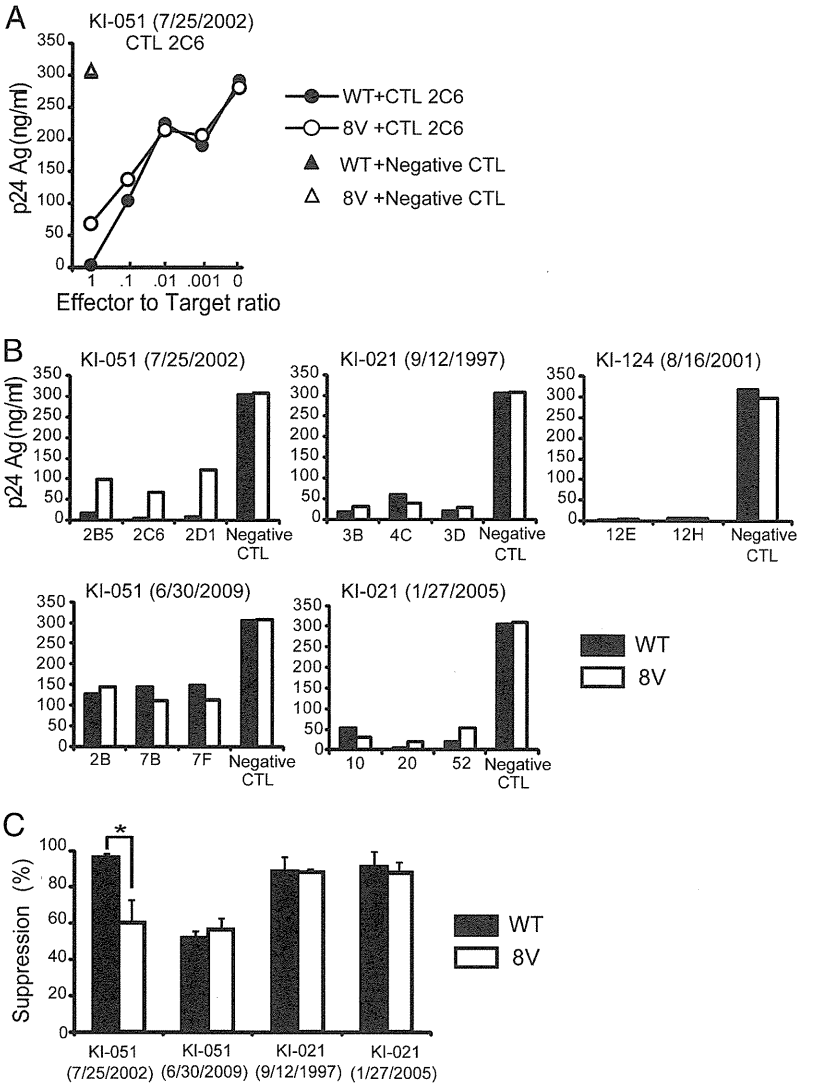

FIGURE 1. Abilities of TI8-specific CTL clones to suppress the replication of the 8Vmutant virus. (**A**) Ability of TI8-specific CTL clone 2C6, which was established from KI-051 in July 2002 when WT virus was still detectable, to suppress the replication of NL432 virus and the 8V virus. $CD4^+$ T cells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

