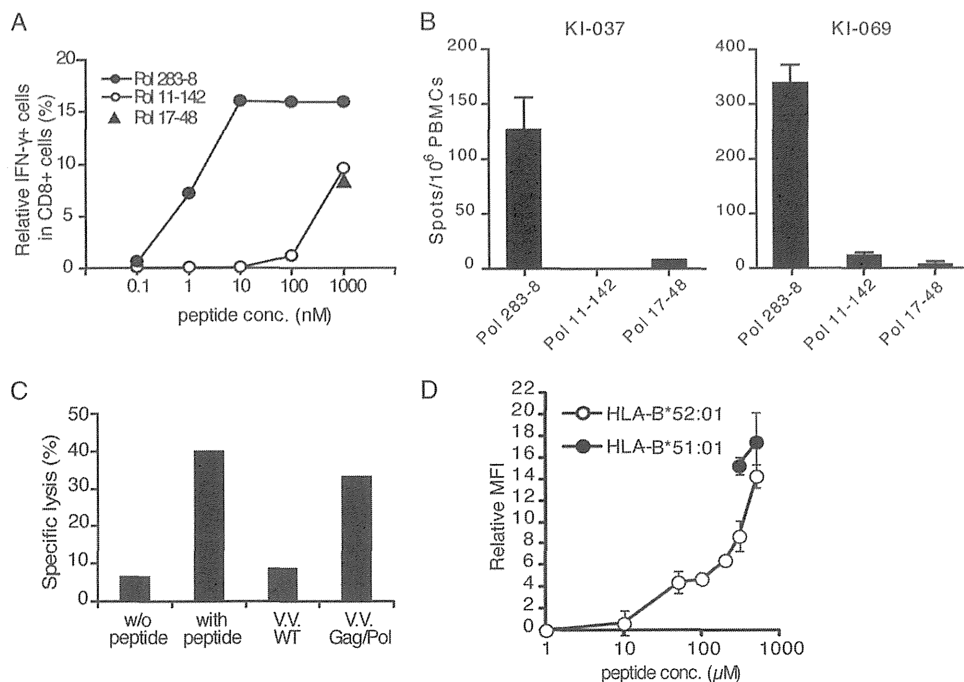

FIG 1 Identification of HLA-B*52:01-restricted Pol epitope. (A) Identification of the epitope peptide recognized by HLA-B*52:01-restricted CD8$^+$ T cells. Bulk T cells were cultured for 2 weeks after stimulation with the Pol17-48 peptide, and then the recognition of C1R-HLA-B*52:01 cells prepulsed with Pol17-48, Pol11-142, or Pol283-8 peptide was assessed by ICS assay. (B) Pol283-8 peptide recognition by T cells *ex vivo*. Recognition of the Pol17-48, Pol11-142, or Pol283-8 peptide by PBMCs from two HLA-B*52:01$^+$ individuals chronically infected with HIV-1 (KI-037 and KI-069) was analyzed by ELISPOT assay. A 100 nM concentration of each peptide was used. (C) Killing activity of Pol283-specific, HLA-B*52:01-restricted CD8$^+$ T cells against cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant. The killing activities of bulk-cultured T cells stimulated with Pol11-142 against target cells infected with a vaccinia virus–HIV-1 Gag/Pol recombinant (Gag/Pol) and against those infected with wild-type vaccinia virus (V.V. WT) are shown. (D) Binding of Pol283-8 peptide to HLA-B*52:01. Binding ability was measured by performing the HLA class I stabilization assay with RMA-S-B*52:01. RMA-S-B*51:01 cells were used as control cells for the Pol283-8 peptide.

TABLE 2 Pol283-8-specific CD8$^+$ T cells in chronically HIV-1-infected, HLA-B*52:01$^+$ individuals

Patient ID	HLA class I alleles		No. of CD4 cells/μl	No. of CD8 cells/μl	Viral load (no. of copies/ml)	Antiretroviral therapy	Relative IFN-γ$^+$/CD8$^+$ % in ICC assay	No. of spots/10^6 PBMCs in ELISPOT[a] assay
KI-037	A*24:02/−	B*52:01/40:02	465	973	76,000	−	64.1	150
KI-090	A*24:02/−	B*52:01/55:01	606	511	≤50	+	40.2	80
KI-106	A*24:02/33:03	B*52:01/07:01	433	890	≤50	+	1.4	<79
KI-126	A*24:02/31:01	B*52:01/40:01	465	NT[b]	36,000	−	60.4	<79
KI-130	A*24:02/−	B*52:01/07:02	351	1,275	14,000	−	0.0	<79
KI-167	A*24:02/−	B*52:01/54:01	455	909	26,000	−	0.0	<79
KI-067	A*24:02/−	B*52:01/48:01	234	1,198	89,000	−	10.9	<79
KI-071	A*24:02/31:01	B*52:01/40:06	292	1,134	48,000	−	0.7	<79
KI-076	A*02:01/24:01	B*52:01/40:01	136	252	14,000	−	61.0	80
KI-114	A*02:01/24:01	B*52:01/27:04	416	463	≤50	+	0.1	<79
KI-056	A*24:02/−	B*52:01/40:02	290	844	8,200	−	-0.1	<79
KI-108	A*24:02/−	B*52:01/−	373	481	NT	−	1.0	<79
KI-028	A*24:02/26:01	B*52:01/48:01	1,351	811	≤50	+	0.5	<79
KI-069	A*24:02/−	B*52:01/40:06	448	1,631	4,400	−	18.1	790

[a] More than the mean number of negative-control spots + 2 SD was defined as a positive response (positive response, >79 spots).
[b] NT, not tested.

HLA-B*52:01. We analyzed the binding of the Pol283-8 peptide to HLA-B*52:01 by using the HLA stabilization assay. The results demonstrated that this peptide bound to HLA-B*52:01 (Fig. 1D). These results together indicate that the Pol283-8 epitope can therefore be presented by both HLA-B*51:01 and HLA-B*52:01.

We investigated whether Pol283-8-specific CD8$^+$ T cells were elicited predominantly in chronically HIV-1-infected HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals. PBMCs from 14 of these individuals were analyzed by ICS assay with Pol283-8 peptide-stimulated culture cells, as well as by ELISPOT assay. The results of the ICS assay showed that 7 of these 14 HLA-B*52:01$^+$ HLA-B*51:01$^-$ patients had Pol283-specific CD8$^+$ T cells, whereas those of the ELISPOT assay with *ex vivo* PBMCs revealed that Pol283-specific CD8$^+$ T cells were detected in only four individuals (Table 2). These results suggest that the three individuals in whom the specific CTLs were detected by the ICS assay but not by the ELISPOT assay may have memory T cells. These results together indicate that Pol283-8 was recognized as an HLA-B*52:01-restricted immunodominant epitope in the HLA-B*52:01$^+$ individuals and support the idea that the I135X mutation was selected by HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cells.

Strong ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cells to suppress HIV-1 replication. A previous study showed that HLA-B*51:01-restricted, Pol283-8-specific T cells have a strong ability to kill HIV-1-infected target cells and to suppress HIV-1 replication (31). Therefore, we expected that the HLA-B*52:01-restricted T cells also would have this strong ability. We generated HLA-B*52:01-restricted, Pol283-8-specific T cell clones and investigated their ability to kill peptide-pulsed or HIV-1-infected target cells. Clone 1E1 effectively killed C1R-B*52:01 cells prepulsed with the Pol283-8 peptide (Fig. 2A) and NL-432-infected CD4$^+$ T cells from an HLA-B*52:01$^+$ individual (Fig. 2B). Additional T cell clones also showed strong killing activity against NL-432-infected HLA-B*52:01$^+$ CD4$^+$ T cells (data not shown). In addition, we investigated the ability of these CTL clones to suppress HIV-1 replication. CD4$^+$ T cells derived from an HLA-B*52:01$^+$ individual were infected with NL-432 or M20A mutant virus, the latter of which has an amino acid substitution at position 20 of Nef and lacks the ability to downregulate the surface expression of HLA-A and -B molecules (Fig. 2C). Representative data on the 1E1 clone and summary data on four clones are shown in Fig. 2D and E, respectively. These CTL clones strongly suppressed the replication of both the NL432 and M20A mutant viruses, indicating that the HLA-B*52:01-restricted CTLs had a strong ability to suppress HIV-1 replication, as was the case with the HLA-B*51:01-restricted ones.

Recognition of I135X mutations by Pol283-8-specific, HLA-B*52:01-restricted CTLs. Four mutations (8T, 8L, 8R, and 8V) were observed predominantly at RT135 in chronically HIV-1-infected HLA-B*52:01$^+$ individuals (Fig. 3). These mutations may have been selected by Pol283-8-specific, HLA-B*52:01-restricted CTLs in these patients. We therefore investigated the ability of HLA-B*52:01-restricted CTLs to suppress the replication of these mutant viruses *in vitro*. The CTL clones failed to suppress the replication of the 8L, 8T, or 8R mutant, though they weakly suppressed that of the 8V virus at an E/T cell ratio of 1:1 (Fig. 4A). These results support the idea that these variants were escape mutations from the HLA-B*52:01-restricted CTLs. To clarify the mechanism by which the CTL clones failed to suppress the replication of these mutant viruses, we investigated the CTL clones for recognition of C1R-B*52:01 cells prepulsed with the mutant peptides. The CTL clones effectively recognized the 8V peptide at the same level as the wild-type peptide and the 8T and 8L peptides at less than that of the wild-type one, whereas they failed to recognize the 8R peptide (Fig. 4B). An ELISPOT assay with *ex vivo* PBMCs from KI-069 showed that Pol283-8-specific CTLs effectively recognized the 8I and 8V variants but not the other three mutant peptides (Fig. 4C), suggesting that Pol283-8-specific CTLs failed to recognize the 8T, 8L, and 8R peptides *in vivo*. The lack of recognition of these mutants by CTLs may be attributable to a failure of T cell receptor (TCR) recognition, the inability of the peptide to bind to HLA-B*52:01, and/or disruption of the processing of the epitope in HIV-1-infected cells.

