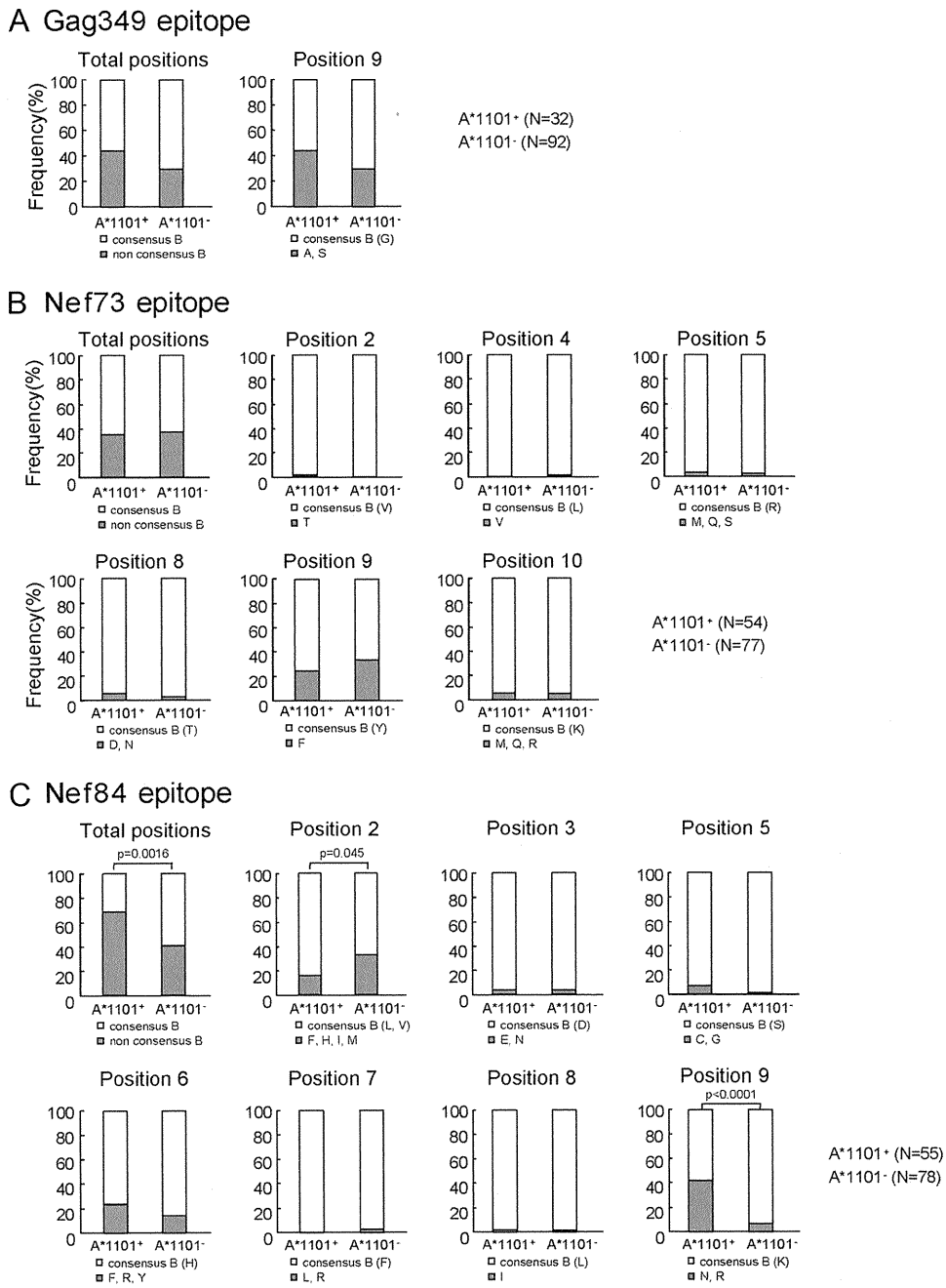

FIG. 4. Frequency of mutations in 3 HLA-A*1101-restricted epitopes. Three epitope sequences, Gag349 (A), Nef73 (B), and Nef84 (C), from HLA-A*1101-positive and HLA-A*1101-negative individuals chronically infected with HIV-1 were analyzed. Consensus sequences of these epitopes in clade B are as follow: Gag349, ACQGVGGPGHK; Nef73, QVPLRPMTYK; Nef84, AVDLSHFLK and ALDLSHFLK. The frequency of mutations in the total sequence of the epitopes was calculated as (number of individuals having the mutation[s]/number of individuals tested) × 100, whereas those at a given position were calculated as (number of individuals having the mutation[s] at a given position/number of individuals tested) × 100. The results were compared between HLA-A*1101-positive and HLA-A*1101-negative individuals, and the *P* values were determined by using Fisher's exact test.

Nef138-2F escape mutants (18). HLA-A*26-restricted Gag169-specific $CD8^+$ T cells also have a strong ability to suppress HIV-1 replication but cannot select any escape mutant (unpublished observation). PD-1 expression on Nef138-specific and Gag169-specific $CD8^+$ T cells from chronically HIV-1-infected individuals was measured by using specific tetramers and anti-PD-1 MAbs. PD-1 expression on Nef138-specific $CD8^+$ T cells was lower than that on the Gag169-specific ones. Taken together, these results show that PD-1 expression on $CD8^+$ T cells that can select escape mutants is significantly lower than that on $CD8^+$ T cells that are unable to select escape mutants (Fig. 6E).

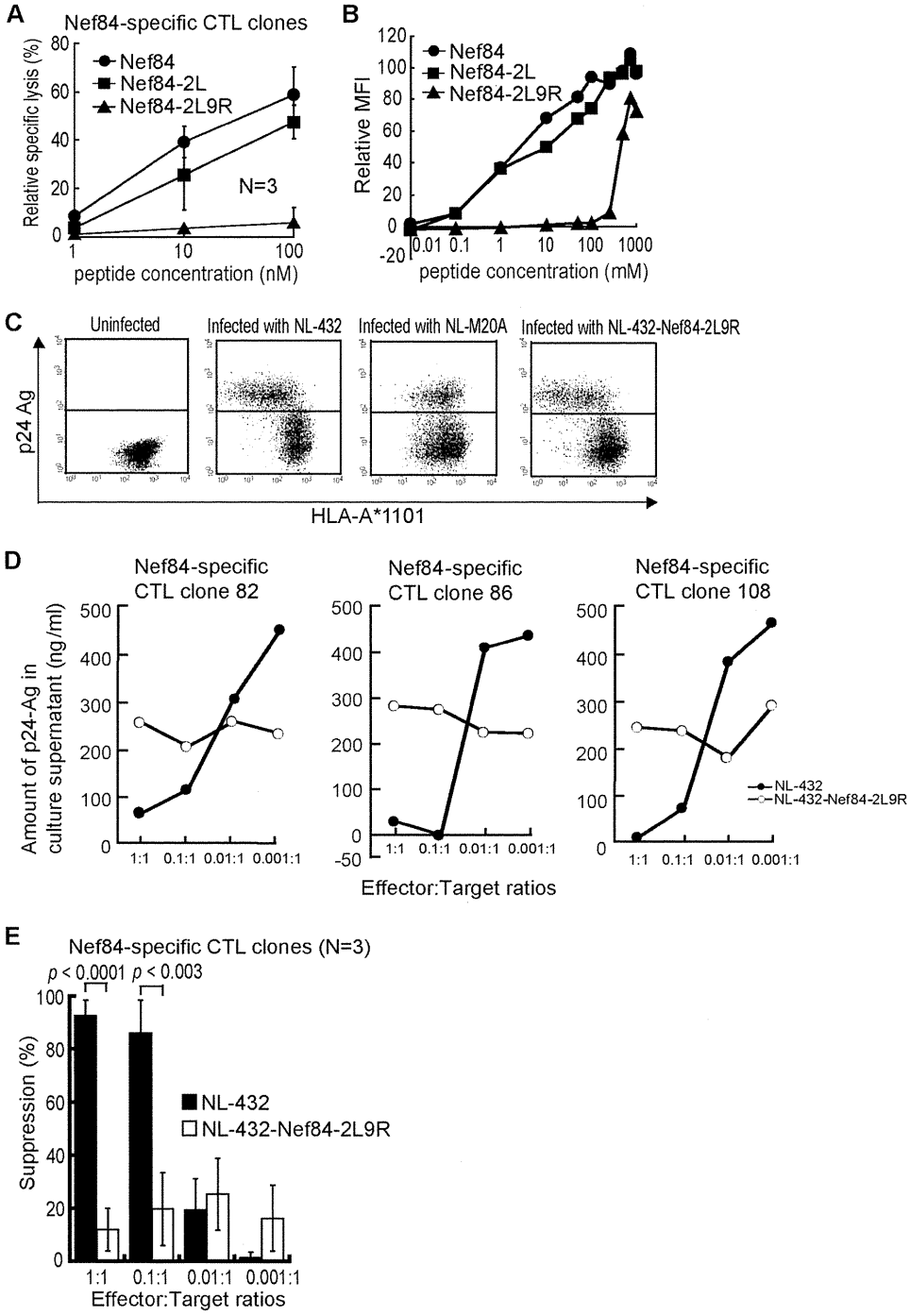

FIG. 5. Ability of Nef84-specific CTLs to suppress replication of HIV-1-Nef84-9R mutant virus. (A) Cytolytic activities of Nef84-specific CTL clones in killing C1R-A*1101 cells pulsed with Nef84-9R peptide. C1R-A*1101 cells were prepulsed with various concentrations of Nef84, Nef84-2L, or Nef84-2L9R peptide. Cytolytic activities of Nef84-specific CTL clones were measured at an effector-to-target ratio of 2:1. (B) Ability of Nef84-2L9R peptide to bind HLA-A*1101. The affinity was measured by a stabilization assay using RMA-S-A*1101 cells. (C) Surface expression of HLA class I molecules on $CD4^+$ T cells infected with NL-432-Nef84-2L9R. (D) Ability of each Nef84-specific CTL clone to suppress NL-432-Nef84-2L9R replication in $CD4^+$ T cells. (E) Analysis of ability of all 3 Nef84-specific CTL clones to suppress replication of NL-432 or NL-432-Nef84-2L9R.

