Table 3. Univariate Analysis of Risks for Kidney Tubular Dysfunction in Patients With HIV Infection Treated With Tenofovir

Characteristic	OR	95% CI	*P* Value
Female sex	1.844	.204–16.67	.586
Age per 1 year	1.165	1.100–1.233	<.001
Weight per 1 kg decrement	1.076	1.021–1.135	.007
CD4 count per 1/μL decrement	1.002	.999–1.004	.261
Baseline eGFR per 1 mL/minutes/1.73 m^2 decrement	1.052	1.016–1.090	.004
Concurrent use of nephrotoxic drugs	1.559	.322–7.555	.581
Hepatitis B	0.721	.156–3.319	.674
C-reactive protein per 1 mg/dL	1.551	.689–3.494	.289
Hypertension	2.234	.843–5.922	.106
Dyslipidemia	0.578	.183–1.823	.349
Duration of treatment with tenofovir disoproxil fumarate (weeks)	0.999	.992–1.007	.888
ABCC2			
−24 CC	10.50	1.369–80.55	.024
1249 AA	7.828	1.609–38.10	.011
−24 CC plus 1249 AA	31.88	3.131–324.5	.003
2934 GG	1.358	.167–11.07	.775
ABCC4			
559 TT	4.912	.837–28.81	.078
912 TT	1.466	.531–4.042	.460
2269 AA	2.756	.530–14.34	.228
3348 GG	1.950	.510–7.463	.329
4135 GG	1.254	.450–3.494	.665
4976 CC	2.462	.925–6.547	.071
ABCC10			
526 GG	1.158	.360–3.725	.805
2759 TT	0.619	.220–1.738	.363
ABCB1			
2677 AA	7.828	1.609–38.10	.011

Abbreviations: CI, confidence interval; eGFR: estimated glomerular filtration rate; HIV, human immunodeficiency virus; OR, odds ratio.

[a] Due to low prevalence of minor alleles, rs56220353, rs11568630, and rs2274407 were not included in this analysis.

associated with tenofovir-induced KTD (OR, 2.497; 95% CI, .902–6.949; $P = .077$).

DISCUSSION

The present study demonstrated that genotype CC at position −24 and genotype AA at position 1249 of *ABCC2* gene are associated with tenofovir-induced KTD in Japanese patients with HIV-1 infection. The effect of SNPs was more evident in patients with both −24 CC and 1249 AA homozygotes than in those with either homozygote only. The findings of this study resolve long-term controversy over the role of genetic

Table 4. Multivariate Analysis for the Risk of Tenofovir-Induced Kidney Tubular Dysfunction With Homozygotes at −24 and 1249 of *ABCC2* in Patients With HIV Infection

ABCC2	Adjusted OR	95% CI	*P* Value
Homozygote at −24 CC	20.08	1.711–235.7	.017
Homozygote at 1249 AA	16.21	1.630–161.1	.017
Homozygotes at −24 CC plus 1249 AA	38.44	2.051–720.4	.015

Each variable was adjusted for sex, age, weight, estimated glomerular filtration rate, and hypertension.

Abbreviations: CI, confidence interval; OR, odds ratio.

polymorphisms in tenofovir-induced KTD and confirm the effect of the SNPs in *ABCC2* gene in tenofovir-induced KTD.

CA haplotype (−24C, 1249A) of *ABCC2* was associated with tenofovir-induced KTD, whereas TG was a protective haplotype (Table 5). Izzedine et al [13] reported the role of CATC haplotype (−24C, 1249A, 3563T, 3972C) of *ABCC2* in KTD. However, 3563T did not play such role in this haplotype analysis, because the prevalence of 3563T is 0% in the Japanese, according to the HapMap data, and haplotype with only −24C plus 1249A still exhibited its effect on tenofovir-induced KTD (Table 5; www.hapmap.org). The reported association between tenofovir-induced KTD and 526G and 2759C of *ABCC10* described by Pushpakom et al [21] was also not reproduced in this study. Furthermore, SNPs in *ABCC4*, *SLC22A6*, and *ABCB1* investigated in the present study did not show a significant association with tenofovir-induced KTD (Table 3).

Three main aspects of our study are important. First, this is the first study to our knowledge that elucidated the effect of SNPs on tenofovir-induced KTD conducted in a country other than European countries or the United States. Our study examined Japanese patients of genetic background different from patients of previous studies, which consisted mostly of whites. While SNPs −24C and 1249A of *ABCC2* have been speculated to correlate with tenofovir-induced KTD in previous studies, the present study confirmed that these SNPs are risk factors for tenofovir-induced KTD in nonwhites.

The result that the SNPs in *ABCC2* are a risk for tenofovir-induced KTD can also be applied to patients with other genetic backgrounds who host SNPs −24C and 1249A. Notably, the impact of SNPs on tenofovir-induced KTD might be more significant in Africans and Indians than in Japanese or whites, considering that the allele frequencies of −24C and 1249A are higher in these population according to the HapMap data (−24C; Africans 96.9%, Indians 92.6%, Japanese 80.8%, whites 81.9%, 1249A; Africans 21.7%, Indians 30.7%, Japanese 8.9%, whites 23.7%; www.hapmap.org).

Second, the study was designed to evaluate the exclusive effect of SNPs on tenofovir-induced KTD by excluding

Table 5. Association Between Haplotype in *ABCC2* and *ABCC4* and Kidney Tubular Dysfunction

SNP Marker/Haplotype	Allele	Allele/Haplotype Frequency, %		OR (95% CI)[a]	*P* Value
		KTD Group (n = 19)	Control Group (n = 171)		
ABCC2					
−24 C → T	C	97.4	78.4	10.22 (1.658–419.8)	.003
1249 G → A	A	28.9	12.3	2.91 (1.345–6.296)	.011
ABCC2 haplotype	CA	28.9	12.3	2.91 (1.295–6.221)	.011
	TG	2.6	21.6	0.098 (.002–.603)	.003
ABCC4					
559 G → T	T	21.1	12.3	1.905 (.705–4.614)	.213
4976 T → C	T	48	55.3	0.746 (.375–1.470)	.399
ABCC4 haplotype					
TT	TT	17.6	7.9	2.497 (.902–6.949)	.077

Abbreviations: CI, confidence interval; KTD, kidney tubular dysfunction; OR, odds ratio; SNP, single-nucleotide polymorphism.

[a] ORs and *P* values are for comparisons of allele/haplotype frequencies between the kidney tubular dysfunction and control groups.

possible predisposing factors for KTD, for example, active infection, malignancies, diabetes mellitus, and preexisting renal impairment, which are known risks for KTD [35]. Patients who showed no HIV-1 viral suppression were also excluded. Furthermore, the enrolled patients were Japanese only, and this helped to examine a study population with comparatively similar genetic background. The study population was also on the same antiretroviral regimen (ritonavir-boosted darunavir plus tenofovir/emtricitabine), and this also helped to evaluate more precisely the effect of SNPs, because plasma concentration of tenofovir is affected by concomitant antiretrovirals and the delta change in plasma tenofovir concentration likely differs in the presence of each concomitant drug [26].