Different pattern of RT135 mutation selection by two HLA alleles. As described above, HLA-B*51:01 and HLA-B*52:01 were associated with I135X in a Japanese population in which the prevalence of HLA-B*51:01 and B*52:01 alleles is relatively high (21.9 and 21.1%, respectively). In a Japanese cohort, out of the five

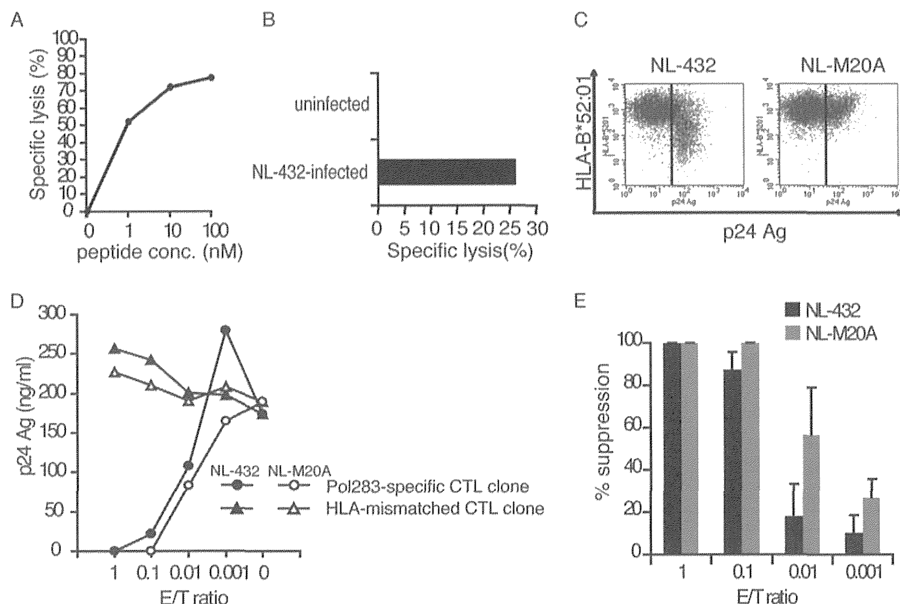

FIG 2 Abilities of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to kill HIV-1-infected CD4$^+$ T cells and to suppress HIV-1 replication. (A) Killing activity of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone against C1R-B*52:01 cells prepulsed with Pol283-8 peptides. The activity of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T clone, 1E1, to kill C1R-B*52:01 cells was measured by performing a ^{51}Cr-release assay. (B) Killing activity of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone 1E1 against CD4$^+$ T cells infected with HIV-1. The ability of the clone to kill CD4$^+$ T cells infected with NL-432 was measured by performing a ^{51}Cr-release assay. (C) Downregulation of HLA-B*52:01 in HIV-1-infected CD4$^+$ T cells. CD4$^+$ T cells derived from an HLA-B*52:01$^+$ donor (HLA-A*11:01/A*24:02, HLA-B*52:01/B*52:01, and HLA-C*12:02/C*14:02) were infected with NL-432 and then cultured for 4 days. The cultured CD4$^+$ T cells were stained with anti-p24 Ag and TÜ109 anti-Bw4 MAbs. (D) Ability of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone to suppress the replication of NL-432 and M20A mutant viruses. Suppressing ability was measured at four different E/T cell ratios (1:1, 0.1:1, 0.01:1, and 0.001:1). HIV-1-infected HLA-B*52:01$^+$ CD4$^+$ T cells were cocultured with an HLA-B*52:01-restricted, Pol283-8-specific CTL clone or an HLA-mismatched CTL clone at various E/T cell ratios. HIV-1 p24 Ag levels in the supernatant were measured on day 6 postinfection. (E) Summary of the ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones ($n = 4$) to suppress the replication of Nl-432 and M20A mutant viruses at four different E/T cell ratios.

amino acid mutations that can be generated by a one-nucleotide mutation from Ile, the T mutation was strongly associated with the presence of HLA-B*51:01 ($P = 4.66 \times 10^{-6}$), whereas HLA-B*52:01 was associated not with any single amino acid substitu-

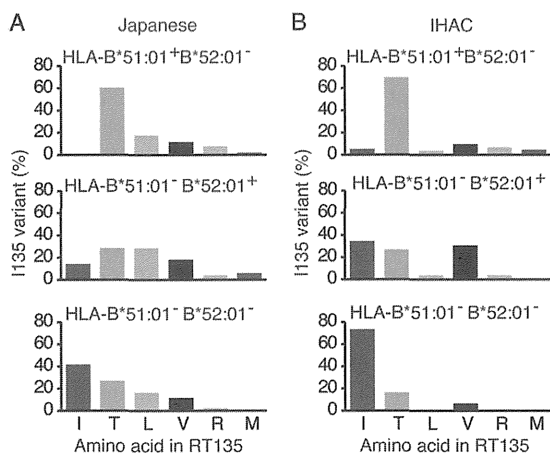

FIG 3 Amino acid variation at RT135 in Japanese individuals. (A) Frequency of the amino acid at RT135 in 51 HLA-B*51:01$^+$ HLA-B*52:01$^-$, 49 HLA-B*51:01$^-$ HLA-B*52:01$^+$, and 151 HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese subjects. (B) Frequency of the amino acid at RT135 in 131 HLA-B*51:01$^+$ HLA-B*52:01$^-$, 26 HLA-B*51:01$^-$ HLA-B*52:01$^+$, and 1195 HLA-B*51:01$^-$ HLA-B*52:01$^-$ subjects in three predominantly Caucasian cohorts from Canada, the United States, and Western Australia (IHAC).

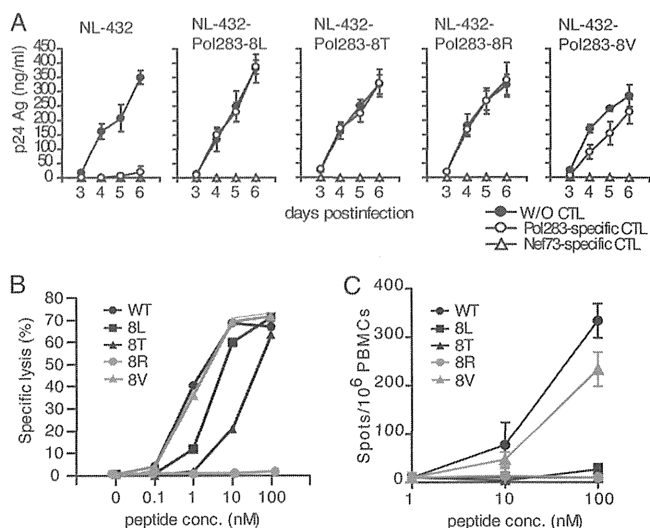

FIG 4 Ability of HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clones to suppress the replication of four mutant viruses. (A) Ability of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone to suppress the replication of four (8L, 8T, 8R, and 8V) mutant viruses and NL-432. The abilities of an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone and an HLA-A*1101-restricted Nef73-specific T cell clone to suppress the replication of these viruses were measured at an E/T cell ratio of 1:1 on days 3 to 6. W/O, without. (B) Recognition by an HLA-B*52:01-restricted, Pol283-8-specific CD8$^+$ T cell clone of C1R-B*52:01 cells prepulsed with any one of the four mutant epitope peptides or the wild-type (WT) peptide (8I). (C) Recognition of mutant epitope peptides by *ex vivo* Pol283-8-specific CTLs. The recognition of the Pol283-8 peptide (WT) or the mutant epitope peptide by PBMCs from KI-069 was analyzed by ELISPOT assay.

tion but only with the non-I mutation ($P = 8.77 \times 10^{-4}$, Table 1). The distribution of amino acid variations at RT135 in the HLA-B*51:01$^+$ HLA-B*52:01$^-$ Japanese subjects was different from that in the HLA-B*51:01$^-$ HLA-B*52:01$^+$ ones (Fig. 3). These results suggest that the HLA-B*51:01-restricted CTLs strongly selected the 135T mutation but that the HLA-B*52:01-restricted ones selected a variety of different amino acids at this position in Japanese individuals.

We also analyzed the association of I135X mutations with HLA-B*52:01 and HLA-B*51:01 in three predominantly Caucasian cohorts from Canada, the United States, and Western Australia (International HIV Adaptation Collaborative [IHAC]) (19) comprising >1,200 subjects (Table 1). HLA-B*51:01 was very strongly associated with the I135X mutation (lnOR of 5.71; $P = 1.58 \times 10^{-51}$). Although only 2.1% of the IHAC cohort subjects expressed HLA-B*52:01, this allele was also associated with I135X (lnOR of 3.06; $P = 2.95 \times 10^{-5}$). The T mutation was strongly associated with HLA-B*51:01 ($P = 1.70 \times 10^{-35}$), whereas the T and V mutations were weakly associated with HLA-B*52:01 ($0.0005 < P < 0.005$). Thus, these results showed a similar selection of RT135 mutations by HLA-B*52:01 in the predominantly Caucasian cohort, despite a substantially lower frequency of HLA-B*52:01. The magnitude of the strength of selection by HLA-B*52:01 and HLA-B*51:01 on RT135 did not differ significantly between the two cohorts (Table 1). These results indicate that HLA-B*51:01 strongly selected 135T but that HLA-B*52:01 selected a variety of substitutions at this site (designated I135X) in both the Japanese and non-Japanese cohorts.

Comparison of TCR affinity and abilities of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTLs to suppress HIV-1 replication. We investigated the TCR affinity of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones by using tetramers of the HLA-B*51:01-Pol283 peptide and the HLA-B*52:01-Pol283 peptide complex (HLA-B*51:01 and HLA-B*52:01 tetramers, respectively). The TCR affinity of these CTL clones was compared in terms of EC_{50}. The EC_{50} of the HLA-B*51:01-restricted CTL clones was significantly lower than that of the HLA-B*52:01-restricted CTL clones (Fig. 5A), suggesting that the former CTL clones had TCRs with a higher affinity for the ligand than those of the latter clones. These results imply that the HLA-B*51:01-restricted CTL clones could recognize the HIV-1-infected targets more effectively than HLA-B*52:01-restricted ones.

Since CD4$^+$ T cells derived from an HLA-B*52:01 homozygous individual were used in the experiment shown in Fig. 2D and E, the ability of the HLA-B*52:01-restricted CTL clones to suppress the replication of NL-432 may have been overestimated. To evaluate and compare the abilities of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones to suppress the replication of NL-432, we used CD4$^+$ T cells from individuals expressing HLA-B*51:01$^+$/B*52:01$^-$, HLA-B*51:01$^-$/B*52:01$^+$, or HLA-B*51:01$^+$/B*52:01$^+$ (Fig. 5B). Two HLA-B*51:01-restricted CTL clones strongly inhibited the replication of HIV-1 in cultures of NL-432-infected HLA-B*51:01$^+$/B*52:01$^-$ CD4$^+$ T cells but not in those of HLA-B*51:01$^-$/B*52:01$^+$ cells, whereas two HLA-B*52:01-restricted CTL clones strongly inhibited the replication of HIV-1 in cultures of NL-432-infected HLA-B*51:01$^-$/B*52:01$^+$ CD4$^+$ T cells but not in those of HLA-B*51:01$^+$/B*52:01$^-$ cells (Fig. 5B, left and middle). The ability of the HLA-B*51:01-restricted CTL clones to suppress the replication of HIV-1 was greater than that of the HLA-B*52:01-restricted CTL clones. This was confirmed by

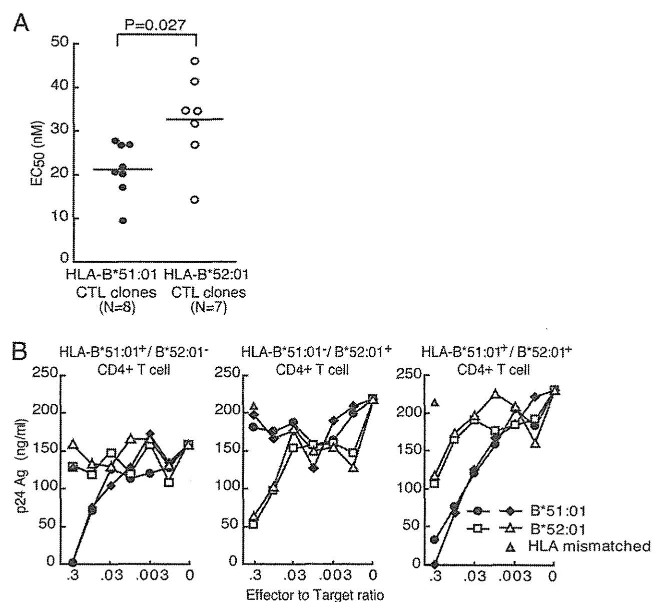

FIG 5 Differences between HLA-B*51:01-restricted and HLA-B*52:01-restricted CD8$^+$ T cell clones in TCR avidity and the ability to suppress HIV-1 replication. (A) TCR avidity of the HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones expressed as EC_{50}. The ability of the TCRs of HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones to bind HLA-B*51:01 tetramers and HLA-B*52:01 tetramers, respectively, was measured in terms of the MFI of each CTL clone stained with the tetramers at concentrations of 5 to 1,000 nM. (B) The ability of two HLA-B*51:01-restricted and two HLA-B*52:01-restricted CD8$^+$ T cell clones to suppress HIV-1 was measured at six different E/T cell ratios (0.3:1, 0.1:1, 0.03:1, 0.01:1, 0.003:1, and 0.001:1). CD4$^+$ T cells from individuals expressing HLA-B*51:01$^+$/B*52:01$^-$, HLA-B*51:01$^-$/B*52:01$^+$, or HLA-B*51:01$^+$/B*52:01$^+$ were infected with NL-432 and then cocultured with a given Pol283-8-specific CTL clone or an HLA-mismatched CTL clone. HIV-1 p24 Ag levels in the supernatant were measured on day 5 postinfection.

the experiment with HLA-B*51:01$^+$/B*52:01$^+$ CD4$^+$ T cells (Fig. 5B, right). Although both HLA-B*51:01-restricted and HLA-B*52:01-restricted CTL clones strongly inhibited the replication of HIV-1 in the cultures of NL-432-infected HLA-B*51:01$^+$/B*52:01$^+$ CD4$^+$ T cells, the former clones exhibited a greater ability to suppress the replication of HIV-1 than did the latter cells. These results indicate that the HLA-B*51:01-restricted CTL clones had a stronger ability to suppress HIV-1 replication than the HLA-B*52:01-restricted clones. Taken together, both our *in vitro* and our *in vivo* (population level HLA-association) data suggest that immune pressure on RT135 by HLA-B*51:01-restricted T cells was stronger than that imposed by HLA-B*52:01-restricted cells.