Different functional abilities between *ex vivo* Nef73-specific and Nef84-specific CTLs. We speculated that Nef84-specific CTLs have a stronger functional ability *in vivo* than Nef73-specific ones. Therefore, we investigated whether Nef84-specific CTLs from *ex vivo* PBMC would respond to the specific epitope more effectively than Nef73-specific ones. To compare functional abilities between these 2 CTLs, we selected 5 individuals who had both Nef73-specific and Nef84-

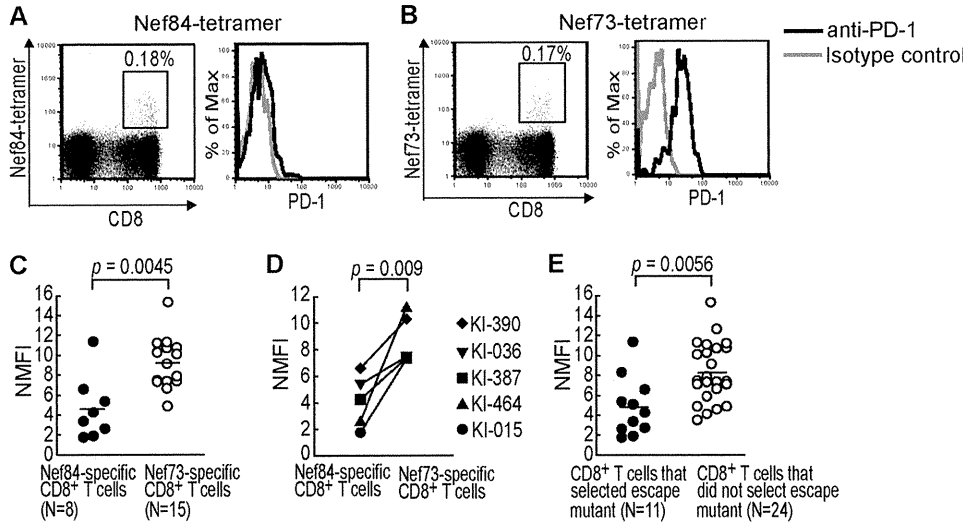

FIG. 6. PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells. (A and B) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells among PBMCs from an HIV-1-infected individual (KI-015). PBMCs from KI-015 were stained with anti-CD3, anti-CD8, anti-PD-1 MAb, and the tetramer. The frequency of $tetramer^+$ $CD8^+$ T cells in the lymphocyte population was plotted (left). The histogram shows PD-1 expression on the specific $CD8^+$ T cells (right). (C) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells in PBMCs of HIV-1-infected individuals. PD-1 expression on the cells from each individual was normalized by the mean fluorescence intensity of the isotype control (NMFI). (D) PD-1 expression on Nef84- and Nef73-specific $CD8^+$ T cells from the same individuals (KI-015, -036, -387, -390, and -464). (E) PD-1 expression on $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* and to select escape mutants. The left part of the plot shows 8 HLA-A*1101-restricted Nef84-specific and 3 HLA-A*2402-restricted Nef-138-specific $CD8^+$ T cells that select escape mutants, and the right part shows 15 HLA-A*1101-restricted Nef73-specific and 9 HLA-A*26-restricted Gag169-specific $CD8^+$ T cells that do not select them.

specific CTLs. IFN-γ production from these T cells among *ex vivo* PBMC was measured after they had been stimulated with Nef84 peptide or Nef73 peptide (Fig. 7A). The results showed that the frequency of IFN-γ-producing cells was higher for Nef84-specific $CD8^+$ T cells than for Nef73-specific ones from each individual. That is, it is significantly higher for the former T cells than for the latter ones (Fig. 7B; see also Fig. S3 in the supplemental material). These results support the idea that Nef73-specific T cells can partially function *in vivo*.

DISCUSSION

Previous studies showed an inverse correlation between the plasma viral load (pVL) and the frequency of some HIV-1-specific CTLs in HIV-1-infected individuals, indicating that these CTLs control HIV-1 *in vivo* (5, 28, 33). However, this correlation was not found in the case of many other HIV-1-specific CTLs (16, 25, 26), suggesting the possibility that the quality of HIV-1-specific CTLs is a critical factor for the control of HIV-1 *in vivo*. However, it is not easy to assess the quality of HIV-1-specifc CTLs. An assay to directly measure the ability of the CTLs to suppress HIV-1 replication *in vitro* is a very useful method to evaluate the ability of the CTLs to control HIV-1. A previous study using this assay demonstrated that the ability of HLA-B*5101-restricted HIV-1-specific CTLs to suppress HIV-1 replication is dependent on the epitope recognized by these CTLs (43). In addition, a recent study showed that HLA-A*2402-restricted Nef138-specific CTLs have a strong ability to suppress HIV-1 replication, whereas HLA-A*2402-restricted Gag133-8-, Pol797-8-, or Gag263-10-specific CTLs showed a weak ability or no ability to suppress HIV-1 replication (18).

TABLE 1. Sequences of Nef73 and Nef84 epitopes in HIV-1 from the 5 subjects whose Nef73- and Nef84-specific CD8 T cells were analyzed for PD-1 expression

Patient ID or sequence description	Sequence[a]																			
	Nef73										Nef84									
Wild type	Q	V	P	L	R	P	M	T	Y	K	A	V	(L)	D	L	S	H	F	L	K
KI-015[b]	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-
KI-036[c]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	-
KI-387[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	-
KI-390[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	R
KI-464[b]	-	-	-	-	-	-	-	-	-	-	-	L		-	-	-	-	-	-	K/R[d]

[a] Sequences were analyzed by the direct sequencing method. "-" indicates agreement with wild-type sequence.
[b] The same sample was analyzed for sequencing and PD-1 expression.
[c] This patient was analyzed for the sequence of HIV-1 on 6 October 2005 and for PD-1 expression on the T cells on 14 July 1999.
[d] The mixture of sequences carrying K or R at position 9 was detected.

The Nef138-specific CTLs select the 2F escape mutation within 1 to 2 years after the start of an HIV-1 infection (18). The frequency of the Nef138-specific CTLs is inversely correlated with pVL in individuals infected with wild-type virus before the virus with the 2F mutant (the 2F virus) is selected. In contrast, it did not correlate with pVL in them after the 2F virus appeared or in individuals originally infected with the 2F virus (18). These observations strongly suggest that Nef138-specific CTLs have a strong ability to suppress the replication of wild-type HIV-1 *in vivo*, such that they can select the 2F escape virus. Thus, a strong ability of HIV-1-specific CTLs to suppress HIV-1 replication is necessary to select CTL escape mutants *in vivo*.

In the present study, we showed that 2 HLA-A*1101-restricted Nef-specific CTLs had a strong ability to suppress

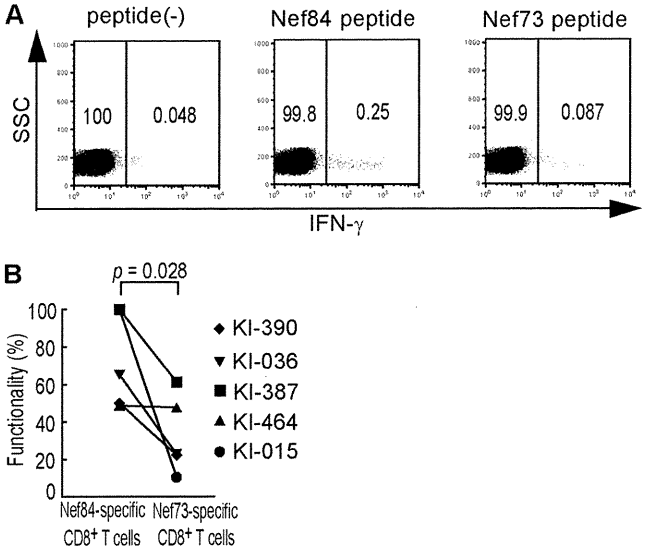

FIG. 7. Functional analysis of *ex vivo* Nef84- and Nef73-specific $CD8^+$ T cells. (A) IFN-γ production of Nef84- and Nef73-specific $CD8^+$ T cells among PBMCs from an HIV-1-infected individual, KI-036. PBMCs from KI-036 were stimulated with Nef73 peptide or Nef84 peptide and stained with anti-CD8, followed by intracellular staining for IFN-γ. The frequency of IFN-γ^+ $CD8^+$ T cells among total $CD8^+$ T cells was plotted. (B) Frequency of Nef84- and Nef73-specific $CD8^+$ T cells producing IFN-γ. The percent functionality was calculated as follows: (frequency of IFN-γ^+ $CD8^+$ T cells among total $CD8^+$ T cells/that of tetramer$^+$ $CD8^+$ T cells among total $CD8^+$ T cells) × 100.

HIV-1 replication. Nef84-specific CTLs selected the escape mutant 9R, whereas Nef73-specific ones did not select any escape mutant. There are several hypotheses to explain the difference in the abilities of these CTLs to select escape mutants. One is that the frequency of mutations is much lower in a part of the Nef73 epitope and its flanking region than in that of the Nef84 epitope and its flanking region. This idea is not likely to be true, however, because the analysis of sequences of HIV-1 isolates reported in the Los Alamos HIV-1 Sequence Database showed that the frequency of mutations in the Nef73 epitope is almost the same as that in the Nef84 one (data not shown). Another possibility is that Nef73-specific CTLs can have a strong ability to suppress HIV-1 replication *in vitro* but not *in vivo*. We analyzed the ability of HIV-1-specific CTLs to suppress HIV-1 replication by using the specific CTL clones. Since CTL clones are established from a small part of the memory or memory effector T-cell population that can effectively proliferate, they may not reflect the CTLs *in vivo*.

Recent studies showed that PD-1 expression on HIV-1-specific T cells is associated with dysfunction of the T cells and disease progression (15, 35, 44, 47). PD-1 is a regulator of virus-specific T-cell survival (4, 8, 24, 31, 38). Therefore, we speculated that Nef73-specific $CD8^+$ T cells express a higher level of PD-1 on their cell surface, such that they lose their ability to suppress HIV-1 replication *in vivo*. Indeed, the expression of PD-1 on Nef73-specific $CD8^+$ T cells was significantly higher than that on Nef84-specific ones. This difference was found in the case of both Nef73-specific and Nef84-specific $CD8^+$ T cells present in the same individuals. In addition, the *ex vivo* analysis of both Nef138-specific and Gag169-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* confirmed that PD-1 was expressed significantly at a lower level on the former T cells, which can select escape mutants, than on those unable to select escape mutants. Thus, since PD-1 expression on the latter cells was much higher than that on the former ones, it is likely that the former could not proliferate and promptly died *in vivo* so that they failed to select escape mutants. A recent study showed that PD-1 expression on HIV-1-specific $CD8^+$ T cells decreased after the variation appeared in the target epitope sequences (39), suggesting that reduced signaling via T-cell receptors (TCR) decreased PD-1 expression. However, the present study showed that lower expression of PD-1 was also found in 4 individuals who had HIV-1 carrying the wild-type Nef84 epitope. Therefore, the T cells in these individuals may not indicate that reduced signaling via TCR decreased the PD-1 expression, because they have wild-type HIV-1. Recent studies suggested that PD-1 expression is a marker of homeostatic stimulation or T-cell differentiation (9, 21, 27, 29, 37). The analysis of the CD27 CD28 CD45RA phenotype of Nef73-specific and Nef84-specific T cells in the 5 individuals excluded the possibility that the difference in expression of PD-1 between these T cells was due to that in differentiation status between these T cells. On the other hand, the present study could not exclude another interpretation, i.e., that the difference between these T cells in ability to suppress HIV-1 replication *in vivo* is due to some mechanism other than that involving PD-1 expression. We showed that *ex vivo* Nef84-specific $CD8^+$ T cells had a stronger ability to recognize the epitope than Nef73-specific ones, suggesting that Nef84-specific $CD8^+$ T cells had a stronger ability to suppress wild-type HIV-1 *in vivo*. Further study of these T cells is necessary to clarify what determines a weak function of Nef73-specific T cells and a strong function of Nef84-specific T cells *in vivo*.

We showed in the present study that 1 of 2 HIV-1-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro* selected escape mutants. In addition, we recently found that 1 of 2 Pol epitope-specific HLA-B*5101-restricted $CD8^+$ T cells and 1 Nef epitope-specific HLA-A*2402-restricted $CD8^+$ T cell having a strong ability to suppress HIV-1 replication *in vitro* could select escape mutants (6; our unpublished observation). Thus, half of HIV-1-specific $CD8^+$ T cells having a strong ability to suppress HIV-1 replication *in vitro*, which were previously and presently analyzed, can select escape mutants *in vivo*, whereas the other half of these $CD8^+$ T cells lose this ability. High expression of PD-1 on the $CD8^+$ T cells may be one explanation for this difference. The mechanism responsible for the presence of 2 types of $CD8^+$ T cells in HIV-1-infected individuals remains unknown.

In the present study, we showed that out of the HIV-1-specific CTLs having the ability to suppress HIV-1 replication *in vitro*, only those having a strong ability to recognize an HIV-1 epitope can select escape mutants. Thus, it is not true that CTL escape mutations are simply selected by CTLs having a strong ability to suppress HIV-1 replication *in vitro*. It is still unknown why a given HIV-1-specific CTL can have a strong ability to recognize the epitope *in vivo* and others cannot, even though both have a strong ability to suppress HIV-1 *in vitro*. Further analysis of the function of HIV-1-specific CTLs *in vivo* will be necessary for clarification of the immunopathogenesis

of AIDS and the development of immunotherapy and an effective AIDS vaccine.

