TABLE 3. Relation Between Demographic and Clinical Factors and the Presence of Transmitted Drug Resistance

	With TDR (n = 58)	Without TDR (n = 1331)	Odds Ratio*	95% CI	*P* Value
Male gender, n (%)	42	831	1.58	0.88 to 2.53	0.13
Age (yrs), n (%)					
<30	24	538	1.00		
30–39	22	557	0.98	0.54 to 1.78	0.95
≥40	12	178	1.67	0.82 to 3.42	0.16
Time since HIV diagnosis, n (%)					
<6 mo	46	923	1.00		
≥6 mo	12	401	0.60	0.31 to 1.15	0.12
Unknown	0	7			
Year of HIV diagnosis					
Before 2008	2	132	1.00		
2008	6	223	1.78	0.35 to 8.93	0.49
2009	13	301	2.85	0.63 to 12.8	0.17
2010	17	293	3.83	0.87 to 16.8	0.08
2011	8	149	3.54	0.74 to 17.0	0.11
2012	12	226	3.50	0.77 to 15.9	0.10
Unknown	0	7			
Year of study enrollment, n (%)					
2008	7	285	1.00		
2009	13	237	2.23	0.88 to 5.69	0.09
2010	16	276	2.36	0.96 to 5.83	0.06
2011	8	286	1.14	0.40 to 3.18	0.80
2012	14	247	2.31	0.92 to 5.81	0.08
Risk of HIV transmission, n (%)					
Heterosexual contact	30	883	0.60	0.33 to 1.09	0.05
Injection drug use	19	367	1.49	0.82 to 2.69	0.19
Other	1	20	1.19	0.16 to 9.07	0.86
Unknown	10	131			
HBs antigen positive, n (%)	12	205	1.43	0.74 to 2.74	0.28
HCV antibody positive, n (%)	19	533	0.72	0.41 to 1.27	0.26
CD4 cell count, cells/μl					
≥100	24	686	1.00		
<100	34	642	1.51	0.89 to 2.58	0.14
Unavailable	0	3			

*Logistic regression model was used for calculating odds ratio.
CI, confidence interval.

found in our study between TDR and various risk factors, the odds ratio was lowest for heterosexual contact, with a marginal *P* value of 0.05, which indirectly suggests that other risk groups, such as IDU or men who have sex with men, is at higher risk of TDR. Meanwhile, the proportion of IDUs in our study had decreased during the 5 years along with the nationwide shift from the concentrated HIV epidemic in male IDUs to the general population. Although we failed to find the statistical impact of HIV risk group on TDR prevalence, TDR prevalence among IDU were

TABLE 4. Prevalence of Transmitted Drug Resistance Mutations in Specific HIV Risk Categories

	Total	2008	2009	2010	2011	2012
Total TDR rate [% (n/total)]	4.18 (58/1389)	2.40 (7/292)	5.20 (13/250)	5.48 (16/292)	2.72 (8/294)	5.36 (14/261)
TDR rate in HIV risk categories [% (n/total)]						
Heterosexual contact alone	3.33 (28/840)	1.40 (2/143)	4.90 (7/143)	3.40 (5/147)	1.92 (4/208)	5.02 (10/199)
IDU alone	5.41 (17/314)	4.10 (3/73)	5.88 (4/68)	6.67 (6/90)	2.27 (1/44)	7.69 (3/39)
IDU plus heterosexual	2.78 (2/72)	3.45 (1/29)	0 (0/5)	0 (0/3)	3.57 (1/28)	0 (0/7)
Homosexual contact alone	0 (0/2)	0 (0/2)	- (0/0)	- (0/0)	- (0/0)	- (0/0)
Other	0 (0/20)	0 (0/13)	0 (0/3)	0 (0/1)	0 (0/3)	- (0/0)
Unknown	7.80 (11/141)	3.13 (1/32)	6.45 (2/31)	9.80 (5/51)	18.2 (2/11)	6.25 (1/16)

TABLE 5. Characteristics of 4 Patients With Drug Resistance Mutations Against Multiple Class Antiretrovirals