Third, SNPs were examined in 190 patients in this study. To our knowledge, the number of enrolled patients is the largest among the studies that have so far examined the effect of SNPs on tenofovir-induced KTD. Thus, this feature provided the study a higher statistical power than previous studies.

Why are polymorphisms in *ABCC2* a risk for tenofovir-induced KTD, even though it is controversial whether MRP2 plays a role in the excretion of tenofovir via the luminal membrane? [18, 20] The exact mechanism has not been determined yet, but we speculate 2 hypotheses. First, there might be unknown endogenous substances that influence tenofovir nephrotoxicity in renal tubular cells, and SNPs in *ABCC2* modulate the function or transportation of such substances [15]. Second, MRP2 may indeed take part in transporting tenofovir, because various substances including methotrexate are reported to be a substrate of MRP2, and *ABCC2* mutation alters excretion of those substances [36, 37]. Further studies are warranted to elucidate the exact mechanism of these SNPs on tenofovir-induced KTD. Furthermore, the impact of these SNPs on KTD with long-term TDF use needs to be evaluated in prospective studies.

Several limitations need to be acknowledged. First, not all polymorphisms in genes of the targeted transporter proteins were examined. Thus, we might have missed other important SNPs on the function of tenofovir transportation. There might be other unknown transporter proteins for tenofovir excretion in the kidney that contribute to susceptibility to tenofovir-induced KTD as well. Second, the diagnostic criteria for TDF-induced KTD are not uniformly established in the field and are different in the published studies. The criteria applied in this study are not entirely similar to the ones used in previous studies that examined the role of SNPs in tenofovir-induced KTD. However, by excluding other predisposing factors for KTD and enrolling a large number of patients, this study succeeded in providing a clear-cut association between SNPs and tenofovir-induced KTD.

In conclusion, the present study demonstrated that SNPs in *ABCC2* associate with tenofovir-induced KTD in Japanese patients, in a setting that excluded other predisposing factors. Assessment of renal tubular function is more cumbersome and costly to monitor than serum creatinine. However, monitoring tubular function is clinically important, because undetected long-term tubular dysfunction might lead to premature osteopenia due to phosphate wasting and accelerated progression of renal dysfunction. Close monitoring of tubular function is warranted in patients with *ABCC2* −24C and 1249A under TDF treatment.

Notes

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Research Letter

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Once-daily darunavir/ritonavir plus abacavir/lamivudine versus tenofovir/emtricitabine for treatment-naïve patients with baseline viral load >100,000 copies/mL

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The efficacy and safety of fixed-dose abacavir/lamivudine against tenofovir/emtricitabine, both with once-daily darunavir/ritonavir, was examined in 80 treatment-naïve patients with baseline HIV-1 viral load >100,000 copies/ml. The time to virologic failure by 48 weeks was not different between the two groups. The percentage of patients with viral suppression was not significantly different with per protocol population. Tenofovir/emtricitabine showed better tolerability; more patients on abacavir/lamivudine changed regimen than those on tenofovir/emtricitabine. A randomized trial to elucidate the efficacy and safety of these two regimens is warranted.

Little information is available on the efficacy and safety of antiretroviral therapy (ART) of ritonavir-boosted darunavir (DRV/r) plus fixed-dose abacavir/lamivudine (ABC/3TC) [1,2]. DRV/r is a protease inhibitor with proven efficacy and safety, and with high barrier to drug resistance [3,4]. ABC/3TC is an alternative choice of nucleoside reverse transcriptase inhibitors (NRTI) in the American Department of Health and Human Services Guidelines [5]. Here we conducted a single-center, observational pilot study to compare the efficacy and safety of DRV/r plus ABC/3TC versus TDF/FTC in patients with baseline HIV-1 viral load (VL) >100,000 copies/ml. Subjects with such VL were chosen because ACTG 5202 demonstrated that the time to virologic failure (VF) was significantly shorter with ABC/3TC than with TDF/FTC in patients with VL >100,000 copies/ml on efavirenz or ritonavir-boosted atazanavir [6]. All subjects were treatment-naïve who commenced once-daily DRV/r plus either fixed-dose ABC/3TC or TDF/FTC from November 2009 to August 2011 at the AIDS Clinical Center, Tokyo. Baseline data (basic demographics, CD4 count, and VL) were collected. VL was measured by Cobas TaqMan HIV-1 real-time PCR version 1.0 assay (Roche Diagnostics, NJ) to the end of November 2011, and later by Cobas TaqMan version 2.0 assay. It was the decision of the attending physician to start ART with either TDF/FTC or ABC/3TC, because the Japanese guidelines consider both TDF/FTC and ABC/3TC the preferred NRTIs [7].

The efficacy outcomes were the time from commencing ART to VF (defined as VL >1,000 copies/ml at or after 16 weeks and before 24 weeks, or >200 copies/ml at or after 24 weeks) [6], and the proportion of patients with VL < 50 copies/ml at 48 weeks regardless of previous VF. The tolerability outcome was the time to any regimen modification. Intent-to-treat (ITT) population, comprising all subjects, was used for all efficacy and tolerability analyses, while per protocol population was used in the efficacy analysis of the suppressed VL. Censored cases represented those who dropped out, referred to other facilities, or reached 48 weeks. Time-to-event distributions were estimated using the Kaplan-Meier method. Uni- and multivariate Cox hazards models estimated the impact of ABC/3TC use over TDF/FTC on the incidence of VF.

The study included 80 patients [ABC/3TC: 21, TDF/FTC: 59, median age: 37.9 years, males: 74 (92.5%), East Asian origin: 72 (90%)], of whom 66 (82.5%) were infected with HIV-1 through homosexual contact. Patients on ABC/3TC had lower baseline CD4 count (46/μl versus 100, $P=0.031$), higher VL (5.75 log10/ml versus 5.58, $P=0.044$), and more likely to have history of AIDS (71.4% versus 37.3, $P=0.010$), than patients with TDF/FTC. All subjects were HLA-B*5701-negative, and all underwent HIV-1 drug-resistance tests before commencement of ART and none had resistant mutations.

The time to VF with ABC/3TC [3 patients (14.3%)] was not significantly different from that with TDF/FTC [4 (6.8%)] by 48 weeks (Fig. 1a), by univariate and multivariate analyses adjusted by CD4 count and VL (HR, 2.651; 95% CI, 0.592–11.88; $P=0.203$, adjusted HR, 1.589; 95% CI, 0.341–7.401; $P=0.555$). At week 48, ITT analysis showed more patients with TDF/FTC had VL of <50 copies/ml (ABC/3TC: 38.1%, TDF/FTC: 64.4%, $P=0.043$) (Fig. 1c), whereas with per protocol analysis, no difference was noted (ABC/3TC: 57.1%, TDF/FTC: 73.1%, $P=0.328$) (Fig. 1d).