Structural basis of the difference in recognition between HLA-B*52:01- and HLA-B*51:01-restricted CTLs. In order to investigate the structural basis of the difference in recognition between HLA-B*52:01- and HLA-B*51:01-restricted CTLs, we performed a crystallographic study of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide. The recombinant HLA-B*52:01 protein was crystallized, and by using the molecular replacement method, the three-dimensional structure of HLA-B*52:01 complexed with the Pol283-8 peptide was successfully determined. The crystal and statistical data are summarized in Table S1 in the supplemental material. The overall structure and peptide-binding mode were similar to those of HLA-B*51:01 complexed with the same Pol283-8 peptide (Fig. 6A and B), which

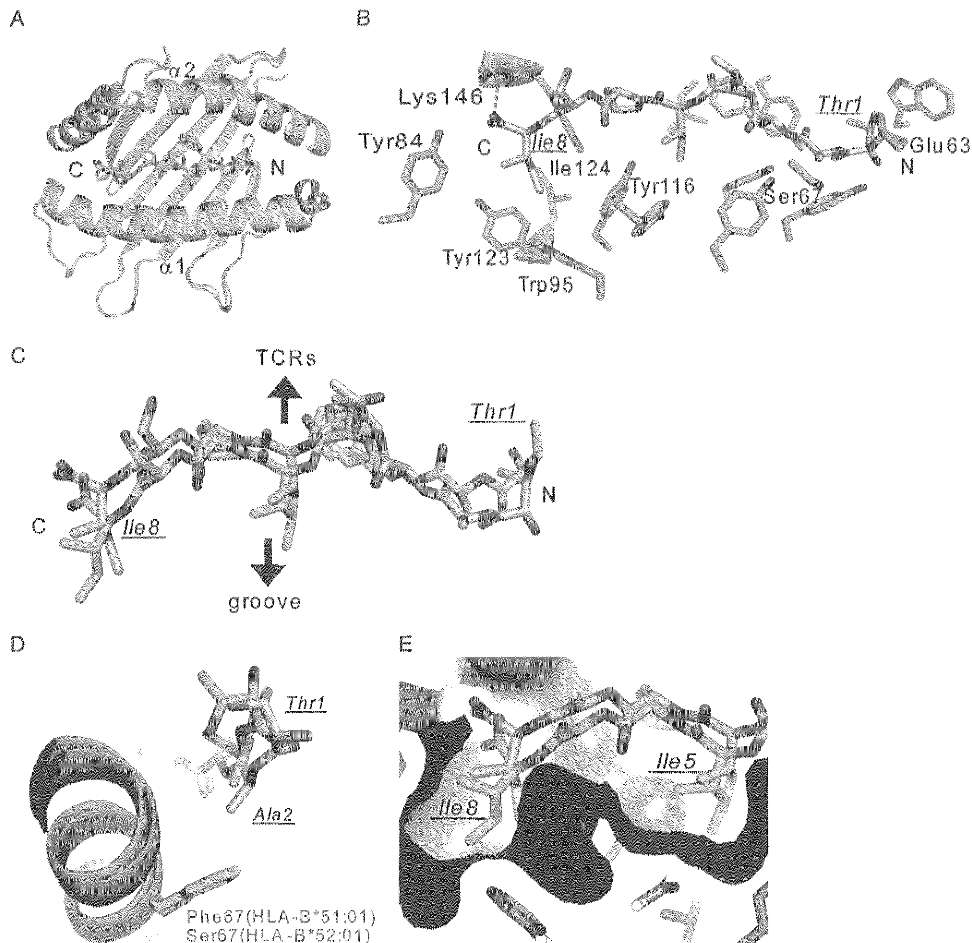

FIG 6 Structural comparison of HLA-B*52:01 and HLA-B*51:01 molecules complexed with the Pol283-8 peptide. (A) Crystal structures of HLA α1-α2 domains complexed with the Pol283-8 peptide (stick model) on the HLA-B*52:01 (green, yellow) and HLA-B*51:01 (cyan, cyan) complexes. This same coloring also applies to panels B to E. (B) Pol283-8 peptide and interacting side chains on the HLA-B*52:01 complex. Hydrogen bonds are shown as blue dotted lines. (C) Comparison of the Pol283-8 peptide conformations of HLA-B*52:01 and HLA-B*51:01 complexes. (D) N-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. (E) C-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. Surface presentation for the α1-α2 domains is shown in gray.

we had previously reported (45). This finding explains the cross presentation of this peptide by both HLA alleles. On the other hand, there was a notable conformational difference in the N-terminal region of the peptide between the two alleles (Fig. 6C and D). The replacement of Phe67 of HLA-B*51:01 with Ser in HLA-B*52:01 makes a local space, causing the N-terminal region of the peptide (T1 and A2) to reside deeper in the peptide-binding groove. Furthermore, the Gln63Glu mutation in HLA-B*52:01 affords a new interaction with the T1 residue of the peptide. These changes would, to some extent, have hidden the side chains of T1 and A2 (flat surface) from the TCRs, which may have reduced their interactions with TCRs on the HLA-B*52:01-restricted CTLs. On the other hand, the conformation of the C-terminal region of the peptide complexed with HLA-B*51:01 or HLA-B*52:01 was similar, even though C-terminal Ile8 of the peptide exhibited shallower penetration of the hydrophobic groove in the case of HLA-B*52:01 than in that of HLA-B*51:01 (Fig. 6C and E). These results may indicate that the relatively flat surface of the N-terminal side of the peptide contributed to the lower affinity for TCRs in the case of HLA-B*52:01.

DISCUSSION

HLA-B*52:01 and HLA-B*51:01 differ by only two residues, at positions 63 and 67 (44). Substitutions at these residues affect the formation of the B pocket in the peptide-binding pocket (45), suggesting the possibility that HLA-B*52:01 has a peptide motif different from that of HLA-B*51:01. Indeed, HLA-B*52:01-binding peptides have P2 primary anchors that are different from HLA-B*51:01-binding ones (30, 46). Since the Pol283-8 epitope carries Ala at its second position and Ile at the C terminus of the peptide, it is likely that this peptide would effectively bind to HLA-B*51:01 but not to HLA-B*52:01. However, the results of the HLA stabilization assay demonstrated that the Pol283-8 peptide did effectively bind to HLA-B*52:01. Since the HLA-B*52:01-binding peptide is known to have Pro as its preferred P2 anchor residue, this peptide carrying Ala at position 2 may be capable of binding to HLA-B*52:01. A previous study showed cross-recognition of allo-reactive T cells between HLA-B*51:01 and HLA-B*52:01 (47, 48), indicating that some self-peptides can be presented by both of these HLA class I molecules. The findings on the crystal structure

TABLE 3 Numbers and frequencies of individuals having I135X mutations in a Japanese cohort and a predominantly Caucasian cohort

	No./total no. (%) of individuals				
Cohort	B*51:01$^+$ B*52:01$^-$	B*51:01$^-$ B*52:01$^+$	B*51:01$^+$ B*52:01$^+$	B*51:01$^-$ B*52:01$^-$	Total
Japanese	51/51 (100)	42/49 (85.7)	5/5 (100)	88/151 (58.3)	186/256 (72.6)
Caucasian	125/131 (95.4)	17/26 (65.4)	0/0	331/1,198 (27.6)	473/1,355 (34.9)

of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide clarified that HLA-B*52:01 could bind to the peptide in a fashion similar to but slightly different from that of HLA-B*51:01. These findings support the presentation of the Pol283-8 peptide by both HLA-B*52:01 and HLA-B*51:01.

Pol283-8-specific CD8$^+$ T cells were detected in 7 of 14 HLA-B*52:01$^+$ HLA-B*51:01$^-$ individuals chronically infected with HIV-1. A previous analysis showed that CD8$^+$ T cells specific for this epitope are frequently detected in HLA-B*51:01$^+$ individuals chronically infected with HIV-1 (49). These results, taken together, indicate that this epitope is immunodominant in both HLA-B*51:01$^+$ and HLA-B*52:01$^+$ individuals. The analysis of 257 Japanese individuals revealed an association between HLA-B*52:01 and a variety of nonconsensus residues at RT codon 135 (I135X). Specifically, variants 8T, 8L, 8R, and 8V predominated in HLA-B*52:01$^+$ individuals, suggesting that these mutations had been selected by HLA-B*52:01-restricted CTLs. The viral suppression assay revealed that the HLA-B*52:01-restricted CTLs failed to suppress the replication of these mutant viruses. These results support the idea that the I135X mutation can be selected by immune pressure via Pol283-8-specific CTLs in HLA-B*52:01$^+$ individuals. Our previous studies showed that the 8L, 8T, and 8R mutations affected the recognition by Pol283-8-specific, HLA-B*51:01-restricted CTL clones (15, 28). These studies, together with the present study, indicate that accumulation of 8L, 8T, and 8R mutations in the HIV-infected Japanese population may be due to immune pressure by both HLA-B*52:01-restricted and HLA-B*51:01-restricted CTLs. Our analysis of the crystal structure of the HLA-B*52:01-peptide complex demonstrated that position 8 of the Pol283-8 peptide was deeply packed into the hydrophobic groove. Whereas the 8L, 8T, and 8R substitutions likely had a relatively large effect on the structure of the complex, the 8V mutation, resulting in only the deletion of the small methyl group, caused only very limited changes. Thus, the structural analysis supports the idea that the 8L, 8T, and 8R mutations affected the TCR recognition of the peptide and/or its binding to HLA-B*52:01.