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Selection of escape mutant by HLA-C-restricted HIV-1 Pol-specific cytotoxic T lymphocytes carrying strong ability to suppress HIV-1 replication

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HIV-1 mutants escaping from HLA-A- or HLA-B-restricted CTL have been well studied, but those from HLA-C-restricted CTL have not. Therefore we investigated the ability of HLA-C-restricted CTL to select HIV-1 escape mutants. In the present study, we identified two novel HLA-Cw*1202-restricted Pol-specific CTL epitopes (Pol328-9 and Pol463-10). CTL specific for these epitopes were detected in 25–40% of chronically HIV-1-infected HLA-Cw*1202$^+$ individuals and had strong abilities to kill HIV-1-infected cells and to suppress HIV-1 replication *in vitro*, suggesting that these CTL may have the ability to effectively control HIV-1 in some HLA-Cw*1202$^+$ individuals. Sequence analysis of these epitopes showed that a V-to-A substitution at the 9th position (V9A) of Pol 463-10 was significantly associated with the HLA-Cw*1202 allele and that the V9A mutant was slowly selected in the HLA-Cw*1202$^+$ individuals. Pol 463-10-specific CTL failed both to kill the V9A virus-infected cells and to suppress replication of the V9A mutant. These results indicate that the V9A mutation was selected as an escape mutant by the Pol463-10-specific CTL. The present study strongly suggests that some HLA-C-restricted CTL have a strong ability to suppress HIV-1 replication so that they can select HIV escape mutants as in the case of HLA-A-restricted or HLA-B-restricted CTL.

Key words: CTL · Escape mutation · Fitness · HLA-C · HIV infection

Supporting Information available online

Introduction

CTL are involved in the control of HIV-1 replication during acute and chronic phases of HIV-1 infections [1–8]. However, CTL cannot completely eradicate HIV-1 because HIV-1 escapes from the cell-mediated immune system of the host by various mechanisms [9–17]. One such mechanism is the appearance of a single amino acid mutation within CTL epitopes, which is crucial for preventing their binding to HLA class I molecules or for the interaction between the TCR of the HIV-1-specific CTL and the peptide-HLA class I complex. The escape mechanisms result in the loss of CTL activities against HIV-1-infected target cells and

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contribute to the selection of viruses capable of escaping from HIV-1-specific CTL [4, 9–11, 18, 19]. Many studies demonstrated that the immune pressure mediated by HIV-1-specific CTL selects escape variants during both acute and chronic HIV-1 infections and that the selection of escape mutants could result in the loss of immune control, leading to progression to AIDS [9, 20–25].

The majority of previous studies concerning HIV-1-specific CTL focused on HLA-A- or HLA-B-restricted ones. However, the role of HLA-C-restricted CTL in HIV infections has not been well documented. It is speculated that HLA-C-restricted CTL do not contribute to the control of HIV-1 replication, because the expression level of HLA-C molecules is approximately 10% of that of HLA-A or -B molecules [26, 27]. In contrast, HIV-1 Nef-mediated HLA class I down-regulation affects HLA-A- or HLA-B-restricted CTL recognition but not the HLA-C-restricted one [28], suggesting a role for the HLA-C allele in HIV-1 infections. In addition, a whole-genome association study indicated that a variant located 35 kb upstream of the HLA-C gene (rs9264942) is associated not only with HLA-C mRNA expression but also with HIV viral load (VL) and AIDS progression [29, 30]. These studies suggest that the HLA-C-restricted immune responses play an important role in the control of HIV-1.

There are very few studies on HIV-1-specific HLA-C-restricted T cells. A previous study using HIV-1-specific HLA-C-restricted CTL clones demonstrated that HLA-HLA-C*03, 07, 15-restricted HIV-1-specific CTL clones effectively suppress HIV-1 replication *in vitro* [28]. A recent study revealed that HLA-C*04-restricted CTL have functional and phenotypic characteristics similar to those of HLA-A or B-restricted CTL [31]. Previous population analyses showed the association of some HLA-C alleles with the substitutions of HIV [32, 33]. Although these studies suggest the possibility that HLA-C-restricted CTL can select HIV-1 escape mutants, they did not directly show that HLA-C-restricted CTL actually do so.

In the present study, we investigated whether HLA-C-restricted CTL could select escape mutants. We focused on HLA-Cw*1202-restricted CTL because this allele, which forms a haplotype with HLA-A*2402 and HLA-B*5201, is frequently detected in Japan. To clarify the role of HLA-Cw*1202-restricted CTL in the selection of escape mutants, we first identified HLA-Cw*1202-restricted epitopes and then measured the ability of the HLA-Cw*1202-restricted CTL to suppress HIV-1 replication. Furthermore, we analysed mutations of HIV that had escaped from the CTL.

Results

Identification of 2 HLA-Cw*1202-restricted HIV-1 Pol-specific CTL epitopes

To identify HLA-Cw*1202-restricted CTL epitopes, we stimulated PBMC from chronically HIV-1-infected donor KI-069 (HLA-A*2402/-, B*5201/4006, Cw*1202/0304) with peptide cocktails including eight 17-mer overlapping peptides from Gag and Pol regions of HIV-1 and cultured the cells for 12–14 days. After stimulation with autologous B-lymphoblastoid cell lines (B-LCL) prepulsed with the corresponding peptide cocktail, each bulk culture was assessed by performing the intracellular cytokine assay. Bulk cultures from KI-069 responded specifically to 1 Gag cocktail, 3 Pol cocktails, and 3 Nef cocktails (data not shown). Further analysis using a single peptide demonstrated that 2 Gag (Gag 17–13 and Gag 17–14) and 3 Pol (Pol 17–40, Pol 17–48, and Pol 17–78) induced specific $CD8^+$ T cells responses (data not shown). HLA restriction of these T-cell responses was subsequently determined using a panel of B-LCL sharing 1 HLA class allele with KI-069. The results showed that $CD8^+$ T-cell responses against Gag 17-13, Pol 17–48, and Pol 17–78 peptides were restricted by a haplotype of HLA-A*2402, HLA-B*5201, and HLA-Cw*1202 (data not shown). Further analysis using C1R transfectant cells expressing each HLA molecule showed that only responses of $CD8^+$ T cells specific for the Pol 17–78 peptide were restricted by HLA-Cw*1202 (Fig. 1A top). Next, we generated a panel of 11-mer peptides covering the 17-mer amino acid sequences of the Pol 17–78 peptide and then tested IFN-γ production of each bulk culture in response to C1R-HLA-Cw*1202 cells prepulsed with these 11-mer peptides. Only the Pol 11–232 peptide induced the specific responses (Fig. 1A middle). To determine minimum length of the epitope, we generated four truncated peptides, Pol 11-232(IV9), Pol 11-232(C9), Pol 11-232(N10), and Pol 11-232(C10). Pol11-232-induced $CD8^+$ T cells recognised Pol 11-232(C10) but neither the IV9 nor the C9 (Fig. 1A bottom), indicating Pol 11-232(C10) to be the optimal epitope.

On the other hand, the ELISPOT assay using 11-mer overlapping Nef, Gag, and Pol peptides for KI-108 carrying HLA-A*2402/A*2402, B*5201/B*5201, and Cw*1202/Cw*1202 showed that 3 Pol peptide cocktails (Pol11-G17, Pol11-G27, and Pol11-G47) induced specific CD8 T-cell responses from this patient (data not shown). Subsequent analysis using single 11-mer peptides demonstrated that Pol11-164, Pol11-263, and Pol11-463 peptide-specific $CD8^+$ T cells were included among the PBMC cultured with Pol11-G17, Pol11-G27, and Pol11-G47, respectively (data not shown). In order to determine HLA class I restriction molecules of these peptide-specific T-cell responses, we employed C1R transfectants expressing each HLA molecule as stimulator cells and found that only Pol11-164-specific T-cell response was restricted by HLA-Cw*1202 (Fig. 1B top). To identify the optimal epitope recognised by $CD8^+$ T cells specific for Pol11-164, we synthesised a set of truncated peptides, Pol 11-164(RY10), Pol 11-164(RI9), Pol 11-164(KY9), Pol 11-164(QY8), and Pol11-164(KQ10) and tested which peptide the bulk cultured cells recognise. Pol 11-164(RY10), Pol 11-164(KY9), and Pol11-164(KQ10) peptides induced high IFN-γ responses of $CD8^+$ T cells in the culture (Fig. 1B middle). In addition, the analysis of peptide titration showed that the Pol11-164(KY9) peptide induced stronger IFN-γ responses of the $CD8^+$