Among the seven patients with VF, three (ABC/3TC: 1, TDF/FTC: 2) achieved sustained VL suppression after week 60 of the initial regimen. The other four underwent drug-resistance tests. One on ABC/3TC was switched to TDF/FTC at week 41; however, viral suppression was not achieved until raltegravir was added at week 74. The

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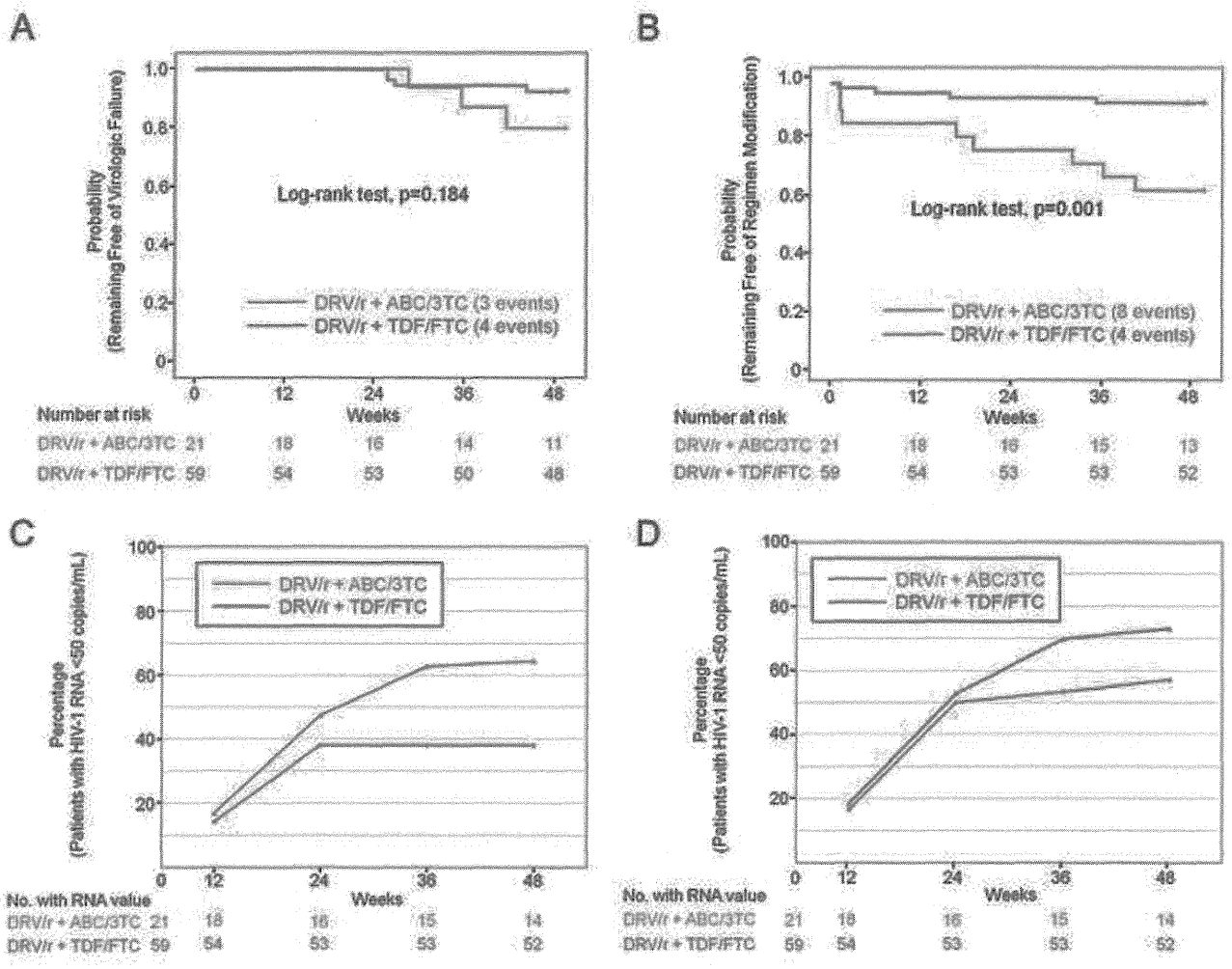

Fig. 1. Efficacy and tolerability results over 48 weeks. (a) Time to protocol-defined virologic failure. (b) Time to tolerability endpoint, defined as first change in treatment regimen. Percent of patients with HIV-1 RNA <50 copies/ml at week 12, 24, 36, and 48, regardless of previous virologic failure, with (c) intention-to-treat population, and with (d) per protocol population.

other with ABC/3TC was switched to TDF/FTC at week 49 and achieved viral suppression despite emergence of protease mutation M46I. Another patient on TDF/FTC had persistent viremia (100–200 copies/ml) without mutation. Another patient on TDF/FTC showed emergence of reverse transcriptase mutation V75L and viremia persisted with 200–500 copies/ml. Reverse transcriptase mutation M184I/T/V did not emerge in any patients.

More patients on ABC/3TC changed or discontinued the initial regimen during the research period [ABC/3TC: 8 (38.1%), TDF/FTC: 4 (6.8%), $P=0.001$] (Fig. 1b). Six [ABC/3TC: 4 (19%), TDF/FTC: 2 (3.4%)] changed ART due to adverse events or VF [ABC/3TC: VF ($n=1$), limb paresthesia ($n=1$), and nausea ($n=2$); TDF/FTC: tenofovir nephrotoxicity ($n=2$)]. None developed ABC-associated hypersensitivity.

This is the first comparison report of the efficacy and safety of ABC/3TC against TDF/FTC with DRV/r in treatment-naïve patients with VL >100,000 copies/ml. The time to VF by 48 weeks was not different between the two groups. Although a higher percentage of patients on TDF/FTC showed viral suppression than those on ABC/3TC at week 48 with ITT population, the difference was not significant with per protocol population. TDF/FTC showed better tolerability, as more patients on ABC/3TC changed regimen than those on TDF/FTC.

These results need to be interpreted with caution, because the baseline characteristics of patients of the two groups were not well-matched due to the nature of the observational study, and this study did not have sufficient power due to the small number of enrolled patients. Because our patients had small stature with median body weight of 58.1 kg, a risk factor for TDF nephrotoxicity, it

is sometimes our practice to avoid TDF in patients with multiple risks, such as advanced HIV-1 infection, to prevent possible acute kidney injury [8–10]. This is presumably the reason for prescribing ABC/3TC to patients with worse disease condition in this study. This allocation bias might have worked as a disadvantage for the efficacy and tolerability results of ABC/3TC.