The present study confirmed previous studies of nine worldwide cohorts (15) and a Chinese cohort (50) that showed a strong association of I135X with HLA-B*51:01. The I135X mutation was found in 58.3 and 27.6% of HLA-B*51:01$^-$ HLA-B*52:01$^-$ Japanese and predominantly Caucasian individuals, respectively (Table 3), supporting greater population level accumulation of this mutation in Japanese than in other cohorts. Since the Japanese cohort included twice as many HLA-B*51:01$^+$ individuals as the IHAC cohort (21.9% of Japanese and 9.4% of Caucasians in IHAC), the difference in the I135X variant frequency between these two cohorts would be driven, to a large extent, by the higher HLA-B*51:01 prevalence in the former than in the latter. The association of HLA-B*52:01 with this mutation was much weaker than that of HLA-B*51:01 in both cohorts but still highly statistically significant (an lnOR of 11.7 [$P = 8.77 \times 10^{-4}$] versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] in the Japanese cohort and an lnOR of 3.06 [$P = 2.95 \times 10^{-5}$] versus an lnOR of 5.71 [$P = 1.58 \times 10^{-51}$] in IHAC). Because of the relatively low B*52:01$^+$ frequency (~2%) in IHAC, the effect of HLA-B*52:01 on the overall prevalence of I135X was relatively low in this cohort. In contrast, in the Japanese cohort, where the HLA-B*52:01$^+$ prevalence was relatively high (>20%), this allele represents a major driving force behind 135X selection in this cohort. Thus, selection pressure from both HLA-B*51:01 and HLA-B*52:01 likely contributed to the observed population level accumulation of I135X mutations in the Japanese population.

Previous studies showed that HLA-B*51:01-restricted, Pol283-8-specific CTLs have a strong ability to suppress HIV-1 replication *in vitro* (28) and that they suppressed the replication of the 8V mutant virus but failed to suppress that of the 8T, 8L, and 8R mutant viruses (15). The frequency of the Pol283-8-specific CTLs was inversely correlated with the plasma viral load in HLA-B*51:01$^+$ hemophiliacs infected with HIV-1 approximately 30 years ago (28). The 8T, 8L, and 8R mutations did not affect replication capacity, whereas the 8V mutation conferred a modest fitness cost (15). These findings support the suppression of the wild-type or 8V mutant virus by Pol283-8-specific CTLs as a major mechanism of slow progression to AIDS in Japanese hemophiliacs. This CTL response was also elicited in Chinese HLA-B*51:01$^+$ individuals infected with the 8V mutant virus; furthermore, a low viral load and a high CD4 count were significantly associated with the presence of at least one of three HLA-B*51:01-restricted CTL responses, including a Pol283-8-specific one (50). Thus, these findings support the idea that Pol283-8-specific CTLs play an important role in the control of HIV-1 infection.

The present study demonstrated that HLA-B*52:01-restricted, Pol283-8-specific CTLs also had a strong ability to suppress HIV-1 replication *in vitro* (80% suppression at an E/T cell ratio of 0.3:1). However, the ability of HLA-B*52:01-restricted CTLs to suppress the replication of HIV-1 was weaker than that of HLA-B*51:01-restricted CTLs (Fig. 5B). Inspection of the crystal structures of both HLA molecules complexed with the Pol283-8 peptide suggests that the relatively shallow penetration of the hydrophobic groove of HLA-B*52:01 by the C-terminal side of the peptide, in contrast to the tightly packed binding with HLA-B*51:01, may have resulted in an unstable conformation of the complex. Furthermore, Ser67 of HLA-B*52:01 would have provided more space and loose interactions with the peptide than in the case of the Phe of HLA-B*51:01. Interestingly, the Pol283-8 peptide would have displayed only side chains of Thr1 and Ser7, and some part of the main chains, to CTLs. Therefore, these results suggest that the unstable backbone conformation and side chain positions in the case of HLA-B*52:01 largely contributed to the lower TCR affinity than that afforded by HLA-B*51:01. These results support that selection pressure *in vivo* via the HLA-B*52:01-restricted CTLs would be weaker than that via the HLA-B*51:01-restricted CTLs. Indeed, the prevalence of I135X mutations in HLA-B*51:

01+ individuals was higher than that in HLA-B*52:01+ individuals. The difference in the pattern of escape mutant selection by these CTLs between the HLA-B*51:01+ and HLA-B*52:01+ individuals might also have been due to the difference in their abilities to suppress HIV-1 replication. However, it still remains unclear why the 8T mutant was predominantly selected in the HLA-B*51:01+ but not in the HLA-B*52:01+ individuals. Further studies are expected to clarify the mechanism to explain how these CTLs selected different patterns of mutations at RT135.

Previous studies showed that the T242N mutant was selected by HLA-B*58:01-restricted and HLA-B*57-restricted CTLs specific for TW10 epitope in HIV-1 clade B-infected and clade C-infected individuals (25–27). Herein we also showed that I135X was selected by Pol283-8-specific CTLs restricted by two different HLA class I molecules. However, the strength and the pattern of the selection of I135X was different between HLA-B*51:01 and HLA-B*52:01. The present study suggests that this difference in the selection pattern was associated with that between the HLA-B*51:01+ and HLA-B*52:01+ individuals in terms of the ability of Pol283-specific CTLs to suppress HIV-1 replication. Thus, we characterized and experimentally validated distinct HIV-1 escape patterns of CTLs with the same epitope specificity and provided evidence that the extremely high prevalence of 135X in circulating Japanese sequences is likely driven not by one but by two HLA-B alleles.

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Selection of TI8-8V Mutant Associated with Long-Term Control of HIV-1 by Cross-Reactive HLA-B*51:01–Restricted Cytotoxic T Cells

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Elite controllers of HIV-1–infected HLA-B*51:01$^+$ hemophiliacs, who remain disease free and have a very low plasma viral load for >30 y, had the 8V mutation at an immunodominant Pol283-8 (TI8) epitope, whereas the 8T mutant was predominantly selected in other HIV-1–infected HLA-B*51:01$^+$ hemophiliacs, suggesting an important role of the 8V mutant selection in long-term control of HIV-1. However, the mechanism of this selection and the long-term control in these elite controllers remains unknown. In this study, we investigated the mechanism of the 8V mutant selection in these controllers. TI8-specific CTLs from these individuals evenly recognized both TI8 peptide–pulsed and TI8-8V peptide–pulsed cells and effectively suppressed replication of wild-type (WT) and the 8V viruses. However, the results of a competitive viral suppression assay demonstrated that CTLs from the individual who had WT virus could discriminate WT virus from the 8V virus, whereas those from the individuals who had the 8V virus evenly recognized both viruses. The former CTLs carried TCRs with weaker affinity for the HLA-B*51:01-TI8-8V molecule than for the HLA-B*51:01-TI-8 one, whereas the latter ones carried TCRs with similar affinity for both molecules. The reconstruction of the TCRs from these CTLs in TCR-deficient cells confirmed the different recognition of the TCRs for these epitopes. The present study showed that the 8V mutant virus could be selected by cross-reactive CTLs carrying TCR that could discriminate a small difference between the two molecules. The selection of the 8V mutant and elicitation of these two cross-reactive CTLs may contribute to the long-term control of HIV-1. *The Journal of Immunology*, **2014, 193: 4814–4822.**

Cytotoxic T lymphocytes play an important role in the control of HIV-1 (1–9). However, HIV-1 can escape from CTL-mediated immune pressure by various mechanisms such as Nef-mediated HLA class I downregulation and mutation to allow escape from HIV-1–specific CTLs (10, 11). The acquisition of amino acid mutations within CTL epitopes and/or its flanking regions leads to reduced ability for peptide binding to HLA class I molecules, impaired TCR recognition, and defective epitope generation (12, 13), resulting in lack of CTL activities to suppress replication of HIV-1 mutant virus as well as in the selection and accumulation of escape mutant viruses (10, 14–19).

A minority of HIV-1–infected individuals, who are known as elite controller or long-term nonprogressors, remain disease free and have a very low viral load (VL), even in the absence of antiretroviral therapy (20–22). A majority of these elite controllers carry the HLA-B*57/58:01, HLA-B*27, or HLA-B*51 allele associated with slow progression to AIDS (23, 24), suggesting that HIV-1–specific CTLs restricted by these HLA alleles control HIV-1 in elite controllers. The mechanism of the control by these CTLs has been well studied in elite controllers and slow progressors carrying HLA-B*57/58:01, HLA-B*27 or HLA-B*13. These studies showed strong Gag-specific CD8$^+$ T cell responses in elite controllers or slow progressors carrying these alleles, suggesting that they may control HIV-1 (15, 16, 25–27). HLA-B*57–mediated immune pressure selects the escape mutation T242N in the Gag TW10 epitope. This mutation impairs viral replication, resulting in control of HIV-1 in these HLA-B*57$^+$ individuals (28, 29). In the case of HLA-B*27$^+$ individuals, the presence of Gag KK10–specific CD8$^+$ T cell is associated with the control of HIV-1 (4, 30–32). The immunodominant KK10 epitope is almost invariably targeted by CD8$^+$ T cells, and the KK10-specific CD8$^+$ T cells display potent effector functions (4, 30, 31, 33). The conservation of this response is thought to account for the control of HIV-1 in these individuals.

A previous study showed that the HLA-B*51:01 allele was associated with long-term control of HIV-1 in HIV-1–infected Japanese hemophiliacs, and the frequency of HLA-B*51:01–restricted Pol283-290 (TI8: TAFTIPSI)–specific CD8$^+$ T cells was inversely associated with plasma VL in HIV-1–infected ones (34), suggesting an important role of TI8-specific CD8$^+$ T cells in the long-term control of HIV-1 infections. Four mutations (8T, 8L, 8R, and 8V) at position 8 of the TI8 epitope were significantly detectable in HLA-B*51$^+$ individuals more than in HLA-B*51$^-$ individuals, suggesting that these mutations were selected by TI8-specific CTLs (35). The 8T mutation is predominantly found in HIV-1–infected HLA-B*51:01$^+$ donors. TI8-specific CTLs have a strong ability to suppress the replication of wild-type (WT) and the 8V mutant viruses in vitro but fail to suppress that of the 8T, 8L, and 8R mutant viruses (35, 36). A study using a Japanese hemophiliac cohort showed that the 8V mutation is found in only

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; RT, reverse transcriptase; VL, viral load; WT, wild-type.

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HLA-B*B51:01$^+$ elite controllers (34), suggesting that the selection of the 8V mutant virus is a critical factor for long-term control of HIV-1 in Japanese hemophiliacs. However, the mechanism underlying selection of the 8V virus in HLA-B*51:01$^+$ elite controllers remains unclear.

In the present study, to clarify the mechanisms of the 8V mutant selection, we investigated how TI8-specific CTLs from elite controllers select the 8V mutant. We established TI8-specific CTL clones from three elite controllers and then analyzed their abilities to suppress the replication of the 8V virus and to select this mutant in vitro. In addition, we assessed the TCR affinity of the CTLs for HLA-B*51:01 with the 8V peptide or WT peptide and evaluated the function of TCRs isolated from the CTL clones by reconstructing them in TCR-deficient cells. In the present study, we clarified the mechanism of the 8V selection and suggested its role in the long-term control of HIV-1 in Japanese hemophiliacs.

Materials and Methods

Patients

Three HIV-1–infected, antiretroviral-naive Japanese hemophiliacs were recruited for the current study, which was approved by the ethics committees of Kumamoto University (RINRI number 540, GENOME number 210) and the National Center for Global Health and Medicine (ID-NCGM-A-000172-00). Written informed consent was obtained from all subjects according to the Declaration of Helsinki.

Cells

C1R cells were purchased from American Type Culture Collection. C1R cells expressing HLA-B*51:01(C1R-B*51:01) were previously generated by transfecting C1R cells with *HLA-A*51:01* genes (37, 38). They were maintained in RPMI 1640 medium containing 5% FBS (R5) and 0.15 mg/ml hygromycin B. TCR-deficient mouse T cell hybridoma cell line TG40 cells were provided by T. Saito (RIKEN Institute, Saitama, Japan). TG40 cells expressing human CD8α (TG40/CD8) were previously established by transfecting TG40 cells with CD8α genes (39). TG40/CD8 and T1 cells, purchased from American Type Culture Collection, were maintained in R5.