Patient ID	Year of HIV Diagnosis	Year of Study Participant	Sex	Risk of HIV Infection	CD4 Count (Cells/μL)	HIV-RNA (Log Copies/mL)	HBs Antigen	HCV Antibody	Resistance Mutations	
									NRTI	NNRTI
08HT0059	2003	2008	M	Heterosexual	10	4.11	Negative	Negative	L74V	V106I, G190A
10HT0136	2010	2010	F	Unknown	283	4.60	Negative	Negative	D67E	Y188C
11HT0201	2011	2011	F	Heterosexual	272	5.98	Positive	Negative	M41L, M184V, T215Y	Y181C, G190A
11HT0299	2011	2011	M	Unknown	147	5.83	Negative	Negative	M184V	V106I, V179D, Y188L

relatively higher, which was above 5% in 2009, 2010, and 2012 and had changed along with the overall TDR prevalence. These findings support that IDU is still important as a TDR risk factor in this population. In this regard, however, our study enrolled 141 patients who were free of possible HIV risk or refused to provide information on their risky behavior. Because their TDR prevalence was high over the study period, their concealment of IDU experience could influence the analysis. Although our study was conducted only in urban area, our findings in individuals at most risk of TDR are useful for the assessment of the situation in the near future of the entire HIV population in Vietnam, including rural area where ART has been rapidly distributed in recent years.

With respect to the drug class, the TDR prevalence was 2.02% for NRTI, 1.37% for NNRTI, and 1.08% for PI. Compared with the TDR rate for CRF01_AE strain in the TDR lists for surveillance[18] (2.9% for NRTI, 0.5% for NNRTI, and 1.5% for PI), the TDR prevalence of NNRTI-related mutations was higher for the entire study period and considered to have increased with ART scale-up. The Vietnamese national guideline for ART recommended nevirapine as one of the first-line regimen in 2005 and either nevirapine and efavirenz since 2009,[6–8] and generally NNRTI-base regimens have low genetic barriers for development of drug resistance. This background provides reasonable explanation of frequent detection of NNRTI-related mutations like in other resource-limited countries. However, TAMs and M184V or I were predominantly seen in NRTI-related mutations, which have clinically significant impact on treatment outcome. Even after changing the first-line NRTI in the national ART guideline from zidovudine (AZT) or stavudine (d4T) into tenofovir (TDF) in 2010, AZT or d4T were still extensively used in Vietnam over the study period. In Western Europe, a decline in the prevalence of TAMs is being observed in treatment-experienced cohort as a consequence of changing prescription patterns and prompt management of treatment failure.[25,26] Therefore, the TDR patterns in Vietnam could be changed with future increase in TDF use and decrease in AZT or d4T use. We should note that 4 individuals in our study had TDR in multiple drug classes, including 1 who had very extensive resistance: M41L, M184V, T215Y in NRTI and Y181C and G190A in NNRTI, which strongly compromise the efficacy of the first-line regimens in Vietnam and could threaten the nationwide ART scale-up program if it spreads. There are multiple factors that influence the prevalence of individual resistance mutations in primary

HIV drug resistance but treatment-experienced persons with resistance might be the main source of such multiple-class TDR. Although continuous TDR surveillance is important to catch TDR expansion, efforts to enhance early diagnosis of treatment failure with improvement in availability of tests for plasma viral load and drug resistance in individuals on treatment, should be encouraged to prevent transmission of drug-resistant HIV.

In conclusion, TDR prevalence in Southern Vietnam remained low during the rapid scale-up of ART in 2008–2012. No demographic factor was statistically related to TDR detection, and the patterns of detected TDRs were similar to those described in previous reports. Although the average TDR prevalence was low, moderate prevalence was noted in part of the study period, and multiple-class TDR was detected in some patients. Because ART will continue to be scaled up, the TDR rate can rise in the future. Our results highlight the importance of TDR surveillance over a long period of time to provide proper assessment of the ART scale-up program.

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

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ABSTRACT

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

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

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

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

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

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

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

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

PATIENTS AND METHODS

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

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

RESULTS

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

Even with Child-Pugh A liver function, the HIV/HCV-coinfected patients showed a worse prognosis than the HCV mono-infected patients. The prognosis was especially poor in those with lower PLT than in the patients with a normal PLT (Table 2). When compared with HCV mono-infected patients with a PLT <150,000 μL, the survival of HIV/HCV-coinfected patients was much shorter (HCV, 5

Table 1. Patient Characteristics

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

CH, chromic hepatitics; LC, liver cirrhosis.

Table 2. Patient Survival after Diagnosis

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

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

years, 97%; 10 years, 86% and HIV/HCV, 5 years, 87%; 10 years, 73%; P < .05).