The usefulness of ABC/3TC has recently received higher recognition than it did in the past; the FDA meta-analysis did not confirm the association between ABC use and myocardial infarction [11], and it became clear that TDF use is associated with decreased bone mineral density and renal dysfunction, both of which might develop into serious complications with long-term TDF use [12–17]. Thus, once-daily DRV/r, a protease inhibitor with high barrier to drug resistance, plus ABC/3TC could be good alternative, especially in patients who cannot tolerate TDF. A randomized trial to elucidate the efficacy and safety of ABC/3TC and TDF/FTC with once-daily DRV/r is warranted.

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ORIGINAL ARTICLE

Assessing recovery of renal function after tenofovir disoproxil fumarate discontinuation

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Abstract Impaired renal function caused by tenofovir disoproxil fumarate (TDF) is considered reversible by discontinuing TDF administration, but there are occasional cases of incomplete recovery. We investigated the recovery of renal function after the discontinuation of TDF. Subjects comprised patients who had been started on TDF but in whom it was later discontinued because of impaired renal function. We investigated renal function until 96 weeks after the discontinuation of TDF, and the duration of TDF administration, up to May 2010. TDF was discontinued because of impaired renal function in 21 of 766 patients (2.7%). Following discontinuation, a significant recovery was seen in eGFR ($p = 0.003$). The median duration of administration was 28 days (6–941 days) in 9 patients whose eGFR recovered to pre-administration levels, 405 days (250–1,379) in 7 patients in whom mild recovery was seen, and 1,110 days (421–1,470) in 5 patients in whom eGFR was much lower than at the time of discontinuation. A significant correlation was seen between the eGFR recovery rate and the duration of TDF administration. TDF administration was discontinued because of renal impairment in 2.7% of patients. The duration of TDF administration was short in patients whose renal function recovered to pre-administration levels, but patients in whom sufficient recovery was not seen after discontinuation had received TDF over long periods and included many whose renal function gradually declined, even after discontinuation. Recovery of renal function after discontinuation of TDF is likely affected by the duration of TDF administration.

Keywords HIV · Antiretroviral therapy · Tenofovir · Renal function · Nephrotoxicity · Reversibility

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Introduction

Tenofovir disoproxil fumarate (TDF) is recommended in the major guidelines as the first-choice nucleoside reverse transcriptase inhibitor (NRTI) that can be administered once daily [1]. However, mild and sometimes severe renal impairment have been reported in patients taking TDF [2, 3], and clear standards for discontinuation have not been defined. Renal impairment caused by TDF is thought to be reversible after the discontinuation of TDF, but cases in which renal function does not recover even after discontinuation are occasionally reported. In this study, we retrospectively investigated the recovery of renal function in patients following the discontinuation of TDF.

Patients and methods

Subjects were Japanese outpatients in the Osaka National Hospital Department of Infectious Disease who began antiretroviral therapy (ART), including TDF, by May 2010

and in whom TDF was discontinued because of renal impairment. The changes in estimated glomerular filtration rate (eGFR)[1] up to 96 weeks after discontinuation of TDF were evaluated using the Wilcoxon signed-rank test. In addition, patients discontinuing TDF administration were divided into three groups based on the eGFR recovery rate until 96 weeks after the discontinuation of TDF: (1) patients whose eGFR recovered to 100% pre-administration levels (recovery group), (2) patients whose eGFR recovered to ≥20% of the level at the start of administration (mild recovery group), and (3) patients in whom no recovery from the level at discontinuation or exacerbation was seen (exacerbation group). The eGFR recovery rate and duration of TDF administration were investigated in these groups, and the correlation between eGFR recovery rate and duration of TDF administration was evaluated using Spearman's rank correlation test.

Results

ART including TDF was started in 766 patients; TDF caused renal impairment and was discontinued in 21 of these patients (2.7%). TDF was discontinued in all these patients because serum creatinine (sCr) had risen to abnormal levels with the administration of TDF.

The characteristics of the patients are shown in Table 1. The median age of patients in whom TDF was discontinued was 45 years (range 25–61), and included 20 men and 1 woman. TDF was the first treatment in 15 patients and the continuing treatment in 6. AIDS had developed in 13 patients. The eGFR (median) at the start of TDF administration was 74.7 mL/min/1.73 m^2 (range 48.1–289.3), the duration of TDF administration (median) was 57 weeks (range 1–210), and the observation period (median) after TDF discontinuation was 131 weeks (range 20–284).

Changes in eGFR for the 21 patients in whom TDF was discontinued are shown in Fig. 1. A decrease was seen from 74.7 mL/min/1.73 m^2 (interquartile range 65.8–83.9) to 48.3 mL/min/1.73 m^2 (interquartile range 45.3–54.3). After the discontinuation of TDF, eGFR recovered rapidly for 12 weeks, after which significant recovery was seen until 96 weeks. At 96 weeks after TDF administration, eGFR was 65.9 mL/min/1.73 m^2 (interquartile range 50.1–82.8) (vs. time of discontinuation, $p = 0.0003$). The most improved eGFR up to 96 weeks after discontinuation was 67.2 mL/min/1.73 m^2 (interquartile range 59.6–85.3) (vs. time of discontinuation, $p < 0.0001$).

The eGFR recovery rate until 96 weeks after the discontinuation of TDF and the duration of TDF administration are shown in Table 2. Nine patients (42.9%; recovery group) showed eGFR recovery after the discontinuation of TDF to the level seen pre-administration. Seven patients (33.3%; mild recovery group) showed eGFR recovery after discontinuation, but not to the level seen at the start of administration. Five patients (23.8%; exacerbation group) showed worsening of the eGFR after discontinuation. Changes in eGFR after discontinuation are shown in Fig. 2. The duration of TDF administration was 28 days (range 6–941 days) in the recovery group, 405 days (range 250–1,379) in the mild recovery group, and 1,110 days (range 421–1,470) in the exacerbation group. Recovery of eGFR was quicker with a shorter duration of TDF administration. Spearman's rank correlation test for eGFR recovery rate and duration of TDF administration showed a significant negative correlation ($r = -0.73$, $p = 0.0002$).

Discussion

Based on its effective anti-HIV activity and the simplicity of being taken once a day, TDF is recommended as the first-choice NRTI in major guidelines and is widely used. However, renal impairment as an adverse reaction has been reported. The renal impairment caused by TDF is thought to be an impairment in renal tubular function, but the detailed mechanism remains unknown. Tenofovir, the active component of TDF, is almost entirely eliminated unchanged in the urine, but a portion is taken up by tubular cells from the blood mainly via organic anion transporters 1 and 3 (OAT1 and OAT3), and eliminated in urine via multidrug resistant protein 4 (MRP4), a carrier protein [4]. It has been suggested that tenofovir contributes to mitochondrial toxicity in tubular cells in this transport process and leads to impairment of renal function [5, 6].

