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

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

Materials and Methods

Patients

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

Cells

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

HIV-1 clones

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

Generation of TI8-specific CTL clones

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

Cytotoxic assay of CTL clones

The cytotoxic activity of TI8-specific CTL clones was determined by the standard ^{51}Cr release assay described previously (36). Briefly, C1R-B*51:01 cells were incubated with 100 μCi Na$_2$^{51}CrO$_4$ in saline for 60 min and then washed three times with R5. Labeled target cells (2 × 10^{3}/well) were added to each well of a U-bottom 96-well microtiter plate (Nunc) with the appropriate amount of the corresponding peptide. After a 1-h incubation, effector cells were added at an E:T ratio of 2:1, and then, the cultures were incubated for 4 h at 37°C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the number of counts per minute in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where cpm exp is the number of cpm in the supernatant in the wells containing both target and effector cells.

HIV-1 replication suppression assay

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

In vitro competitive viral suppression assay

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

Tetramer binding assay

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

Sequencing of plasma RNA

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

TCR clonotype analysis

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

Reconstruction of TCRs on TCR-deficient T cells

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

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

IL-2 secretion assays

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

Results

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

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

Recognition of the 8V mutant by TI8-specific CTLs

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

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

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

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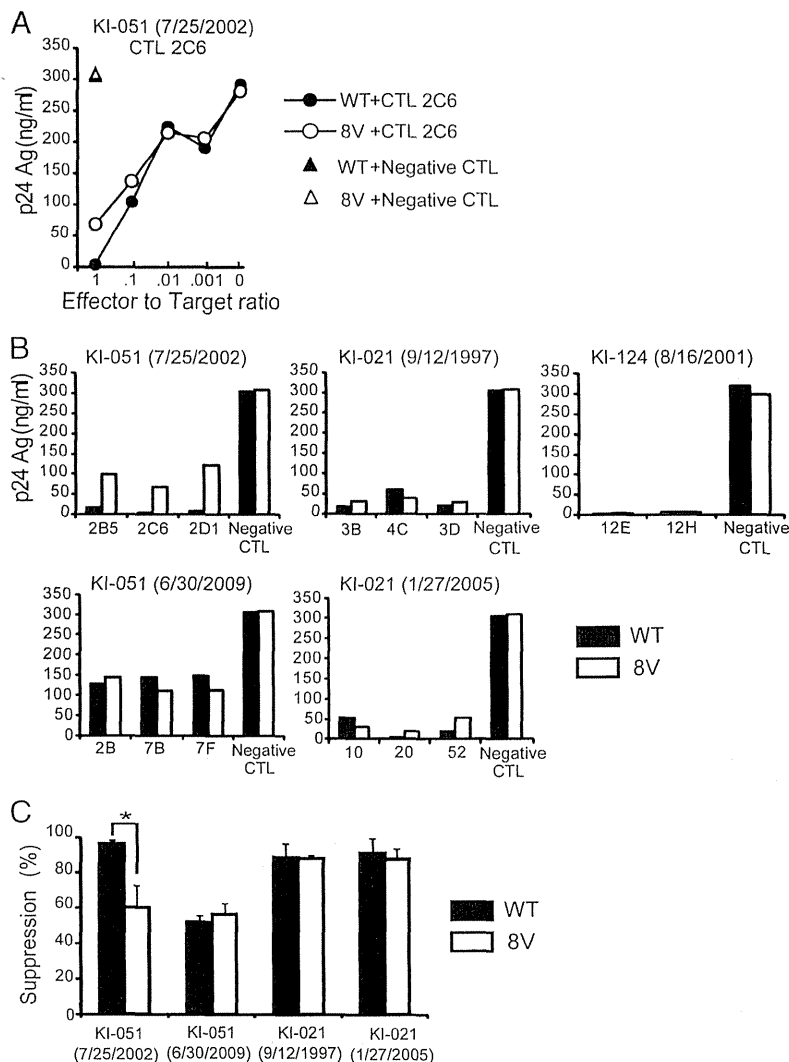[d]NT, not tested.

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

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

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

confirming that all CTL clones used in this experiment strongly suppressed the replication of both viruses.

TCR affinity of TI8-specific CTL clones

We found two types of TI8-specific CTL clone for the 8V recognition. To further characterize the 8V recognition by these CTL clones, we investigated the TCR affinity of these clones by using tetramers of HLA-B*51:01 with TI8 peptide (WT tetramer) and with the 8V mutant peptide (8V tetramer). Clone 2B5 exhibited significantly weaker affinity for the 8V tetramer than for the WT one (EC_{50}: 60.7 ± 14.3 nM for WT and 332.5 ± 32.7 nM for 8V; $p < 0.00019$; Fig. 3A). In contrast, clone 2B from KI-051 after the emergence of the 8V mutant and clone 3B from KI-021 showed almost the same affinity for both tetramers (EC_{50}: clone 2B, 116.3 ± 52.3 nM for WT and 115.1 ± 39.2 nM for 8V; $p < 0.98$; clone 3B, 104.5 ± 16.5 nM for WT and 112.5 ± 56.1 nM for 8V, $p < 0.82$; Fig. 3A). The TCR affinity for the 8V tetramer of all CTL clones was compared in terms of EC_{50} ratio of WT to 8V tetramer. The EC_{50} ratio of the CTL clones from KI-051 in July 2002 was significantly lower than that of the CTL clones from KI-051 in June 2009 and from KI-021 in September 1997 and January 2005 (Fig. 3B). These results taken together indicate that 2B5 carried TCR's with weaker affinity for the 8V tetramer than for the WT one, whereas 2B and 3B carried TCR's with similar affinity for both tetramers.
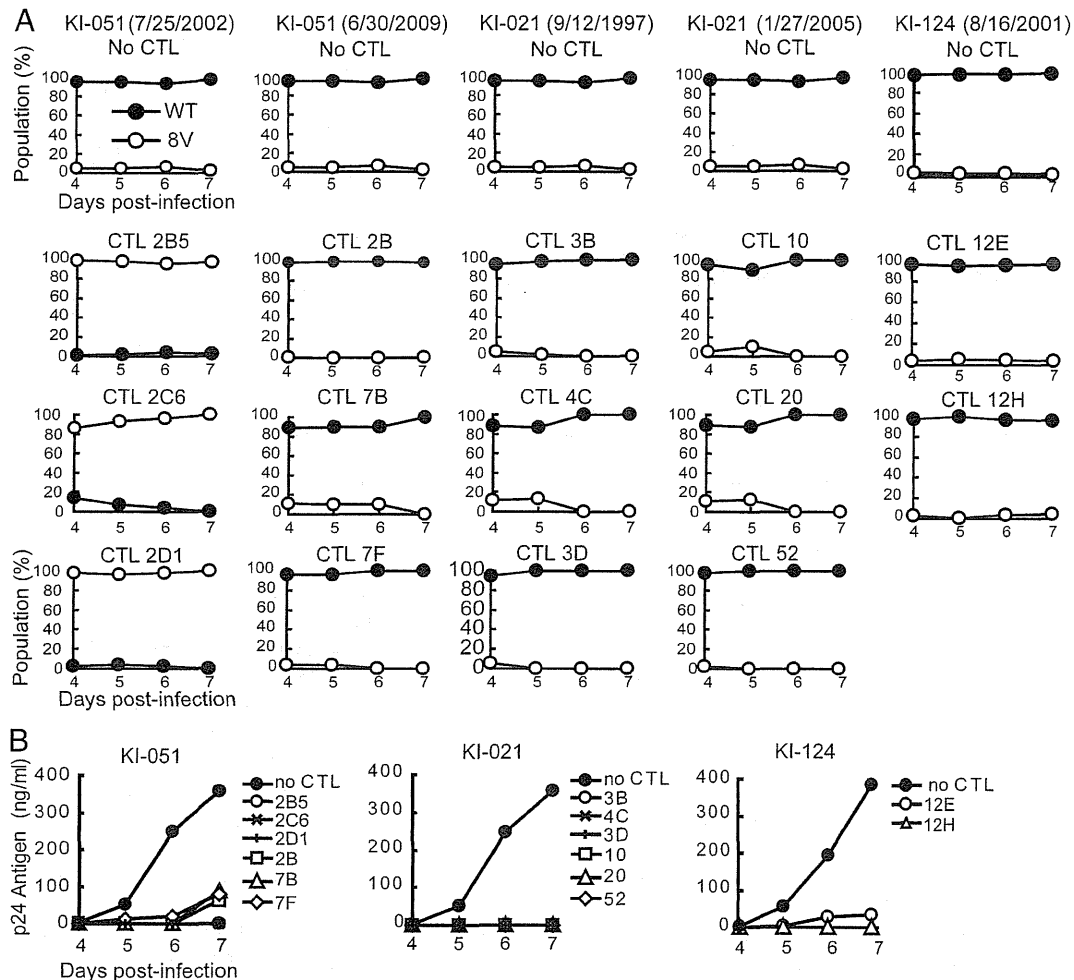
FIGURE 2. In vitro selection of the 8V mutant virus by TI8-specific CTL clones. (**A**) Ability of TI8-specific CTL clones to select the 8V mutant. T1 cells expressing HLA-B*51 and CD4 molecules were infected with NL-432 or NL432-Pol283-8V virus together at a ratio of 9:1. The infected cells were cocultured with TI8-specific CTL clones at an E:T ratio of 0.05:1. The culture supernatants were collected from days 4 to 7 postinfection. The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram. (**B**) Ability of TI8-specific CTL clones to suppress the replication of HIV-1. HIV-1 p24 Ag levels in the supernatant were measured by use of an enzyme immunoassay (sensitivity: 7.8 pg/ml). The concentrations of HIV-1 p24 Ag in the collected supernatants were >0.1 ng/ml.