DISCUSSION

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

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

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

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

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

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

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Short communication

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

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Abstract

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

1. Introduction

Treatment with effective antiretroviral therapy (ART) results in a decline in viral load and increase in CD4$^+$ T cell count in the majority of HIV-1-infected individuals [1]; whereas the presence of drug-resistance mutations can contribute to increased risk of virologic failure [2]. Many such mutations occur in regions of HIV-1 Pol, which encompasses a number of previously identified cytotoxic T lymphocyte (CTL) epitopes [3,4]. If drug-resistance mutations enhance the immunogenic antigenicity of the CTL epitope, drug treatment might drive the CTL response towards HIV control; otherwise, they may cause an immunologically uncontrollable HIV infection if they affect the CTL responses. On the other hand, HIV-1-specific CTLs, especially those against HIV-1 Gag and

Pol, play a major role in controlling replication of HIV-1 [5,6]. However, HIV-1 escapes from the host immune system by various mechanisms [7]. The appearance of CTL escape mutations is one of them [5,6]. If such CTL escape mutations occur in the drug-target proteins, including reverse transcriptase, protease, and integrase, they may alter the drug sensitivity or modify the patterns of drug-resistance mutations [8,9].

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

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

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

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

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

2.2. Synthetic peptides

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

2.3. Cells

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

2.4. Generation of CTL clones

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

2.5. HIV-1 clones

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

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

Cytotoxic activity was measured by the standard ^{51}Cr-release assay, as previously described [6]. Target cells (2×10^5) were incubated for 1 h with 100 μl of Ci Na$_2^{51}$CrO$_4$ in saline and then washed 3 times with RPMI 1640 medium containing 10% FCS. Labeled target cells $(2 \times 10^3$/well) were added to 96-well round-bottomed microtiter plates (Nunc) along with the appropriate amount of the corresponding peptide. After 1 h of incubation, effector cells were added; and then incubation was carried out for 4 h at 37 °C. The supernatants were collected and analyzed with a gamma counter. Spontaneous ^{51}Cr release was determined by measuring the

counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum ^{51}Cr release was determined by measuring the cpm in supernatants from wells containing target cells in the presence of 2.5% Triton X-100 (cpm max). Specific lysis was defined as (cpm exp − cpm spn)/(cpm max − cpm spn) × 100, where "cpm exp" is the counts per minute in the supernatant in the wells containing both target and effector cells. Relative specific lysis of peptide-pulsed target cells was defined as (specific lysis of exp − specific lysis of cells without peptide).

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

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

2.8. ELISpot assay

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

2.9. Sequencing of plasma RNA

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

Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by use of an ABI 3500 genetic analyzer.

3. Results

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

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

3.2. Ex vivo CD8^{+} T-cells fail to recognize EL11—E92Q peptide

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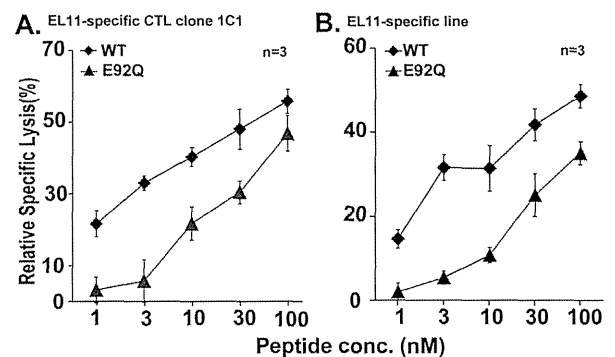

Fig. 1. Relative cytolytic activity of EL11-specific CTLs against target cells pulsed with the WT (EL11) or mutant (EL11—E92Q) peptide. An EL11-specific CTL clone and cell line were generated from PBMCs by stimulating them with WT peptide from a HLA-B*40:02 patient, KI-400, infected with WT virus. The antiviral activities of an EL11-specific CTL clone 1C1 (A) and the EL11-specific cell line (B) were analyzed. Relative cytotoxic activity toward C1R-B*40:02 cells prepulsed with the WT or EL11-E92Q peptide at concentrations of 1—100 nM was measured. The cytotoxic activity assay was performed at an E:T ratio of 1:1. The error bars indicate standard deviations.
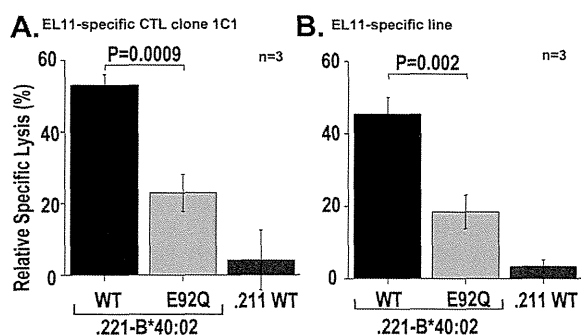
Fig. 2. Relative cytolytic activity of EL11-specific CTLs against target cells infected with WT (NL-432) or mutant virus (NL432-E92Q). Relative cytotoxic activity against 721.221-CD4-B*40:02 cells infected with NL-432 (WT virus) or NL-432-E92Q (E92Q virus) was assessed. (A) WT virus-infected (43.7% of total cells were p24 Ag$^+$) and E92Q virus-infected (59.1% of total cells were p24 Ag$^+$) cells were used as target cells. (B) WT virus-infected (59.2% of total cells were p24 Ag$^+$) and E92Q virus-infected (44.5% of total cells were p24 Ag$^+$) cells were used as target cells. NL-432 virus-infected 721.221-CD4$^+$ (.221 WT) cells were used as a negative control. The antiviral activities of an EL11-specific CTL clone, 1C1 (A), and an EL11-specific cell line (B) were analyzed. The cytotoxic activity was measured at E:T ratio of 1:1. The error bars indicate standard deviations. P values were determined by using Student's T-test.