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

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

TCR affinity of TI8-specific CTL clones

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

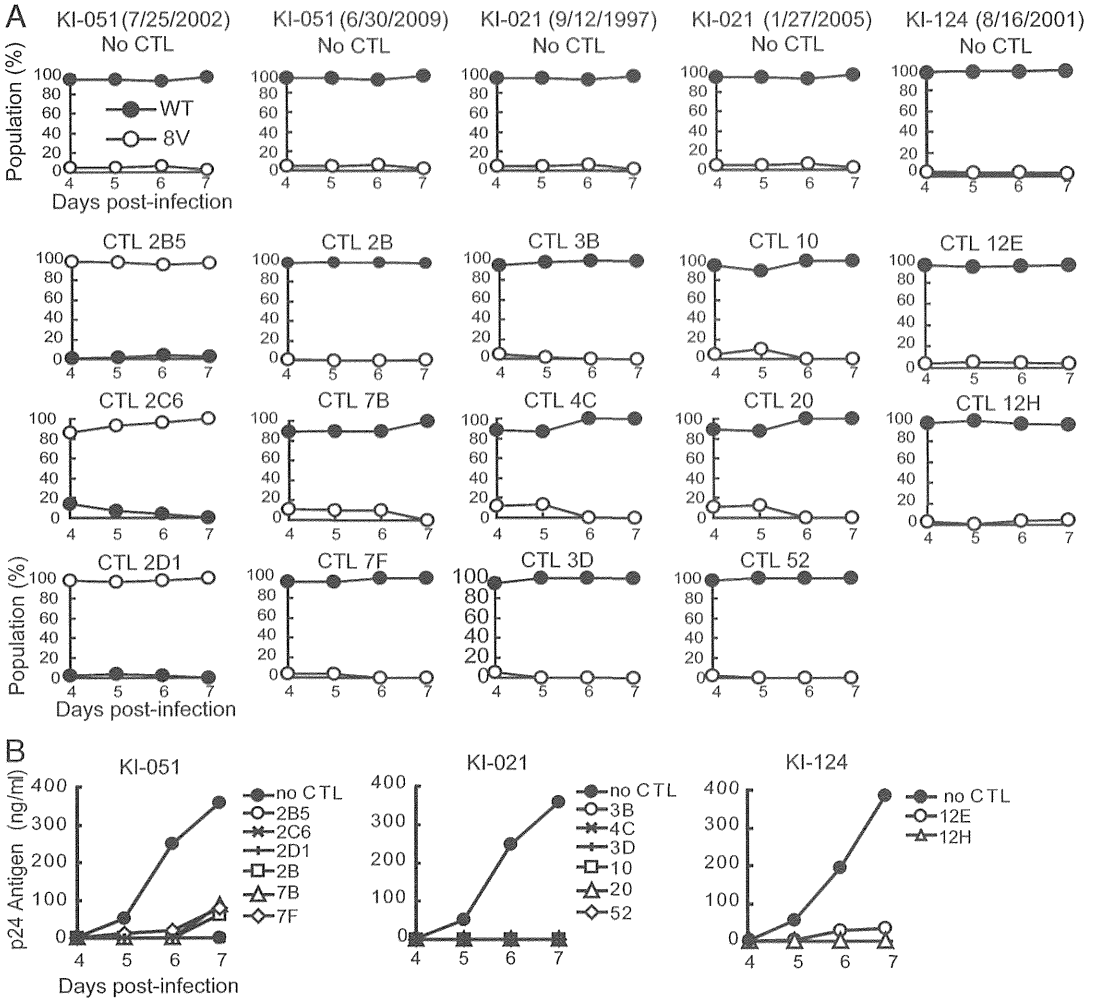

FIGURE 2. In vitro selection of the 8V mutant virus by TI8-specific CTL clones. (**A**) Ability of TI8-specific CTL clones to select the 8V mutant. T1 cells expressing HLA-B*51 and CD4 molecules were infected with NL-432 or NL432-Pol283-8V virus together at a ratio of 9:1. The infected cells were cocultured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

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

Reconstruction of the TCR function in TCR-deficient cells

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

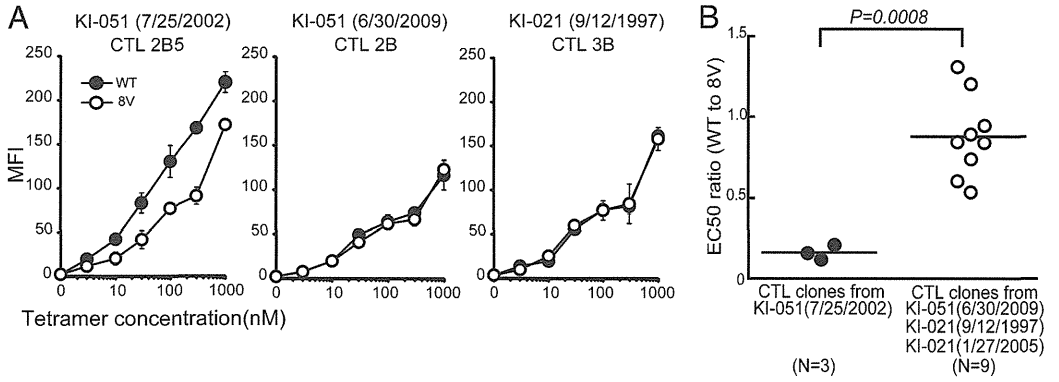

FIGURE 3. Difference in TCR affinity for HLA-B*51:01 with TV8 peptide complex among TI8-specific CTL clones. (**A**) The ability of the TCRs on TI8-specific CTL clones to bind WT or 8V tetramer was measured in terms of the mean fluorescence intensity (MFI) of each CTL clone stained with the tetramers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

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

Discussion

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

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

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

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

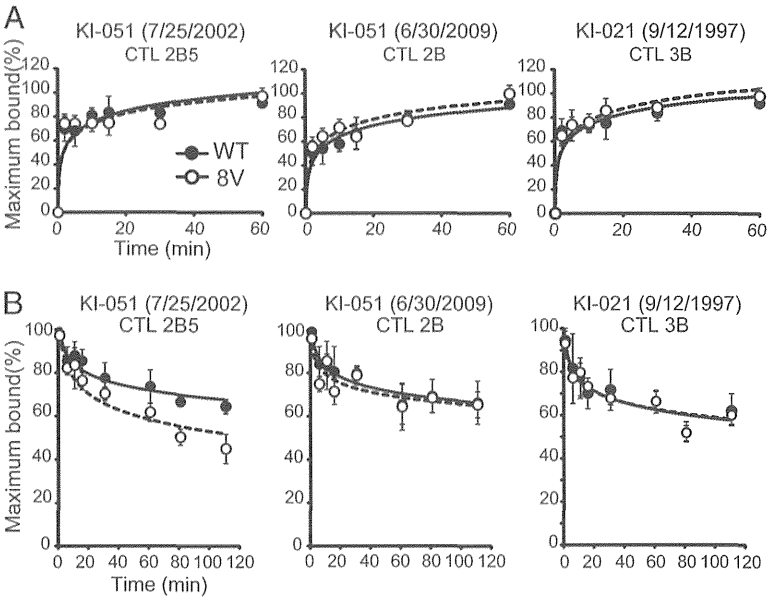

FIGURE 4. Kinetics of interaction between HLA-B*51:01–peptide complex and TI8-specific CTL clones. (**A**) Kinetics of tetramer association with TCR of TI8-specific CTL clones. The CTL clones were incubated with WT or 8V tetramer at a concentration of 1000 nM and taken periodically (2, 5, 10, 15, 30,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