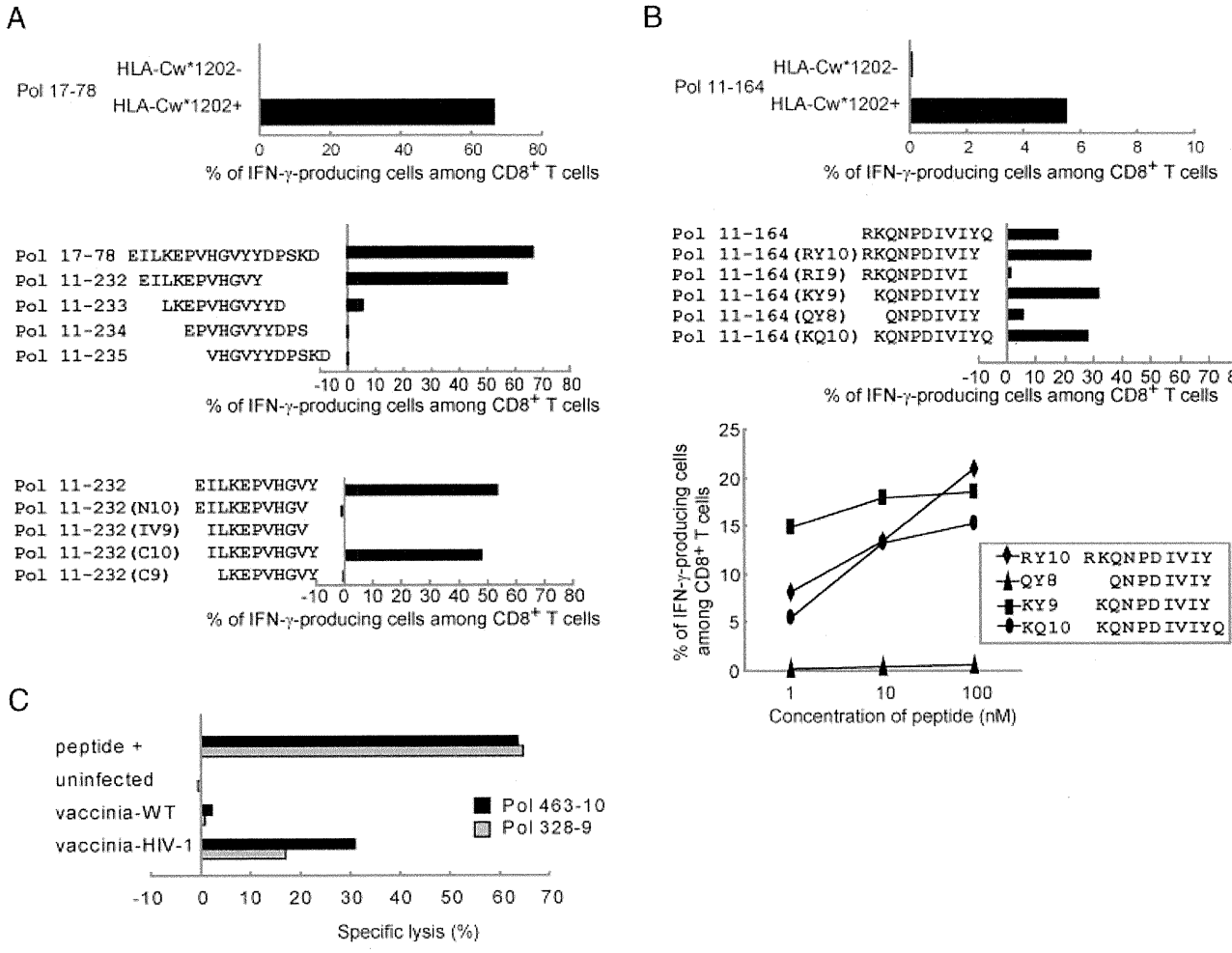

Figure 1. Identification of two HIV-1 Pol-specific epitopes using overlapping peptides. Candidates of HLA-Cw*1202-restricted HIV-1 CTL epitopes were identified using overlapping 17-mer or 11-mer HIV-1 peptides. PBMC from HLA-Cw*1202$^+$ HIV-1-seropositive individuals (KI-069 and KI-108) were stimulated with the 17-mer peptide cocktails and the 11-mer peptide cocktails, respectively, and then cultured for 12–14 days. (A) Top: A candidates of 17-mer Pol epitope peptide. The cultured PBMC cells form KI-069 were stimulated with the corresponding peptide-pulsed C1R cells expressing HLA-Cw*1202 (Cw*1202$^+$) or C1R cells (Cw*1202$^-$). Middle: Identification of 11-mer HIV-1 Pol peptides including HLA-Cw*1202-restricted epitope. The 17-mer cocktail peptide-specific bulk CD8$^+$ T cells were stimulated with C1R-Cw*1202 cells prepulsed with each of four overlapping 11-mer peptides. Bottom: Recognition of the 9-mer and 10-mer truncated peptides by the 11-mer-specific CD8$^+$ T cells. The 11-mer cocktail peptide-specific bulk CD8$^+$ T cells were stimulated with C1R-Cw*1202 prepulsed with each 8- to 10-mer truncated peptide. Peptide-specific CD8$^+$ T cells were detected using the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown at each figure. Each bar presents the data from one bulk T cells in a single experiment. (B) Top: A candidates of 11-mer Pol epitope peptides. The cultured PBMC cells from KI-108 were stimulated with the corresponding peptide-pulsed C1R cells expressing HLA-Cw*1202 (Cw*1202$^+$) or C1R cells (Cw*1202$^-$). Middle and Bottom: Pol11-164-specific bulk CTL were co-cultured with C1R-Cw*1202 prepulsed with each truncated peptide at concentrations of 100 nM (middle) or from 1 to 100 nM (bottom). The responsiveness of the bulk CD8$^+$ T cells toward each truncated peptide was measured by conducting the intracellular IFN-γ staining assay. The percentages of IFN-γ-producing cells among CD8$^+$ T cells are shown at each figure. Each bar or graph presents the data from one bulk T cells in a single experiment. (C) Presentation of two Pol epitopes by HLA-Cw*1202 on C1R-Cw*1202 cells infected with HIV-1 recombinant-HIV-1 vaccinia virus. The CTL activity of Pol 328-9-specific and Pol 463-10-specific bulk T cells against C1R-Cw*1202 cells prepulsed with a 1 μM concentration of the epitope peptide (peptide+) or infected with recombinant vaccinia virus expressing the corresponding HIV-1 Gag/Pol proteins (vaccinia-HIV-1) or WT vaccinia virus (vaccinia WT) was tested at an E:T ratio of 2:1. Each bar presents the data from one bulk T cells in a single experiment.

T cells than Pol the 11-164(RY10) or Pol 11-164(KQ10) one (Fig. 1B bottom). These results indicate that Pol 11-164(KY9) is the optimal epitope.

To clarify whether these two peptides were endogenously processed and presented by HLA-Cw*1202, we generated CTL clones specific for Pol 11-164(KY9) [9-mer peptide starting from position 328: Pol 328-9] or Pol 11-232(C10) [10-mer peptide starting from position 463: Pol 463-10], and then investigated whether these CTL clones could kill C1R-HLA-Cw*1202 cells infected with recombinant vaccinia virus expressing the HIV-1 Gag/Pol protein. These CTL clones effectively killed C1R-Cw*1202 infected with the recombinant vaccinia virus, but not those cells infected with WT vaccinia or uninfected cells (Fig. 1C), indicating that Pol 328-9 and Pol 463-10 are naturally processed CTL epitopes.

Frequency of HLA-Cw*1202-restricted HIV-1-specific CD8$^+$ T cells in chronically HIV-1-infected individuals

Next we investigated the frequency of the two Pol-specific HLA-Cw*1202-restricted CTL in chronically HIV-1-infected individuals carrying HLA-Cw*1202 in order to clarify whether they were immunodominant epitopes. We detected Pol 328-9-specific and Pol 463-10-specific T cells *ex vivo* among CD8$^+$ T cells from the HLA-Cw*1202$^+$ individuals by performing the ELISPOT assay (Fig. 2). Ten of 25 individuals tested showed positive responses for the Pol328-9 epitope. Since Pol 463-9 (ILKEPVHGV) is reported to be an HLA-A*02 epitope [34], we selected HLA-Cw*1202$^+$ individuals who did not have HLA-A*02 for Pol 463-10-specific T cells. Four of 15 individuals carrying HLA-Cw*1202 but not HLA-A*02 showed positive responses for Pol463-10. These indicate that the specific T cells were frequently elicited in chronically HIV-1-infected individuals carrying HLA-Cw*1202.

Strong abilities of HLA-Cw*1202-restricted CTL clones to suppress HIV-1 replication

To investigate the ability of HLA-C*1202-restricted HIV-1-specific CTL to suppress HIV-1 replication, we measured the ability of CTL clones specific for Pol 328-9 or Pol 463-10 to suppress HIV-1 replication in primary CD4$^+$ T cells infected with HIV-1 NL432. Three Pol 328-9-specific HLA-Cw*1202-restricted and three Pol 463-10-specific HLA-Cw*1202-restricted CTL clones completely suppressed HIV-1 replication at an E:T ratio of 1:1 (Fig. 3). Our previous studies showed that approximately 70% of HLA-A-restricted or HLA-B-restricted CTL clones weakly suppress HIV-1 replication (less than 50% suppression at E:T ratio of 1:1), whereas others such as HLA-B*5101-restricted Pol 283-specific and HLA-A*2402-restricted Nef 138-specific ones strongly suppress it (Supporting Information Table 1) [12, 35–37]. These results show that the two HLA-Cw*1202-restricted CTL had a strong ability to suppress HIV-1 replication *in vitro*.

Pol 463-10-9A is a mutant that escaped from Pol 463-10-specific CTL

To clarify whether Pol 328-9-specific or Pol 463-10-specific CTL select escape mutants at the population level, we analysed the sequences of these epitopes and their flanking regions in viruses from HLA-Cw*1202$^+$ and HLA-Cw*1202 HIV-1 infected-donors. Analysis of 16 HLA-Cw*1202$^+$ and 66 HLA-Cw*1202$^-$ individuals showed that several mutations were found in the Pol 328-9 epitope region (data not shown), but these mutations were not significantly associated with HLA- Cw*1202 ($p > 0.05$). We also analysed the sequence of Pol 463-10 from 33 HLA-Cw*1202$^+$ and 108 HLA-Cw*1202$^-$ HIV-1 infected-donors. Several mutations were found at positions 3 and 9 (Fig. 4A). The frequency of the 9A mutation was significantly higher in the HLA-Cw*1202$^+$ donors than in the HLA-Cw*1202$^-$ ones ($p = 0.001$, Fig. 4A), suggesting that the 9A was a mutant that escaped from the Pol 463-10-specific CTL. Since Pol 463-9 (ILKEPVHGV) is known to be an HLA-A*02 epitope [34], the 9A may be selected by Pol 463-9-specific HLA-A*02-restricted CTL. To clarify this possibility, we analysed the sequences at this position from 55 HLA-A*02$^+$ and 88 HLA-A*02$^-$ HIV-1-infected donors. Frequencies of HLA-A*02$^+$ and HLA-A*02$^-$ individuals having the 9A are 10.9 and 19.8%, respectively, indicating that HLA-A*02 is not significantly associated with the 9A mutation. Indeed, the 9A mutation has not been reported as escape mutant from Pol 463-9-specific HLA-A*02-restricted CTL. Further analysis of 26 HLA-Cw*1202$^+$ HLA-A*02$^-$ and 60 HLA-Cw*1202$^-$ HLA-A*02$^-$ HIV-1-infected-donors showed that the frequency of the 9A mutation was significantly higher in the HLA-Cw*1202$^+$ HLA-A*02$^-$ donors

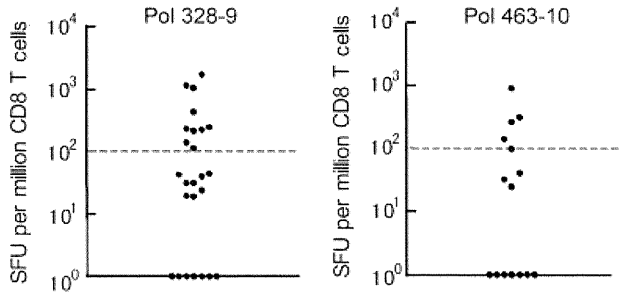

Figure 2. Frequency of HLA-Cw*1202-restricted Pol epitopes-specific CD8$^+$ T cells in chronically HIV-1-infected individuals. The frequencies of Pol 328-9-specific and Pol 463-10-specific CD8$^+$ T lymphocytes in chronically HIV-1-infected HLA-Cw*1202$^+$ individuals were measured using IFN-γ ELISPOT. Ten of 25 individuals tested showed positive responses for Pol328-9 epitope (mean SFU = 234), whereas four of 15 individuals who did not have HLA-A2 tested showed positive responses for Pol463-10 (mean SFU = 121). The subjects revealing a response of less than 100 SFU were evaluated as non-responders. SFU: spot-forming unit. Each dot represents one individual.

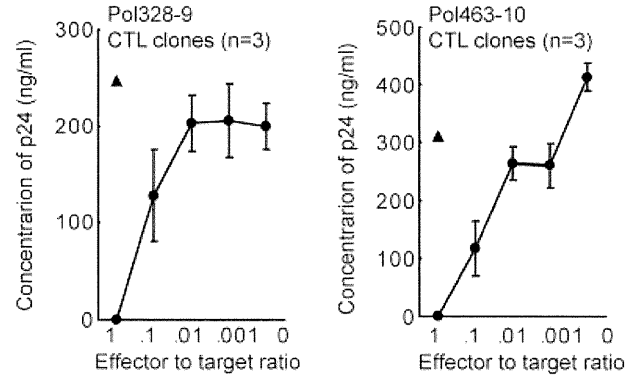

Figure 3. Strong abilities of two HIV-1 Pol-specific CTL to suppress HIV-1 replication. CD4$^+$ T cells from an HLA-Cw*1202$^+$ donor were infected with NL-432, and then co-cultured with the Pol-specific CTL clones ($n = 3$) at E:T ratios of 1:1, 0.1:1, 0.01:1, and 0.001:1 (circles). As a negative control, HLA-A*1101-restricted Pol675-specific CTL clone ($n = 1$) was used at an E:T ratio of 1:1 (triangle). HIV-1 p24 Ag in the supernatant were measured on day 7 after infection by an enzyme immunoassay. The data shown are the means and SD of assays for three HIV-1-specific CTL clones. They were from one out of two independent experiments.

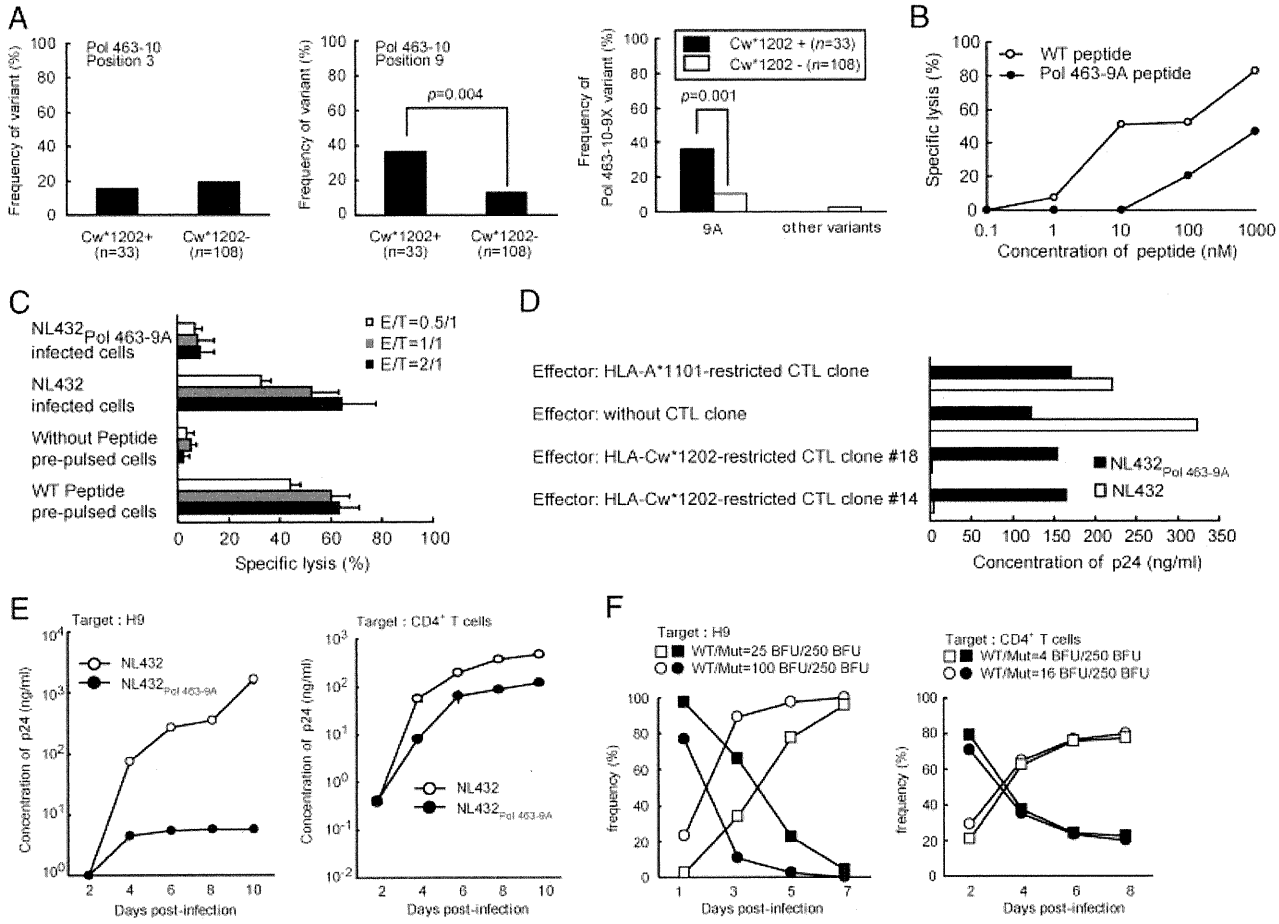

Figure 4. Characterization of escape Pol 463-9A escape mutation. (A) Frequency of mutations in Pol 463-10 epitope among chronically HIV-1-infected HLA-Cw*1202$^+$ and HLA-Cw*1202$^-$ HIV-1-infected individuals. The sequence of Pol 463-10 epitope was analysed in a single sample from both HLA-Cw*1202-positive or HLA-Cw*1202-negative individuals chronically infected with HIV-1. Thirty-three HLA-Cw*1202-positive or 108 HLA-Cw*1202-negative individuals were analysed. The consensus sequence of the Pol 463-10 epitope in clade B is ILKEPVHGVY. The frequency of mutations at positions 3 and 9 of the epitope are shown for both HLA-Cw*1202-positive and HLA-Cw*1202-negative donors. Frequency of Ala mutation at position 9 was significantly higher in HLA-Cw*1202-positive donors than in HLA-Cw*1202-negative donors. The p values were determined by Fisher's exact test. (B) Cytotoxic activities of Pol463-10-specific CTL clone toward C1R-HLA-Cw*1202 cells pulsed with Pol 463-10 or Pol 463-10-9A peptide. C1R-Cw*1202 cells were prepulsed with various concentrations of Pol 463-10 or Pol 463-10-9A peptide. Cytotoxic activity of a Pol463-10-specific CTL clone was measured at an E:T ratio of 2:1. The results were from a single T cell clone in one of two independent experiments. (C) Cytotoxic activity of Pol 463-10-specific CTL clones against 721.221-CD4-HLA-Cw*1202 cells infected with the 9A mutant virus; 721.221-CD4- HLA-Cw*1202 cells were infected with NL-432 or NL-432 $_{\text{Pol 463-10-9A}}$ mutant virus. NL-432-infected or NL-432 $_{\text{Pol 463-10-9A}}$-infected 721.221-CD4-HLA-Cw*1202 were used as target cells at an E:T ratio of 2:1, 1:1, or 0.5:1. The data shown are the means and SD of assays for three HIV-1-specific CTL clones. They were from one of two independent experiments. (D) Ability of HIV-1-specific CTL clones to suppress HIV-1 replication in the 9A mutant virus-infected CD4$^+$ T cells. CD4$^+$ T cells from an HLA-Cw*1202$^+$ HLA-A*1101$^-$ healthy donor were infected with NL-432 or NL-432 $_{\text{Pol 463-10-9A}}$, and then co-cultured with the Pol 463-10-specific CTL clone (clone #14 or clone #18) or HLA-A*1101-restricted CTL clone at an E:T ratio of 2:1 or without the CTL clone. HIV-1 p24 Ag in the supernatant were measured on day 7 after infection by performing an enzyme immunoassay. The results were from one T-cell clone in one of two independent experiments. (E) Fitness of the 9A mutant virus. Production of p24 Ag in culture supernatant was determined by an enzyme immunoassay. Profiles of replication kinetics (p24 production) of NL-432 (closed circles), NL-432 $_{\text{Pol 463-10-9A}}$ (open circles) were determined with H9 cells and CD4$^+$ T cells. The data shown are the means and SD of triplicates in one of two independent experiments. (F) A competitive HIV-1 replication assay using the 9A mutant and WT virus. To compare the replication kinetics of NL-432 (open symbols) and NL-432 $_{\text{Pol 463-10-9A}}$ (closed symbols), H9 cells (left), and CD4$^+$ T cells (right) were infected with both viruses at different viral titers. The frequency of each virus at day 1, 3, 5, and 7 (H9 cells) or at day 2, 4, 6, and 8 (CD4$^+$ T cells) was determined from the relative peak height on sequencing electrograms. The data were from one sample in a single experiment.

than in the HLA-Cw*1202$^-$HLA-A*02$^-$ ones (34.6 *versus* 14.4%, $p = 0.037$). These results together suggest that HLA-Cw*1202-restricted Pol 463-10-specific CTL selected the 9A mutant.

To clarify whether the 9A was indeed an escape mutant of Pol 463-10-specific CTL, we investigated the ability of Pol 463-10-specific CTL to recognise the Pol 463-10-9A mutant epitope. We first tested the activity of Pol 463-10-specific CTL clones to kill target cells prepulsed with the Pol 463-10-9A mutant peptide. Three Pol 463-10-specific CTL clones effectively killed target cells prepulsed with the Pol 463-10 WT peptide but showed reduced ability to kill those prepulsed with the Pol 463-10-9A mutant peptide (Fig. 4B), suggesting that the 9A mutant had escaped

Table 1. Longitudinal analysis of Pol 463-10 epitope sequence in HIV-1-infected individuals

ID	HLA-Cw*1202	Sample date Month/day/year	Sequence ILKEPVHGVY
KI-037	Positive	01/29/2002	----------
		06/17/2004	--------A-
KI-163	Positive	08/30/2002	----------
		06/28/2004	----------
		08/29/2005	--------A-
		02/27/2006	--------A-
KI-428	Positive	03/12/2003	----------
		06/28/2006	----------
		09/27/2006	----------
		07/12/2007	--------A-
KI-452	Positive	12/30/1999	----------
		01/30/2007	--------A-
KI-097	Negative	04/25/2003	--------A-
		02/18/2005	--R-------
KI-091	Negative	09/10/2001	--------A-
		07/09/2003	--R-------
KI-161	Negative	07/25/2002	--E-----A-
		05/07/2004	----------
		09/29/2004	----------

from Pol 463-10-specific CTL. Therefore we generated the 9A mutant virus from NL432 (NL-432-$_{\text{Pol 463-10-9A}}$) to further analyse the ability of Pol 463-10-specific CTL to kill target cells infected with the 9A mutant virus. The Pol 463-10-specific CTL clones effectively killed the target cells infected with NL-432 whereas they failed to kill those infected with NL-432-$_{\text{Pol 463-10-9A}}$ (Fig. 4C). In addition, by performing a replication suppression assay we analysed whether these CTL could suppress the replication of mutant virus and WT virus *in vitro*. Pol 463-10-specific CTL clones (clone #14 and #18) effectively suppressed the replication of the WT virus, whereas they failed to suppress that of the 9A mutant virus (Fig. 4D). These results indicate that the 9A is indeed escape mutant of Pol 463-10-specific CTL.

We performed longitudinal analysis of the Pol 463-10 epitope in 14 HLA-Cw*1202$^+$ individuals. Four HLA-Cw*1202$^+$ individuals showed the WT sequence of Pol 463-10 in the early phase and the Pol 463-10-9A mutant appeared more than 3 years later (Table 1), supporting that the 9A is escape mutant from Pol 463-10-specific CTL. KI-037 is a haemophiliac patient who had been infected with HIV-1 before 1985, indicating that the 9A mutant appeared more than 17 years after HIV-1 infection. Thus, this mutant may be slowly selected in HLA-Cw*1202$^+$ individuals.

Reversion of the 9A mutant

To examine the effect of the 9A mutation on viral fitness, we compared the replication ability of NL432 (WT) and the 9A mutant using the p24 production assay. The results using H9 cells and primary CD4$^+$ T cells as target cells showed that fitness cost of the 9A was much higher than that of WT (Fig. 4E). In addition, we performed a competitive HIV-1 replication assay for further comparison of replication kinetics in H9 cells and primary CD4$^+$ T cells. During 7 days culture, we observed that the 9A had higher fitness cost than WT in both cells (Fig. 4F). These results suggest that this mutant is able to revert to WT in HLA-Cw*1202$^-$ HIV-1-infected individuals. To clarify the reversion, we performed a longitudinal analysis of this epitope sequence on five HLA-Cw*1202$^-$ individuals who could be followed from early stage of the infection and had the 9A mutation at the early stage. Three of these five HLA-Cw*1202$^-$ individuals showed the reversion within approximately 2 years after the 9A had been found (Table 1). These results support the finding that the 9A mutant did not remarkably accumulate in the HLA-Cw*1202$^-$ individuals.

Discussion

HLA-C molecules are believed to play a less important role in the presentation of various Ag than HLA-A and -B ones, because the former molecules are expressed on the cell surface at a level that is approximately 10% of that of the latter molecules [26, 27]. On the other hand, HLA-A and -B molecules are down-regulated in HIV-1-infected cell mostly due to the effect of Nef whereas HLA-C molecules are not, implying that HLA-C-restricted HIV-specific CTL can be elicited and have some role in the control of HIV-1. A previous study demonstrated that HLA-C-restricted responses are elicited in an African cohort infected with HIV-1 clade C, although it showed that HLA-B-restricted T-cell responses are much stronger than those of HLA-C-restricted or HLA-A-restricted ones [38]. A study using HLA-C-restricted HIV-1-specific CTL clones previously demonstrated that the ability of HLA-C-restricted CTL to suppress HIV-1 replication *in vitro* is similar

to that of HLA-A- or HLA-B-restricted CTL [28]. The present study also demonstrated that two HLA-C-restricted CTL had a strong ability to suppress HIV-1 replication *in vitro*. These findings suggested the possibility that some HLA-C-restricted T cells can control HIV-1 *in vivo*.

Previous studies demonstrated that HLA-A-restricted or HLA-B-restricted HIV-1-specific CTL recognised target cells infected with NL-432 M20A mutant (one amino acid substitution of Ala for Met at residue 20 of Nef), which lost the ability to down-regulate HLA-A and HLA-B molecules in HIV-1-infected cells, much more than those infected with NL-432 [12, 35, 39]. For example, Gag263-10-specific and Rev77-9-specific CTL showed approximately 50% suppression of the M20A virus replication but did not suppress NL432 replication (Supporting Information Table 1). Thus, HLA-C-restricted CTL, which is not affected by the Nef-mediated HLA down-regulation, have an advantage in the recognition of HLA–epitope complex on HIV-1-infected cells *in vivo*.

Previous population studies analysing HIV sequences in African cohorts demonstrated that some amino acid substitutions of HIV-1 Gag, Pol, and Nef are associated with HLA-C alleles [32, 33]. These studies suggested possibility that these substitutions are escape mutations selected by HLA-C-restricted T cells. However, since they did not demonstrate that specific CTL failed to recognise these substitutions, it still remained unclear whether HLA-C-restricted T cells could select escape mutant. We demonstrated here that the Pol463-10-specific CTL failed to kill the 9A mutant-infected cells but effectively killed WT HIV-1-infected ones. In addition, the CTL had a strong ability to suppress replication of a WT of HIV-1 but no ability to suppress that of the 9A mutant. The longitudinal analysis of HLA-Cw*1202$^+$ HIV-1-infected individuals showed the mutation from the WT to the 9A mutant. These results together support the idea that HLA-C-restricted CTL selected this escape mutant *in vivo*.

A previous study on a cohort infected with HIV-1 clade C virus demonstrated that HLA-C allele-associated Pol mutations are associated with low VL [33], suggesting these mutations increase fitness cost. The present study also demonstrated that NL-432 carrying the 9A mutant had a higher fitness cost than NL-432. However, the analysis of HLA-Cw*1202$^+$ individuals having and not having this mutation showed no association between VL and the presence of this mutation (data not shown). These suggest the possibility that a complementary substitution may compensate the effect of the 9A in terms of fitness cost. Another explanation is that fitness cost of the 9A mutant virus is not so much higher than that of the WT virus *in vivo*. Indeed, the difference in fitness cost between the two viruses in primary CD4$^+$ T cells is much smaller than that between them in the cell lines.

Both Pol 328-9-specific and Pol 463-10-specific CTL had strong ability to suppress HIV replication *in vitro*. However, the latter CTL selected escape mutants whereas the former CTL did not. It remains unknown why the one could select an escape mutant but the other could not. A recent study demonstrated that HLA-A*1101-restricted Nef73-specific and Nef84-specific CTL clones have strong ability to suppress HIV-1 replication *in vitro* but that the latter CTL can select an escape mutant whereas the former one did not [37]. *Ex vivo* analysis of these CTL showed that Nef84-specific CTL have a stronger ability to recognise the epitope than the Nef73-specific CTL [37]. That study suggested that only CTL having a strong ability to recognise the epitope can suppress HIV-1 replication *in vivo* so that escape mutants may be selected. This might be the case also for these HLA-Cw*1202-restricted CTL.

A variant 35 kb upstream of the *HLA-C* gene (*−35C/T*) was previously shown to be associated with the HLA-C mRNA expression level and steady-state plasma HIV RNA levels [29]. A recent study analysing 1698 European American individuals demonstrated that the *−35CC* allele is a proxy for high cell surface expression of HLA-C and that individuals with this allele progress more slowly to AIDS and control viremia significantly better than those without this low allele [40]. HLA-Cw*1202 is frequently found in east-Asia including Japan and forms a haplotype with HLA-A*2402 and HLA-B*5201. HLA-Cw*1202 is known to be highly associated with *−35CC* allele [40]. Therefore, we speculate that HLA-Cw*1202 is associated with a slow progression to AIDS.

In the present study, we demonstrated that HLA-Cw*1202-restricted Pol 463-10-specific CTL, which had a strong ability to suppress HIV-1 replication, selected an escape mutant, indicating that HLA-C allele-restricted HIV-specific CTL also play an important role in the generation of HIV-1 polymorphism. Further analysis of HLA-C-restricted CTL is expected to clarify the role of HLA-C alleles in HIV-1 infections.

Materials and methods

Samples of HIV-1-infected individuals

Plasma and PBMC were separated from whole blood of chronically HIV-1-infected individuals. The National Center for Global Health and Medicine and the Kumamoto University Ethical Committee approved this study. Informed consent was obtained from all subjects according to the Declaration of Helsinki.

HLA-typing

The HLA type of the chronically HIV-1-infected individuals was determined by standard sequence-based genotyping.

Synthetic peptides

We previously designed and generated overlapping peptides consisting of 11-mer or 17-mer amino acids in length and spanning Gag, Pol, and Nef of HIV-1 clade B consensus sequences [41, 42]. Each 11-mer and 17-mer peptide was overlapped by at least 9 and 11 amino acids, respectively.

Sequence of autologous virus

Viral RNA was extracted from plasma samples from HIV-1-infected individuals using a QIAamp MinElute virus spin kit (QIAGEN). cDNA was synthesised from the viral RNA using Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). The Pol regions including the two epitopes was amplified by nested PCR, and amplified products were used for sequencing reaction by BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). DNA sequencing was performed by ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Cells

The EBV-transformed B-LCL were generated by transforming B cells from PBMC of healthy volunteers and HIV-1-seropositive individuals, KI-069, and KI-108. C1R cells expressing HLA-Cw*1202 (C1R-HLA-Cw*1202) were generated by transfecting C1R cells with the HLA-Cw*1202 gene; 721.221-CD4-HLA-Cw*1202 cells were generated by transfecting 721.221-CD4 cells with HLA-Cw*1202 genes. These transfectants were cultured in RPMI 1640 supplemented with 10% FBS and 0.15/mL hygromycin B. H9 cells were cultured in RPMI 1640 supplemented with 10% FBS. MGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-gal cells) were cultured in DMEM supplemented with 10% FBS as described previously [43].

Generation of 2 HLA-Cw*1202-restricted HIV-1-specific CTL clones

The two Pol-epitope-specific CTL clones were generated from bulk CTL specific for Pol328-9 or Pol463-10 epitopes as described previously [37].

Generation of NL−432$_{-Pol\ 463-10-9A}$ mutant clones

The NL-432$_{-Pol\ 463-10-9A}$ mutant virus was generated by introducing the Pol463-10-9A mutation into NL-432 using site-directed mutagenesis (Invitrogen).

Intracellular cytokine assay

PBMC from HLA-Cw*1202-positive HIV-1-infected patients were stimulated with HIV-1-derived peptide (1 μM) in culture medium (RPMI 1640 medium supplemented with 10% FBS and 200 U/mL recombinant human IL-2). After 14 days in culture, the cells were assessed for IFN-γ production using a FACS Calibur (BD Bioscience). Briefly, bulk cultures were stimulated with HLA-Cw*1202-expressing cells pulsed with HIV-1-derived peptide (1 μM) for 2 h at 37°C. Brefeldin A (10 μg/mL) was added, and incubated for a further 4 h. The cells were collected and stained with PE-labelled anti-CD8 mAb (Dako, Glostrup, Denmark). Cells were fixed with 4% paraformaldehyde solution, and permeabilised with permeabilization buffer (0.1% saponin and 20% Newborn Calf Serum in PBS) at 4°C for 10 min, followed by staining with FITC-labelled anti-IFN-γ mAb (PharMingen, San Diego, CA).

CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity of HIV-1-specific CTL was measured by the standard ^{51}Cr release assay, as previously described [12]. Briefly, target cells were labelled by $Na_2^{51}CrO_4$, then washed three times with RPMI 1640-10% FBS. ^{51}Cr-labelled target cells were plated 96-U plate with or without 1 μM peptide, and incubated for 1 h. After 1 h of incubation, CTL clones were added and incubated for 4 h. The supernatants were harvested and measured by a γ counter.

CTL assay for target cells infected with HIV-1

721.221-CD4-HLA-Cw*1202 cells were exposed to NL-432 or NL-432$_{-Pol\ 463-10-9A}$ for 3–6 days. Infection rate of these cells were measured by staining HIV-1 p24 Ag (KC57-FITC; Beckman Coulter). When approximately 30–60% of cells were infected, ^{51}Cr-labeled infected cells were co-cultured with CTL clones for 6 h. The supernatants were harvested and measured by a γ counter.

Replication suppression assay

The ability of HIV-1-specific CTL to suppress HIV-1 replication was examined as previously described [41]. Briefly, $CD4^+$ T cells were incubated with a given HIV-1 clone for 6 h at 37°C. After three washes with RPMI 1640-10% FBS, the cells were co-cultured with HIV-1-specific CTL clones. From day 3 to day 9 post infection, 10 μL of culture supernatant was collected; and the concentration of p24 Ag in it was measured with an enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix, Buffalo, NY). The percentage of suppression of HIV-1 replication was calculated as follows: % suppression = (1–concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured with HIV-1-specific CTL/concentration of p24 Ag in the supernatant of HIV-1-infected $CD4^+$ T cells cultured without the CTL) × 100.

p24 production assay

H9 cells (8×10^5) and $CD4^+$ T cells (8×10^5) were exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 6 h, washed twice with PBS, and cultured in 5 mL of complete medium [43]. The culture supernatants (0.2 mL) were harvested every other day, and the volume removed was replaced with fresh medium. The concentration of p24 Ag was measured with of an enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix). Replication kinetics assays were performed in duplicate.

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) and $CD4^+$ T cells (3×10^5) were exposed for 2 h to mixtures of paired virus preparations (various blue cell-forming units) for examination of their replication ability, washed twice with PBS, and cultured as described previously [43]. Every other day the supernatant was harvested, and then cDNA was synthesised and sequenced. The change in viral population was determined from the relative peak height on sequencing electrograms.

ELISPOT assay

ELISPOT assay was performed as previously described [37]. Briefly, cryopreserved PBMC of 25 HLA-Cw*1202$^+$ HIV-1-infected individuals were plated in 96-well polyvinylidene plates precoated with 0.5 μg/mL of anti-IFN-γ mAb 1-DIK (Matbech, Stockholm, Sweden). The appropriate amount of Pol 328-9 or Pol 463-10 peptide and PBMC were added at 1×10^5 cells/well and then the plates were incubated for 40 h. After the addition of biotinylated anti-IFN-γ mAb at 0.5 μg/mL, plates were incubated at room temperataure for 100 min. and then washed with PBS. Subsequently, streptavidin-conjugated alkaline phospatase was added, followed by 40 min incubation at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA).

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Abbreviations: **B-LCL:** B-lymphoblastoid cell lines · **V9A:** V-to-A substitution at the 9th position · **VL:** viral load

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Selection and Accumulation of an HIV-1 Escape Mutant by Three Types of HIV-1-Specific Cytotoxic T Lymphocytes Recognizing Wild-Type and/or Escape Mutant Epitopes

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It is known that cytotoxic T lymphocytes (CTLs) recognizing HIV-1 escape mutants are elicited in HIV-1-infected individuals, but their role in the control of HIV-1 replication remains unclear. We investigated the antiviral ability of CTLs recognizing the HLA-A*24:02-restricted Gag28 -36 (KYKLKHIVW) epitope and/or its escape mutant (KYRLKHIVW) elicited in the early and chronic phases of the infection. Wild-type (WT)-epitope-specific CTLs, as well as cross-reactive CTLs recognizing both WT and K30R (3R) epitopes, which were predominantly elicited at early and/or chronic phases in HLA-A*24:02$^+$ individuals infected with the WT virus, suppressed the replication of the WT virus but failed to suppress that of the 3R virus, indicating that the 3R virus was selected by these 2 types of CTLs. On the other hand, cross-reactive and 3R-specific CTLs, which were elicited in those infected with the 3R virus, did not suppress the replication of either WT or 3R virus, indicating that these CTLs did not contribute to the control of 3R virus replication. High accumulation of the 3R mutation was found in a Japanese population recently recruited. The selection and accumulation of this 3R mutation resulted from the antiviral ability of these Gag28-specific CTLs and high prevalence of HLA-A*24:02 in a Japanese population. The present study highlighted the mechanisms for the roles of cross-reactive and mutant-epitope-specific CTLs, as well as high accumulation of escape mutants, in an HIV-1-infected population.

Human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) play an important role in the control of HIV-1 during the acute and chronic phases of an HIV-1 infection (22, 40). However, HIV-1-specific CTLs cannot completely eliminate HIV-1-infected cells, because HIV-1 escapes from CTL-mediated immune pressure by various mechanisms, such as selection of escape mutations, Nef-mediated HLA class I downregulation, and skewed maturation of memory HIV-specific CD8$^+$ T lymphocytes (5, 8, 9). The most documented escape mechanism is acquisition of amino acid mutations within the CTL epitope and/or its flanking regions. These mutations lead to reduced ability of peptide to bind to HLA class I molecules, impaired T cell receptor (TCR) recognition, and defective epitope generation (21, 31). These escape mechanisms are involved in impaired activities of HIV-1-specific CTLs to kill target cells infected with escape mutant virus and to suppress HIV-1 replication, contributing to the selection of escape mutant viruses (5, 10, 13, 20, 29, 35, 41).

There is growing evidence that escape mutations selected by HLA class I-restricted CTLs accumulate at the population level (7, 28, 36). The accumulation of escape mutants may affect the clinical outcomes for HIV-1-infected individuals (11, 37, 38). On the other hand, it is known that CTLs recognizing escape mutants are elicited after the emergence of the escape mutant selected by wild-type (WT) epitope-specific CTLs (2, 4, 12, 15, 33, 39). The escape mutant-specific CTLs were also elicited in new hosts carrying the same restricted HLA allele when they were infected with the mutant (15). Several studies showed that CTLs cross-recognizing the WT and its escape mutant epitopes are elicited before or after the emergence of the escape mutant in the same hosts (18, 25, 26, 33, 34). However, the antiviral abilities of these cross-reactive CTLs remain unknown, since the recognition of cross-reactive CTLs for synthesized epitope peptides was characterized by using the enzyme-linked immunosorbent spot assay (ELISPOT) or ^{51}Cr cytotoxic assay in those studies. We previously showed that HLA-A*24:02-restricted Nef 138-specific CTLs recognizing an escape mutant had weaker ability to suppress the replication of the mutant virus than that of the WT virus (15). However, it still remains unclear whether cross-reactive or escape mutant-specific CTLs contribute to the control of HIV-1, since the CTLs have not been analyzed in detail.

To clarify the abilities of cross-reactive and escape mutant-specific CTLs to recognize HIV-1-infected cells, we analyzed CTLs specific for HLA-A*24:02-restricted HIV-1 Gag28-36 (KYKLKH IVW; Gag28), which is the only immunodominant Gag epitope presented by this HLA class I allele (24). Since HLA-A*24:02 is found in approximately 70% of the Japanese population (42), the mutants of HLA-A*24:02-restricted epitopes may accumulate in HIV-1-infected Japanese individuals. We previously suggested that K30R (3R) in the Gag28 epitope is an escape mutation from HLA-A*24:02-restricted Gag28-specific CTLs (30) and that CTLs recognizing 3R are elicited in HIV-1-infected HLA-A*24:02$^+$ individuals (46). From these studies, we hypothesized that cross-reactive CTLs recognizing WT and 3R mutant epitopes and/or 3R-specific CTLs are elicited in HLA-A*24:02$^+$ HIV-1-infected individuals after the 3R mutant is selected and in new 3R virus-infected hosts carrying HLA-A*24:02. Here, we investigated the elicitation of Gag28-specific CTLs in 12 HLA-A*24:02$^+$ HIV-1-

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infected Japanese individuals who could be monitored from the early phase to the chronic phase of an HIV-1 infection, as well as the abilities of cross-reactive, 3R mutant-specific, and WT-specific CTLs to kill WT or 3R virus-infected cells and to suppress the replication of the WT or 3R virus. In addition, we investigated the accumulation of the 3R mutation in HIV-1-infected nonhemophiliac Japanese individuals, as well as in Japanese hemophiliacs who had been infected around 1983. The results clarified the role of CTLs recognizing the WT and/or 3R epitope in high accumulation of the 3R mutant in HIV-1-infected Japanese individuals.

MATERIALS AND METHODS

Samples from HIV-1-infected individuals. This study was approved by the ethics committee of Kumamoto University and the National Center for Global Health and Medicine. Informed consent was obtained from all individuals according to the Declaration of Helsinki. For sequence analysis, blood specimens were collected in EDTA. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood. HLA types were determined by standard sequence-based genotyping. Twelve HLA-A*24:02$^+$ individuals who could be monitored from the early to the chronic phase of an HIV-1 infection were recruited for CTL analysis. Early HIV-1 infection was confirmed by seroconversion within 6 months or by an increasing number and density of bands on Western blots. Four-hundred fifty-one chronically HIV-1-infected individuals were also recruited for sequence analysis.

Cells. C1R cells expressing HLA-A*24:02 (C1R-A2402) and 721.221 cells expressing CD4 and HLA-A*24:02 (721.221-CD4-A2402) were previously generated (27, 30). These cells were cultured in RPMI 1640 medium containing 5 to 10% fetal bovine serum (FBS) and 0.15 mg/ml hygromycin B. MAGIC-5 cells (CCR5-transfected HeLa-CD4/long terminal repeat–β-galactosidase [LTR–β-Gal] cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS as described previously (17).

Induction of Gag28-specific T cells. PBMCs from HIV-1-infected HLA-A*24:02$^+$ individuals were stimulated with WT or 3R peptide (1 μM) in culture medium (RPMI 1640 containing 10% FBS and 200 U/ml human recombinant interleukin-2 [rIL-2]). After 14 days, the cultured PBMCs were tested for gamma interferon (IFN-γ) production by performing an intracellular cytokine staining (ICC) assay.

ICC assay. C1R-A2402 cells were prepulsed or not with the WT or 3R peptide at concentrations from 0.1 to 1,000 nM at 37°C for 1 h and then were washed twice with RPMI 1640 containing 10% FBS. PBMCs cultured for 2 weeks after peptide stimulation were incubated with the C1R-A2402 cells in a 96-U plate (Nunc) at 37°C. Brefeldin A (10 μg/ml) was added after a 2-h incubation, and then the cells were incubated for an additional 4 h. Subsequently, the cells were stained with Pacific-blue-conjugated anti-CD8 monoclonal antibody (MAb) (BD Biosciences) and 7-aminoactinomycin D (7-AAD) (BD Biosciences) at 4°C for 30 min, after which the cells were fixed with 4% paraformaldehyde solution and rendered permeable with permeabilization buffer (0.1% saponin and 10% FBS in phosphate-buffered saline) at 4°C for 10 min. Thereafter the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-IFN-γ MAb (BD Biosciences) at 4°C for 30 min and then washed twice with the permeabilization buffer. The percentage of CD8$^+$ cells producing IFN-γ was analyzed by flow cytometry (FACSCanto II).

Generation of Gag28-specific CTL clones. Gag28-specific CTL clones were generated from Gag28-specific bulk-cultured T cells by limiting dilution in 96-U plates, together with 200 μl of cloning mixture (1×10^6 irradiated allogeneic PBMCs from healthy donors and 1×10^5 irradiated C1R-A2402 cells prepulsed with the WT or 3R peptide at a concentration of 1 μM in RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% phytohemagglutinin [PHA] soup). After 14 to 21 days in culture, the growing cells were tested for cytotoxic activity by performing the standard chromium release assay. Since TCRs on these CTL clones were not sequenced, it is still possible that they were oligonucleotide clones.

HIV-1 clones. An infectious provirus, HIV-1 pNL-432, was reported previously (1). NL-432gagSF2 and NL-432gagSF2-3R were previously generated (30).

Assay of cytotoxicity of CTL clones toward target cells prepulsed with the epitope peptide. The cytotoxic activities of Gag28-specific CTL clones were determined by use of the standard chromium release assay, as described previously (15). Briefly, 721.221-CD4-A2402 cells were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ in saline for 1 h and then washed 3 times with RPMI 1640 containing 10% newborn calf serum. The labeled target cells (2×10^3/well) were prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM for 1 h and then cocultured at 37°C for 4 h with effector cells at an effector-to-target (E:T) ratio of 1:1 in 96-U plates (Nunc). The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the 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 counts per minute in the supernatant in the wells containing both target and effector cells.

Assay of cytotoxicity of CTL clones toward target cells infected with HIV-1. 721.221-CD4-A2402 cells were infected with WT or 3R virus, and then the infection rates were determined by detecting intracellular p24 antigen (Ag)-positive cells stained with FITC-conjugated anti-p24 Ag MAb (KC57-FITC; BD Biosciences). When approximately 50% of the total cells were p24 Ag-positive cells, they were used as target cells. The ^{51}Cr-labeled target cells (2×10^3/well) were cocultured with effector cells at E:T ratios of 0:1 to 2:1 in 96-U plates at 37°C for 6 h. The supernatants were collected and analyzed with a gamma counter.

Generation of HLA-peptide tetrameric complexes. HLA class I-peptide tetrameric complexes (tetramers) were synthesized as previously described (3). The WT or 3R peptide was added to the refolding solution containing the biotinylation sequence-tagged extracellular domain of the HLA-A*24:02 molecule and β2 microglobulin. The purified monomer complexes were mixed with phycoerythrin (PE)-labeled streptavidin (Molecular Probes) at a molar ratio of 4:1.

Tetramer binding assay. CTL clones were stained with PE-conjugated tetramer at concentrations of 1 to 100 nM at 37°C for 30 min. After 2 washes with RPMI 1640 containing 10% FBS (R10), the cells were stained with FITC-conjugated anti-CD8 MAb and 7-AAD at 4°C for 30 min. Thereafter, the cells were washed twice with R10 and then analyzed by flow cytometry (FACSCanto II). The mean fluorescence intensity (MFI) of tetramer-positive cells among CD8-positive cells was calculated.

Replication suppression assay. The ability of Gag28-specific CTLs to suppress HIV-1 replication was examined as previously described (43). CD4$^+$ T cells were isolated from PBMCs of healthy HLA-A*24:02$^+$ donors and incubated with a given HIV-1 clone at 37°C for 6 h. After 3 washes with R10, the cells (3×10^4/well) were cocultured with Gag28-specific CTL clones at E:T ratios of 0.1:1 to 1:1 in R10 containing 1% nonessential amino acid solution and, 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. From day 3 to day 7 postinfection, a 30-μl volume of culture supernatant was collected, and the volume removed was replaced with fresh medium. The concentration of p24 Ag was measured by using an enzyme-linked immunosorbent assay (ELISA) (HIV-1-p24-Ag ELISA kit; ZeptoMetrix).

Replication kinetics assay. The replication kinetics of the WT and 3R viruses were examined as previously described (17). After CD4$^+$ T cells (2×10^6) had been exposed to each infectious virus preparation (500 blue cell-forming units in MAGIC-5 cells) for 2 h and washed twice with R10, they were cultured in 1 ml of R10 containing 1% nonessential amino acid solution and 1% 100 mM sodium pyruvate (complete medium) plus 200 U/ml rIL-2. Then, 0.1 ml of the culture supernatant was collected from

day 2 to day 10 postinfection, and the volume removed was replaced with fresh medium. The concentration of p24 Ag in the supernatant was measured by using ELISA. Replication kinetics assays were performed in triplicate.

Sequence of autologous virus. Viral RNA was extracted from plasma samples from HIV-1-infected individuals by using a QIAamp MinElute virus spin kit (Qiagen). For clone sequencing, cDNA was synthesized from the RNA with SuperScript III and Random Primers (Invitrogen), and the Gag region was amplified by nested PCR with *Taq* DNA polymerase (Promega). Then, the PCR products were gel purified and cloned with a TOPO TA cloning kit (Invitrogen). For bulk sequencing, the Gag region was amplified from the RNA by using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen) and Gag-specific primers, and then the second PCR was done. We prepared the Gag-specific primer sets shown below. For clone sequencing, 5′-TTTTT GACTAGCGGAGGCTAGAA-3′ and 5′-CACAATAGAGGGTTGCTAC TGT-3′ were used for the first PCR and 5′-GGGTGCGAGAGCGTCGG TATTAAGC-3′ and 5′-TAAGTTCTTCTGATCCTGTCTG-3′ for the second PCR. For bulk sequence, 5′-TCTCTCGACGCAGGACTC-3′ and 5′-AGGGTTCCTTTGGTCCTTGT-3′ were employed for the reverse transcription (RT)-PCR and 5′-TCTCTCGACGCAGGACTC-3′ and 5′-TCTCCTACTGGGATAGGTG-3′ for the second PCR. All DNA sequencing was performed by using a BigDye Terminator cycle-sequencing kit (Applied Biosystems) and an ABI Prism 310 or 3100 genetic analyzer.

RESULTS

Selection of the 3R mutation by WT epitope-specific CD8$^+$ T cells in individuals infected with WT virus. We investigated 12 HIV-1-infected HLA-A*24:02$^+$ individuals who could be monitored from the early to the chronic phases of their infections. We first analyzed the sequence of the Gag28 epitope at an early phase in the 12 HIV-1-infected HLA-A*24:02$^+$ individuals. The WT sequence of the Gag28 epitope was detected in 4 of these individuals, whereas 3R was found in the other 8, suggesting that the former and the latter individuals had been infected with WT and 3R viruses, respectively (Table 1). This is consistent with a previous finding that the 3R mutant is found in approximately 70% of HIV-1-infected HLA-A*24:02$^+$ individuals (30). We investigated the elicitation of Gag28-specific CD8$^+$ T cells in the individuals infected with WT virus. PBMCs from these individuals at early and chronic phases were stimulated with WT or 3R peptide and then cultured for 2 weeks. The frequency of Gag28-specific CD8$^+$ T cells among the cultured cells was measured by performing the ICC assay using WT and 3R peptides. Gag28-specific CD8$^+$ T cells were detected at the early phase in 3 of the 4 individuals when the PBMCs were stimulated with WT peptide (Table 2). In 2 individuals, i.e., KI-092 and KI-161, Gag28-specific CD8$^+$ T cells were much more WT specific than 3R mutant specific, whereas in KI-158 they recognized both peptides, but especially the WT peptide (Fig. 1). On the other hand, cross-reactive CD8$^+$ T cells were induced in KI-092 and KI-161 when their PBMCs had been stimulated with 3R peptide, although the frequency of cross-reactive CD8$^+$ T cells induced by stimulation with 3R peptide was lower than that of WT-specific cells induced by stimulation with WT peptide. The 3R peptide failed to induce Gag28-specific CD8$^+$ T cells in PBMCs from KI-158. Thus, WT-specific CD8$^+$ T cells were predominantly elicited at an early phase in the individuals infected with WT virus, although a small but significant number of cross-reactive T cells were also elicited in them.

To clarify the specificity of Gag28-specific CD8$^+$ T cells at the early phase in KI-092 and KI-161, we generated Gag28-specific CTL clones by stimulating early-phase PBMCs from KI-092 and KI-161 with the WT peptide. The CTL clones from KI-092 showed a much greater ability to kill cells prepulsed with WT peptide than to kill those prepulsed with the 3R peptide (Fig. 2A), suggesting that they were WT-specific CTLs. To further clarify the specificity of these T cell clones, we investigated the binding affinity of the clones for WT peptide-binding HLA-A*24:02 tetramer (WT tetramer) and 3R peptide-binding HLA-A*24:02 tetramer (3R tetramer). These clones exhibited much greater binding ability to the WT tetramer than to the 3R tetramer (Fig. 2B). These results together indicate that these were WT-specific CTL clones. We further analyzed the abilities of these clones to recognize HIV-1-infected cells. These CTL clones effectively killed WT-virus-infected cells, but not the 3R virus-infected cells (Fig. 2C), and showed the ability to suppress the replication of WT virus, but not to suppress that of the 3R virus (Fig. 2D). WT-specific CD8$^+$ T cell

TABLE 1 Sequence at Gag30 in 12 HLA-A*24:02$^+$ individuals with an early-phase HIV-1 infection

Patient ID[a]	Sampling date (mo/day/yr)	Gag30 sequence	Method
KI-091	12/13/2000	3R	Cloning
	12/27/2000	3R	Direct
	1/7/2002	3R	Direct
	7/9/2003	3R	Cloning
	9/29/2004	3R	Cloning
	8/4/2005	3R	Cloning
KI-092	1/22/2001	WT	Cloning
	11/21/2001	WT	Cloning
	12/10/2002	WT/3R	Cloning
	8/14/2003	3R	Cloning
KI-102	5/11/2001	WT	Direct
	7/5/2004	WT	Direct
	3/28/2005	WT	Direct
KI-126	7/19/2001	3R	Direct
	1/18/2002	3R	Direct
	11/15/2004	3R	Direct
	9/12/2005	3R	Direct
KI-134	10/25/2001	3R	Direct
	6/30/2004	3R	Direct
KI-136	10/29/2001	3R	Direct
	7/10/2003	3R	Direct
KI-140	11/08/2001	3R	Direct
KI-151	5/2/2001	3R	Direct
	8/28/2003	3R	Direct
KI-154	4/12/2002	3R	Direct
KI-158	6/14/2002	WT	Direct
	10/11/2002	WT	Direct
	8/25/2003	WT	Direct
	11/14/2003	WT/3R	Direct
	2/23/2004	3R/WT	Direct
	11/1/2004	3R	Direct
	4/4/2005	3R	Direct
KI-161	2/15/2002	WT	Direct
	9/12/2002	WT	Direct
	3/4/2003	WT	Direct
	9/30/2003	WT/3R	Direct
	5/6/2004	3R	Direct
	1/27/2005	3R	Direct
	6/16/2005	3R	Cloning
KI-163	8/30/2002	3R	Direct
	9/27/2004	3R	Direct

[a] ID, identifier.

TABLE 2 Responses of CD8$^+$ T cells from individuals infected with WT virus to WT or 3R peptide

Patient ID	Virus sequence [mo/day/yr (type)]		PBMC sampling date (mo/day/yr)	PBMCs cultured with:	% IFN-γ-producing cells specific for each peptide among CD8$^+$ T cells[a]		
	Early phase	Chronic phase			Without	WT	3R
KI-092	1/22/2001 (WT)	8/14/2003 (3R)	5/24/2001	WT	0.2	**34.4**	**13.7**
				3R	0.1	**12.1**	**16.8**
			2/3/2003	WT	0.2	**5.8**	**4.2**
				3R	0.6	0.3	0.3
KI-102	5/11/2001 (WT)	3/28/2005 (WT)	7/11/2001	WT	1.0	0.6	1.1
				3R	1.1	1.5	2.0
			7/5/2004	WT	0.2	**28.7**	**9.3**
				3R	0.6	0.7	0.6
KI-158	6/14/2002 (WT)	4/4/2005 (3R)	10/11/2002	WT	1.4	**19.3**	**24.6**
				3R	0.1	0.5	0.4
			4/4/2005	WT	0.3	**23.3**	**23.8**
				3R	0.4	**18.8**	**20.9**
KI-161	2/15/2002 (WT)	6/16/2005 (3R)	7/26/2002	WT	0.0	**74.5**	**8.0**
				3R	0.2	**55.1**	**41.8**
			5/6/2004	WT	0.1	**21.4**	**4.9**
				3R	0.2	**42.5**	**43.9**

[a] Without, without peptide. Boldface, positive IFN-γ-producing response.

clones established from early-phase PBMCs of KI-161 also showed a similar ability to kill WT virus-infected and 3R virus-infected cells (Fig. 3). In these individuals, the 3R mutant virus became dominant 1 to 2 years after the early phase (Table 1). Taken together, these findings suggest that the 3R mutation was selected by WT-specific CTLs.

The 3R virus was not detected by approximately 4 years postinfection in KI-102, who had been infected with the WT virus (Table 1). This individual did not have Gag28-specific CD8$^+$ T cells at an early phase of the HIV-1 infection (Fig. 1). Interestingly, only WT-specific CD8$^+$ T cells were induced from PBMCs of this patient 2.5 year later. Thus, WT-specific CD8$^+$ T cells did not select 3R within about 2 years after the WT-specific CD8$^+$ T cells had been elicited in the patient.

Cross-reactive CD8$^+$ T cells in individuals who had been infected with WT virus and had selected 3R virus. We investigated whether the 3R-specific or cross-reactive CD8$^+$ T cells were elicited after the 3R mutant had been selected in individuals who had been infected with the WT virus. In KI-158, no Gag28-specific CD8$^+$ T cells were induced from early-phase PBMCs stimulated with the 3R peptide, whereas cross-reactive CD8$^+$ T cells were induced from chronic-phase PBMCs stimulated with WT peptide or 3R peptide (Fig. 1). In KI-161, Gag28-specific CD8$^+$ T cells recognizing WT peptide more than the 3R peptide were induced from early-phase PBMCs stimulated with WT peptide or the 3R peptide, whereas cross-reactive CD8$^+$ T cells were predominantly induced from chronic-phase PBMCs stimulated with the 3R peptide (Fig. 1). These results indicate that cross-reactive CD8$^+$ T cells became dominant in the Gag28-specific CD8$^+$ T cell population after the emergence of the 3R virus in these 2 individuals.

To investigate the function of these cross-reactive CD8$^+$ T cells, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-161 by stimulating them with the 3R peptide. The CTL clones evenly recognized both WT and the 3R peptides (Fig. 3A) and showed the same binding affinity to the 2 tetramers (Fig. 3B). These results suggest that the two peptides had the same binding affinity for HLA-A*24:02. They effectively killed WT-virus-infected cells and weakly killed the 3R virus-infected cells (Fig. 3C), whereas they suppressed the replication of the WT virus but not that of the 3R virus (Fig. 3D). These results indicate that these cross-reactive CTLs contributed to the selection of the 3R virus. In addition, the results strongly suggest weak presentation of the 3R peptide in the cells infected with 3R virus, because the cross-reactive CTL clones had TCR with the same binding affinity for both HLA-A*24:02-WT peptide and HLA-A*24:02-3R peptide complexes and because WT and 3R peptides had the same binding affinity for HLA-A*24:02. This reduced presentation may have affected the control of 3R virus by the cross-reactive CTLs.

Gag28-specific T cell repertoire in an individual infected with WT virus. The results in Fig. 1 suggest that both WT-specific and cross-reactive CD8$^+$ T cells were elicited at an early phase of HIV-1 infection in 3 individuals infected with WT virus (KI-092, KI-158, and KI-161). To characterize Gag28-specific CTLs elicited at that time, we established Gag28-specific CTL clones from PBMCs at an early phase in KI-161 by stimulating them with the WT peptide. We found 3 types of CTL clones among the 8 clones analyzed. As shown in Fig. 3A, 3 clones effectively recognized the WT peptide but not the 3R peptide (WT specific), 3 clones recognized the WT peptide more than the 3R peptide (WT dominant), and 2 clones evenly recognized both peptides (cross-reactive). We next investigated the binding affinity of TCRs on these clones to WT tetramer and 3R tetramer. The results confirmed the specificity of these 3 types of CTL clones (Fig. 3B). These results together indicate that KI-161 had a multiple T cell repertoire for the Gag28 epitope before the 3R virus had been selected.

Next, we analyzed the abilities of these T cell clones to kill HIV-1-infected cells. The WT-specific and WT-dominant CTL clones effectively killed the target cells infected with WT virus but failed to kill those infected with the 3R virus (Fig. 3C, left and right graphs under early phase). On the other hand, cross-reactive CTL clones weakly killed the target cells infected with the 3R virus and effectively killed those infected with the WT virus (Fig. 3C, middle