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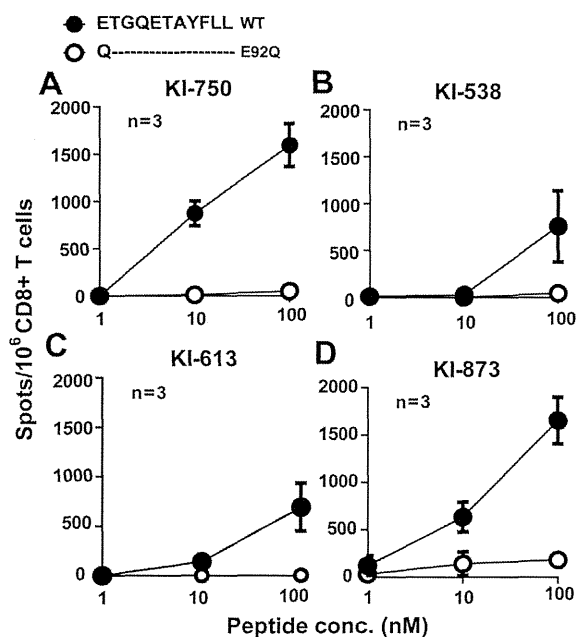

Fig. 3. *Ex vivo* CD8$^+$ T-cells response specific for WT (EL11) or mutant (EL11—E92Q) peptides in chronically HIV-1-infected HLA-B*40:02$^+$ individuals. CD8$^+$ T cell responses to EL11 or EL11—E92Q peptides by PBMCs from 5 HLA-B*40:02$^+$ chronically HIV-1-infected individuals were analyzed by performing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

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

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

4. Discussion

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

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

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

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

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

Conflict of interest

The authors have no conflicting financial interests.

Acknowledgments

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Clinical Importance of Hyper-Beta-2-Microglobulinuria in Patients With HIV-1 Infection on Tenofovir-Containing Antiretroviral Therapy

To the Editors:

A single-tablet once-daily regime of elvitegravir (EVG), cobicistat (COBI), tenofovir disoproxil fumarate (TDF), and emtricitabine (FTC) showed non-inferior safety and efficacy compared with 2 current first-line regimens recommended for antiretroviral treatment (ART)-naive HIV-1–infected patients,[1,2] and it is now listed as an alternative regimen in the ART guidelines.[3,4] Although TDF is considered to be nephrotoxic, the assessment of renal dysfunction based on the monitoring of serum creatinine may be somewhat difficult during EVG/COBI/TDF/FTC-ART, because COBI inhibits the active tubular secretion of creatinine, resulting in an increase in serum creatinine and a reduction in estimated creatinine clearance without actually affecting glomerular function.[5] Therefore, routine monitoring of not only serum creatinine but also urinary protein, and periodic monitoring of serum phosphorus in high-risk patients, are recommended during the EVG/COBI/TDF/FTC-ART in the guidelines and prescribing information.[3,6] These recommendations seem to be reasonable because tubular dysfunction usually precedes the decline in the glomerular filtration rate in TDF-induced nephrotoxicity. How-

ever, specific markers of renal tubular function may be more sensitive.[7,8] We identified previously urinary β2-microglobulin (U-β2MG) as a useful marker for monitoring TDF-induced tubular dysfunction, and we currently measure it routinely in TDF-treated patients.[9,10] To address whether the measurement of U-β2MG enables the detection of TDF-induced tubular dysfunction, we compared the values of U-β2MG in TDF-treated patients with those of urinary protein and serum phosphorus. We also determined the clinical significance of hyper-β2-microglobulinuria in patients with TDF-induced tubular dysfunction.