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

ognition 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
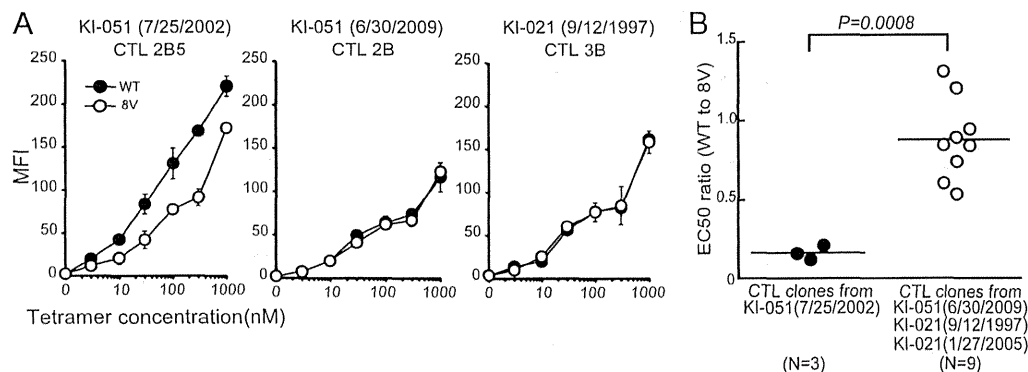
FIGURE 3. Difference in TCR affinity for HLA-B*51:01 with TV8 peptide complex among TI8-specific CTL clones. (**A**) The ability of the TCRs on TI8-specific CTL clones to bind WT or 8V tetramer was measured in terms of the mean fluorescence intensity (MFI) of each CTL clone stained with the tetramers 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.
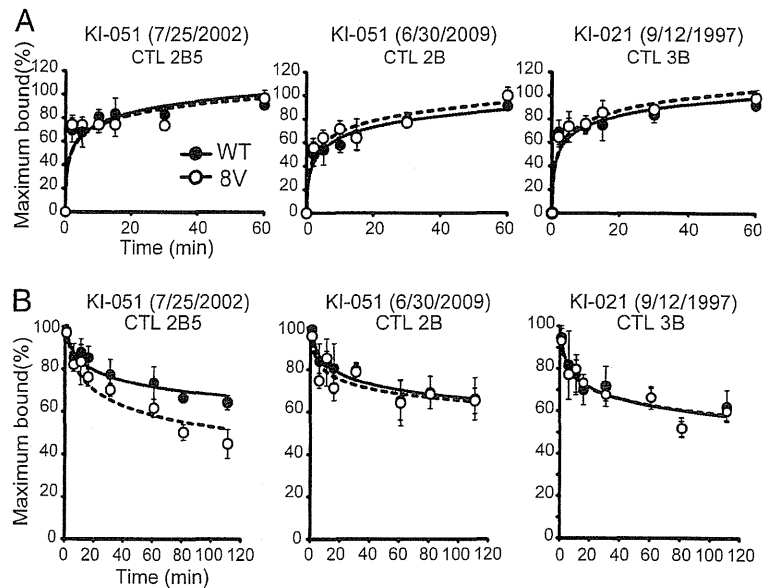
by the TI8-specific cross-reactive CTL response in our three elite controllers. We speculate that the CTLs elicited in these three elite controllers strongly inhibited HIV-1 replication in the acute phase of HIV-1 infection and maintained VL at a very low level for the long time so that selection of 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
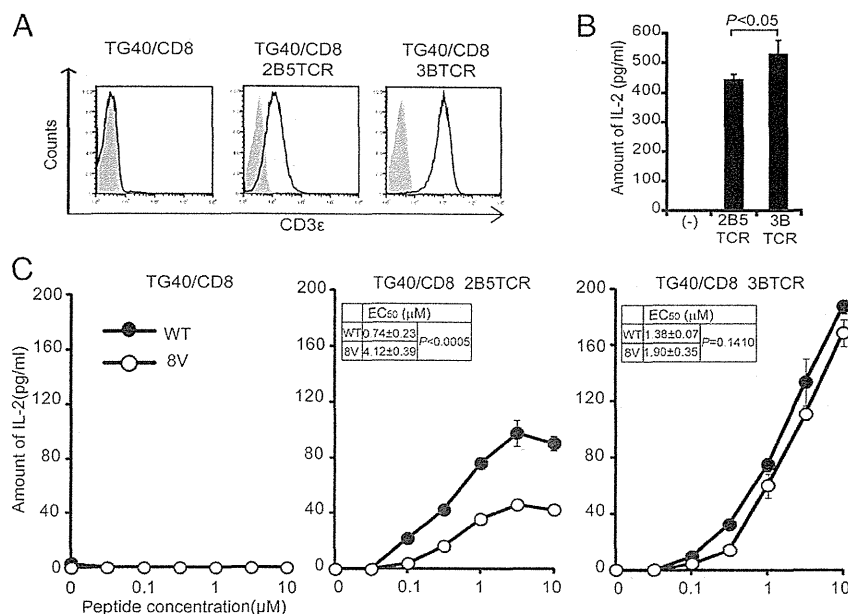

FIGURE 5. Reconstruction and recognition of TI8-specific TCRs on TCR-transfected TG40/CD8 cells. (**A**) CD3 expression in TG40/CD8 cells transfected with 2B5 TCR or 3B TCR. The TCR-transfected cells were stained with anti-CD3ε mAb (open histogram) or with an isotype control (shaded histogram), and then, 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.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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Superimposed Epitopes Restricted by the Same HLA Molecule Drive Distinct HIV-Specific CD8+ T Cell Repertoires

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Superimposed epitopes, in which a shorter epitope is embedded within a longer one, can be presented by the same HLA class I molecule. CD8+ CTL responses against such epitopes and the contribution of this phenomenon to immune control are poorly characterized. In this study, we examined HLA-A*24:02–restricted CTLs specific for the superimposed HIV Nef epitopes RYPLTFGWCF (RF10) and RYPLTFGW (RW8). Unexpectedly, RF10-specific and RW8-specific CTLs from HIV-1–infected HLA-A*24:02+ individuals had no overlapping Ag reactivity or clonotypic compositions. Single-cell TCR sequence analyses demonstrated that RF10-specific T cells had a more diverse TCR repertoire than did RW8-specific T cells. Furthermore, RF10-specific CTLs presented a higher Ag sensitivity and HIV suppressive capacity compared with RW8-specific CTLs. Crystallographic analyses revealed important structural differences between RF10– and RW8–HLA-A*24:02 complexes as well, with featured and featureless conformations, respectively, providing an explanation for the induction of distinct T cell responses against these epitopes. The present study shows that a single viral sequence containing superimposed epitopes restricted by the same HLA molecule could elicit distinct CD8+ T cell responses, therefore enhancing the control of HIV replication. This study also showed that a featured epitope (e.g., RF10) could drive the induction of T cells with high TCR diversity and affinity. *The Journal of Immunology*, 2014, 193: 77–84.

C ytotoxic CD8+ T lymphocytes recognize target cells through the recognition of peptides 8–11 aa long that are presented by MHC class I (MHCI) molecules (1–3). Of note, two epitopes in which a shorter one is embedded within a longer one are defined as superimposed epitopes, and they have been shown to be presented by the same MHCI molecule (4–6). A number of studies have reported CTL responses against such superimposed peptides in the context of an HIV-1 infection (7–10). For instance, these responses include CTLs specific for HLA-B57–restricted p24 Gag-derived peptides, for example, KI8 (residues 162–169, KAFSPEVI) and KF11 (residues 162–172, KAFSPEVIPMF), as well as for HLA-B*35:01–restricted Nef-derived peptides, for example, VY8 (residues 74–81, VPLRPMTY) and RY11 (residues 71–81, RPQVPLRPMTY). However, the biological relevance of this phenomenon remains unclear. Indeed, although it is speculated that CTLs can show cross-reactivity toward superimposed

epitopes and work together effectively against HIV-infected targets, the functional synergism of these cells has not been studied in detail.

HLA-A*24:02 is the most frequent HLA class I allele in Japan, being found in ~70% of Japanese individuals (11) and of those infected with HIV-1 (12, 13). This allele also occurs in the range from ~25–64% in other Asian countries and in 18% in white populations (14, 15). Therefore, the study of immune responses to epitopes restricted by this allele is important for our understanding of HIV pathogenesis and vaccine development. We previously reported that Nef138-10 (RYPLTFGWCF, RF10) is an immunodominant CTL epitope in HLA-A*24:02+ Japanese individuals chronically infected with HIV-1 (16, 17). Of interest, Nef138-8 (RYPLTFGW, RW8) is also defined as an optimal epitope presented by HLA-A*24:02 in white individuals (18). Although these superimposed epitopes elicit effective specific CTL responses important for the control of HIV-1 replication in HLA-A*24:02+ individuals, the overlap in terms of reactivity and antiviral ability between RF10- and RW8-specific CTLs remains unknown.

In the present study, we performed a comprehensive analysis of CTL responses specific for RW8- and RF10-superimposed epitopes by using multiple approaches. We used RF10 and RW8 tetramers to identify and isolate cells from chronically HIV-1–infected HLA-A*24:02+ Japanese individuals, α- and β-chain TCR repertoire analyses at the single-cell level to assess the degree of overlap between responses, and crystallographic approaches to reveal the structural basis of RF10– and RW8–HLA-A*24:02 interactions. We report unanticipated differences between RF10- and RW8-specific CTLs, that is, the elicitation of totally distinct CTL responses against superimposed HIV-1 epitopes restricted by the same HLA molecule, as well as distinct TCR repertoires between the featured (RF10) and featureless (RW8) epitope-specific CTLs.

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The online version of this article contains supplemental material.