In observational studies in other countries investigating renal impairment caused by TDF, Nelson et al. reported elevated sCr of grade 1 or higher in 2.2% of patients [7], and Madeddu et al. [8] reported elevated sCr of grades 2–4 in 2.5% of patients. The incidence of renal impairment in these studies was similar to that in the Japanese patients in the present study.

In the investigation at our hospital, there was a significant decrease in eGFR from the level at the start of administration in the 21 patients in whom TDF was discontinued compared with all patients (median: all patients 120.0 mL/min/1.73 m^2 vs. discontinuation patients 74.7 mL/min/1.73 m^2; $p < 0.0001$). When administering TDF to patients with decreased renal function, it is advisable to select a drug after adequately evaluating renal function using eGFR or other parameters. In addition, when other causes of renal

[1] GFR calculation for Japanese (2008 Japanese Society of Nephrology calculation): eGFR (mL/min/1.73 m^2) = $194 \times Cr^{-1.094} \times age^{-0.287}$ (for women, ×0.739).

Table 1 Characteristics of patients

Variable	No. of patients (%)	
	All patients (n = 766)	TDF discontinuation patients (n = 21)
Sex		
Male	745 (97.3)	20 (95.2)
Female	21 (2.7)	1 (4.8)
Subjects		
Naïve patients	529 (69.1)	15 (71.4)
Experienced patients	237 (30.9)	6 (28.6)
Development of AIDS	167 (21.8)	13 (61.9)
Third drug		
EFV	268 (35.0)	4 (19.0)
ATV/r	240 (31.3)	7 (33.3)
LPV/r	136 (17.8)	7 (33.3)
Others	403 (52.6)	3 (14.3)
Variable	Median (range)	
	All patients (n = 766)	TDF discontinuation patients (n = 21)
Age (years)	37 (18–73)	45 (25–61)
HIV RNA (copies/mL)	55,000 (<40–31,700,000)	95,000 (<40–3,500,000)
CD4 cell count (cells/mm^3)	205 (0–1,700)	110 (6–647)
eGFR (mL/min/1.73 m^2)	120.0 (37.4–326.7)	74.7 (48.1–289.3)
TDF duration (weeks)	107 (1–393)	57 (1–210)
Observation period after TDF discontinuation (weeks)	–	131 (20–284)

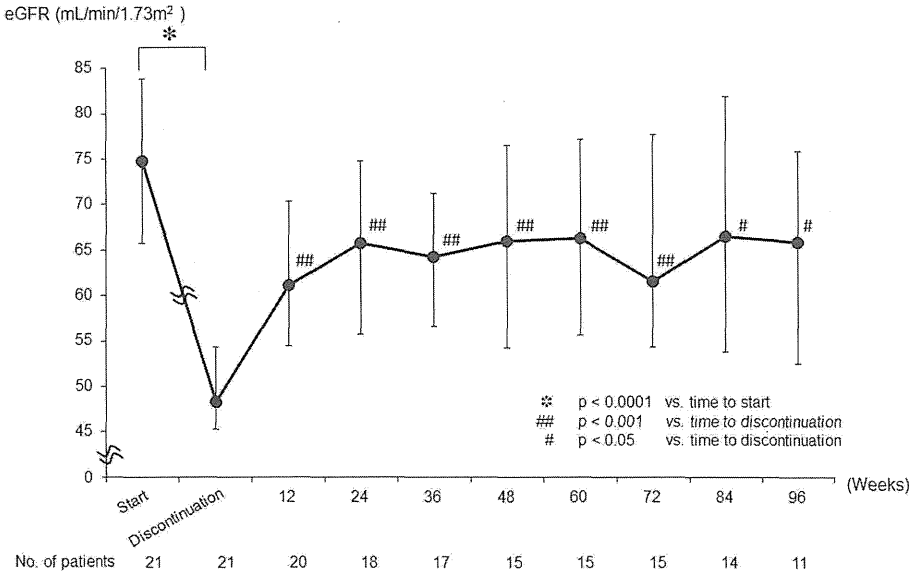

Fig. 1 Changes in eGFR (median ± interquartile range) in TDF discontinuation patients. The trend in eGFR (median ± interquartile range) after the discontinuation of TDF in 21 patients is shown

impairment were investigated in the 21 patients in whom TDF was discontinued, 13 (61.9%) had developed AIDS and received nephrotoxic drugs for treating opportunistic infections, 8 (38.1%) smoked, 5 (23.8%) had dyslipidemia, 2 (9.5%) had hypertension, and 2 (9.5%) had diabetes. Among these 21 patients, AIDS was particularly common, and it presented with *Pneumocystis jirovecii* pneumonia (n = 8), cytomegalovirus infection (n = 3), oropharyngeal candidiasis (n = 2), progressive multifocal leukoencephalopathy (n = 2), mycobacterium tuberculosis infection (n = 1), cryptococcal infection (n = 1), and *Mycobacterium avium* complex infection (n = 1). Furthermore, the most commonly used treatment drug for these opportunistic infections was trimethoprim-sulfamethoxazole (n = 7),

Table 2 eGFR recovery rate and duration of TDF administration in TDF discontinuation patients

	No. of patients (%)	Median (interquartile range)	
		eGFR at TDF discontinuation (mL/min/1.73 m^2)	Duration of TDF administration (days)
(1) Recovery group			
Patients with 100% recovery of eGFR after TDF discontinuation	9 (42.9%)	48.1 (46.6–57.7)	28 (6–941)
(2) Mild recovery group			
Patients with mild recovery of eGFR after TDF discontinuation	7 (33.3%)	48.2 (41.4–51.7)	405 (250–1,379)
(3) Exacerbation group			
Patients with exacerbation of eGFR after TDF discontinuation	5 (23.8%)	49.2 (48.3–52.3)	1,110 (421–1,470)