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

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

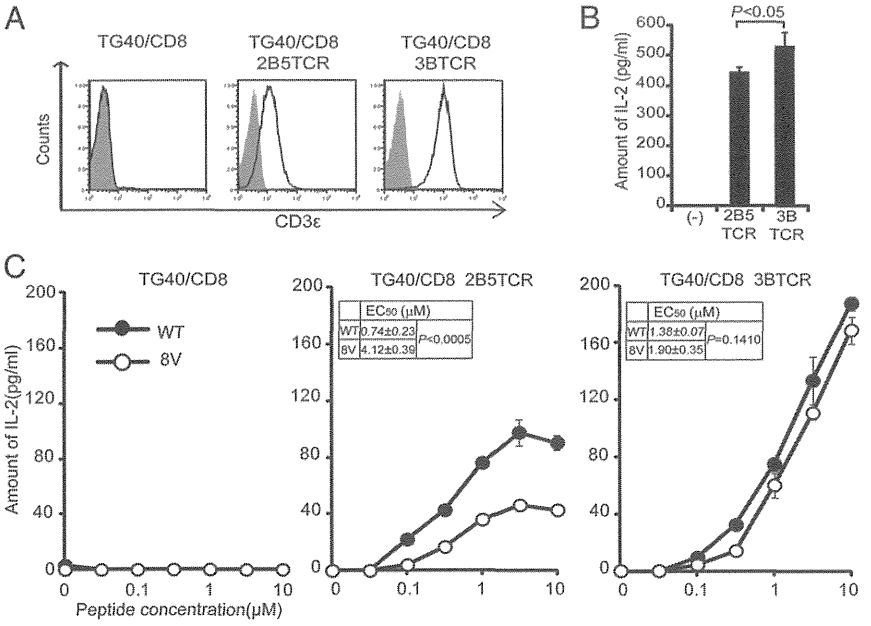

FIGURE 5. Reconstruction and recognition of TI8-specific TCRs on TCR-transfected TG40/CD8 cells. (**A**) CD3 expression in TG40/CD8 cells transfected with 2B5 TCR or 3B TCR. The TCR-transfected cells were stained with anti-CD3ε mAb (open histogram) or with an isotype control (shaded histogram), and then,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

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

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

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

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

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Disclosures

The authors have no financial conflicts of interest.

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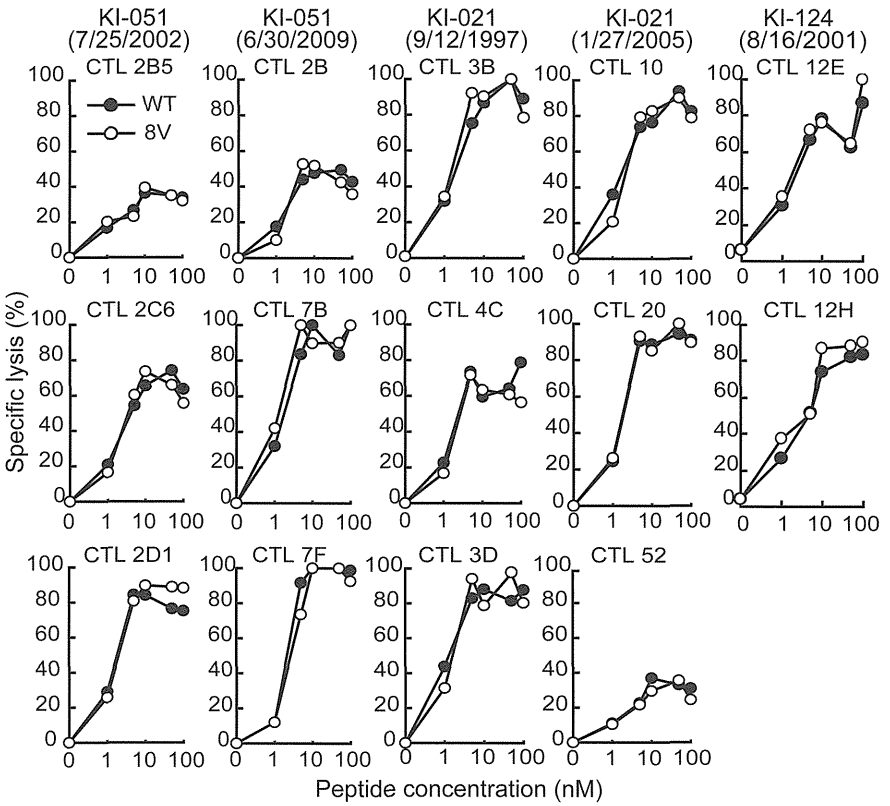
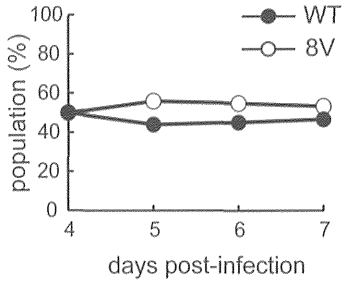


Supplementary Figure 1:

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

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



Supplementary Figure 2:

Replication capacity of WT and the 8V mutant viruses.

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

ELSEVIER

Analysis of the Hepatic Functional Reserve, Portal Hypertension, and Prognosis of Patients With Human Immunodeficiency Virus/Hepatitis C Virus Coinfection Through Contaminated Blood Products in Japan

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ABSTRACT

Background. As the survival of human immunodeficiency virus (HIV)-infected individuals has improved due to the widespread use of antiretroviral therapy, the mortality rate due to hepatitis C virus (HCV)-related liver disease has increased in HIV/HCV-coinfected patients.

Aim. The aims of this study were to establish the appropriate therapeutic strategy for HIV/HCV-coinfected patients by evaluating the liver function, including the hepatic functional reserve and portal hypertension, and to investigate the prognosis of HIV/HCV-coinfected patients in Japan.