Abbreviations used in this article: MFI, mean fluorescence intensity; MHCI, MHC class I; tet, tetramer.

Materials and Methods

Patient samples

The study was approved by the Ethics Committees of Kumamoto University and the National Center for Global Health and Medicine. Informed consent

was obtained from all individuals according to the Declaration of Helsinki. Twenty-three HLA-A*24:02[+] treatment-naive individuals chronically infected with HIV-1 and eight HLA-A*24:02[+] HIV-1 seronegative individuals were recruited (Supplemental Table I). Their plasma and PBMCs were separated from whole blood. HLA types were determined by standard sequence-based genotyping.

HIV-1–specific CTL clones

Ag-specific CTL clones were generated as previously described (17). Briefly, RW8- and RF10-specific CTL cell lines were first obtained by stimulating PBMCs from patient KI-158 with cognate peptides. Peptide-specific CTL clones were then generated from the cell lines by limiting dilution in 96-well U-shaped plates cocultured with 1×10^6 irradiated feeder PBMCs from healthy donors and 1×10^5 irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide at 1 μM concentration. All CTL clones were cultured in 200 μl cloning medium (RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% PHA soup) and stimulated weekly with irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide.

Tetramer staining

HLA-A*24:02 tetrameric complexes were synthesized as previously described (19). For tetramer-binding assays, CTL clones were stained with PE-conjugated RW8 or RF10 tetramers at various concentrations (0–1000 nM) at 37°C for 30 min before staining with FITC-conjugated anti-CD8 mAb at 4°C for 30 min. The cells were analyzed by using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star). For TCR avidity measurements, the tetramer concentration that yielded the EC_{50} mean fluorescence intensity (MFI) was calculated by probit analysis.

Cell line

C1R-A*2402 and RMA-S-A*2402 cells were previously generated by transfecting HLA-A*24:02 genes into C1R cells and RMA-S cells, respectively (5, 20, 21). The C1R cell line is a human B cell lymphoblastoid line lacking surface expression of HLA-A and partially HLA-B molecules. It was derived from a normal B cell line, Hmy2, through three rounds of mutagenesis and selection with anti-HLA mAb (22). RMA-S cells are a TAP2 deficiency cell line derived from RMA cells. They express high levels of empty MHC molecules (i.e., not carrying endogenous peptides on the cell surface) when cultured at 26°C and very low levels when cultured at 37°C (23). RMA-S-A*2402 and C1R-A*2402 cells were cultured in RPMI 1640 medium containing 10% FCS and 0.2 mg/ml hygromycin B.

Peptide-binding assay

The binding of peptides to HLA-A*24:02 molecules was tested as previously described (24). Briefly, RMA-S-A*2402 cells were precultured at 26°C for 14–18 h and then incubated at the same temperature for 1 h with either RW8 or RF10 peptide at various concentrations (0–100 nM). Thereafter, they were incubated at 37°C for 3 h. After incubation, the peptide-pulsed cells were stained with anti-HLA class I α3 domain mAb TP25.99 (19) and subsequently with FITC-conjugated sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The MFI was measured by flow cytometry (FACSCanto II).

Replication suppression assay

Two HIV-1 virus laboratory strains, NL-432-10F and NL-M20A-10F, were used in these assays. They were generated from NL-43 or NL-M20A by site-directed mutagenesis to carry the RYPLTFGWCF sequence (17). The ability of HIV-1–specific CTLs to suppress HIV-1 replication was examined as previously described (17). Briefly, primary CD4^{+} T cells were infected with NL-432-10F and NL-M20A-10F, respectively, for 6 h before being washed with R10 medium. The cells were then cocultured with HIV-1–specific CTL clones at various E:T ratios. Ten microliters of culture supernatant was collected at day 6, and the concentration of p24 Ag in it was determined by performing p24 ELISA (ZeptMetrix, Buffalo, NY).

[51]Cr-release assay

The cytotoxic potential of CTL clones against C1R-A*2402 prepulsed with appropriate peptide at various concentrations (0–100 nM) was determined as previously described (25). Briefly, C1R-A*2402 cells were labeled with 100 μl ^{51}Cr for 1 h before washing and then pulsed for 1 h with peptides. Effector cells were cocultured for 4 h at 37°C with target cells (2×10^3/ well) at an E:T ratio of 2:1. After centrifugation, 100 μl supernatant was collected and analyzed with a gamma counter. The specific lysis was calculated as [(cpm experiment − cpm supernatant)/(cpm maximum − cpm supernatant)] × 100.

Ex vivo single-cell TCR repertoire analysis and assessment of TCR diversity

Cryopreserved PBMC samples from patients were thawed, divided, and immediately stained with RW8 or RF10 tetramers, followed by staining with anti-CD3 mAb (Pacific Blue), anti-CD8 mAb (FITC), and 7-amino-actinomycin D. RW8 and RF10 tetramer^{+}CD3^{+}CD8^{+}7-aminoactinomycin D^{−} cells were sorted into a 96-well plates (Bio-Rad) by using a FACSAria I (BD Biosciences). Unbiased identification of TCR α- and β-chain usage was assessed as previously described (26). An Illustra ExoStar (GE Healthcare, Little Chalfont, U.K.), which contains alkaline phosphatase and exonuclease 1, was used to remove unincorporated primers and nucleotides from amplification reaction for the subsequent tailing reaction. The names of all identified TCR genes were given based on the international ImMunoGeneTics information system nomenclature (27). The diversity of TCR clonotypes was calculated by using both the number of different clonotypes and Simpson's diversity index for both α- and β-chains and the formula $D_s = 1 - \Sigma\{[n_i(n_i - 1)]/[N(N - 1)]\}$, where n_i is the TCR clone size of the ith clonotype and N is the total number of TCR sequences sampled. This index uses the relative frequency of each clonotype to calculate a diversity index ranging between 0 and 1, with 0 being minimal and 1 being maximal diversity (28).

Crystallization, data collection, and processing

Soluble peptide–HLA-A*24:02 complexes were prepared as previously described (29). HLA-A*24:02 molecules were purified by Superdex 200 10/300 GL gel-filtration chromatography (GE Healthcare). All crystallization attempts were performed by the hanging drop vapor diffusion method at 18°C with a protein/reservoir drop ratio of 1:1. Crystals were seen after 3–5 d in 0.1 M MES (pH 6.5) and 12% (w/v) polyethylene glycol at 20,000 g/mol. The crystals were briefly soaked in reservoir solution containing 17% (v/v) glycerol, mounted on an x-ray machine with a nylon loop, and then flash-cooled in a stream of gaseous nitrogen. Diffraction data were collected by using beamline NE3A in the KEK Synchrotron Facility (Tsukuba, Japan) and an ADSC Q270 imaging-plate detector at a wavelength of 1.0 Å. Data were indexed, integrated, and scaled by using HKL2000. The data collection statistics are shown in Table I. Data were analyzed by molecular replacement by use of Phaser in CCP4. We used the A24VYG molecule as the search model (Protein Data Bank accession no. 2BCK, http://www.rcsb.org/pdb/home/home.do). All of the structures were further refined by several rounds of refinement made by using the PHENIX program. The refinement statistics are given in Table I.

Results

Effective induction of RW8- or RF10-specific CTL responses in HIV-1–infected HLA-A*24:02[+] patients

To assess the degree of overlap between CTL responses specific for two superimposed Nef epitopes (RW8 and RF10), we first generated HLA-A*24:02 tetramers with RW8 or RF10 peptides (RW8-tet and RF10-tet, respectively) and compared ex vivo frequencies of 8-mer– or 10-mer–specific CD8^{+} T cells in 23 treatment-naive HLA-A*24:02^{+} individuals with chronic HIV-1 infection. In eight HIV-1 seronegative HLA-A*2402^{+} donors, the frequencies of RW8 and RF10 tetramer^{+} CD8^{+} T cells were 0.080 ± 0.009 and 0.045 ± 0.022% (mean ± SD), respectively (Fig. 1A). We evaluated the mean ± 3 SD as positive staining and therefore considered 0.10 and 0.11% of tetramer[+] CD8+ cells as positive values for RW8-specific and RF10-specific T cells, respectively (Fig. 1B, dashed line). Among the 23 HIV-1–infected individuals studied, 14 and 19 were positive for RW8-specific CTLs and RF10-specific CTLs, respectively (Fig. 1A). Thirteen of the 23 individuals analyzed (56.5%) presented both RW8- and RF10-specific T cells (Fig. 1B). The magnitude of RW8-specific and RF10-specific T cells correlated with one another across individuals (Fig. 1B). However, this correlation was modest, indicating that these populations did not overlap entirely. In fact, the frequency of RF10 tetramer^{+} CD8^{+} cells was significantly higher than that of RW8 tetramer^{+} CD8^{+} cells (Fig. 1A). Taken together, these results indicate that both RW8-specific and RF10-specific CTLs could be effectively elicited in HLA-A*24:02[+] individuals with a chronic HIV-1 infection; however,
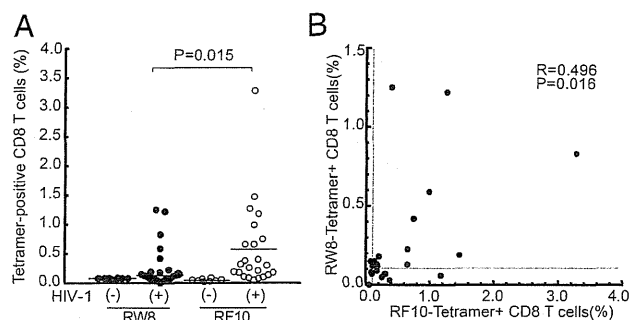
FIGURE 1. Frequencies of RW8- and RF10-specific CD8^{+} T cells in individuals with chronic HIV-1 infection. (**A**) Frequencies of total RW8 and RF10 tetramer^{+} CD8^{+} T cells in 23 chronically HIV-1-infected treatment-naive HLA-A*24:02^{+} individuals and in 8 HLA-A*24:02^{+} uninfected controls. Statistical analyses were conducted by using the nonparametric Mann–Whitney U test. (**B**) Correlations between RW8 and RF10 tetramer^{+} CD8^{+} T cell frequencies in HIV-1–infected HLA-A*24:02^{+} individuals. Of tetramer^{+} CD8^{+} cells, 0.10 and 0.11% were considered as positive values for RW8-specific and RF10-specific T cells, respectively (the dashed line indicates the threshold), as described in the text. The correlation was determined by using the Spearman rank test.

they do not appear to be equivalent, which begs the question of their cross-reactive potential.