Urinary protein and U-β2MG levels, measured by the dip stick method and latex agglutination turbidimetric immunoassay, respectively, were available from the medical record of 943 patients who had been on TDF-containing ART for >3 months in the Outpatient Clinic of AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan, between 2010 and 2012. The distribution of the peak value of U-β2MG in each patient is shown in Figure 1A. U-β2MG was persistently within the normal range (<300 μg/L) in only 231 patients (24.5%), but transiently or persistently abnormal in the other patients (75.5%), indicating that at least mild tubular dysfunction is common in TDF-treated patients. Severe tubular dysfunction (U-β2MG >10,000 μg/L) was observed in 76 patients (8.1%). Their urine protein measured at the same time is shown in the left bar graph of Figure 1B. Proteinuria (+, 2+, and 3+) was observed in 17 patients (22.4%) only, indicating that the dipstick method is not sufficiently sensitive to detect tubular dysfunction associated with TDF use. Serum phosphorus values were measured in 55 patients with a peak U-β2MG of >10,000 μg/L (Fig. 1B, right bar). Of these, only 5 (9.1%) patients had hypophosphatemia, indicating that serum phosphorus is not a useful marker for TDF-caused tubular dysfunction. Considered together, measurements of urinary protein and serum phosphorus levels do not seem sufficient for monitoring TDF-treated patients, and measurement of markers specific for renal tubular dysfunction may be necessary.

FIGURE 1. A, Peak levels of U-β2MG in tenofovir-treated patients. Data are the distribution of peak levels of U-β2MG in 943 patients treated with tenofovir for >3 months. B, Urinary protein and serum phosphorus levels in patients with severe renal tubular damage associated with tenofovir use. Urinary protein in 76 and serum phosphorus in 55 patients with U-β2MG of >10,000 μg/ L are shown. C, Tenofovir withdrawal rate. Nine hundred forty-three tenofovir-treated patients were divided into 4 groups according to the peak level of U-β2MG, and the rate of tenofovir withdrawal in each group is shown. U-Pro, urinary protein.
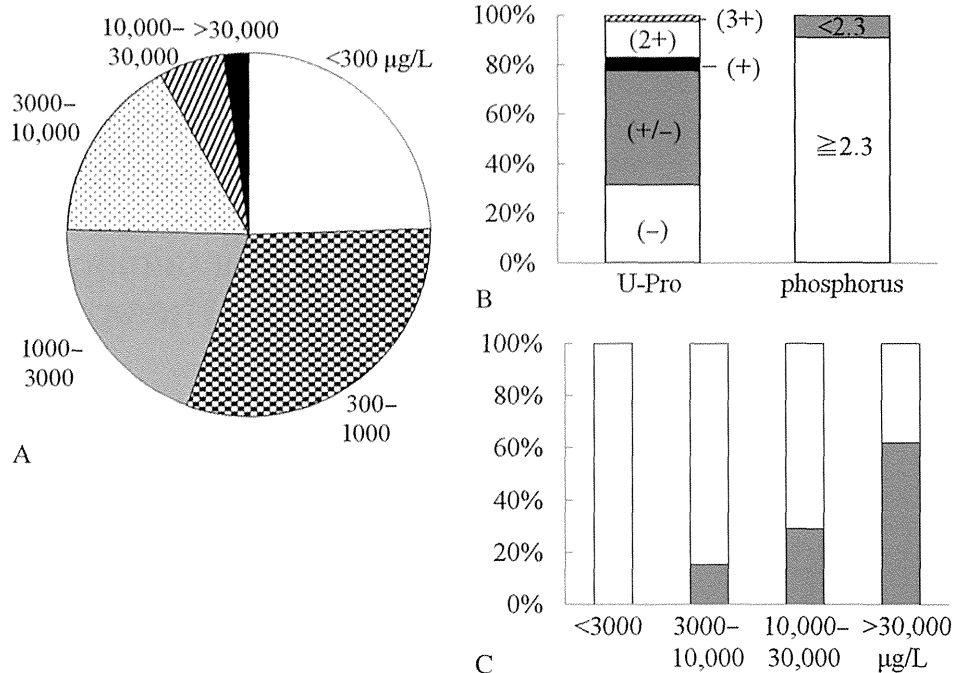
Next, to define the clinical significance of U-β2MG, we divided the patients based on the peak value of U-β2MG and analyzed the rate of TDF discontinuation associated with renal damage. TDF-containing ART was discontinued during the observation period in none of 711 patients with U-β2MG persistently <3000 μg/L, suggesting that U-β2MG level <3000 μg/L is not clinically significant in TDF-treated patients (Fig. 1C). The peak values of U-β2MG were >3000 and <10,000 μg/L (3000–10,000) in 156 TDF-treated patients, and TDF was discontinued because of renal damage in 24 (15.4%) patients. The peak U-β2MG levels were >10,000 and <30,000 μg/L (10,000–30,000) in 55 TDF-treated patients, and TDF was discontinued in 16 (29.1%) of these patients. These data suggest that the close monitoring of tubular dysfunction and renal damage is necessary when U-β2MG increases beyond 10,000 μg/L. In the remaining 21 patients, the peak U-β2MG levels were >30,000 μg/L (>30,000), and TDF was withdrawn in 13 patients (61.9%) of them, indicating that TDF withdrawal should be considered when U-β2MG is ≥30,000 μg/L.