HIV-1 clones

A previously reported infectious proviral clone of HIV-1, pNL-432, was used (40). Pol283-8V mutant viruses were previously generated on the basis of pNL-432 (34, 35).

Generation of TI8-specific CTL clones

HLA-B*51:01–restricted TI8-specific CTL clones were generated from HIV-1–specific bulk-cultured T cells by limiting dilution in U-bottom 96-well microtiter plates (Nunc). Each well contained 200 μl cloning mixture (~1 × 10^6 irradiated allogeneic PBMCs from healthy donors and 1 × 10^5 irradiated C1R-B*51:01 cells prepulsed with the corresponding peptide at 1 μM in RPMI 1640 medium containing 10% FBS [R10], 200 U/ml human rIL-2, and 2.5% PHA soup). These CTL clones were cultured in RPMI 1640 medium containing 10% FBS, 200 U/ml human rIL-2, and 2.5% PHA soup. The CTL clones were stimulated biweekly with irradiated target cells pulsed with the peptide.

Cytotoxic assay of CTL clones

The cytotoxic activity of TI8-specific CTL clones was determined by the standard ^{51}Cr release assay described previously (36). Briefly, C1R-B*51:01 cells were incubated with 100 μCi $Na_2{}^{51}CrO_4$ in saline for 60 min and then washed three times with R5. Labeled target cells (2 × 10^3/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc) with the appropriate amount of the corresponding peptide. After a 1-h incubation, effector cells were added at an E:T ratio of 2:1, and then, the cultures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the number of counts per minute in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the number of cpm in the supernatant in the wells containing both target and effector cells.

HIV-1 replication suppression assay

The ability of TI8-specific CTL clones to suppress HIV-1 replication was examined as described previously (41). CD4$^+$ T cells were isolated from PBMCs of HLA-B*51:01$^+$ healthy donors and incubated with the desired HIV-1 clones for 4 h at 37°C. After three washes with R5, the cells were cocultured with TI8-specific CTL clones. From days 3 to 7 post infection, culture supernatants were collected, and the concentration of p24 Ag in them was measured by use of an ELISA kit (HIV-1 p24 Ag ELISA kit; ZeptoMetrix).

In vitro competitive viral suppression assay

T1 cells (HLA-B*51:01$^+$) were coinfected with NL-432 and NL432-Pol283-8V mutant viruses at a ratio of 9:1. The infected cells were incubated with TI8-specific CTL clones at an E:T ratio of 0.05:1. From days 4 to 7 postinfection, culture supernatants were collected; and the concentration of p24 Ag in these supernatants was measured by using the HIV-1 p24 Ag ELISA kit (ZeptoMetrix). Viral RNA was extracted from the culture supernatant by using a QIAamp Viral RNA Mini kit (Qiagen) and subjected to RT-PCR by use of a SuperScript III One-Step RT-PCR System (Invitrogen). Nested PCR was subsequently performed for direct sequencing. The ratio of WT to mutant virus was determined by the relative peak height on the sequencing electrogram.

Tetramer binding assay

HLA class I–peptide tetrameric complexes (tetramers) were generated as described previously (34). CTL clones were stained with PE-conjugated tetramers at 37°C for 30 min. The cells were then washed twice with R5, followed by staining with FITC-conjugated anti-CD8 mAb and 7-amino-actinomycin D (7-AAD) at 4°C for 30 min. Finally, they were washed twice with R5. For the analysis of tetramer association, CTL clones were stained with PE-conjugated tetramer at a concentration of 1 μM at 37°C for various times (0, 2, 5, 10, 15, 30, and 60 min) and then washed as described above. For the analysis of tetramer dissociation, CTL clones were stained with PE-conjugated tetramer at a concentration of 1 μM at 37°C for 30 min. A portion of the cells was then removed periodically (0, 5, 10, 15, 30, 60, 80, and 110 min), and the cells were washed as described above. The cells stained with tetramer were then analyzed by flow cytometry (FACSCanto II).

Sequencing of plasma RNA

Viral RNA was extracted from the plasma of chronically HIV-1–infected individuals by using a QIAamp Mini Elute Virus spin kit (Qiagen). cDNA was synthesized from the RNA with Superscript III and random primer (Invitrogen). We amplified HIV reverse transcriptase (RT) sequences by nested PCR with RT-specific primers 5′-ACACCTGTCAACATAATTGG-3′ and 5′-TGTATGTCATTGACAGTCCA-3′ for the first-round PCR and 5′-GGGCCTGAAAATCCATACAA-3′ and 5′-GGTGATCCTTTCCATCCC-TG-3′ for the second-round PCR. PCR products were sequenced directly or cloned with a TOPO TA cloning kit (Invitrogen) and then sequenced. Sequencing was done with a BigDye Terminator version 1.1. cycle sequencing kit (Applied Biosystems) and analyzed by use of an ABI PRISM 310 or 3100 Genetic Analyzer.

TCR clonotype analysis

CTL clones were stained with PE-conjugated tetramers, anti-CD8 mAb, and 7-AAD, and then, tetramer$^+$CD8$^+$7-AAD$^-$ cells were sorted by using a FACSAria. Unbiased identification of TCR-αβ-chain usage was assessed as described previously (42). TCR gene designations were based on the ImMunoGeneTics database.

Reconstruction of TCRs on TCR-deficient T cells

The cDNAs encoding full-length TCRα and TCRβ of TI8-specific CTL clones were obtained by a previously described method (42). cDNA was then amplified by nested PCR with TCR-α–specific primers 5′-GGAATTCGCCGCCACCATGCTCCTGCTGCTCGTCCCAG-3′ and 5′-ATTTGCGGCCGCAGATCTCAGCTGGACCACAGCCGCAG-3′ or 5′-GGAATTCGCCGCCACCATGGAAACTCTCCTGGGAGTGT-3′ and with TCR-β–specific 5′-GGAATTCGCCGCCACCATGGCCTCCCTGCTCTT-CTTCT-3′ and 5′-ATTTGCGGCCGCCTAGCCTCTGGAATCCTTTCTC-TTGA-3′ or 5′-GGAATTCGCCGCCACCATGGGCACCAGGCTCCTCT-GCT-3′. The amplified genes were separately cloned into a retrovirus vector pMX and used to transfect TG40/CD8, as described previously (39). Briefly, the genes of TCR were subcloned into the retroviral vector pMX. First, the ecotropic virus packaging cell line Platinum-E was transfected with the constructs. Two days later, the culture supernatant containing recombinant

virus was collected and then incubated with TG40/CD8 cells in the presence of 10 μg/ml polybrene for 6 h. The cells were cultured for an additional 2 d for analysis of TCR gene expression. Finally, the cells showing bright staining with PE-conjugated anti-mouse $CD3^+$ mAb (2C11; BD Pharmingen) were sorted by using the FACSAria.

IL-2 secretion assays

TG40/CD8 cells transfected with TCRs (4×10^4/well) were cultured with C1R-B*51:01 (4×10^4/well) in 200 μl R5 in the presence of various concentrations of peptides in U-bottom 96-well microtiter plates (Nunc) for 48 h at 37°C. The culture supernatants were then collected, and the concentration of IL-2 in them was measured by use of an IL-2 ELISA kit (eBioscience).

Results

*Slow selection of Pol283-8V mutant in HLA-B*51:01⁺ Japanese elite controllers*

We previously revealed that only 3 (patients KI-021, KI-051, and KI-124) of 108 Japanese hemophiliacs who had survived without antiretroviral therapy for ~15 y, from 1983 to 1998, exhibited a strong inhibition of HIV-1 for an additional 10 y (34). All three of these elite controllers recruited from 1997 to 1999 had HLA-B*51:01 and the 8V mutation at position 8 in the TI8 epitope. To identify when the 8V emerged, we performed longitudinal sequence analysis of the TI8 epitope in these three patients. KI-021 and KI-124 already had the 8V mutant in September 1997 and January 1998, respectively. KI-051 had only the WT sequence in October 1999, the 8V mutant in 63% of the clones analyzed in July 2002, and then only the 8V mutant in October 2006 (Table I). Since KI-051 had been infected with HIV-1 before 1985, these results indicate that the WT virus predominantly existed for >17 y after HIV-1 infection. Thus, this mutant was slowly selected and had accumulated in this patient.

Recognition of the 8V mutant by TI8-specific CTLs

A previous study showed that the 8V mutation weakly reduced the recognition of TI8-specific CTL clones established from KI-051 in July 2002 (34, 35). We first reconfirmed this finding by using three TI8-specific CTL clones (2B5, 2C6, and 2D1), which were established from KI-051 in July 2002 when the WT virus was still detectable. The results for a representative CTL clone (2C6 clone) are shown in Fig. 1A. This clone effectively suppressed both WT and 8V mutant viruses but revealed slightly weaker ability to suppress the replication of the 8V mutant than the WT virus at higher E:T ratios. All three CTL clones revealed significantly weaker ability to suppress the replication of the 8V mutant than the WT virus at an E:T cell ratio of 1:1 (Fig. 1B, 1C). We further established 11 TI8-specific CTL clones from three elite controllers (KI-051 in June 2009, KI-021 in September 1997 and January 2005, and KI-124 in August 2001) and investigated the ability of these TI8-specific CTL clones to recognize the WT and the mutant epitopes. We first measured the killing activity toward target cells prepulsed with the WT or the 8V mutant peptide. All 11 CTL clones as well as 2B5, 2C6, and 2D1 clones showed the same killing activity toward target cells prepulsed with the 8V peptide as that of those prepulsed with the WT peptide (Supplemental Fig. 1). We next analyzed the ability of these CTL clones to suppress the replication of the 8V virus. CTL clones 2B, 7B, and 7F, which were established from KI-051 in June 2009 when only the 8V virus was detectable, exhibited a similar ability to suppress both the 8V and the WT virus at the same level (Fig. 1B, 1C). The CTL clones from KI-021 and KI-124 also showed characteristics similar to those of these clones from KI-051 (Fig. 1B, 1C).

Table I. Sequence of Pol283-8 epitope in three HLA-B*51:01⁺ elite controllers of HIV-1–infected Japanese hemophiliacs

Patient	HLA Allele						Sample Date (month/date/year)	Pol283-8 Epitope			VL (copies/ml)	CD4 (cells/ml)	Name of CTL Clone
	A Allele		B Allele		C Allele			Sequence	Frequency				
								TAFTIPSI[a]	Direct (%)[b]	Cloning[c]			
KI-021	2402	2602	5101	6701	0702	1402	9/12/1997	- - - - - - - - V	100	12/12	<400	727	3B, 4C, 3D
							3/27/1998	- - - - - - - - V	100	12/12	<400	808	
							1/27/2005	- - - - - - - - V	100	12/12	<50	646	10, 20, 52
KI-051	0206	3101	4002	5101	1402	1502	10/26/1999	- - - - - - - - -	100	NT[d]	<400	629	
							7/25/2002	- - - - - - - - -	37	6/12	63	911	2B5, 2C6, 2D1
								- - - - - - - - V	63	6/12			
							10/24/2006	- - - - - - - - V	75	NT[d]	<50	966	
								- - - - V - - V	25	NT[d]			
							6/30/2009	- - - - - - - - V	90	9/12	<50	1040	2B, 7B, 7F
								- - - - V - - V	10	3/12			
KI-124	1101	0206	5101	1501	0401	1402	1/13/1998	- - - - - - - - V	100	15/15	<400	745	
							8/16/2001	- - - - - - - - V	100	18/18	600	511	12E, 12H

[a]The sequences for KI-021 in March 1998 and January 2005 were obtained from proviral DNA, whereas those for all other patients and KI-021 in September 1997 were from plasma RNA.
[b]Direct, direct sequence.
[c]Number of clones carrying the indicated sequence/number of clones tested.
[d]NT, not tested.