Patients and Methods. In addition to regular liver function tests, the hepatic functional reserve of 41 patients with HIV/HCV coinfection was evaluated using the indocyanine green retention rate and liver galactosyl serum albumin-scintigraphy. The data for 146 patients with HIV/HCV coinfection through blood products were extracted from 4 major HIV centers in Japan. In addition to liver function tests, the platelet counts (PLT) were evaluated as a marker of portal hypertension.

Results. In spite of the relatively preserved general liver function test results, approximately 40% of the HIV/HCV-coinfected patients had an impaired hepatic functional reserve. In addition, while the albumin and bilirubin levels were normal, the PLT was <150,000/μL in 17 patients. Compared with HCV mono-infected patients with a PLT <150,000/μL, the survival of HIV/HCV-coinfected patients was shorter (HCV, 5 years, 97%; 10 years, 86% and HIV/HCV, 5 years, 87%; 10 years, 73%; $P < .05$).

Conclusion. These results must be taken into account to establish an optimal therapeutic strategy, including the appropriate timing of liver transplantation in HIV/HCV-coinfected patients in Japan.

FROM 1970 until the early 1980s, blood products were imported to Japan, and contaminated blood products were unknowingly used to treat patients with hemophilia. It was later revealed that these patients were sometimes infected with both human immunodeficiency virus (HIV) and hepatitis C virus (HCV; HIV/HCV coinfection) [1].

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However, as the survival of HIV-infected people has improved due to the widespread use of antiretroviral therapy, the mortality due to HCV-related liver disease has increased in HIV/HCV-coinfected patients [2,3].

The main aims of this investigation were to investigate the status of portal hypertension and the prognosis in HIV/HCV-coinfected patients, and to establish an appropriate therapeutic strategy for HIV/HCV-coinfected patients, including the timing of liver transplantation, in Japan.

PATIENTS AND METHODS

Routine hematology and blood chemistry tests (general liver function), abdominal ultrasonography, and contrast-enhanced computed tomography (CT) were performed for 30 patients with HIV/HCV coinfection at Nagasaki University Hospital. To investigate the hepatic functional reserve, liver GSA-scintigraphy and the indocyamine green retention test at 15 minutes were performed. In addition, upper gastrointestinal tract endoscopy to diagnose gastroesophageal varices was performed.

The data of the 146 patients who had acquired HIV/HCV coinfection through blood products were extracted from 4 major HIV centers in Japan, including the AIDS Clinical Center, Osaka National Hospital, Yokohama Municipal Hospital, and Kyushu Medical Center. In addition to liver function tests, platelet counts (PLT) were evaluated as a marker of portal hypertension. As a control, HCV mono-infected patients from Nagasaki Medical Center were used for comparison.

RESULTS

In spite of the relatively well-maintained general liver functions, approximately 40% of the HIV/HCV-coinfected patients had an impaired hepatic functional reserve (Table 1). In addition, in spite of maintained albumin and bilirubin levels, the PLT was <150,000/μL in 17 coinfected patients, indicating the presence of ongoing portal hypertension.

Even with Child-Pugh A liver function, the HIV/HCV-coinfected patients showed a worse prognosis than the HCV mono-infected patients. The prognosis was especially poor in those with lower PLT than in the patients with a normal PLT (Table 2). When compared with HCV mono-infected patients with a PLT <150,000 μL, the survival of HIV/HCV-coinfected patients was much shorter (HCV, 5 years, 97%; 10 years, 86% and HIV/HCV, 5 years, 87%; 10 years, 73%; $P < .05$).

Table 1. Patient Characteristics

Child-Pugh A/B/C	38 (93%)/1 (2%)/2 (5%)
ICG R15 (%)	
<10/10–20/20–30/30<	24 (59%)/8 (20%)/3 (7%)/6 (14%)
GSA schincigram LHL15	
>0.9/0.8–0.9/0.8>	28 (69%)/6 (15%)/7 (16%)
Liver configuration on CT	
Normal/CH/LC	10 (24%)/17 (42%)/14 (34%)
Splenomegaly	
Yes/no	26 (63%)/15 (37%)
Esophageal varices	
Yes/no	13 (32%)/28 (68%)

CH, chromic hepatitics; LC, liver cirrhosis.

Table 2. Patient Survival after Diagnosis

	5Y OS	10Y OS	
HCV mono-infection (Child-Pugh A)	97%	86%	
HIV/HCV coinfection (Child-Pugh A)			
PLT > 150,000	94%	85%	
PLT < 150,000	87%	73%	$P < .05$ vs HCV mono-infection

5Y OS, 5 year patient survival; 10Y OS, 10 year patient survival.

DISCUSSION

In HIV/HCV-coinfected patients, liver failure due to HCV hepatitis was previously reported to be enhanced by antiretroviral therapy ART-related hepatotoxicity, especially manifesting as noncirrhotic portal hypertension (NCPH) [4,5]. One of the ART drugs, Didanosin (DDI), has been suspected to be related to the serious morbidity observed in coinfected patients [6]. Thus, not only in patients with deteriorated liver function, such as in Child-Pugh B or C cases, but also even in Class A cases, the patients' liver function can easily deteriorate abruptly [7]. The natural course of pure NCPH is unknown because it can be modulated by HCV or other causes, and has only been reported as case series. An important study of "NCPH in HIV Mono-Infected Patients Without HCV" was published in 2012 [8]. All 5 patients had portal hypertensive symptoms, such as ascites or variceal bleeding, after receiving antiretroviral therapy.