TDF-related renal damage is more common in Japanese patients than in whites, because low body weight is one

of the risk factors for TDF-related nephrotoxicity.[11,12] Considering that TDF use has expanded in Asia and Africa where patients have lower body weights compared with whites, appropriate monitoring methods for TDF-induced renal damage should be recognized globally. Measurement of not only serum creatinine and urinary protein but also renal tubular damage–specific markers, such as U-β2MG, is recommended.

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Reply

To the Editors:

We appreciate the interest from Carrieri et al and their comments regarding our article about the effect of different alcohol consumption levels on HIV surrogate markers in the Swiss HIV Cohort Study (SHCS).[1] Carrieri et al conducted a similar longitudinal analysis using data from the French ANRS APROCO-COPILOTE CO-08 cohort. In contrast to our study, they found in 1108 individuals with 11 years of follow-up that low alcohol consumption levels were associated with higher CD4 cell counts, whereas alcohol abstinence and higher levels of alcohol consumption were associated with lower CD4 counts. No significant association between alcohol consumption and virological failure was found in both cohorts.

Some important issues raised merit a few comments.

Follow-up period was indeed shorter in our study (median follow-up 2.5 years) compared with that in the French cohort (median follow-up 6.9 years). This time difference could possibly explain why in the SHCS no influence of alcohol on CD4 cell counts was observed because the toxic effect of alcohol needs time to evolve. However, despite the shorter follow-up period, we had a larger sample size (n = 2982 vs. n = 1108 in the French cohort) and our cohort also has an excellent follow-up where individuals are

asked about alcohol consumption every 6 months and only very few missing data are recorded (<1%). Carrieri et al did not specify the frequency of the alcohol assessments and the level of missing data in the French cohort.

Most importantly, we would like to highlight that the 2 studies cannot be compared one to one because different baselines were used. Individuals in our study were antiretroviral therapy (ART) naive and initiated first ART. The French cohort, however, used "protease inhibitor initiating date" as baseline and only 45% were ART naive at baseline. This could cause a problem in model fitting because individuals at different stages of ART treatment have different trajectories for both HIV viral load and CD4 cell counts. In particular, the definition for virological failure used in both studies would only be appropriate in the ART initiation period. As a consequence, individuals in the French cohort were more heterogeneous with a longer period since HIV diagnosis (3.8 years vs. 1.0 years) and more AIDS events (21% vs. 13%). Furthermore, the French cohort had more injecting drug users (17% vs. 7%) and individuals co-infected with hepatitis C virus (22% vs. 10%). One could argue that individuals in the French cohort were sicker and more often at risk of severe alcohol drinking and therefore of nonadherence to ART. However, we do not have information on treatment interruption and adherence to ART in the French cohort.

Furthermore, in the French cohort, different categories for alcohol consumption were used from what we used. We applied the categories of the World Health Organization[2]: low consumption was <20 g/d for women and <40 g/d for men, moderate 20–40 g/d for women and 40–60 g/d for men, and severe >40 g/d for women and >60 g/d for men, whereas in the French study, low alcohol consumption was <10 g/d for both men and women, moderate 10–30 g/d for women and 10–40 g/d for men, and severe >30 g/d for women and >40 g/d for men, respectively. Therefore, if the French definition would be applied to our cohort, there would be fewer individuals categorized in the moderate drinking group in favor of

the high drinking group. Compared with the French study, we had 46% nondrinkers (vs. 19%), 46% light health risk drinkers (vs. 54%), 5% moderate (vs. 21%), and 2% severe health risk drinkers (vs. 6%). Thus, in our cohort, there were significantly more nondrinkers and indeed fewer individuals with severe health risk drinking, limiting possibly the power in the latter category; the same was true for moderate health risk drinkers. But we would also like to rectify the comment made by Carrieri et al that we collapsed the categories of low and moderate health risk drinking, which is not the case. We fused the categories of none and light health risk drinkers, because both of them gave similar results for CD4 cell counts and HIV viral loads in the single analysis.