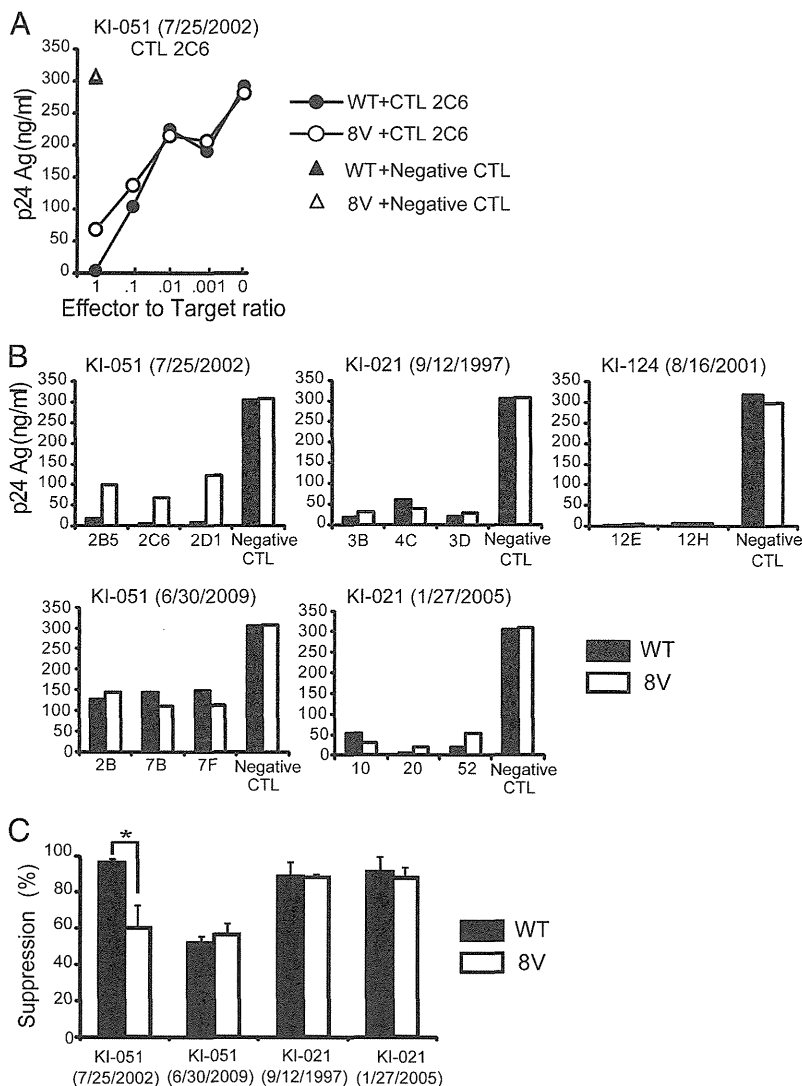

FIGURE 1. Abilities of TI8-specific CTL clones to suppress the replication of the 8Vmutant virus. (**A**) Ability of TI8-specific CTL clone 2C6, which was established from KI-051 in July 2002 when WT virus was still detectable, to suppress the replication of NL432 virus and the 8V virus. $CD4^+$ T cells from an HLA-B*51:01$^+$donor were infected with NL432 or NL432-Pol283-8V virus and then cocultured with clone 2C6 at different E:T ratios. An HLA-mismatched (HLA-A*11:01–restricted Pol675 specific) CTL clone was used as negative control at an E:T cell ratio of 1:1. HIV-1 p24 Ag levels in the supernatant were measured on day 6 postinfection by performing an enzyme immunoassay. (**B**) Summary of the ability of other TI8-specific CTL clones established from elite controllers (KI-051, KI-021, or KI-124). The results at the E:T ratio of 1:1 are shown. (**C**) Percent inhibition of three clones. Statistical analysis was performed by using the paired *t* test. *$p < 0.05$.

In vitro selection of the 8V mutant by TI8-specific CTLs

The results described above strongly suggest that the 8V was an escape mutant selected by TI8-specific CTLs having the ability to discriminate this mutant from WT. The 8V virus had the same replication capacity as the WT virus in a competitive proliferation assay for 7 d (Supplemental Fig. 2). Therefore, to confirm this selection by the TI8-specific CTLs, we investigated whether TI8-specific CTL clones could select this mutant virus in vitro by competitive viral suppression assay for 7 d. The TI8-specific CTL clones were cultured with HLA-B*51:01–positive $CD4^+$ T cells infected with NL-432 and the 8V mutant virus together at a ratio of 9 to 1. The ratio of the 8V mutant virus to the WT one increases if the TI8-specific CTLs have ability to suppress WT virus more than the mutant virus. Indeed, CTL clones 2B5, 2C6, and 2D1 from KI-051, which exhibited weaker ability to suppress replication of the 8V virus compared with that to suppress that of the WT (Fig. 1), selected the 8V mutant virus in this competitive viral suppression assay (Fig. 2A). In contrast, other CTL clones, which exhibited similar ability to recognize the 8V as the WT (Fig. 1), did not select the 8V mutant virus (Fig. 2A). These results indicate that the 8V mutant was selected as an escape mutant by CTLs that could discriminate the 8V virus from the WT virus but not by other CTLs, which could not do so. HIV-1 p24 Ag levels in the culture supernatant in the presence of the CTL clones were very low compared with those in the absence of these clones (Fig. 2B), confirming that all CTL clones used in this experiment strongly suppressed the replication of both viruses.

TCR affinity of TI8-specific CTL clones

We found two types of TI8-specific CTL clone for the 8V recognition. To further characterize the 8V recognition by these CTL clones, we investigated the TCR affinity of these clones by using tetramers of HLA-B*51:01 with TI8 peptide (WT tetramer) and with the 8V mutant peptide (8V tetramer). Clone 2B5 exhibited significantly weaker affinity for the 8V tetramer than for the WT one (EC_{50}: 60.7 ± 14.3 nM for WT and 332.5 ± 32.7 nM for 8V; $p < 0.00019$; Fig. 3A). In contrast, clone 2B from KI-051 after the emergence of the 8V mutant and clone 3B from KI-021 showed almost the same affinity for both tetramers (EC_{50}: clone 2B, 116.3 ± 52.3 nM for WT and 115.1 ± 39.2 nM for 8V; $p < 0.98$; clone 3B, 104.5 ± 16.5 nM for WT and 112.5 ± 56.1 nM for 8V, $p < 0.82$; Fig. 3A). The TCR affinity for the 8V tetramer of all CTL clones was compared in terms of EC_{50} ratio of WT to 8V tetramer. The EC_{50} ratio of the CTL clones from KI-051 in July 2002 was significantly lower than that of the CTL clones from KI-051 in June 2009 and from KI-021 in September 1997 and January 2005 (Fig. 3B). These results taken together indicate that 2B5 carried TCR's with weaker affinity for the 8V tetramer than for the WT one, whereas 2B and 3B carried TCR's with similar affinity for both tetramers.

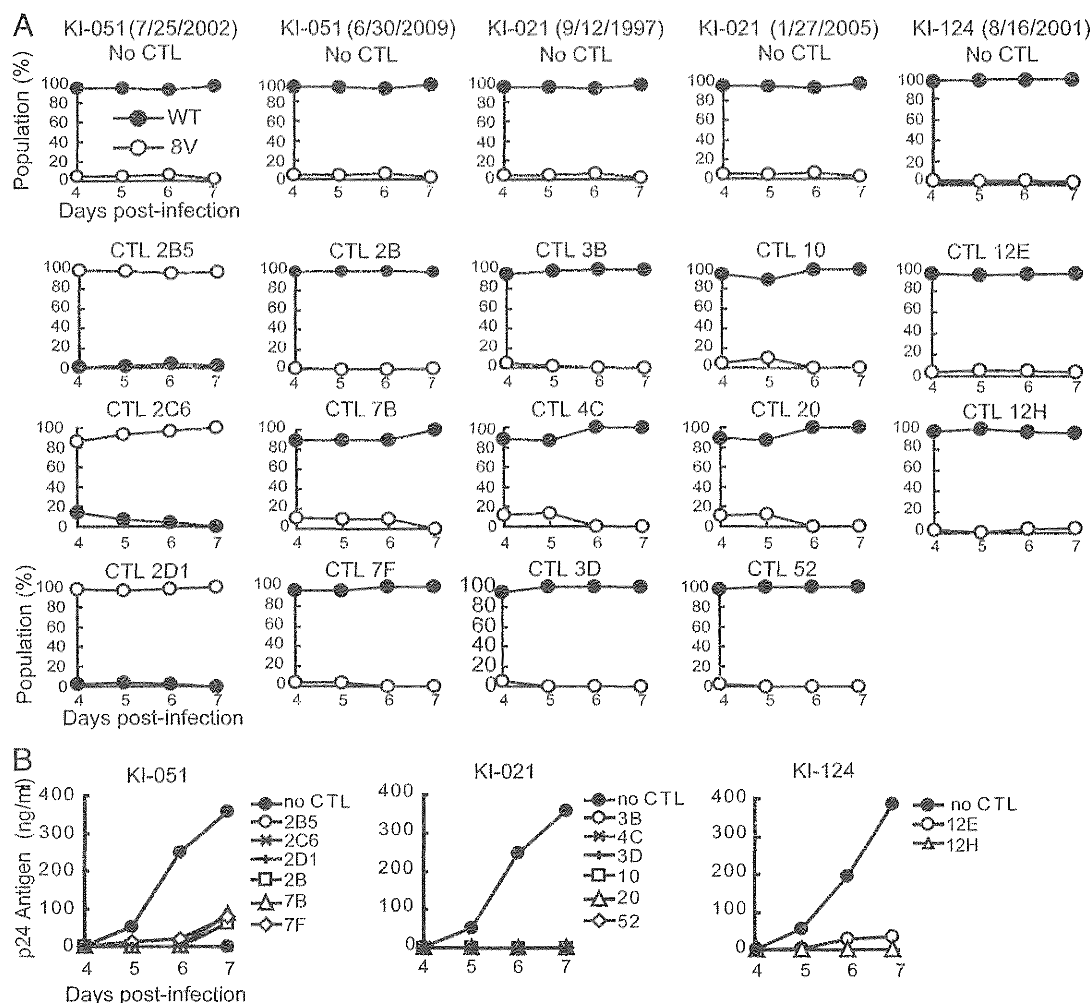

FIGURE 2. In vitro selection of the 8V mutant virus by TI8-specific CTL clones. (**A**) Ability of TI8-specific CTL clones to select the 8V mutant. T1 cells expressing HLA-B*51 and CD4 molecules were infected with NL-432 or NL432-Pol283-8V virus together at a ratio of 9:1. The infected cells were cocultured with TI8-specific CTL clones at an E:T ratio of 0.05:1. The culture supernatants were collected from days 4 to 7 postinfection. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram. (**B**) Ability of TI8-specific CTL clones to suppress the replication of HIV-1. HIV-1 p24 Ag levels in the supernatant were measured by use of an enzyme immunoassay (sensitivity: 7.8 pg/ml). The concentrations of HIV-1 p24 Ag in the collected supernatants were >0.1 ng/ml.