Therefore, all HIV/HCV-coinfected patients should be carefully followed up so as not to miss an opportunity for liver transplantation (LT) [9]. The prognosis for HIV/HCV-coinfected patients was reported to be worse than that for HCV mono-infected patients [10]. In the present study, coinfected patients with a PTL <150,000 μL had an especially poor prognosis, with a shorter survival than mono-infected patients. Our results should be taken into account to establish a therapeutic strategy, while also considering the appropriate timing of LT in HIV/HCV-coinfected patients.

In 2013, based on the evidence of rapid progression of the liver cirrhosis and portal hypertension in patients with HIV/HCV coinfection, a rank-up system for the waiting list for deceased donor LT was set up in Japan. Even HIV/HCV-coinfected liver cirrhotic patients with Child-Pugh class A can be listed for LT as "point 3" because of the NCPH (noncirrhotic portal hypertension) nature. Coinfected patients with Child-Pugh class B and C disease can be listed as "point 6" and "point 8," respectively, based on the data collected by the HIV/acquired immunodeficiency syndrome (AIDS) project team of the Ministry of Health, Labor, and Welfare of Japan, and the published literature [11]. This primarily covers victims who received contaminated blood products for hemophilia.

Future perspectives on LT for HIV/HCV coinfection include the following: new anti-HCV agents should be

developed to improve the control against HCV; new ART drugs, such as Raltegravir, should facilitate post-transplantation immunosuppressive therapy; noninvasive tests for portal hypertension, such as the fibroscan, should be performed for hemophilic patients; and the development of guidelines for the management hemophilia in the peri-operative period should facilitate better outcomes.

In conclusion, the present results should be taken into account to establish an optimal therapeutic strategy, including the appropriate timing of LT in HIV/HCV-coinfected patients.

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Short communication

Raltegravir and elvitegravir-resistance mutation E92Q affects HLA-B*40:02-restricted HIV-1-specific CTL recognition

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Abstract

Interplay between drug-resistance mutations in CTL epitopes and HIV-1-specific CTLs may influence the control of HIV-1 viremia. However, the effect of integrase inhibitor (INI)-resistance mutations on the CTL recognition has not been reported. We here investigated the effect of a raltegravir and elvitegravir-resistance mutation (E92Q) on HLA-B*40:02-restricted Int92-102 (EL11: ETGQETAYFLL)-specific CTLs. EL11-specific CTLs recognized E92Q peptide-pulsed and E92Q mutant virus-infected cells less effectively than EL11 peptide-pulsed and wild-type virus-infected cells, respectively. *Ex vivo* ELISpot analysis showed no induction of E92Q-specific T cells in chronically HIV-1-infected individuals. Thus, we demonstrated that EL11-specific CTL recognition was affected by the INI-resistance mutation.

Keywords: Integrase inhibitor; CTL; HIV

1. Introduction

Treatment with effective antiretroviral therapy (ART) results in a decline in viral load and increase in $CD4^+$ T cell count in the majority of HIV-1-infected individuals [1]; whereas the presence of drug-resistance mutations can contribute to increased risk of virologic failure [2]. Many such mutations occur in regions of HIV-1 Pol, which encompasses a number of previously identified cytotoxic T lymphocyte (CTL) epitopes [3,4]. If drug-resistance mutations enhance the immunogenic antigenicity of the CTL epitope, drug treatment might drive the CTL response towards HIV control; otherwise, they may cause an immunologically uncontrollable HIV infection if they affect the CTL responses. On the other hand, HIV-1-specific CTLs, especially those against HIV-1 Gag and Pol, play a major role in controlling replication of HIV-1 [5,6]. However, HIV-1 escapes from the host immune system by various mechanisms [7]. The appearance of CTL escape mutations is one of them [5,6]. If such CTL escape mutations occur in the drug-target proteins, including reverse transcriptase, protease, and integrase, they may alter the drug sensitivity or modify the patterns of drug-resistance mutations [8,9].

Several studies have demonstrated CTL responses to HIV-1 drug-resistance mutations. Some protease inhibitor (PI)-resistance mutations (G48V, M46I, I47A, and I50V) abolish CTL recognition [10]; whereas other PI-resistance mutations (L63P and L10I) enhance it [4]. HIV-1 viruses with the nucleoside/nucleotide reverse transcriptase inhibitor (NRTI)-resistance M184V mutation show reduced viral replication capacity compared to the wild-type virus; whereas individuals having an M184V-specific CTL response have a lower viral load than those without this CTL response [11], suggesting that M184V-specific CTLs may suppress the replication of this mutant

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HIV-1. Thus, the M184V mutation may have the effect of controlling HIV-1. Most NRTI-resistance mutations (M41L, L74V, M184V, and T215Y/F) do not impair CTL recognition [3]. In contrast, T cells fail to recognize wild type but effectively recognize the non-nucleoside reverse-transcriptase inhibitors (NNRTIs)-resistance mutation K103N in some patients [12], indicating that some drug-resistance mutations have a positive effect on CTL recognition. Thus, drug-resistance mutations have various effects on CTL recognition. The combined effect of CTL escape mutations I135T/L/R and drug-resistance mutation E138K confers significant resistance towards NNRTIs, though separately they have only a mild resistance effect toward NNRTI [9], suggesting that CTL pressure sometimes generates more potent drug-resistance mutations.

Raltegravir (RAL), the first integrase inhibitor (INI), was approved in 2007, followed by elvitegravir (EVG) in 2012; although EVG shows extensive cross-resistance with RAL. Both RAL and EVG are important options for first-line therapy as well as for the treatment of highly ART-experienced patients. INIs can suppress virus replication in HIV-1 patients harboring viruses resistance to other ARTs and constitute a valuable option for salvage therapy. INIs have a relatively low genetic barrier to resistance; and a single mutation is able to confer resistance to INIs [13]. Despite the potency, tolerability, and durability of INIs, signature resistance mutations against RAL (N155H, Q148H/K/R, Y143C/H/R, E92Q, and a few others) were detected in 60% of patients who experienced virologic failure in clinical trials involving highly treatment-experienced patients [14]. Moreover, the most common EVG-resistance mutations that emerged in clinical trials were E92Q, Q148R/H/K, and N155H [15]. E92Q alone reduces susceptibility to EVG more than 20 fold and causes limited (<5 fold) cross resistance to RAL [16]. As these drugs are frequently used to treat HIV-1 patients, and resistance mutations are appearing in clinical isolates, analysis of the interaction between CTL and INI-resistance mutations is important in studies concerning the effect of drug-resistance mutations on immune-recognition. In the present study, we investigated the effect of the INI-resistance mutation E92Q on EL11-specific HLA-B*40:02-restricted CTL recognition in chronically HIV-1-infected Japanese individuals having HLA-B*40:02 (The frequency of this allele is 16.6% in Japan).

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

This study was approved by the National Center for Global Health and Medicine and the Kumamoto University Ethical Committee. Informed consent was obtained from all subjects according to the Declaration of Helsinki. Plasma and peripheral blood mononuclear cells (PBMCs) were separated from heparinized whole blood. The HLA type of the patients was determined by standard sequence-based genotyping. Five HLA-B*40:02^{+} chronically HIV-1-infected individuals were recruited for this study.

2.2. Synthetic peptides

INI-resistance peptide EL11–E92Q was synthesized by utilizing an automated multiple peptide synthesizer and purified by high-performance liquid chromatography (HPLC). The purity was examined by HPLC and mass spectrometry. Peptides with more than 90% purity were used in the present study.

2.3. Cells

C1R cells expressing HLA-B*40:02 (C1R–B*4002), 721.221 cells expressing CD4 (721.221-CD4), and 721.221-CD4 cells expressing HLA-B*40:02 (721.221-CD4-B*4002) were previously generated [17]. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 2.0 mg/ml hygromycin B.

2.4. Generation of CTL clones

Peptide-specific CTL clones were generated from established peptide-specific bulk CTLs by seeding 0.8 cells/well into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) together with 200 ml of cloning mixture (RPMI 1640 medium containing 10% FCS, 200 U/ml human recombinant interleukin-2, 5×10^5 irradiated allogeneic PBMCs from a healthy donor, and 1×10^5 irradiated C1R–B*40:02 cells pulsed with a 1 μM concentration of the appropriate HIV-1-derived peptides. Wells positive for growth after about 2 weeks were examined for CTL activity by performing the intracellular cytokine staining assay. All CTL clones were cultured in RPMI 1640 containing 10% FCS and 200 U/ml recombinant human interleukin-2. CTL clones were stimulated biweekly with irradiated target cells pulsed with the corresponding peptides.

2.5. HIV-1 clones

An HIV-1 mutant (NL-432-E92Q) was generated by introducing the EL11–E92Q mutation into NL-432, which is an infectious proviral clone of HIV-1. Site-directed mutagenesis (Invitrogen) based on overlap extension was used for the generation of this virus.

2.6. CTL assay for target cells pulsed with HIV-1 peptide

Cytotoxic activity was measured by the standard ^{51}Cr-release assay, as previously described [6]. Target cells (2×10^5) were incubated for 1 h with 100 μl of Ci $Na_2^{51}CrO_4$ in saline and then washed 3 times with RPMI 1640 medium containing 10% FCS. Labeled target cells (2×10^3/well) were added to 96-well round-bottomed microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After 1 h of incubation, effector cells were added; and then incubation was carried out for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the

counts per minute (cpm) 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. Relative specific lysis of peptide-pulsed target cells was defined as (specific lysis of exp − specific lysis of cells without peptide).

2.7. CTL assay for target cells infected with HIV-1

721.221-CD4-B*40:02 cells were exposed to NL-432 or NL-432-E92Q viruses, and 721.221-CD4 cells were exposed to NL-432, for several days. The cells were used as target cells for CTL assays once approximately 40–60% of the cells had been infected, which were confirmed by intracellular staining for HIV-1 p24 antigen. Infected cells were labeled with ^{51}Cr as described above. Labeled target cells were added along with effector cells into round-bottomed microtiter plates (Nunc), and the mixtures were incubated for 6 h at 37 °C. Relative specific lysis of target cells infected with HIV-1was defined as (specific lysis of exp − specific lysis of uninfected cells)/(percentage of infected cell used).

2.8. ELISpot assay

The appropriate amount of EL11 or EL11–E92Q peptides and PBMCs from HLA-B*40:02$^+$ individuals chronically infected with HIV-1 were added to 96-well polyvinylidene plates (Millipore, Bedford, MA) that had been pre-coated with 5 mg/mL anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). The plates were incubated for 16 h at 37 °C in 5% CO_2 and then washed with PBS before the addition of biotinylated anti-IFN-γ mAb (Mabtech) at 1 mg/mL. After the plates had been incubated at room temperature for 90 min, they were subsequently incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 60 min at room temperature. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-idolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). The CD8$^+$ T cells without peptide stimulation were used as a negative control.

2.9. Sequencing of plasma RNA

Viral RNA was extracted from the plasma of HIV-1-infected individuals by using a QIAamp MinElute Virus Spin Kit (QIAGEN). cDNA was synthesized from the RNA with SuperScript III First-Strand Synthesis System for RT-PCR and random primer (Invitrogen). HIV-1 Pol gene was amplified by nested PCR using Taq polymerase (Promega). Sequencing reactions were performed with a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by use of an ABI 3500 genetic analyzer.

3. Results

3.1. INI-resistance E92Q mutation affects recognition of EL11-specific CTLs

The INI-resistance E92Q mutation is located in the HLA-B*40:02-restricted EL11 (ETGQETAYFLL) CTL epitope [17]. We therefore examined whether this mutation would affect the recognition by EL11-specific CTLs. The EL11-specific CTL clone and line, which were generated from an HIV-1-infected HLA-B*40:02$^+$ individual, effectively killed the wild-type peptide-pulsed C1R-B*40:02 cells, whereas they showed a reduced ability to kill the target cells pulsed with the E92Q mutant peptide (Fig. 1). To clarify the recognition of E92Q mutant-infected cells by EL11-specific CTLs, we generated an HIV-1 mutant virus by introducing the E92Q mutation into NL-432 (NL-432-E92Q) and then examined whether EL11-specific CTLs could kill target cells infected with the E92Q mutant virus. The EL11-specific CTL clone and cell line killed both WT-infected target cells and the NL-432-E92Q-infected ones, though the killing activity of the clone and cell line for the latter cells was significantly reduced as compared with that for the former ones (Fig. 2). These results indicate that INI-resistance E92Q mutation reduced EL11-specific CTL recognition.

3.2. Ex vivo CD8$^+$ T-cells fail to recognize EL11–E92Q peptide

To clarify the *ex vivo* CD8$^+$ T cell response to EL11-E92Q, we measured the responses in 5 HLA-B*40:02$^+$ individuals chronically infected with HIV-1 by performing ELISpot assays using EL11 and EL11-E92Q peptides. A strong T cell

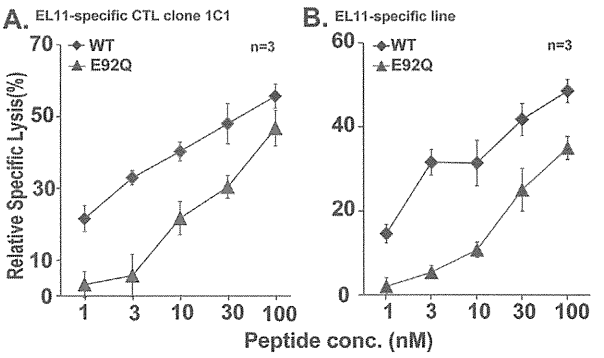

Fig. 1. Relative cytolytic activity of EL11-specific CTLs against target cells pulsed with the WT (EL11) or mutant (EL11–E92Q) peptide. An EL11-specific CTL clone and cell line were generated from PBMCs by stimulating them with WT peptide from a HLA-B*40:02 patient, KI-400, infected with WT virus. The antiviral activities of an EL11-specific CTL clone 1C1 (A) and the EL11-specific cell line (B) were analyzed. Relative cytotoxic activity toward C1R-B*40:02 cells prepulsed with the WT or EL11-E92Q peptide at concentrations of 1–100 nM was measured. The cytotoxic activity assay was performed at an E:T ratio of 1:1. The error bars indicate standard deviations.

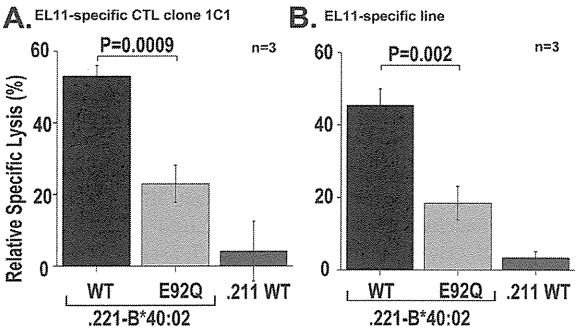

Fig. 2. Relative cytolytic activity of EL11-specific CTLs against target cells infected with WT (NL-432) or mutant virus (NL432-E92Q). Relative cytotoxic activity against 721.221-CD4-B*40:02 cells infected with NL-432 (WT virus) or NL-432-E92Q (E92Q virus) was assessed. (A) WT virus-infected (43.7% of total cells were p24 Ag^+) and E92Q virus-infected (59.1% of total cells were p24 Ag^+) cells were used as target cells. (B) WT virus-infected (59.2% of total cells were p24 Ag^+) and E92Q virus-infected (44.5% of total cells were p24 Ag^+) cells were used as target cells. NL-432 virus-infected 721.221-$CD4^+$ (.221 WT) cells were used as a negative control. The antiviral activities of an EL11-specific CTL clone, 1C1 (A), and an EL11-specific cell line (B) were analyzed. The cytotoxic activity was measured at E:T ratio of 1:1. The error bars indicate standard deviations. *P* values were determined by using Student's *T*-test.

response to the EL11 peptide was found in 4 of the 5 individuals (Fig. 3). However, these individuals did not have any response to the EL11–E92Q peptide. These results indicate that the HLA-B*40:02$^+$ individuals failed to recognize the INI-resistance E92Q mutation *ex vivo*.

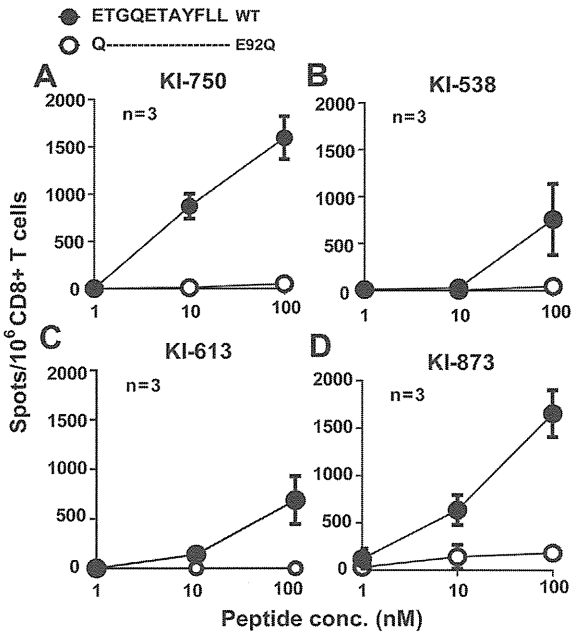

Fig. 3. *Ex vivo* $CD8^+$ T-cells response specific for WT (EL11) or mutant (EL11–E92Q) peptides in chronically HIV-1-infected HLA-B*40:02$^+$ individuals. $CD8^+$ T cell responses to EL11 or EL11–E92Q peptides by PBMCs from 5 HLA-B*40:02$^+$ chronically HIV-1-infected individuals were analyzed by performing ELISpot assays using WT (EL11) or mutant (E92Q) peptides at 1 nM–100 nM. A response eliciting greater than 200 spots was taken as a positive response. Four of the 5 individuals showed positive responses to the E11 peptide but not to the mutant one.

3.3. Frequency of the INI-resistance E92Q mutation in treatment-naïve Japanese individuals

The E92Q mutation was reported to occur in 2.2% of INI-treated patients, whereas the mutation was not found in treatment-naïve ones [18]. To clarify the accumulation of the E92Q mutation in Japanese individuals, we analyzed this part of the HIV-1 sequence in 363 treatment-naïve chronically HIV-1-infected Japanese patients and found no E92Q mutation in these individuals (data not shown). Thus, so far the E92Q mutation has not accumulated in the Japanese population.

4. Discussion

The RAL and EVG-resistance E92Q mutation is accumulating in INI-treated HIV-1 clade B patients [18]. In contrast, INI-resistance mutations, which are currently clinically relevant, are absent or highly infrequent in INI treatment-naïve patients [19]. We presently analyzed the HIV-1 sequence in 363 Japanese treatment-naïve patients and did not observe this INI-resistance E92Q mutation in any members of this population. These observations indicate that this mutation has not yet accumulated in treatment-naïve patients. INI-resistance E92Q was reported to be associated with significant fitness cost [20]. It could easily revert to the wild type in the absence of drug pressure after transmission from INI-containing ART-failing patients to untreated ones. However, INIs are relatively new; and the frequency of INI-resistance mutations might increase with future extensive use of such drugs.

Mutations in the anchor residues of a peptide can diminish the binding affinity for HLA class I molecules by changing the conformation of the peptide, though non-anchor residues also have an important role in peptide binding to HLA molecules [21]. HLA-B*40:02 binding peptide has an anchor residue at P2 [22]. INI-resistance E92Q mutations occur in the non-anchor P1 position of the EL11 epitope. The INI pressure replacing glutamic acid (E), having a negatively charged side chain, in the P1 position with glutamine (Q), having a polar uncharged side chain, might change the epitope conformation, resulting in weakened peptide binding to HLA-B*40:02 or impaired antigen presentation [23]. Changes in the non-anchor P1 position of a peptide might lead to significant unfavorable contacts with residues of the TCR.

We observed that EL11-specific CTLs killed both wild-type virus-infected cells and E92Q mutant virus-infected ones *in vitro*, although the CTLs killed more effectively the former cells than the latter cells. In contrast, EL11-specific $CD8^+$ T cells failed to recognize the mutation *ex vivo*. As *ex vivo* data reflects more closely the *in vivo* than the *in vitro* data, these results suggest that EL11–E92Q epitope would not be recognized by T cells *in vivo*. CTLs have high antigen sensitivity towards EL11 [17]; and $CD8^+$ T cells specific for the EL11 epitope were detected in 4 of the 5 HLA-B*40:02$^+$ individuals examined, indicating EL11 to be an immunodominant epitope. However, since the INI-resistance EL11-E92Q epitope may not be recognized by the $CD8^+$ T cells *in vivo*,

this INI-resistance mutation could hamper the eradication of HIV-1.

In the present study, we observed the effect of an INI-resistance mutation on CTL recognition. The data presented here demonstrated that the INI-resistance E92Q mutation affected EL11-specific $CD8^+$ T cell recognition both *in vitro* and *ex vivo* in the HLA-B*40:02$^+$ individuals. The CTL epitope having incorporated this drug-resistance mutation showed reduced immunogenicity, suggesting that this INI-resistance mutation affected HIV-1 control by the CTLs. Virologic failure in RAL-containing ART is associated with integrase mutations in at least 3 genetic pathways (Q148H/K/R, N155H, and Y143R/H/C pathways), N155H pathway includes the E92Q mutation [24]. Considering that RAL-resistance pathways at failure are not predicted by baseline viral mutations, host immune pressure could be one of the determinants of the resistance pathway [25]. In order to answer the question as to whether the INI-resistance E92Q mutation can emerge more frequently in HLA-B*40:02$^+$ individuals than in those negative for it during INI-containing ART, large clinical trials may be necessary. Further research is warranted.

Conflict of interest

The authors have no conflicting financial interests.

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