Besides the alcohol drinking quantity, the drinking pattern also plays an important role. Binge drinking is known to be associated with worse prognosis concerning morbidity and mortality from alcohol-induced problems and adherence to ART.[3,4] Furthermore, the type of the consumed alcohol might also be important in predicting health outcomes.[5] For the French paradox, red wine was shown to be associated with lower cardiovascular mortality, whether this is true for hard liquor as well is unknown.[6,7] As in both cohorts neither the drinking pattern nor the type of the consumed alcohol is known, no conclusions can be drawn.

The U-shaped curve between alcohol consumption levels and CD4 cell counts as shown by Carrieri et al[8] was found by the same research team for cardiovascular mortality, the so-called French paradox. According to our pathophysiological understanding and also in animal models, it is difficult to explain the U-shaped association between alcohol and CD4 cell counts. It is not clear how a postulated cytotoxic effect of alcohol can be protective for CD4 cells at a certain low level, but being toxic below and above this level.[9–11]

Overall, this French study contributes to the ongoing controversy whether alcohol has an influence on HIV surrogate markers or not. Both studies are well and carefully performed and

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Host-Specific Adaptation of HIV-1 Subtype B in the Japanese Population

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ABSTRACT

The extent to which HIV-1 clade B strains exhibit population-specific adaptations to host HLA alleles remains incompletely known, in part due to incomplete characterization of HLA-associated HIV-1 polymorphisms (HLA-APs) in different global populations. Moreover, it remains unknown to what extent the same HLA alleles may drive significantly different escape pathways across populations. As the Japanese population exhibits distinctive HLA class I allele distributions, comparative analysis of HLA-APs between HIV-1 clade B-infected Japanese and non-Asian cohorts could shed light on these questions. However, HLA-APs remain incompletely mapped in Japan. In a cohort of 430 treatment-naive Japanese with chronic HIV-1 clade B infection, we identified 284 HLA-APs in Gag, Pol, and Nef using phylogenetically corrected methods. The number of HLA-associated substitutions in Pol, notably those restricted by HLA-B*52:01, was weakly inversely correlated with the plasma viral load (pVL), suggesting that the transmission and persistence of B*52:01-driven Pol mutations could modulate the pVL. Differential selection of HLA-APs between HLA subtype members, including those differing only with respect to substitutions outside the peptide-binding groove, was observed, meriting further investigation as to their mechanisms of selection. Notably, two-thirds of HLA-APs identified in Japan had not been reported in previous studies of predominantly Caucasian cohorts and were attributable to HLA alleles unique to, or enriched in, Japan. We also identified 71 cases where the same HLA allele drove significantly different escape pathways in Japan versus predominantly Caucasian cohorts. Our results underscore the distinct global evolution of HIV-1 clade B as a result of host population-specific cellular immune pressures.

IMPORTANCE

Cytotoxic T lymphocyte (CTL) escape mutations in HIV-1 are broadly predictable based on the HLA class I alleles expressed by the host. Because HLA allele distributions differ among worldwide populations, the pattern and diversity of HLA-associated escape mutations are likely to be somewhat distinct to each race and region. HLA-associated polymorphisms (HLA-APs) in HIV-1 have previously been identified at the population level in European, North American, Australian, and African cohorts; however, large-scale analyses of HIV-1 clade B-specific HLA-APs in Asians are lacking. Differential intraclade HIV-1 adaptation to global populations can be investigated via comparative analyses of HLA-associated polymorphisms across ethnic groups, but such studies are rare. Here, we identify HLA-APs in a large Japanese HIV-1 clade B cohort using phylogenetically informed methods and observe that the majority of them had not been previously characterized in predominantly Caucasian populations. The results highlight HIV's unique adaptation to cellular immune pressures imposed by different global populations.