We next examined the kinetics of the TCR/HLA–peptide interaction. We first measured the time for half-maximal binding of these CTL clones by using the tetramers. The results showed that the time of this binding was nearly identical among these three clones (time of half-maximal binding: clone 2B5, 1.70 ± 0.48 min for WT and 1.71 ± 0.23 min for 8V; $p < 0.99$; clone 2B, 3.30 ± 0.66 min for WT and 3.06 ± 1.78 min for 8V; $p < 0.83$; clone 3B, 1.81 ± 0.36 min for WT and 1.61 ± 0.13 min for 8V; $p < 0.43$; Fig. 4A). Because the affinity of the TCR/HLA–peptide interaction is mainly controlled by its dissociation rate (43, 44), we additionally performed a tetramer dissociation assay to compare the stabilities of tetramer–TCR binding among these three clones. Clone 2B5 showed a faster dissociation rate of the 8V tetramer compared with that of the WT one (half-lives: 1530 ± 407 min for WT and 140 ± 53 min for 8V; $p = 0.027$; Fig. 4B), whereas clone 2B and 3B showed similar dissociation kinetics of both tetramers (half-lives: 2B, 1347 ± 75 min for WT and 2058 ± 382 min for 8V; $p = 0.50$; 3B, 300 ± 68 min for WT and 471 ± 189 min for 8V; $p = 0.50$; Fig. 4B). These results demonstrated that the binding stability between clone 2B5 TCR and HLA-B*51:01-TV8 peptide was weaker than that between the TCR and HLA-B*51:01-TI8, suggesting that the reduction in the 8V recognition and selection of the 8V virus resulted from this lower stability.

Reconstruction of the TCR function in TCR-deficient cells

To characterize TI8-specific CTLs at the molecular level, we analyzed the TCR-αβ genes of a TI8-specific CTL clone. Clone 2B5 expressed the TRAV8-2/TRBV24-1 clonotype, whereas clone 3B expressed the TRAV17/TRBV7-3 one (45). The TRAV17/TRBV7-3 clonotype is a public clonotype in TI8-specific CTLs and predominantly detected in KI-021 and KI-051 (45). This TCR clonotype was expressed on all CTL clones from KI-021, whereas the TRAV8-2/TRBV24-1 clonotype was on all three clones from KI-051 in July 2002 (data not shown). To confirm TCR function, we cloned their TCRs and then reconstructed them in TCR-deficient mouse T cell line TG40 transfected with human CD8α (TG40/CD8). TG40/CD8 cells transfected with 2B5-TCR or 3B-TCR genes (TG40/CD8-2B5 TCR or TG40/CD8-3B TCR) expressed CD3 (Fig. 5A), suggesting that these TCRs had been successfully reconstructed on the surface of the TG40/CD8 cells. In addition, TCR-transfected TG40/CD8 cells could produce IL-2 in response to stimulation with anti-CD3mAb (Fig. 5B), confirming functional TCR/CD3-mediated signaling in these cells. To

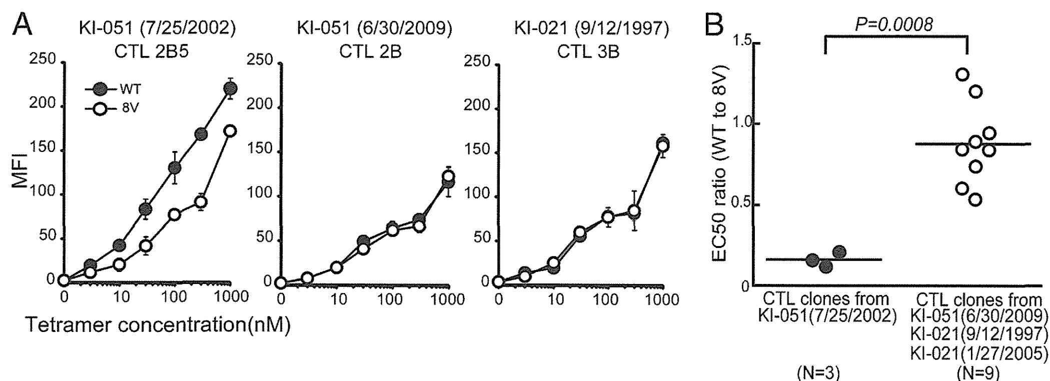

FIGURE 3. Difference in TCR affinity for HLA-B*51:01 with TV8 peptide complex among TI8-specific CTL clones. (**A**) The ability of the TCRs on TI8-specific CTL clones to bind WT or 8V tetramer was measured in terms of the mean fluorescence intensity (MFI) of each CTL clone stained with the tetramers at concentrations of 3–1000 nM. Data are shown as mean ± SD of n = 3 samples. An independent experiment gave similar results. (**B**) Comparison of TCR affinity for 8V tetramer of TI8-specific CTL clones in terms of EC_{50} ratio for WT to 8V tetramer (EC_{50} for WT tetramer/that for 8V tetramer). ● and ○, CTL clones from KI-051 in July 2002 and CTL clones from KI-051 in June 2009 and from KI-021, respectively. Statistical analysis was performed by using the t test. Bar indicates the average.

investigate whether 2B5 TCR and 3B TCR had Ag specificity, we coincubated TCR-transfected cells with C1R-B*51:01 prepulsed with various concentrations of the TI8 peptide. Both TG40/CD8-2B5 TCR and TG40/CD8-3B TCR cells showed IL-2 secretion in response to the peptide (Fig. 5C), indicating that TRAV8-2/TRBV24-1 and TRAV17/TRBV7-3 TCRs, respectively, were functionally expressed on these transfected cell lines. We further analyzed the recognition by these cells for the 8V mutant peptide. The cells expressing TRAV17/TRBV7-3 TCR exhibited a similar response to 8V as that to WT (EC_{50}: 1.38 ± 0.07 μM for WT and 1.90 ± 0.35 μM for 8V; p = 0.14), whereas the cells expressing TRAV8-2/TRBV24-1 exhibited a weaker response to 8V than that to WT (EC_{50}: 0.74 ± 0.23 μM for WT and 4.12 ± 0.39 μM for 8V; p = 0.0002) (Fig. 5C). These results confirmed that the recognition of the 2B5 TCR for 8V was weaker than that for WT and that 3B TCR evenly recognized both 8V and WT.

Discussion

We established TI8-specific CTL clones from three elite controllers at various time points and then analyzed the function of these clones. All CTL clones established from two of the elite controllers (KI-021 and KI-124) having only the 8V mutant virus and those from KI-051 after the emergence of the 8V mutant had almost the same ability to suppress the replication of the 8V as they did that of the WT one, whereas CTLs established from KI-051 having the WT virus had weaker ability to suppress the replication of the 8V virus than that of the WT one. These findings indicate that two types of CTLs for recognition of the 8V mutation were elicited in these elite controllers. Furthermore, the results of the in vitro competitive viral suppression assay revealed that the CTL clones established from KI-051 having the WT virus could select the 8V virus, whereas those from KI-051 having the 8V virus and other patients could not. Taken together, these results indicate that the 8V was an escape mutant selected by the former CTLs that could discriminate the 8V from WT and that HIV-1 was controlled by the latter ones after the emergence of the 8V virus.

The tetramer binding assay revealed that clone 2B5 carried TCR's with a little weaker affinity for the 8V tetramer than for the WT one, whereas clones 2B and 3B carried TCRs with similar affinity for both ligands. In addition, the tetramer dissociation assay demonstrated that the half-life of binding of the 2B5 TCR to the 8V tetramer was ~10-fold shorter than that to the WT one but that the 2B and 3B TCRs exhibited similar half-lives to both tetramers. Thus, the weaker recognition of clone 2B5 for the 8V mutant epitope resulted from a reduced binding stability between 2B5 TCR and HLA-B*51:01-TV8 peptide. To confirm the ability of these TCRs to recognize WT and mutant viruses, we reconstructed 2B5 TCR or 3B TCR on the surface of TG40/CD8 cells by transfecting the cells with these genes. The analysis using the cells expressing these TCRs also demonstrated the reduced ability of the 2B5 TCR to recognize the 8V peptide. These results confirmed that 2B5 TCR recognized the 8V peptide weaker than the WT one and that 3B TCR evenly recognized both peptides.

Our recent study demonstrated that 2B5 and 3B CTLs expressed TRAV8-2/TRBV24-1 and TRAV17/TRBV7-3 TCR clonotypes, respectively (45). The latter clonotype was predominantly detected in TI8-specific CTLs from KI-021 and KI-051 after emergence of the 8V virus, whereas the former was found in seven TI8-specific CTL clones established from KI-051 when WT virus was still detectable (45). The CTL clones expressing TRAV8-2/TRBV24-1 clonotype were predominantly established from PBMCs in July 2002 from KI-051 but not detected in ex vivo PBMCs (45), suggesting that the CTLs expressing this clonotype were in the minority but had a strong ability to proliferate by Ag stimulation. Unlike the TCR detected in HLA-B27–restricted KK10-specific CTLs, the 3B TCRs exhibited very weak affinity for HLA-B*51:01-TI8 as compared with pathogenic epitope-specific TCRs. However, the parental CTLs had strong ability to suppress the replication of HIV-1 in vitro. These findings indicate that even CTLs having weak TCR affinity could effectively control HIV-1.

The substitution from Val (GTA) to Leu (TTA or CTA) is easily produced by only a single nucleotide substitution because Val at position 8 is encoded by the "GTA" nucleotide codon, whereas more than two nucleotide substitutions are required for the change from Val to Thr (ACA) or to Arg (CGA or AGA). TI8-specific CTLs having the ability to discriminate the 8V mutant from WT highly cross-recognized the 8L mutant peptide but failed to suppress the replication of the 8L mutant virus (34, 35), suggesting that the 8L mutation has a deleterious effect on Ag processing of TI8 epitope. Therefore, it is likely that TI8-specific CTLs established from KI-051 at the different time points or from the other patients also failed to suppress the 8L virus. These facts suggest that the 8L mutant can be selected by a second immune response. Indeed, a previous study of a Chinese cohort infected with the 8V mutant virus as the founder virus showed that most of the HLA-B*51:01^{+} patients had the 8L mutation, not the 8T one (46). However, selection of the 8L from the 8V mutant was not driven

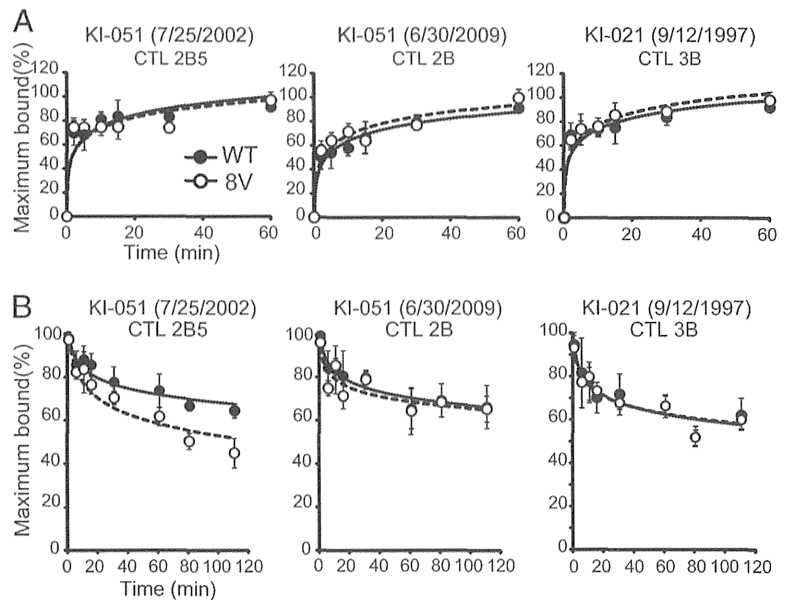

FIGURE 4. Kinetics of interaction between HLA-B*51:01–peptide complex and TI8-specific CTL clones. (**A**) Kinetics of tetramer association with TCR of TI8-specific CTL clones. The CTL clones were incubated with WT or 8V tetramer at a concentration of 1000 nM and taken periodically (2, 5, 10, 15, 30, and 60 min), fixed, and analyzed for the intensity of tetramer staining. Tetramer staining is expressed as a percentage of maximal staining intensity (maximum bound). Data are shown as the mean ± SD of $n = 3$ samples. (**B**) Kinetics of tetramer dissociation from TCRs of TI8-specific CTL clones. The CTL clones were stained with WT or 8V tetramer at a concentration of 1000 nM for 30 min, washed, and resuspended in R5. The cells were then taken periodically (5, 10, 15, 30, 60, 80, and 110 min), fixed, and analyzed for the intensity of tetramer staining. Tetramer staining is expressed as percentage of maximal staining intensity (time 0 min: 100%). Data are shown as the mean ± SD of $n = 3$ samples.

by the TI8-specific cross-reactive CTL response in our three elite controllers. We speculate that the CTLs elicited in these three elite controllers strongly inhibited HIV-1 replication in the acute phase of HIV-1 infection and maintained VL at a very low level for the long time so that selection of the mutant virus may have been very slow in these patients. The exact mechanism by which the 8L mutant was not selected in these elite controllers remains unclear. Therefore, further study is required to clarify it.