Distinct reactivity of RW8- and RF10-specific CTLs

To investigate whether RW8- or RF10-specific CTLs could cross-recognize the superimposed epitopes, we first performed concurrent RW8 and RF10 tetramer (RW8-tet and RF10-tet, respectively) staining of PBMCs from HIV-1–infected donors. In patients presenting both RF10- and RW8-specific CD8^{+} T cells, these cells did not seem to be RW8 and RF10 cross-reactive, as they failed to stain for both tetramers simultaneously. A representative case (patient KI-

158) is shown in Fig. 2A. To analyze further the fine reactivity toward these epitopes, we next established CTL clones from patient KI-158 presenting both RF10- and RW8-specific CD8^{+} T cells upon initial selection and stimulation with RW8 or RF10 peptides. RF10- and RW8-specific clones were clearly discriminated by using both tetramers together at the same concentration (Fig. 2B). We performed staining using different concentrations of the specific tetramers to measure the TCR avidity of representative RF10- or RW8-specific clones. CTL52 clone (RW8-specific) exhibited a strong affinity for RW8-tet but not for RF10-tet, whereas the CTL173 (RF10-specific) clone exhibited a strong affinity for RF10-tet but not RW8-tet (Fig. 2C), indicating that CTL52 and CTL173 clones had TCRs with high affinity for RW8 peptide–HLA-A*24:02 and RF10 peptide–HLA-A*24:02 complexes, respectively.

Next, we tested the functional avidity of RF10- and RW8-specific clones. RF10-specific clones (CTL170 and CTL173) effectively killed RF10 peptide–pulsed cells but failed to kill RW8 peptide–pulsed targets even at a high concentration of RW8 peptide (Fig. 2D), indicating that these RF10-specific clones did not cross-recognize the 8-mer peptide. RW8-specific clones (CTL52 and CTL72) recognized both RW8 peptide– and RF10 peptide–pulsed targets, but the cytotoxic activity of these clones against RW8 peptide–pulsed target cells was 10- to 50-fold higher than that against the RF10 peptide–pulsed ones (Fig. 2E). Although RW8 clones presented some cross-reactivity toward RF10, they recognized the RW8 peptide with greater efficiency than RF10. Altogether, these results indicate that RW8-specific and RF10-specific CTLs displayed no or poor cross-reactivity for their respective superimposed epitopes.

Different TCR usage between RW8- and RF10-specific CD8^{+} T cells

The lack of cross-reactivity between RW8-specific and RF10-specific CD8^{+} T cells implies that distinct clonotypes should
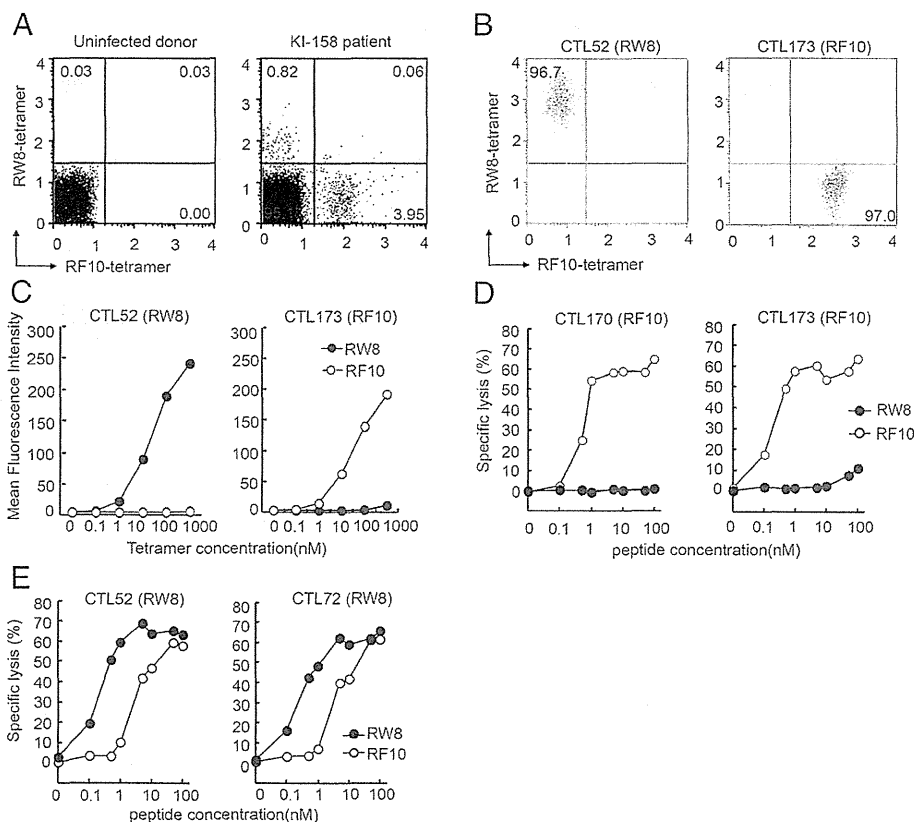

FIGURE 2. Recognition of RW8 and RF10 peptides by RW8- or RF10-specific CTL clones. (**A**) Simultaneous RW8 and RF10 tetramer staining of PBMCs from a representative HIV-1–infected donor and an HIV-1–uninfected healthy individual. The percentage of tetramer^{+} cells among CD8^{+} cells is shown. (**B**) Simultaneous RW8 and RF10 tetramer staining of CTL52 and CTL173 clones. The percentage of tetramer^{+} cells among CD8^{+} cells is shown. (**C**) RW8 and RF10 tetramer staining of CTL52 and CTL173 clones at various concentrations. Tetramer binding to CTL52 and CTL173 clones was analyzed by flow cytometry, and the data are shown as MFI. (**D** and **E**) Cytolytic activities of HLA-A*24:02–restricted RF10 (CTL170/CTL173)-specific (D) and RW8 (CTL52/CTL72)-specific (E) CTL clones toward HLA-A*24:02^{+} target cells (C1R-A*2402) prepulsed with the indicated peptide at various concentrations.

comprise these populations. To verify this point, we performed TCR repertoire analysis at the single-cell level to expose the degree of overlap between the two responses at the clonotypic level. Most previous studies on TCR repertoires in virus infection or tumor studies focused mainly on characterization of the TRB gene (30–32). However, analysis of TRB genes provides only a partial account of the TCR repertoire, because the same TRB gene can pair with different TRA genes (26, 33). In the present study, we sequenced both α- and β-chains of the TCR on RW8-specific and RF10-specific single CD8$^+$ T cells sorted by FACS from three patients presenting both RW8- and RF10-specific CD8$^+$ T cells, as well as from two individuals with either one (Supplemental Table II). This analysis showed that RW8- and RF10-specific CD8$^+$ T cells consisted indeed of entirely distinct sets of clonotypes (Fig. 3, Supplemental Fig. 1). Of note, TRBV7-9 clonotypes were often detected among RW8-specific T cells, whereas TRBV28-1 was frequently found among RF10-specific T cells. Additionally, we observed that the number of distinct clonotypes as well as clonotypic diversity (based on both α- and β-chains) among RW8-

specific CD8$^+$ T cells was significantly lower than for RF10-specific ones (Fig. 4). Overall, the differences in α- and β-chain TCR gene usage and overall clonotypic diversity between RW8- and RF10-specific CD8$^+$ T cells supported significantly distinct modes of TCR recognition of the RW8 peptide– and RF10 peptide–HLA-A*24:02 molecule complexes.

Structure of RW8 peptide– and RF10 peptide–HLA-A*24:02 molecular complexes

Considering the potential impact of peptide–MHCI structural constraints on TCR repertoire composition, we next aimed at investigating the molecular basis of the interaction between the HLA-A*24:02 molecule and the RW8 or RF10 peptide to elucidate the determining factor for the lack of overlap between RW8- and RF10-specific CD8$^+$ T cell populations. We therefore determined the crystal structure of HLA-A*24:02 in complex with RW8 (HLA-A*24:02-RW8) and HLA-A*24:02-RF10 complex (HLA-A*24:02-RF10) (Table I). The two superimposed epitopes, RF10 and RW8, showed dramatically different conformations when bound to HLA-

A Paired T cell receptor usage of RW8-specific CTLs

Sample	TRAV	TRAJ	CDR3α	TRBV	TRBD	TRBJ	CDR3β	Frequency
KI-026	TRAV12-2*03	TRAJ40*01	CAVPRTGTYKYIF	TRBV7-9*03	TRBD2*01	TRBJ2-2*01	CASSLTSGANTGELFF	10/18
	TRAV12-2*03	TRAJ23*01	CAVSFYNQGGKLIF	TRBV7-9*03	TRBD1*01	TRBJ1-2*01	CASSPRDKPNYGYTF	8/18
KI-158	TRDV1*01	TRAJ40*01	CALGELGAPGTYKYIF	TRBV20-1*01	TRBD2*01	TRBJ2-7*01	CSARDPVSTYEQYF	27/31
	TRAV12-2*02	TRAJ50*01	CAAFKTSYDKVIF	TRBV20-1*02	TRBD2*01	TRBJ2-7*01	CSARDPIRLISYEQYF	4/31
KI-654	TRAV8-4*03	TRAJ30*01	CAVSDEVIF	TRBV7-9*07	TRBD1*01	TRBJ2-5*01	CASSIRDRVPETQYF	13/43
	TRAV9-2*01	TRAJ16*01	CALFLDGQKLLF	TRBV7-9*07	TRBD2*01	TRBJ2-2*01	CASSDTSGANTGELFF	7/43
	TRAV12-2*02	TRAJ40*01	CAVPVPGTYKYIF	TRBV7-9*07	TRBD2*01	TRBJ2-2*01	CASSDTSGANTGELFF	23/43
KI-102	TRAV8-1*01	TRAJ10*01	CAVIFTGGGNKLTF	TRBV7-9*03	TRBD1*01	TRBJ2-5*01	CASSQRDSQETQYF	65/65

B Paired T cell receptor usage of RF10-specific CTLs

Sample	TRAV	TRAJ	CDR3α	TRBV	TRBD	TRBJ	CDR3β	Frequency
KI-026	TRAV9-2*01	TRAJ21*01	CALGVDFNKFYF	TRBV19*01	TRBD1*01	TRBJ2-2*01	CASKGTVTGELFF	7/27
	TRDV1*01	TRAJ9*01	CALGELTNTGGFKTIF	TRBV11-2*03	TRBD2*01	TRBJ2-7*01	CASSYDRGYEQYF	6/27
	TRDV1*01	TRAJ9*01	CALGELSRTGGFKTIF	TRBV28*01	TRBD2*02	TRBJ2-7*01	CASLPSVGKGAYEQYF	6/27
	TRDV1*01	TRAJ24*02	CALWIMTTDSWGKLQF	TRBV4-1*01	TRBD1*01	TRBJ2-1*01	CASSQSPGQGVGEQFF	4/27
	TRDV1*01	TRAJ13*02	CALGELSSGGYQKVTF	TRBV6-1*01	TRBD1*01	TRBJ1-2*01	CASSDVGQGSSNYGYTF	2/27
	TRDV1*01	TRAJ36*01	CALGVLDQTGANNLFF	TRBV28*01	TRBD1*01	TRBJ1-2*01	CASSSPGQGYGYTF	1/27
	TRAV21*02	TRAJ7*01	CAVWYYGNNRLAF	TRBV28*01	TRBD2*02	TRBJ2-2*01	CASSLMGLAGVPGELF	1/27
KI-158	TRDV1*01	TRAJ9*01	CALGELSGTGGFKTIF	TRBV6-1*01	TRBD1*01	TRBJ2-1*01	CASSEGFGQGGIEQFF	19/32
	TRDV1*01	TRAJ53*01	CALGELLRGGSNYKLTF	TRBV6-2*01	TRBD2*01	TRBJ2-7*01	CASSYSHRLGLHEQYF	11/32
	TRAV8-6*02	TRAJ48*01	CAVLSLISNFGNEKLTF	TRBV19*01	TRBD2*01	TRBJ2-7*01	CASSISAGEGVPEQYF	2/32
KI-118	TRDV1*01	TRAJ9*01	CALGELSSTGGFKTIF	TRBV28*01	TRBD1*01	TRBJ2-1*01	CASTSFGQGTNEQFF	12/14
	TRAV13-1*02	TRAJ20*01	CAALNDYKLSF	TRBV19*01	TRBD2*01	TRBJ2-1*01	CASSIDPPGLADNEQFF	2/14
KI-102	TRDV1*01	TRAJ9*01	CALGELSHTGGFKTIF	TRBV11-2*01	TRBD1*01	TRBJ2-7*01	CASSYDRSYEQYF	4/30
	TRDV1*01	TRAJ9*01	CALGELTNTGGFKTIF	TRBV6-6*01	TRBD1*01	TRBJ1-2*01	CASSYSIGTGVNNYGYTF	3/30
	TRDV1*01	TRAJ36*01	CALGVLDQTGANNLFF	TRBV28*01	TRBD1*01	TRBJ1-2*01	CASSSPGQGYGYTF	7/30
	TRDV1*01	TRAJ54*01	CALGVIGIQGAQKLVF	TRBV28*01	TRBD1*01	TRBJ1-5*01	CASSPSTGKGNQPQHF	7/30
	TRAV25*01	TRAJ23*01	CPFYNQGGKLIF	TRBV19*01	TRBD1*01	TRBJ1-2*01	CASSTALRTGNYGYTF	4/30
	TRAV26-1*01	TRAJ28*01	CVVNSGAGSYQLTF	TRBV6-1*01	TRBD1*01	TRBJ2-7*01	CASSETGGTYEQYF	3/30
	TRAV8-3*02	TRAJ37*01	CAVDEGKLIF	TRBV4-1*01	TRBD1*01	TRBJ1-1*01	CASSQRDRGTDTEAFF	1/30
	TRAV14/DV4*01	TRAJ33*01	CAMQDSNYQLIW	TRBV7-9*03	TRBD2*02	TRBJ2-1*01	CASSLVSGRGNEQFF	1/30

FIGURE 3. Clonotypic analysis of RW8- and RF10-specific CD8$^+$ T cells. Single RW8- or RF10-specific CD8$^+$ T cells from five chronically HIV-1–infected HLA-A*24:02$^+$ individuals were FACS sorted, and TCR α- and β-chain sequencing was performed. Paired TCR α- and β-chain usage, CDR3 amino acid sequences, and individual clonotype frequencies are shown. (**A**) Paired T cell receptor usage of RW8-specific CTLs. (**B**) Paired T cell receptor usage of RF10-specific CTLs.
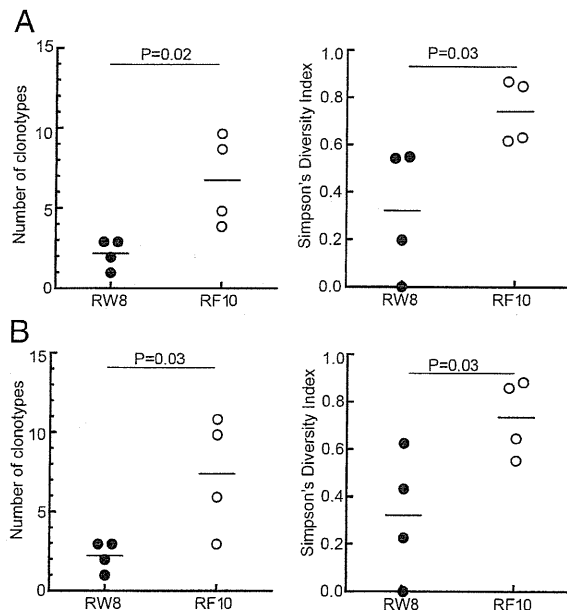
FIGURE 4. TCR repertoire diversity of RW8- and RF10-specific CD8^{+} T cells. TCR repertoire diversity was assessed by using both the number of clonotypes and Simpson's diversity index for α- (**A**) and β-chain (**B**). Statistical analysis was conducted by use of the unpaired *t* test.

the necessity to elicit different TCR repertoires to recognize these HLA-A*24:02 superimposed epitopes.

The total hydrogen bonds and van der Waal's interactions between HLA-A*24:02 and the RW8 or RF10 peptide were analyzed in detail (Fig. 5B, Supplemental Table III). The first 3 aa (P1–P3 residues) of both RW8 and RF10 peptides displayed almost identical main-chain conformations. However, the N-terminal anchor residue (Pn) tyrosine (Y) at position P2 formed a hydrogen bond with H70 in the B pocket of HLA-A*24:02. One additional hydrogen bond between P2 residue and Lys[66] and another hydrogen bond between the P1 residue and Arg[170] were also observed in the RF10 peptide. Moreover, whereas the Pc residue Trp[8] of the RW8 peptide formed four hydrogen bonds with the HLA molecule, the subanchor residue Trp[8] and Pc residue Phe[10] of the RF10 peptide formed one more hydrogen bond. This additional hydrogen bonding likely impacted the binding affinity of RF10 for HLA-A*24:02, making it greater than that of RW8.

Superior HIV-suppressive capacity of RF10-specific CD8^{+} CTL clones

Measurements of the binding affinity of RF10 and RW8 peptides for HLA-A*24:02 molecules indeed revealed that the RF10 affinity was ~10-fold higher than the RW8 affinity (Fig. 6A). Such differences in peptide–MHCI binding affinity may eventually have affected the efficiency of T cells to recognize their specific targets. We thus investigated the ability of RW8- and RF10-specific CD8^{+} CTL clones to suppress HIV-1 replication in cultures of virus-infected CD4^{+} T lymphocytes. To this end, we used two viruses, NL-432-10F and NL-M20A-10F, both carrying the RYPLTFGWCF sequence. In contrast to NL-432-10F, NL-M20A-10F does not downregulate cell-surface expression of HLA class I molecules (34). A previous study demonstrated the epitope-dependent effect of Nef-mediated HLA class I downregulation on the capacity of HIV-1 specific CTLs to suppress HIV-1 replication. The capacity may be dependent on the expression level of HLAI molecules

A*24:02 (Fig. 5A). The RW8 peptide was buried in the binding groove. In contrast, the two extra amino acids at the C terminus of the RF10 peptide caused a switch of the Pc anchor residues from Trp[8] in RW8 to Phe[10] in RF10, so that the central region (P4–P7) of RF10 protruded out of the groove. Therefore, the solvent-accessible surface area of the central region of RF10 significantly diverged from that of RW8. These dramatic conformational differences explain the lack of cross-reactivity between RW8- and RF10-specific CTLs, as well as

Table I. Statistics for crystallographic data collection and structure refinement

	A24, 8-Mer	A24, 10-Mer
Data collection		
Space group	$P2_1$	C2
Cell dimensions		
a, b, c (Å)	71.95, 152.52, 90.17	161.74, 65.06, 50.42
α, β, γ (°)	90.00, 90.12, 90.00	90.00, 90.23, 90.00
Resolution (Å)	50.0–2.4	50.0–2.4
R_{merge}	0.087 (0.554)[a]	0.102 (0.469)
$I/\sigma I$	13.0 (2.2)	20.0 (2.6)
Completeness (%)	99.3 (99.3)	97.2 (92.6)
Redundancy	3.1 (3.1)	4.1 (3.6)
Refinement		
Resolution (Å)	36.0–2.4	42.7–2.4
No. of reflections	70,383	20,284
R_{work}/R_{free}	0.1952/0.2185	0.1882/0.2223
No. of atoms		
Protein	12,528	3,148
Water	609	238
B factors		
Protein	46.5	38.4
Water	38.0	40.0
R.m.s.d.		
Bond lengths (Å)	0.004	0.003
Bond angles (°)	0.857	0.689
Ramachandran plot (%)		
Most favored regions	87.9	96.3
Allowed regions	12.1	3.7
Disallowed regions	0	0

[a]Values in parentheses are for the highest resolution shell.
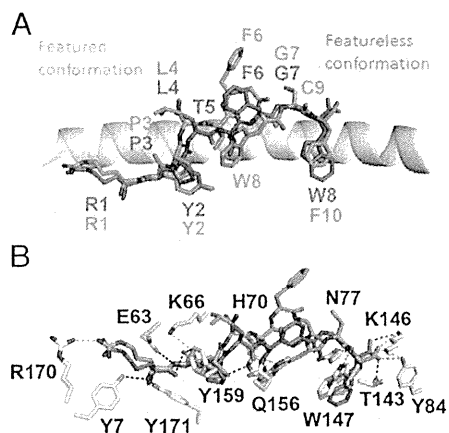R.m.s.d., root mean square deviations.

FIGURE 5. Comparison between the structure of the HLA-A24:02 molecule in complex with the RW8 or RF10 epitope peptide. (A) Superposition of HLA-A*24:02 in complex with RW8 (pink) or RF10 (green) peptides. The peptides are shown in stick-model form. RF10 displays a featured conformation with the residue F6 exposed to the solvent, whereas RW8 displays a featureless conformation, with the residue F6 hidden in the groove. (B) Hydrogen bond interactions between HLA-A*24:02 and RW8 (pink) or RF10 (green) peptide. The hydrogen bonds in common between the two peptides are shown in black.

carrying the epitope peptide (35). RF10-specific CTL clones 170 and 173 completely suppressed the replication of both NL-432-10F and NL-M20A-10F at E:T ratios of 1:1 and 0.1:1, respectively, indicating that RF10-specific CTLs could strongly suppress HIV-1 replication regardless of Nef-mediated downregulation of HLA class I molecules (Fig. 6B). In comparison, the ability of RW8-specific CTL clones 52 and 72 to suppress NL-432-10F was

weaker than that of RF10-specific CTLs, even at an E:T ratio of 1:1, although their respective capacities to suppress NL-M20A-10F were comparable (Fig. 6B). These results indicate that RF10-specific CTLs presented higher Ag sensitivity than did RW8-specific CTLs, implying that the RF10 peptide is more presented on the cell surface than is the RW8 peptide.

In addition to the higher binding affinity of HLA-A*24:02 for the RF10 peptide, we wanted to compare the TCR avidity of RW8- and RF10-specific CD8+ T cell clones. We thus measured TCR avidity of these clones by using the tetramer dilution assay. Twelve clones of each specificity were stained with different concentrations of RW8-tet or RF10-tet (Fig. 6C). The EC50 values of the RF10-specific CTL clones were significantly lower than those of the RW8-specific ones (Fig. 6D), indicating that RF10-specific CD8+ T cells had a higher TCR avidity than did the RW8-specific CD8+ T cells.

Discussion

Screening for optimal CTL epitopes is central for the characterization of antiviral or antitumoral CD8+ T cell responses (36–38). It is not unusual to observe CTL reactivity toward peptides of 8–12 aa in length around an optimal epitope. This observation is thought to reflect the flexibility of TCR–MHC pairing to accommodate peptides close to the optimal one, such that the same CD8+ T cells are able to recognize these peptides (39–41). In the present study, we examined CD8+ T cell responses against two superimposed HIV nef epitopes (RW8 and RF10) restricted by HLA-A*24:02. Using RW8 and RF10 tetramers, we could discriminate between T cells specific for these two peptides and could show that these cells represented two distinct populations with independent reactivity. Furthermore, we applied single-cell TCR analysis to characterize both TCR α- and β-chain repertoires directly
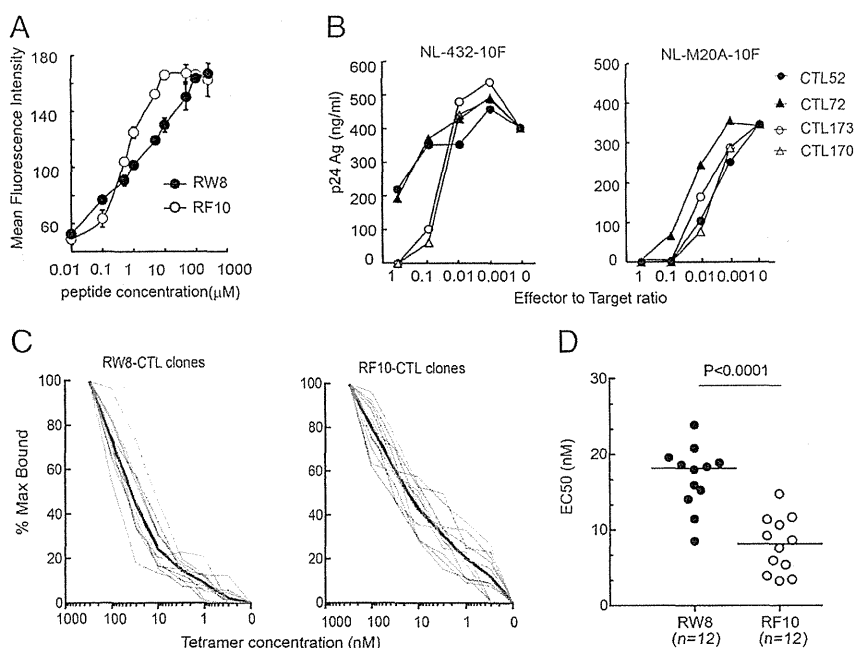

FIGURE 6. Efficacy of superimposed epitope-specific CTLs to suppress HIV-1 replication. (A) Binding of RW8 and RF10 peptides to HLA-A*24:02 molecules on RMA-S-A*2402 cells quantified by use of the HLA-A*24:02 stabilization assay. (B) HIV suppressive capacity of two RW8-specific CTL clones (CTL 52 and 72) and two RF10-specific CTL clones (CTL 170 and 173). Cultured CD4+ T cells derived from an HLA-A*24:02+ donor were infected with NL-432-10F or NL-M20A-10F and then cocultured with the indicated CTL clones at various E:T ratios. HIV-1 p24 Ags in the supernatant were measured on day 6 postinfection by performing an enzyme immunoassay. (C) Normalized MFI of staining with specific tetramers at concentrations from 0.3 to 300 nM for RF10- and RW8-specific CTL clones was used to calculate TCR avidity. Individual clones (gray lines) and the mean value of each group (black line) are shown. (D) TCR avidity of RF10- and RW8-specific CTL clones. The EC50 of tetramer staining (MFI) was calculated for each clone from (C). The statistical comparison was conducted by using the nonparametric Mann–Whitney U test.

from cryopreserved PBMCs and could show that different TCR repertoires were elicited as responses against the two superimposed epitopes. RW8 and RF10 epitopes presented by HIV-1–infected cells are therefore recognized by independent specific T cells. Thus, RYPLTFGWCF (RF10) presented two epitopes to HLA-A*24:02, with each eliciting a distinct CTL response. We analyzed in the present study the ability of CTL clones from a single individual to recognize these epitopes and to suppress HIV-1. Additional analyses using CTL clones from other individuals would be useful to confirm the conclusion of the present study.

Previous studies indicated that HLA-B57–restricted KI8 and KF10 or HLA-B35–restricted VY8 and RY11 superimposed epitopes induce independent CTL responses (7, 10), but that HLA-B54–restricted FV9 and FP10 superimposed epitopes elicit mainly cross-reactive CTLs in HIV-1–infected patients (8). Although the detailed mechanisms remain unclear, these studies suggest that different lengths or conformations of a peptide may determine the nature of the CTL response. Our comprehensive analysis of RW8-specific and RF10-specific CD8$^+$ T cells, showing no overlap or cross-reactivity between these two populations, is in line with a recent report that peptide length determines the outcome of TCR/peptide–MHCI engagement (42). This study shows indeed that a given TCR is predisposed to engage peptides of a defined length so that TCR plasticity and cross-reactivity are strictly restricted to a single MHCI–peptide length.

Emerging evidence also indicates that conformational features of peptides presented in the groove of HLA molecules can partially determine the diversity of the TCR repertoire (43), although consensus is still lacking. It was reported that epitopes with featured conformations are associated with a highly diverse TCR repertoire (44–46) and that a featureless epitope results in the generation a less diverse TCR repertoire (47). However, the opposite result was also reported, with a featureless epitope (HCMV pp65, FPTKDVAL) being associated with diverse TCR usage (48). In the present study, we examined two immunodominant superimposed epitopes, derived from the same antigenic source and restricted through the same MHC allele, and we used unbiased single-cell *TRA* and *TRB* sequence analyses to compare TCR α- and β-chain repertoire diversity in the same individuals. Compared to previous studies, the present one was therefore particularly appropriate for investigating the effect of epitope conformation on TCR repertoire diversity. Our data showed that featured (RF10) and featureless (RW8) epitope conformations were indeed associated with a diverse and restricted TCR repertoire, respectively, in line with the putative availability of clonotypes in the naive T cell pool able to recognize the epitopes.

A diverse TCR repertoire is thought to facilitate the selection of CTLs with high avidity and therefore to influence their functional properties and efficacy against viruses (49–53). We indeed found that the binding affinity of specific tetramers for RF10-specific CTL clones was significantly higher than that for RW8-specific ones, suggesting that the former CTLs had higher TCR avidity than did the latter ones. Moreover, RF10-specific clones presented a stronger ability to suppress HIV-1 in vitro than did RW8-specific clones, and the frequency of RF10-specific CTLs was higher than that of RW8-specific CTLs in HIV-1–infected individuals. Taken together, our data support the idea that the selection of high-avidity TCRs is associated with TCR repertoire diversity and suggest that RF10-specific CTLs exert a superior control of HIV-1 replication in vivo compared with RW8-specific CTLs.

In conclusion, we investigated HLA-A*24:02–restricted CTLs specific for superimposed Nef epitopes, RF10 and RW8, by using multiple approaches. We demonstrated that RW8 and RF10 peptides bound to HLA-A*24:02, resulting in different peptide conformations. This difference was responsible for the induction of totally different CTL responses, that is, no cross-reactivity, distinct TCR repertoires, and different functional avidity. Our study provides a clear demonstration that superimposed epitopes restricted by the same HLA molecule could elicit entirely different CD8$^+$ T cell responses. We show that this difference was linked to featured versus featureless epitope conformations, yielding distinct TCR repertoires for the two CTL populations. The featured RF10 epitope was associated with the induction of T cells carrying TCRs with high diversity and avidity. This finding is directly relevant to our understanding of CD8$^+$ T cell–mediated control of HIV-1, as well as to the choice of immunogens for vaccine design. Our findings indicate that targeting a single viral sequence, for example, RF10, can lead to the induction of two immune responses against HIV and thus enhance the suppression of its replication.

Disclosures
The authors have no financial conflicts of interest.

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Low Prevalence of Transmitted Drug Resistance of HIV-1 During 2008–2012 Antiretroviral Therapy Scaling up in Southern Vietnam

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Background: The recent expansion of antiretroviral therapy (ART) program in resource-limited setting has raised concern about possible transmission of drug resistance (TDR). We assessed the prevalence of TDR over a 5-year period among treatment-naive individuals in Southern Vietnam during rapid ART scale-up.

Methods: Drug resistance mutations among antiretroviral-naive HIV-1–infected patients in Ho Chi Minh City were evaluated prospectively from 2008 to 2012 by HIV-1 pol gene sequencing. TDR was defined according to the World Health Organization list for surveillance of transmitted HIV-1 drug resistance in 2009.

Results: Pol sequence was obtained in 1389 individuals (median age: 30 years, males: 52.3%). Risks of HIV-1 infection included heterosexual contact in 60.7%, injection drug use in 22.4% and both 5.2%. The majority was infected with CRF01_AE (97%), whereas 19 were infected with subtype B. Over the 5-year study period, TDR was detected in 58 individuals (4.18%): 28 (2.02%) against nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 (0.29%) against both NRTIs and NNRTIs. The most common TDR was K103N (0.5%) for NNRTI. The annual prevalence of TDR remained low to moderate (2008: 2.4%; 2009: 5.2%; 2010: 5.48%; 2011: 2.72%; 2012: 5.36%), and there was no clear trend over time.

Conclusions: There was no increase in TDR prevalence in Southern Vietnam during and after the 2008–2012 rapid scale up of ART.

Key Words: HIV, transmitted drug resistance, Vietnam

(J Acquir Immune Defic Syndr 2014;66:358–364)

INTRODUCTION

The recent roll-out campaigns in resource-limited settings to scale up antiretroviral therapy (ART) seem to have improved the morbidity and mortality of HIV-infected individuals. In Vietnam, where the HIV epidemic affected 249,660 individuals including 52,325 AIDS-related deaths up to the end of 2011, a national effort to facilitate ART supply has been implemented, and the ART coverage rate has rapidly increased from 18.1% in 2006 to 53% in 2011, saving 18,110 lives from AIDS-related deaths between 2000 and 2009.[1]

The expansion of ART program, however, has been accompanied by concerns on HIV drug resistance and risk of subsequent transmission of drug resistance (TDR) in new cases of HIV infection.[2] The WHO recommends surveillance of TDR where ART is being scaled up[3,4] and the Vietnam Authority of HIV/AIDS Control issued in 2008 a 5-year plan to assess and prevent HIV drug resistance. Because the large part of HIV epidemic in Vietnam has been driven by intravenous drug users (IDUs),[1,5] it is theoretically possible that the transmission of drug-resistant HIV spreads fast by sharing contaminated needles. The recent increase in HIV transmission by sexual intercourse in Vietnam also makes the TDR problem more difficult to control.[5] In addition, the pattern of antiretroviral drug use has been changing according to the global policy on ART recommendations or increased availability of second-line ART.[6–9] It is therefore important to monitor the prevalence of TDR and its pattern in Vietnam on a regular basis. Previous surveys and studies demonstrated low-to-moderate prevalence of TDR in Vietnam.[10–17] However, those studies were conducted using a cross-sectional setting or included monitoring for only a short period of time. To the best of our knowledge, there are no data on long-term monitoring of the prevalence of TDR in Vietnam.

This study was designed to assess the prevalence of TDR over a 5-year period in HIV-infected treatment-naive

individuals from Southern Vietnam during the 2008–2012 rapid ART scale-up.

METHODS

Study Population

Antiretroviral-naive individuals who visited the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, were enrolled in the study from 2008 until 2012. The enrollment of consecutive antiretroviral-naive patients started in October and ended when 300 enrollments had been achieved. In 2009 and 2012, the enrollment was stopped at 250 and 270, respectively, for the operational reasons. After securing written informed consent, plasma samples were collected and stored at −80°C. At the end of the year's sampling, the frozen plasma samples were shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo, Japan, for genotypic resistance testing. Patients with history of exposure to any antiretroviral drug, including mono or dual therapy were excluded. The study protocol was approved by the institutional ethical review boards of both Hospital for Tropical Diseases in Vietnam and NCGM in Japan (NCGM#360).

Genotypic HIV-1 Resistance Testing and Subtype Determinations

Drug resistance genotyping was performed using in-house protocols at NCGM. Briefly, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription–polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify the pol-reverse transcriptase (RT) and protease (PR) region. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 11.0 (Software Development, Tokyo). The subtypes of HIV-1 were determined by using RT gene with "Genotyping/NCBI" tool using BLAST algorithm (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Drug resistance mutations were identified from the list for surveillance of transmitted drug resistance mutations.[18] All sequences obtained from the study have been deposited in the DNA Data Bank of Japan database (accession no: AB894875 to AB896651).

Statistical Analysis

Differences between 2 groups were tested for statistical significance by using χ^2 test for categorical data and the Mann–Whitney test for continuous variables. Logistic regression model was used to identify the factors associated with infection by TDR. Differences were considered significant if the P value was less than 0.05. Statistical analyses were performed using IBM SPSS Statistics software version 21J (IBM Japan, Inc, Tokyo, Japan).

RESULTS

Characteristics of Study Population

The study enrolled 1426 individuals but 20 were later found to be ineligible after providing written informed consents (previous ART exposure, n = 17, insufficient blood withdrawn, n = 2 had, negative for HIV infection, n = 1). The remaining 1406 participants were assigned to the drug resistance test. The characteristics of these subjects are summarized in Table 1. Approximately 63% of the study participants were men, and the latter were older than females (31 years vs 29 years; $P < 0.001$). The most frequently reported HIV transmission route was heterosexual contact (65.9%), followed by injection drug use (IDU) (29.6%). Very few (0.1%) declared homosexual contact as a risk for HIV infection. The majority of patients with IDU were men, and the percentage of IDUs was greater in men than that in women (men: 42%; women: 3.4%; $P < 0.001$). The proportion of IDUs had decreased over time (35% in 2008, 17.6% in 2012) and the prevalence of hepatitis C infection, which reflects possible multiple needle sharing, had decreased simultaneously. These changes in the study population reflect preponderance of HIV epidemic in male IDUs in the early phase and recent expansion to the general population in Vietnam.[1]

Prevalence of Transmitted Drug Resistance Mutations

Among the 1406 individuals who underwent HIV drug resistance genotyping, we obtained the complete sequences of both PR and RT in 1389 individuals. The majority were infected with CRF01_AE (98%), whereas 17 were infected with subtype B. Over the 5-year study period, drug resistance mutations were detected in 58 individuals (4.18%): 28 (2.02%) against nucleos(t)ide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 against both NRTIs and NNRTIs. Table 2 summarizes the prevalence of the specific drug resistance mutations. The annual prevalence of TDR was persistently low during the study period, ranging from 2.40% to 5.48%, and no clear trend was noted over time. In thymidine analog mutations (TAMs), mutations at codon 215 were the most frequent (0.36%) followed by K219Q (0.22%). In other NRTI-related mutations, V75M and mutations at codon 74 and 184 were relatively frequent, of which V75M was reported previously as frequent d4T-resistance-related mutation among CRF01_AE.[19] We did not identify mutations related to Q151M complex or insertions at codon 69. The most common NNRTI mutation was K103N (0.5%), followed by Y181C (0.43%), G190A and E (0.36%), and mutations at codon 188 (0.22%). The most common PI-associated mutations was M46L (0.43%) and M46I (0.29%) but both were considered polymorphisms.[20,21] All other PI-associated mutations were rare; only 1 among 1389 sample (0.07%) harbored each mutation. Of those, D30N, L76V, and L90M were major mutations, whereas F53Y was not major mutation[20,21] and not clinically significant when it occurred alone without any other PI mutations.

TABLE 1. Patients' Characteristics

		Year of Sampling				
	Total	2008	2009	2010	2011	2012
Patients, n	1406	298	250	294	297	267
Male gender, n (%)	881 (62.7)	213 (71.5)	150 (60)	184 (62.6)	154 (51.9)	180 (67.4)
Age, median (range)	30 (16–66)	29 (16–58)	29 (20–60)	30 (17–55)	31 (19–66)	33 (18–65)
Living in HCMC, n (%)	735 (52.3)	163 (54.7)	132 (52.8)	148 (50.3)	150 (50.5)	142 (53.2)
Time since HIV diagnosis, n (%)						
<6 mo	975 (69.3)	224 (75.2)	181 (72.4)	233 (79.3)	138 (46.5)	199 (74.5)
≥6 mo	431 (30.7)	74 (24.8)	69 (27.6)	61 (20.7)	159 (53.5)	68 (25.5)
Risk of HIV transmission, n (%)						
Heterosexual contact, alone	854 (60.7)	148 (49.7)	143 (57.2)	149 (50.7)	210 (70.7)	204 (76.4)
IDU, alone	315 (22.4)	73 (24.5)	68 (27.2)	90 (30.6)	44 (14.8)	40 (15.0)
Heterosexual and IDU	73 (5.2)	29 (9.7)	5 (2)	3 (1)	29 (9.8)	7 (2.6)
Homosexual contact	2 (0.1)	2 (0.7)	0	0	0	0
Other/unknown	162 (11.5)	46 (15.4)	34 (13.6)	52 (17.7)	14 (4.7)	16 (6.0)
HIV-1 subtype, n (%)						
CRF01_AE	1378 (98.0)	295 (99.0)	246 (98.4)	289 (98.3)	289 (97.3)	255 (95.5)
Subtype B	19 (1.5)	1 (0.7)	4 (1.6)	2 (1)	6 (2)	6 (2.2)
Other/unclassified	9 (0.8)	0	0	2 (0.7)	1 (0.6)	6 (2.2)
HBs antigen positive, n (%)	217 (15.4)	42 (14.1)	43 (17.2)	49 (16.7)	47 (15.8)	36 (13.5)
Anti-HCV antibody positive, n (%)	557 (39.6)	148 (49.7)	106 (42.4)	117 (39.8)	105 (35.4)	81 (30.3)
CD4 cell count, cells/µL, median (range)	110 (1–1322)	70 (1–1042)	115 (1–753)	95 (1–1048)	253 (2–1322)	47 (1–1211)
Plasma HIV-1 RNA levels, log copies/mL, median (range)	5.01 (1.59–6.90)	4.81 (1.69–5.70)	4.38 (1.69–5.70)	5.23 (1.59–6.61)	5.02 (2.31–6.90)	5.38 (1.60–6.83)

HCMC, Ho Chi Minh City; CRF01_AE, circulating recombinant form01_AE; HBs antigen, hepatitis B virus surface antigen; anti-HCV antibody, anti-hepatitis C virus antibody.

The presence of TDR did not correlate with any specific demographic factor, risk group, or year of study enrollment, although the odds ratio of acquiring TDR was relatively low in heterosexual individuals (Table 3). Annual trends of TDR prevalence in particular HIV risk categories are shown in Table 4. TDR prevalence in heterosexual contact alone, IDU alone, and IDU plus heterosexual contact were 3.33%, 5.41%, and 2.78% respectively, which were not statistically different. Although no significant annual trend was noted over the study period among them, the TDR prevalence in the HIV risk group of IDU alone were higher than the WHO first threshold 5% in the year 2009, 2010, and 2012 (4.10% in 2008, 5.88% in 2009, 6.67% in 2010, 2.27% in 2011%, and 7.69% in 2012). Phylogenetic tree analysis showed no clustering of sequences from the study participants with TDR. Details of the 4 individuals with TDR in more than 1 group of antiretrovirals are listed in Table 5. One individual had very extensive resistance: M41L, M184V, T215Y in NRTI-associated mutations, and Y181C and G190A in NNRTI-associated mutations. Overall, persistently low prevalence of TDR during the last 5 years of ART expansion was noted. However, individuals with multiple-drug resistances were identified during ART expansion. This finding highlights the importance of TDR and undermines the efficacy of currently scaled up ART regimens.

DISCUSSION

In this study, we traced the prevalence of TDR over a relatively long period of time (from 2008 to 2012) in treatment-naive individuals in Southern Vietnam during rapid ART scaling up program. Our result of 4.18% of overall TDR prevalence was similar to those described previously in Vietnam.[10–17] However, the study covered longer period of time and demonstrated the stability of TDR prevalence over this period. In comparison, all the other previous surveillance studies conducted in Vietnam were shorter in duration. Primary HIV drug resistance is one of the main concerns in any ART program because it can compromise the clinical outcome of ART, especially in countries with limited ART options. Our data of persistently low prevalence of TDR in Southern Vietnam possibly reflect the success of the recent ART scale-up program in this country.

The TDR rate in our study, however, ranged from 2.4% to 5.5%, reaching the threshold of low prevalence according to the WHO definition (<5%) in 2009, 2010, and 2012.[4] Considering lower viral replication fitness of strains harboring drug resistance mutations than that of wild-type strain, the rate of pretreatment resistance in chronic HIV infection could underestimate the real drug resistance transmission with time since HIV infection. In particular, the low-level prevalence of M184V[22] despite widespread use of lamivudine, which is sometimes used for treatment of hepatitis B virus infection, could be related to the lower viral fitness. Of note, the percentage of individuals diagnosed as HIV positive more than 6 months before study enrollment was higher in 2011 (53.5%) than that in other study periods, and the TDR prevalence in 2011 was lower (2.72%) than that in 2009, 2010, and 2012. Most cases had chronic HIV infection at the time of HIV

TABLE 2. Prevalence of Transmitted Drug Resistance Mutations

	Total	2008	2009	2010	2011	2012
Study population (n)	1389	292	250	292	294	261
Any TDR [n (%)]	58 (4.18)	7 (2.40)	13 (5.20)	16 (5.48)	8 (2.72)	14 (5.36)
RT in total [n (%)]	43 (3.10)	7 (2.40)	9 (3.60)	14 (4.79)	4 (1.36)	10 (3.83)
NRTI [n (%)]						
Any	28 (2.02)	3 (1.03)	6 (2.40)	11 (3.76)	3 (1.02)	5 (1.92)
Thymidine analog mutations						
M41L	2 (0.14)			1	1	
D67N	1 (0.07)		1			
D67E	1 (0.07)			1		
K70E	1 (0.07)			1		
T215Y	1 (0.07)				1	
T215I	1 (0.07)		1			
T215S	1 (0.07)				1	
T215D	2 (0.14)		2			
K219Q	3 (0.22)		1	2		
Others						
K65R	2 (0.14)			2		
L74V	1 (0.07)	1				
L74I	4 (0.29)	1		2		1
V75M	6 (0.43)	1		2		3
M184V	3 (0.22)		1		2	
M184I	2 (0.14)			1		1
NNRTI [n (%)]						
Any	19 (1.37)	5 (1.71)	3 (1.20)	4 (1.37)	3 (1.02)	4 (1.53)
K101E	4 (0.29)	1	2	1		
K103N	7 (0.50)	1	1	1		4
Y181C	6 (0.43)	1		2	1	2
Y188L	1 (0.07)				1	
Y188H	1 (0.07)			1		
Y188C	1 (0.07)			1		
G190A	4 (0.29)	2		1	1	
G190E	1 (0.07)				1	
PI [n (%)]						
Any	15 (1.08)	0	4 (1.60)	2 (0.68)	4 (1.36)	5 (1.92)
D30N	1 (0.07)				1	
M46I	4 (0.29)		2			2
M46L	6 (0.43)		1		3	2
M46I/L	1 (0.07)			1		
F53Y	1 (0.07)			1		
L76V	1 (0.07)					1
L90M	1 (0.07)		1			

diagnosis, and the exact latency from infection to diagnosis or to study enrollment was unavailable. Thus, the longer duration from diagnosis to study participation allows more frequent reversion from TDR into wild-type virus. This should be taken into account in the interpretation of the results of the study.

Although our study participants did not represent the national HIV-infected population in Vietnam but were rather HIV-infected individuals living in or near Ho Chi Minh City (HCMC), their age, sex, and the distribution of HIV risks were almost comparable with the national HIV-infected population in Vietnam. Notably, HCMC accounts for approximately 50% of the entire population receiving ART in Vietna,[12] and ART had been widely accessible in HCMC since the early phase of ART scale-up or even before ART scale-up at private clinics. Since previous studies had predicted increased TDR rates after 5–8 years of ART scale-up,[2] HIV-infected individuals in HCMC are considered to be at higher risk of TDR compared with those in other areas of Vietnam. In addition, a previous study conducted in HCMC showed that 73% of patients on ART reported having injected drugs,[1] and the sentinel surveillance in 2009 showed that HCMC had high HIV prevalence among IDUs (46%).[1] Since IDU is considered a risk factor for poor adherence and emergence of drug resistance,[23,24] patients in HCMC are considered the key population for TDR monitoring. Although no statistical relationship was