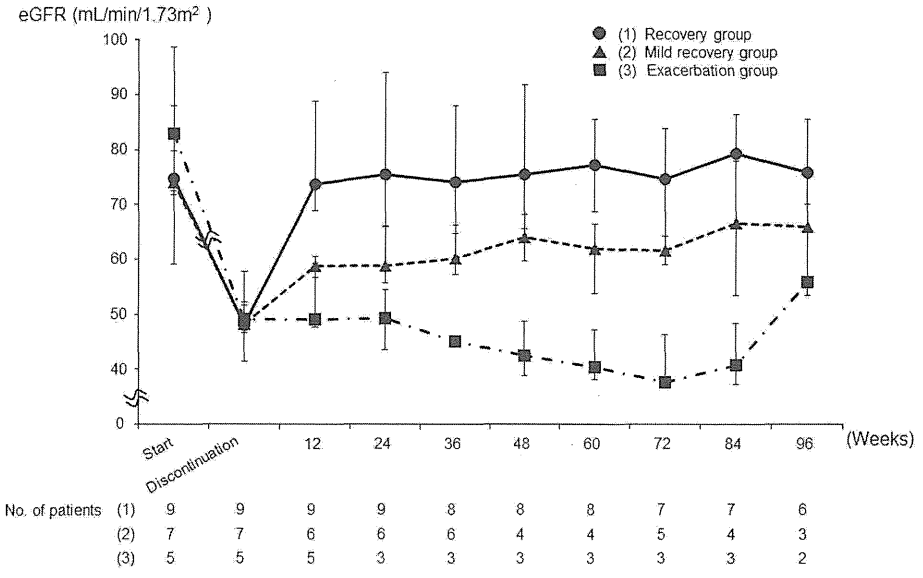

Fig. 2 Changes in eGFR (median ± interquartile range) in each eGFR recovery rate group of TDF discontinuation patients. TDF discontinuation patients were divided into a recovery group (9 patients), mild recovery group (7 patients), and exacerbation group (5 patients), and the changes in eGFR (median ± interquartile range) after discontinuation are shown for each group

followed by ganciclovir and fluconazole ($n = 4$ each), rifabutin, pentamidine, sulfamethoxazole, and azithromycin ($n = 2$ each), and isoniazid, ethambutol, amikacin, and clarithromycin ($n = 1$ each). Most patients received combination therapy with 2–4 of these drugs. In AIDS patients, there is a possibility of deceased renal function both from AIDS itself and from drugs used to treat opportunistic infections; thus, sufficient caution is needed in these patients.

The renal impairment caused by TDF is thought to be reversible with the discontinuation of TDF [9–12], but in investigations using eGFR, there are patients in whom a full recovery is not seen following the discontinuation of TDF [13, 14]. In the present results, eGFR recovered rapidly for 12 weeks after discontinuation, and significant recovery was seen until 96 weeks. However, examining individual cases revealed that some patients exhibited only a mild recovery of eGFR after the discontinuation of TDF, or even exacerbated decreases in eGFR from the level at the time of discontinuation. Recovery of eGFR to the level at the start of TDF was seen in 42.9% of patients, similar to the 42% reported by Wever et al. [14]. The fact that patients are occasionally seen in whom renal function does not recover even after discontinuation suggests the possibility that impairment of renal function may be irreversible after the discontinuation of TDF, depending on the patient.

To investigate the possible factors related to this irreversible impairment of renal function, we classified the trends in eGFR following TDF discontinuation into a recovery group, a mild recovery group, and an exacerbation group, and investigated the effect of duration of TDF administration. The recovery of eGFR was quicker with shorter durations of TDF administration. Full recovery was not seen in patients who received TDF over a long period and in whom renal function declined gradually. In 5 of 9 patients in the recovery group, renal function declined rapidly within 1 month of the start of TDF administration, and in each of these 5 cases, eGFR recovered quickly after discontinuation to the level at the start of administration.

In the mild recovery and exacerbation groups, however, none of the patients showed a rapid decline in renal function within 1 month. The decline was gradual over a long period in nearly all of these patients, and insufficient recovery or an exacerbation in eGFR was seen after discontinuation. Therefore, in patients who receive TDF over a long period, a state of gradual decline in renal function continues with accumulating damage to renal tubules from TDF, which is a likely factor in the irreversibility of damage to renal function after the discontinuation of TDF. The analysis performed in this study was done with a limited number of patients, so the factors related to irreversible damage to renal function after discontinuation of TDF could not be fully clarified. However, a correlation was confirmed between the duration of TDF administration and the eGFR recovery rate.

In recent years, Japanese and other Asians, who have a small build, have been reported to be susceptible to renal impairment caused by TDF [15, 16]. We investigated whether the recovery of renal function after the discontinuation of TDF varied depending on the eGFR equation used. No differences were observed between the eGFR equation for Japanese individuals (2008 Japanese Society of Nephrology calculation) and the eGFR equation that used a Cockcroft–Gault (CG) equation that considered the effects of body weight, and a similar trend was seen in the eGFR recovery rate after the discontinuation of TDF. On the other hand, body weight data could not be obtained at all measurement points during follow-up in the present study, and an analysis of eGFR using the CG equation and an investigation of the effects of body weight therefore could not be sufficiently conducted. In the future, it may be necessary to investigate relationships to body weight and body surface area, as well as the effects of TDF blood concentrations, in Japanese patients, who have a small build, and AIDS patients, who have a tendency to lose weight.

Our findings suggest that when discontinuation criteria for TDF are established based on sCr or eGFR, renal function may not recover following the discontinuation of TDF, particularly among patients who have received long-term administration of TDF and exhibit a gradual decline in renal function. Urinary β_2-microglobulin and tubular reabsorption of phosphate have been reported to be useful markers for detecting renal tubular dysfunction caused by TDF at an earlier stage [13]. Because long-term administration of TDF is expected to continue in the future, it is important to investigate the use of markers that enable earlier and more sensitive detection of renal impairment caused by TDF to complement the assessment of renal function using eGFR.

With advances in treatment, HIV infection has gone from being a fatal condition to being a chronic disease that can be managed medically. At the same time, the various side effects that accompany long-term medication are becoming clear, and measures to improve the long-term prognosis of HIV-infected patients will be an issue in the coming years. In the guidelines of the United States Department of Health and Human Services, revised January 10, 2011, the first recommendation as an NRTI is TDF/emtricitabine (FTC) alone [1]. Thus, the number of patients who continue ART including long-term TDF administration is predicted to increase, and management of renal function will be important. It is reported that approximately 30% of patients with HIV infection have pre-existing renal abnormalities, and factors in renal disease are reported to be adverse effects from anti-HIV drugs or agents to treat opportunistic infection and complications such as HIV-associated nephritis, diabetes mellitus, and hypertension [17, 18]. Given the possibility that the duration of TDF administration affects recovery from renal impairment caused by TDF, it is important when using TDF to consider the increased risk factors for concomitant diseases such as diabetes and hypertension with aging of the patient, in addition to avoiding renal impairment from drugs, such as concurrent medications. Moreover, when renal function declines gradually in patients who receive long-term administration of TDF, sufficient care must be exercised in the management of renal function and in attempts to improve the long-term outcome.

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

Assay of cytotoxicity of CTL clones toward target cells prepulsed with the epitope peptide. The cytotoxic activities of Gag28-specific CTL clones were determined by use of the standard chromium release assay, as described previously (15). Briefly, 721.221-CD4-A2402 cells were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ in saline for 1 h and then washed 3 times with RPMI 1640 containing 10% newborn calf serum. The labeled target cells (2 × 10^3/well) were prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM for 1 h and then cocultured at 37°C for 4 h with effector cells at an effector-to-target (E:T) ratio of 1:1 in 96-U plates (Nunc). The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the counts per minute in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells.

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

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

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

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

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

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

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

RESULTS

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

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

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

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

[a] ID, identifier.

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

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

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

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

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

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

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

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

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

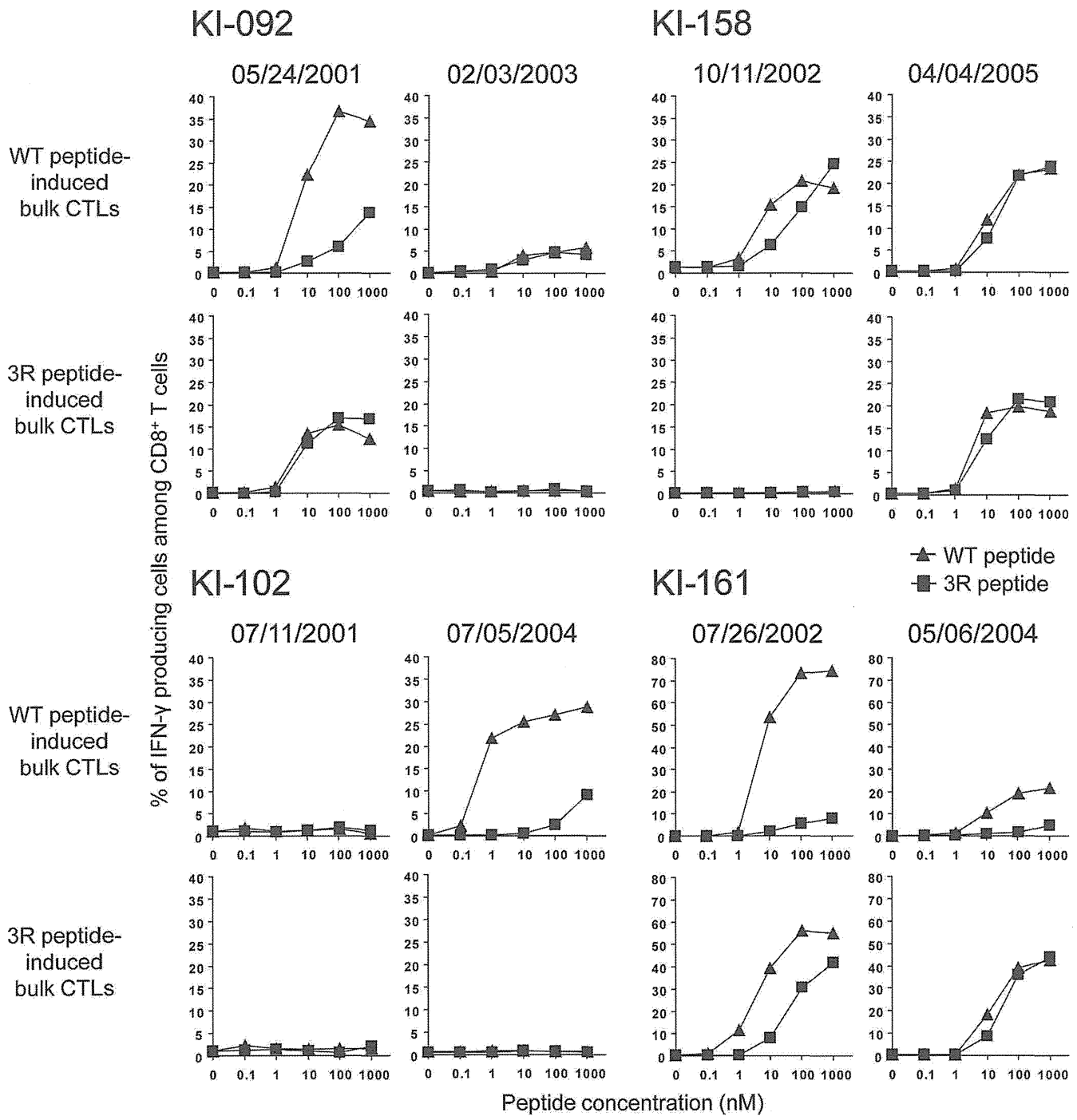

FIG 1 Gag28-specific CD8$^+$ T cells from individuals infected with WT virus at early and chronic phases. Gag28-specific CD8$^+$ T cells were induced by stimulating PBMCs from early and chronic phases in 4 WT-virus-infected HLA-A*24:02$^+$ individuals with WT or 3R peptide. The responses of these bulk-cultured cells to C1R-A2402 cells prepulsed with WT or 3R peptide at concentrations of 0.1 to 1,000 nM were analyzed by using the ICC assay.

graphs under early phase). Then, we analyzed the abilities of these CTL clones to suppress HIV-1 replication. Both WT-specific and cross-reactive CTL clones effectively suppressed the replication of the WT virus, whereas WT-specific and cross-reactive CTL clones exhibited no and weak ability, respectively, to suppress that of the 3R virus (Fig. 3D). These results indicate that WT-specific and cross-reactive CTLs could suppress the replication of the WT virus but that the former CTLs could not suppress the 3R virus *in vivo*. The latter CTLs may weakly suppress 3R virus *in vivo*. Interestingly, the WT-dominant CTL clones exhibited much weaker ability to suppress the replication of WT virus than did the WT-specific and cross-reactive CTLs (Fig. 3D), although no difference in killing activity against WT-virus-infected cells was found among these 3 CTL clones. Overall, KI-161 had a multiple Gag28-

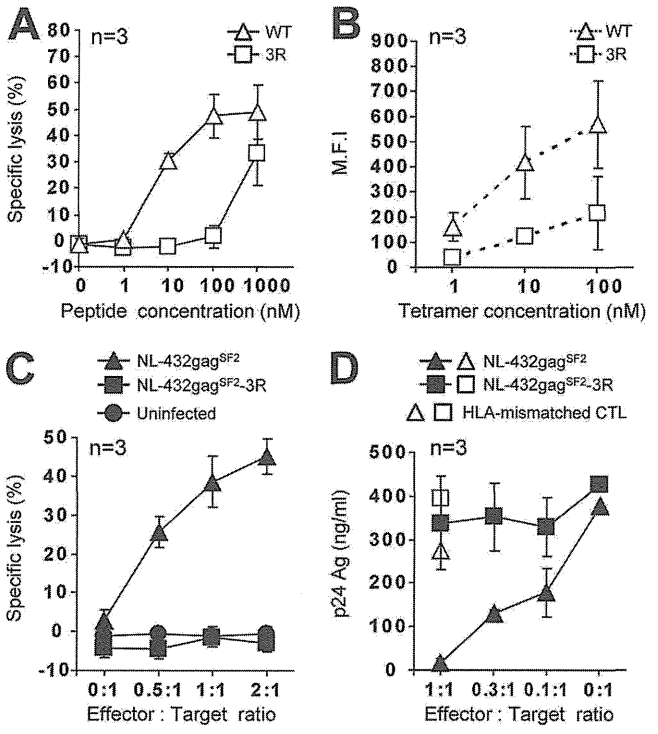

FIG 2 Antiviral activity of Gag28-specific CTL clones generated from early-phase PBMCs from patient KI-092, infected with WT virus. Gag28-specific CTL clones were generated from early-phase PBMCs from KI-092 by stimulating them with WT peptide. The activities of 3 CTL clones ($n = 3$) were analyzed. (A) Cytotoxic activity toward 721.221-CD4-A2402 cells prepulsed with the WT or 3R peptide at concentrations of 1 to 1,000 nM. The cytotoxic activity was measured at an E:T ratio of 1:1. (B) Binding affinity to WT and 3R tetramers at concentrations of 1 to 100 nM. The MFI values of the T cell clones are shown. (C) Cytotoxic activity against 721.221-CD4-A2402 cells infected with NL-432gag^{SF2} (WT virus) or NL-432gag^{SF2}-3R (3R virus). WT-virus-infected (49.1% of total cells were p24 Ag$^+$) and 3R virus-infected (48.6% of total cells were p24 Ag$^+$) cells were used as target cells. The cytotoxic activity was measured at E:T ratios of 0.5:1, 1:1, and 2:1. (D) Abilities of the clones to suppress the replication of WT or 3R viruses. The ability was tested at different E:T ratios. The error bars indicate standard deviations.

specific CTL repertoire at an early phase of HIV-1 infection, but only 2 types of Gag28-specific CTLs, which were the majority among the Gag28-specific CTLs, contributed to the suppression of WT virus replication.

Cross-reactive CD8$^+$ T cells and 3R-specific CD8$^+$ T cells in individuals who were infected with 3R virus. Next, we analyzed the elicitation of Gag28-specific CD8$^+$ T cells in 5 individuals infected with the 3R virus. Gag28-specific CD8$^+$ T cells were detected at both early and chronic phases in 3 individuals, whereas they were found at only the chronic phase in the other 2 (Table 3). Cross-reactive CD8$^+$ T cells were induced by stimulating KI-091 PBMCs from both early and chronic phases, not only with 3R peptide, but also with WT peptide. To characterize Gag28-specific CD8$^+$ T cells in KI-091, we generated Gag28-specific CTL clones from PBMCs at a chronic phase in KI-091 by stimulating them with 3R peptide. We investigated the recognition of 3 CTL clones for WT and 3R peptides. These CTL clones evenly recognized both peptides (Fig. 4A) and revealed the same binding affinity for the 2 tetramers (Fig. 4B), indicating that they were cross-reactive CTLs. They moderately killed target cells infected with either WT or 3R virus (Fig. 4C) but did not suppress the replication of the WT and 3R viruses (Fig. 4D). Thus, Gag28-specific CD8$^+$ T cells elicited in KI-091 had no ability to suppress the replication of WT and 3R viruses. Further analysis of 13 other clones revealed similar characteristics (data not shown), supporting the data indicating that cross-reactive CTLs were predominantly elicited in KI-091.

In the chronic phase, KI-091 had cross-reactive CD8$^+$ T cells, whereas 3R-specific CD8$^+$ T cells were found in 4 other individuals (Table 3). To characterize these 3R-specific CD8$^+$ T cells, we generated 3R-specific CTL clones from KI-163 PBMCs at the chronic phase by stimulating them with 3R peptide. All 3 clones recognized the 3R peptide much more effectively than the WT peptide (Fig. 4A). These CTL clones bound to 3R tetramer, but not to WT tetramer (Fig. 4B), indicating that these CTL clones carried a 3R-specific TCR. In addition, we analyzed the abilities of these CTL clones to recognize virus-infected cells and found that they effectively killed target cells infected with 3R virus, but not those infected with WT virus (Fig. 4C). However, they failed to suppress the replication of either 3R or WT virus (Fig. 4D). These results indicate that Gag28-specific CD8$^+$ T cells elicited in all individuals infected with 3R virus had no ability to suppress the replication of WT or 3R virus. Thus, Gag28-specific CD8$^+$ T cells seem to have failed to control the 3R virus, although they were elicited in individuals infected with the 3R virus.

High accumulation of the 3R variant in the Japanese population. The results described above strongly suggest that WT-specific and cross-reactive CD8$^+$ T cells selected the 3R mutation in the individuals infected with the WT virus and that 3R-specific and cross-reactive CD8$^+$ T cells failed to control the 3R virus in the individuals infected with it. Therefore, we assume that this 3R mutation has accumulated in the HLA-A*24:02$^+$ individuals. In addition, since HLA-A*24:02 is found in approximately 70% of Japanese, we speculate that the mutation has accumulated to high levels in the Japanese population.

A previous study analyzed the frequency of 3R in only 32 HLA-A*24:02$^+$ and 26 HLA-A*24:02$^-$ individuals chronically infected with HIV-1 and showed that the frequency of 3R was significantly higher in HLA-A*24:02$^+$ individuals than in the HLA-A*24:02$^-$ individuals (30). To confirm the association of this mutation with HLA-A*24:02, we analyzed a large number of chronically HIV-1-infected nonhemophiliac individuals (220 HLA-A*24:02$^+$ and 154 HLA-A*24:02$^-$ individuals) recruited from April 2008 to March 2011 (2008 to 2011 cohort). The results confirmed that the frequency of 3R was significantly higher in HLA-A*24:02$^+$ individuals than in the HLA-A*24:02$^-$ individuals ($P < 0.0005$) (Fig. 5). Since 3R was found in 74.7% of the HLA-A*24:02$^-$ individuals in this cohort, we speculate that the mutation has been accumulating in the Japanese population. Therefore, we analyzed HIV-1-infected nonhemophiliac Japanese individuals who had been recruited from 1996 to 2002 (1996 to 2002 cohort), as well as Japanese hemophiliacs who had been infected around 1983 (hemophiliac cohort), and then compared them to the 2008 to 2011 cohort (Fig. 5). The association of this mutation with HLA-A*24:02 was also found in both the 1996 to 2002 cohort and the hemophiliac cohort ($P < 0.01$ and $P = 7.4 \times 10^{-7}$, respectively). The frequency of this mutation in HLA-A*24:02$^-$ individuals significantly increased from 0% in the hemophiliac cohort to 50.0% in the 1996 to 2002 cohort ($P = 0.0084$) and to 74.7% in the 2008 to 2011 cohort ($P = 2.6 \times 10^{-7}$). These results indicate that the 3R mutation was strongly selected by Gag28-specific CTLs and has