It is well known that escape mutants are selected by WT epitope-specific CTLs that cannot recognize the mutant virus (10, 16, 18, 47). In the present study, we demonstrated that the 8V virus was selected by TI8-specific CTLs that could discriminate 8V from WT, although the CTLs could effectively recognize the 8V mutant. A similar mutation is known in the KK10 epitope. The L268M mutation in the KK10 epitope restricted by the HLA-B*27 allele is selected by WT KK10-specific CTLs under the control of virus replication, and then, cross-reactive KK10-specific CTLs effectively recognizing WT and L268M are elicited after the emergence of this L268M mutation (48, 49). The cross-reactive TI8-specific CTLs evenly recognizing 8V and WT epitopes were elicited after the emergence of the 8V mutant, but they failed to select further escape mutants such as the 8L and 8T. In contrast to TI8-specific CTLs, cross-reactive CTLs together with WT KK10-specific CTLs finally select the R264K mutant with the compensatory S173A

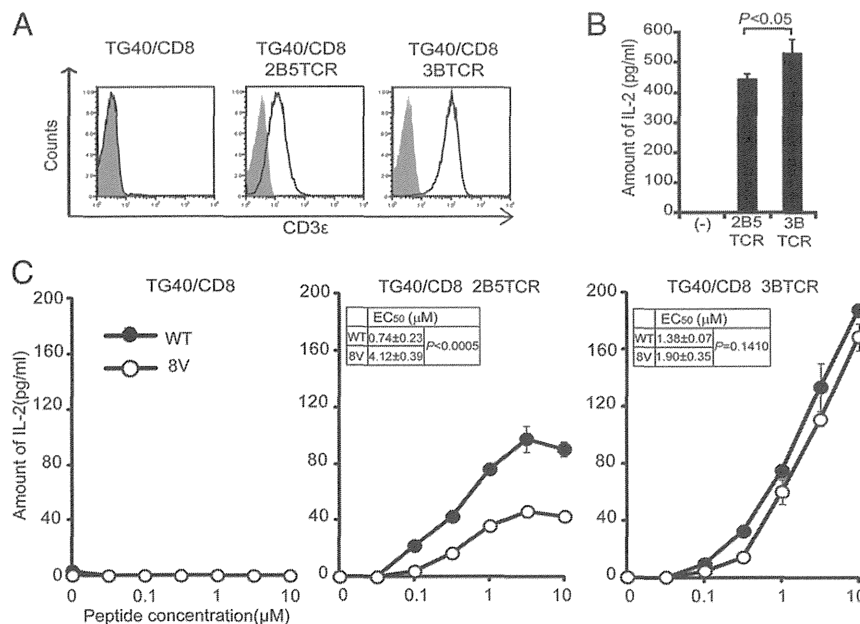

FIGURE 5. Reconstruction and recognition of TI8-specific TCRs on TCR-transfected TG40/CD8 cells. (**A**) CD3 expression in TG40/CD8 cells transfected with 2B5 TCR or 3B TCR. The TCR-transfected cells were stained with anti-CD3ε mAb (open histogram) or with an isotype control (shaded histogram), and then, CD3 expression on the cells was analyzed by flow cytometry. (**B**) IL-2 secretion by 2B5 TCR- or 3B TCR-TG40/CD8 cells stimulated with anti-CD3ε mAb. The cells were cultured in CD3ε mAb–coated wells for 48 h, and the amounts of IL-2 in supernatants were measured by use of an enzyme immunoassay. The data are shown as the means and SD of triplicates (2B5 TCR: 445 ± 16 pg/ml; 3B TCR: 529 ± 47 pg/ml). Another independent experiment gave similar results. (**C**) IL-2 secretion by 2B5 TCR- or 3B TCR-TG40/CD8 cells stimulated with TI8 (WT) or TV8 peptide. These cells were coincubated for 48 h with C1R-B*51:01 cells prepulsed with various concentrations of TI8 or TV8 peptide (0.03–10 μM). The amounts of IL-2 in supernatants were measured by the enzyme immunoassay. The data are shown as the means and SD of triplicates. Another independent experiment gave similar results.

mutation (49, 50). These mutations (S173A/R264K/L268M) critically reduce the binding to HLA-B*27 so that WT and cross-reactive KK10-specific CTLs fail to control the escape mutant virus (16, 17, 51). Thus, the events of the 8V selection and the control of HIV-1 by TI8-specific CTLs were quite different from those in the case of the KK10-specific CTLs.

A previous study showed that the 8T and the 8R mutation were selected within only 6 and 12 mo after the first test, respectively, in acutely infected HLA-B*51:01$^+$ patients who had been infected with the WT virus (35). Longitudinal sequence analysis of the TI8 epitope in KI-051, who had been infected with HIV-1 around 1983, revealed that 37% of the HIV-1 isolates were the WT virus in July 2002, with the 8V mutant being predominantly detected at this time, indicating that the WT virus had existed for ~20 y in this patient. Thus, the 8V mutant was very slowly selected in KI-051, whereas the L268M mutation was mostly selected within 1–3 y after HIV-1 infection by highly effective KK10-specific CTLs recognizing the WT but not the L268M mutant (49, 52). We found that CTLs had a strong ability to inhibit the 8V virus in vitro and that elite controllers carrying the 8V mutant maintained a very low level of VL for a long time. However, because the highly effective WT-specific and L268M cross-reactive KK10-specific CTLs can select new escape mutants (49), other mechanisms may be involved in maintenance of the 8V in HLA-B*51:01$^+$elite controllers.

The HLA-B*51:01 allele was associated with slow progression to the disease in HIV-1–infected Japanese hemophiliacs, and the frequency of HLA-B*51:01–restricted TI8-specific CTLs is inversely correlated with the plasma VL in chronically HIV-1–infected Japanese hemophiliacs (34). These findings indicate that the TI8-specific CTLs control HIV-1 in these patients. In contrast, the escape mutations 8T, 8R, and 8L were found to accumulate in several populations (35). A recent analysis using Japanese cohorts also showed that the WT sequence 8I is present in only 13.5% of chronically HIV-1–infected Japanese individuals (53). These findings suggest that TI8-specific CTLs are hardly elicited in HLA-B*51:01$^+$ individuals recently infected with HIV-1 because the escape mutations in the TI8 epitope accumulate in epidemic HIV-1. Thus, the HLA-B*51:01 allele is then no longer associated with slow progression to AIDS. Indeed, this allele is not associated with the slow progression in Japanese individuals recently infected with HIV-1 (54).

In conclusion, we found two different TI8-specific CTLs recognizing the 8V mutant in HLA-B*51:01$^+$ elite controllers. We showed that 8V was an escape mutant selected by cross-reactive CTLs having weaker ability to recognize 8V virus–infected cells than WT virus–infected ones and that this 8V mutant could elicit CTLs evenly recognizing 8V virus–infected cells and WT virus–infected ones in the elite controllers. These two cross-reactive CTLs effectively suppressed HIV-1 for a long time. Our findings provide a novel mechanism concerning selection of the 8V mutant and long-term control of HIV-1 in HLA-B*51:01$^+$ elite controllers. Further studies clarifying why these elite controllers carrying the 8V mutant do not select other escape mutants such as the 8L mutant may impact on the fields of pathogenesis and immunotherapy in AIDS research.

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Disclosures

The authors have no financial conflicts of interest.

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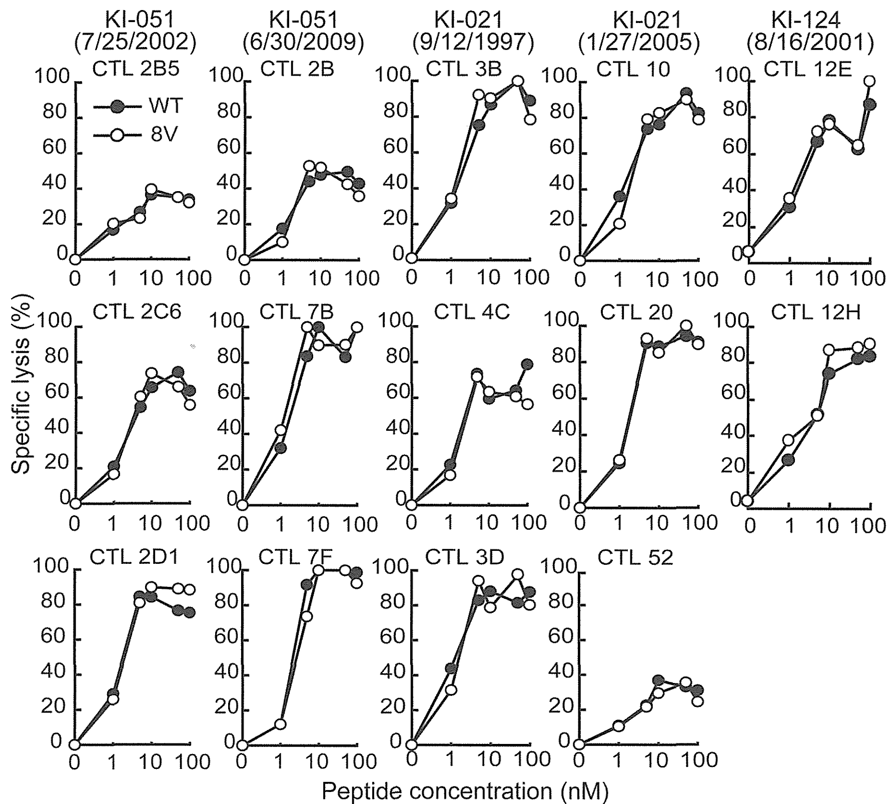
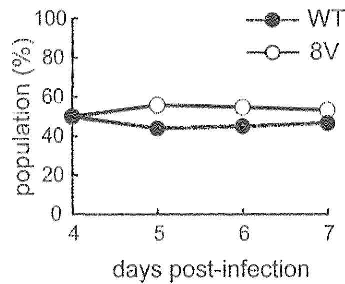


Supplementary Figure 1:

Recognition of TI8 (WT) and TV8 (8V) peptides by TI8-specific CTL clones.

Cytotoxic activity toward C1R-B*51:01 cells prepulsed with TI8 or TV8 peptide at concentrations of 1 to 100 nM was determined. The cytotoxic activity was measured at an E:T ratio of 2:1.



Supplementary Figure 2:

Replication capacity of WT and the 8V mutant viruses.

A competitive proliferation assay using WT and the 8V mutant viruses. T1 cells were infected with WT and the 8V mutant viruses at a ratio of 1:1. The culture supernatants were collected from day 4 to day 7 post infection. Viral RNA extracted from the supernatants was subjected to RT-PCR followed by nested PCR for direct sequencing. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram.