Therefore, we generated 2 truncated peptides (AL9: ATDIQTKEL and TL8: TDIQTKEL). Bulk $CD8^+$ T cells induced by Pol919-929 peptide recognized all 3 peptides (Fig. 1A) and showed higher sensitivity to TL8 than to the other 2 peptides (Fig. 1F), indicating that Pol921-928 (TL8) was the optimal epitope. Similarly we speculated TL8 to be optimal epitope for Pol921-931 (TQ11: TDIQTKELQKQ), because no hydrophilic residue is found in the C-terminus of HLA-class I-restricted epitopes. Although bulk $CD8^+$ T cells induced by Pol921-931 peptide recognized both TQ11 and TL8 peptides (Fig. 1A), they showed higher sensitivity to TL8 than to TQ11 (Fig. 1F). These findings indicate that Pol919-929 and Pol921-931 11-mer peptides included the same epitope, Pol921-928(TL8).

Thus, we identified 4 HLA-B*4002-restricted optimal peptides. Interestingly, these 4 Pol epitopes were all derived from integrase.

*3.3. Generation and antigen sensitivity of HLA-B*4002-restricted Pol-specific CTL clones*

To analyze the $CD8^+$ T cells specific for these 4 integrase epitopes, IT10 (Pol799-808), EL11 (Pol807-817), GI8 (Pol912-919), and TL8 (Pol921-928), we established the specific $CD8^+$ T cell clones and analyzed them for their antigen sensitivity by using the ICC assays. The result was shown in Fig. 2. The T cell clones and their EC_{50} values were as follows: Pol799-808-specific T cells (27.7), Pol807-817-specific T cells (191.7), Pol912-919-specific T cells (443.1), and Pol921-928-specific T cells (7.6). These results indicate that Pol799-808-specific and Pol921-928-specific $CD8^+$ T cell clones had higher antigen sensitivity than Pol807-817-specific and Pol912-919-specific ones.

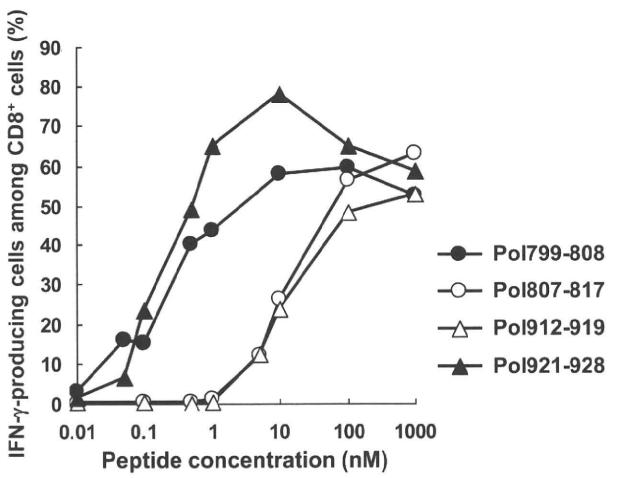

Fig. 2. Antigen Sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells. Antigen sensitivity of 4 HIV-1 integrase-specific $CD8^+$ T cells was examined by using the ICC assay. The responsiveness of these CTL clones was examined for C1R-B*4002 cells pre-pulsed with each truncated peptide at concentrations from 0.01 to 1000 nM.

3.4. Recognition of HIV-1-infected cells by specific T cells

To clarify whether Pol799-808, Pol807-817, Pol912-919, and Pol921-928 were naturally occurring peptides and whether CTLs specific for these epitopes had the ability to recognize HIV-1-infected cells, we investigated the response of these peptide-specific $CD8^+$ T cell clones toward HIV-1 (NL-432)-infected .221-CD4 cell lines expressing HLA-B*4002. NL-432 includes wild-type sequences of these 4 epitopes. .221-CD4 cell lines and those expressing HLA-B*4002 were infected with NL-432, and then cultured for 4 days. The responses of the T cell clones toward these infected cells were measured by using the ICC assay. The percentage of the HIV-1-infected cells was determined by staining intracellular HIV-1 p24 (Fig. 3A). The Pol799-808-specific, Pol807-817-specific, Pol912-919-specific, and Pol921-928-specific CTL clones responded to .221-CD4-B*4002 cells infected with HIV-1 but not to uninfected .221-CD4-B*4002 cells or to HLA-B*4002-negative .221-CD4 cells infected with HIV-1. These results indicate that Pol799-808, Pol807-817, and Pol921-928 peptides were naturally processed and presented by HLA-B*4002 and that the T cells specific for these epitopes could recognize HIV-1-infected cells (Fig. 3B). On the other hand, the responses of Pol807-817-specific and Pol912-919-specific CTL clones was much weaker than those of the other CTL clones (Fig. 3B), indicating that the former CTLs only weakly recognized HIV-1-infected cells.

4. Discussion

There is only 1 amino acid substitution, at residue 97, on the peptide binding floor between HLA-B*4001 and HLA-B*4002. A previous study on the peptide motif of HLA-B*4001 showed that HLA-B*4001-binding peptide anchors are Glu at P2 (2E) and Leu at the C-terminus [20]. Indeed, 7 of 8 reported HLA-B*4001-restricted HIV-1-specific T cell epitopes have 2E and Leu at their C-terminus [11–13]. Although no HLA-B*4002-binding peptide motif had not yet been identified, we speculated that this motif would be similar to the HLA-B*4001-binding one. Indeed, all 7 HLA-B*4002-restricted epitopes previously reported have 2E (Table 1). However, 2 of the 4 epitopes identified in the present study did not have the 2E anchor. In addition, only 5 of 11 HLA-B*4002-restricted epitopes had Leu at their C-terminus. These findings suggest that the substitution from Ser to Arg at residue 97 may partially affect the structure of the F and B pockets. Pol807-817 (ETGQETAYFLL) does not have the 2E anchor. QL8 (QETAYFLL) is speculated to be an HLA-B*4002-restricted epitope because this peptide has 2E. However, the antigen sensitivity of the T cells specific for QL8 is much weaker than that for EL11. This result excludes the possibility that QL8 is the epitope peptide. Thr at position 2 of Pol807-817 may bind to the residues facing the B-pocket by hydrogen-bonding. Nine of the 11 HLA-B*4002-restricted epitopes have 2E, suggesting that the 2E is still anchor residue for HLA-B*4002.

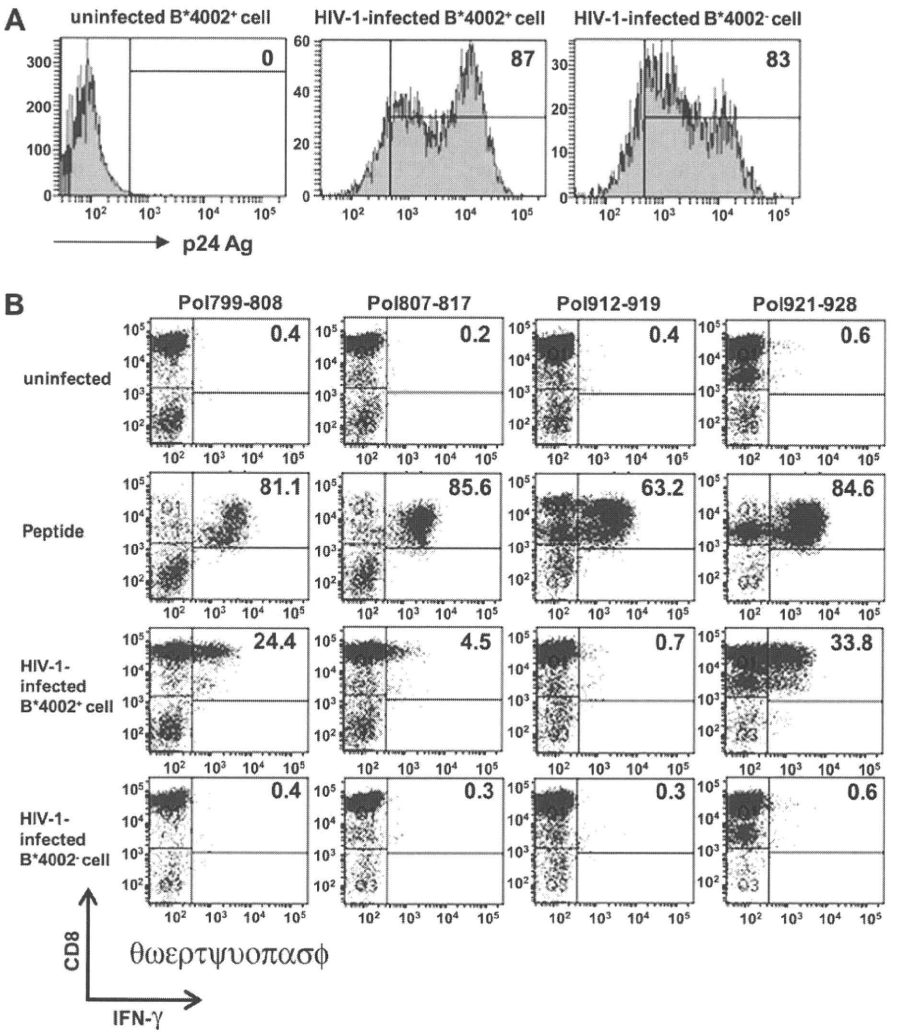

Fig. 3. Ability of 4 HIV-1 integrase-specific $CD8^+$ T cells to recognize HIV-1-infected cells. A. The .221-CD4 and $B*4002^+$.221-CD4 cell lines were infected with HIV-1 (NL-432) and cultured for 4 days. The frequency of HIV-1-infected cells was detected by using staining of intracellular p24 with anti-p24 mAb. The percentage of HIV-1-infected cells is shown in each figure. B. Recognition of HIV-1-infected cells by the Pol799-808-, Pol807-817-, Pol912-919- or Pol921-928-specific $CD8^+$ T cell clones. The activities of these peptide-specific $CD8^+$ T cell clones to recognize $B*4002^+$.221-CD4 cell lines infected with HIV-1 or those pre-pulsed with the corresponding peptide (1000 nM) were measured by use of the ICC assay. The percentages of IFN-γ-producing cells among $CD8^+$ T cells are shown in each figure.

Although the 7 HLA-B*4002-restricted epitopes previously reported do not include Pol-derived ones, we identified novel 4 HLA-B*4002-retricted Pol-specific T cell epitopes in the present study. Interestingly, all of these Pol epitopes were derived from integrase. Though 29 integrase epitopes were reported as 20 different HLA class I-restricted epitopes (Los Alamos HIV Molecular Immunology Data), integrase epitopes were not found among HLA-B*4001-restricted Pol epitopes. Regarding the integrase epitopes, HLA-B*4201 and HLA-B*1503 present 3 different epitopes, whereas the other 18 alleles present 1 or 2 epitopes. Thus, HLA-B*4002 is so far the only HLA-class I allele that can present more than 3 integrase epitopes.

Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, whereas Pol807-817-specific and Pol912-919-specific ones weakly recognized these cells. Antigen sensitivity of the former T cells was much higher than that of the latter ones. Thus, the ability to recognize HIV-1-infected cells was associated with the antigen sensitivity. However, it is difficult to clarify why the 2 T cells weakly recognize HIV-1-infected cells because we did not measure the bindings of these epitope peptides to HLA-B*4002 molecules and of the specific tetramers to the specific T cells. We can suggest 2 possibilities from the data shown in Fig. 2 and Fig. 3: 1) The former T cells may have higher affinity TCR and/or 2) these former epitope peptides are more highly presented than the latter by HLA-B*4002 in HIV-1-infected cells. Since Pol799-808-specific and Pol921-928-specific T cells strongly recognized HIV-1-infected cells, we proposed that they would effectively recognize and kill HIV-1-infected cells *in vivo*.

HLA-B*4001 and HLA-B*4002 are found in 10.8% and 16.6% of the Japanese population, respectively. Since both

Table 1
A list of HLA-B*4002-restricted epitopes identified previously and in this study.

Sequence	Protein	Reference
GELDRWEKI	Gag (p17)	*15
KETINEEAA	Gag (p24)	*15
AEWDRVHPV	Gag (p24)	*15
AEAMSQVTNS	Gag (p2p7p1p6)	*16
TERQANFL	Gag (p2p7p1p6)	*15
REPHNEWTL	Vpr	*14
KEKGGLEGL	Nef	*15
IEAEVIPAET	Pol (Integrase)	This study (Pol799-808)
ETGQETAYFLL	Pol (Integrase)	This study (Pol807-817)
GERIVDII	Pol (Integrase)	This study (Pol912-919)
TDIQTKEL	Pol (Integrase)	This study (Pol921-928)

HLA-class I alleles are detected in approximately 25% of Japanese individuals, T cell epitopes presented by these alleles are useful for studies on HIV-1 immunopathogenesis and the development of AIDS vaccines.

Acknowledgments

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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 $CD8^+$

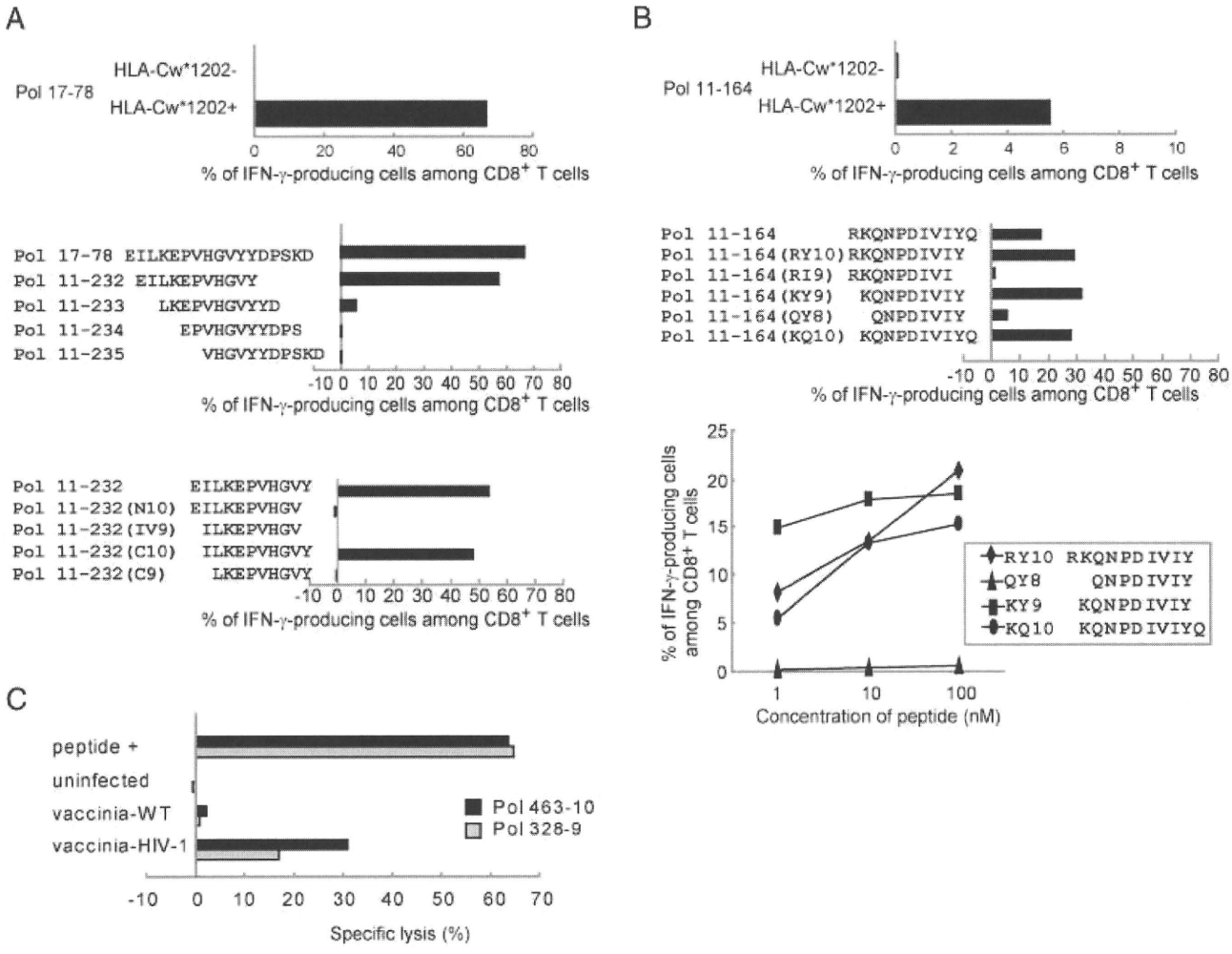

Figure 1. Identification of two HIV-1 Pol-specific epitopes using overlapping peptides. Candidates of HLA-Cw*1202-restricted HIV-1 CTL epitopes were identified using overlapping 17-mer or 11-mer HIV-1 peptides. PBMC from HLA-Cw*1202$^+$ HIV-1-seropositive individuals (KI-069 and KI-108) were stimulated 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

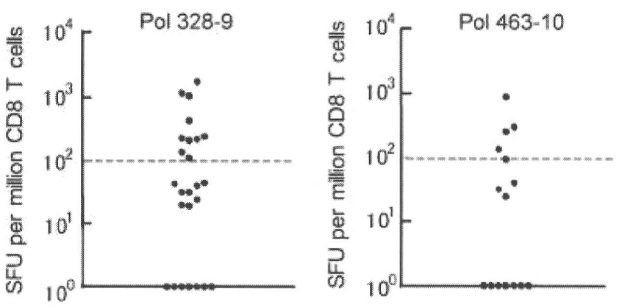

Figure 2. Frequency of HLA-Cw*1202-restricted Pol epitopes-specific CD8$^+$ T cells in chronically HIV-1-infected individuals. The frequencies of Pol 328-9-specific and Pol 463-10-specific CD8$^+$ T lymphocytes in chronically HIV-1-infected HLA-Cw*1202$^+$ individuals were measured using IFN-γ ELISPOT. 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.

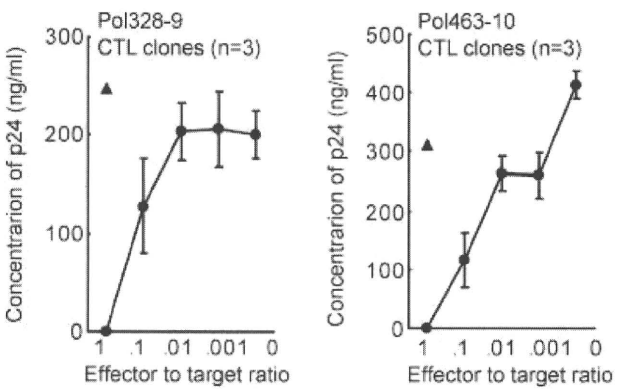

Figure 3. Strong abilities of two HIV-1 Pol-specific CTL to suppress HIV-1 replication. CD4$^+$ T cells from an HLA-Cw*1202$^+$ donor were infected with NL-432, and then co-cultured with the Pol-specific CTL clones ($n = 3$) at E:T ratios of 1:1, 0.1:1, 0.01:1, and 0.001:1 (circles). As a negative control, 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.

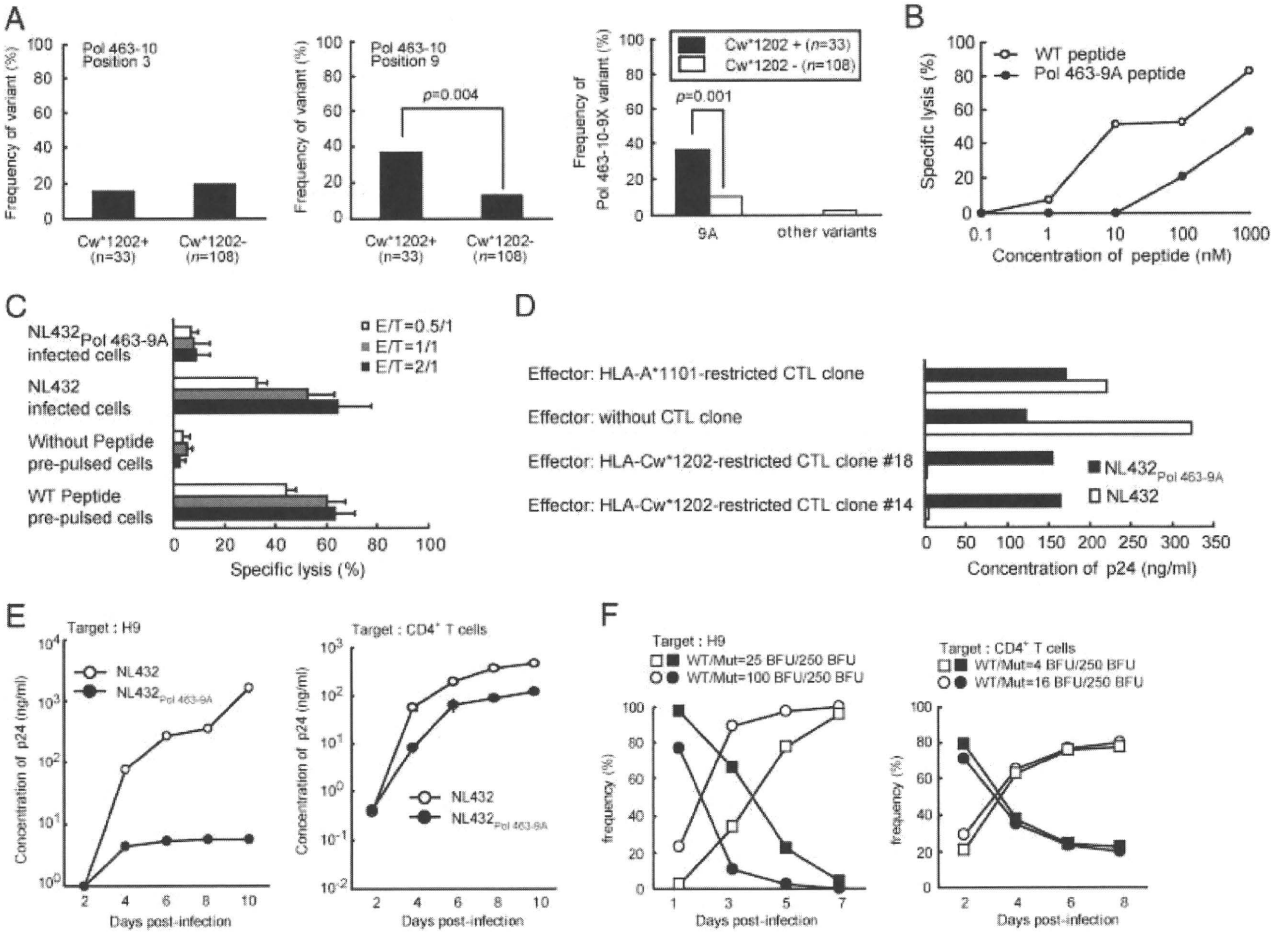

Figure 4. Characterization of escape Pol 463-9A escape mutation. (A) Frequency of mutations in Pol 463-10 epitope among chronically HIV-1-infected HLA-Cw*1202$^+$ and HLA-Cw*1202$^-$ HIV-1-infected individuals. The sequence of Pol 463-10 epitope was analysed in a single sample from both HLA-Cw*1202-positive 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$_{-\text{Pol 463-10-9A}}$ mutant clones

The NL-432-$_{\text{Pol 463-10-9A}}$ 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-$_{\text{Pol 463-10-9A}}$ 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 phosphatase 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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