H IV cytotoxic T lymphocyte (CTL) escape occurs in a manner that is highly reproducible in the context of the HLA class I alleles expressed by the host (1–8). By extension, HIV sequences circulating in a given host population exhibit polymorphisms that reflect the HLA allele distribution of that population (9). Because HLA class I allele distributions differ among racial and ethnic groups worldwide (10), the pattern and diversity of HLA-associated escape mutations are also likely to be somewhat distinct to each race and region. Numerous population-based studies identifying HLA-associated polymorphisms (HLA-APs) have been conducted in European, North American, Australian, and African cohorts (2, 6, 8). However, fewer have been undertaken in Asian cohorts, where HIV-1 prevalence is also substantial (11). Since Asian populations differ in their HLA allele distributions from the cohorts previously studied, it is important to identify and analyze HLA-APs to achieve a better understanding of HIV-1 pathogene-

sis in Asia and to inform future HIV vaccine design efforts targeted to these populations. The Japanese epidemic is unique in Asia. While clades A/E and C predominate in many Asian countries (12–14), the Japanese HIV-1 epidemic comprises 80% clade B infections (12). As such, the analysis of Japanese cohorts also pro-

vides the opportunity to undertake comparative analyses of HLA-APs between Asian and non-Asian populations infected with HIV clade B.

Previous studies have investigated differential HLA-driven HIV evolution across human populations. For example, a study of HLA-specific adaptations in HIV Pol in a Mexican cohort identified "unique" HLA-APs in this population that were not present in an international cohort from Canada, the United States, and Australia, even though both cohorts harbored HIV clade B (15). Most of the unique Mexican HLA-APs were restricted by HLA alleles particular to this population (e.g., HLA-B*39) but that were underrepresented or absent in the international cohort (15). This study, therefore, illustrates population-specific HIV adaptation in its most intuitive manifestation, i.e., where distinctive HLA-associated polymorphisms are observed in a population due to the presence (or comparatively high frequency) of an HLA allele in that population compared to another.

What remains unknown, however, is the extent to which the same HLA allele may drive divergent escape pathways in different human populations. Two critical features are required to address this question. First, the identification of HLA-APs must be undertaken at the HLA subtype level. This is because the majority (>60%) of HLA-associated polymorphisms are best defined at the subtype level (16), even for closely related HLA subtype members that present the same or similar peptide epitopes (16, 17, 18, 19). Comparative studies undertaken at allele level (two-digit) resolution cannot disentangle whether population-specific HLA-APs are attributable to differential HLA subtype distributions between cohorts or whether they are "true" cases where the same HLA subtype drives different escape pathways across populations. Indeed, a study investigating >500 Americans with chronic HIV-1 clade B infection observed distinct patterns of HLA-APs among white, black, and Hispanic individuals that were likely attributable to the differential distribution of closely related HLA subtypes among these groups (18) rather than true differential escape. The present study is therefore undertaken at subtype level resolution. Second, the identification of population-specific escape pathways driven by the same HLA allele requires a method to do so. Here, we adapt phylogenetically corrected statistical methods originally developed to assess differential escape among related HLA subtypes (17) and apply them to investigate differential escape across host populations.

The present study is divided into two parts, each with a specific major objective. Our first objective was to identify and characterize HLA-APs in HIV-1 Gag, Pol, and Nef proteins in a cohort of 430 chronically clade B-infected Japanese individuals using phylogenetically informed approaches (20) and to investigate their associations with clinical parameters (CD4$^+$ T cell count [CD4 count] and plasma viral load [pVL]). Importantly, HLA genotyping (and thus HLA-AP identification) was undertaken at subtype level resolution, allowing us to analyze the effects of genetic differences among closely related HLA subtypes on the selection of HLA-APs in the Japanese cohort as part of this objective. Our second major objective was to perform a comparative analysis of HLA-APs identified in Japan and those identified in a large international (Canada/United States/Australia) cohort of antiretroviral-naive, chronically clade B-infected, predominantly Caucasian individuals. As expected, a substantial proportion of Japanese HLA-APs were restricted by alleles unique to (or highly enriched in) Japan compared to the non-Asian cohort. Notably, we also observed numerous cases where the same HLA allele drove significantly different—sometimes opposing—escape pathways in these two populations. Our results highlight HIV's unique adaptation to cellular immune pressures imposed by different global populations.

MATERIALS AND METHODS

Ethics statement. This study was approved as part of the study of immunological and virological analysis in HIV-1 infection (number 540) by the ethics committee for epidemiology and general study in the Faculty of Life Science of Kumamoto University and the National Center for Global Health and Medicine (NCGHM). All studied individuals were adults. Written informed consent was obtained from all studied individuals according to the Declaration of Helsinki.

Subjects. Four-hundred thirty treatment-naive Japanese individuals with chronic HIV-1 clade B infection were enrolled in the NCGHM from 2008 to 2011. The HLA alleles of these individuals were determined at the four-digit level by a probe-based sequence-specific oligonucleotide (SSO) typing method (HLA Laboratory, Kyoto, Japan). The median CD4 count and pVL at the first visit to the NCGHM were 321 cells/μl (interquartile range [IQR], 190 to 440 cells/μl) and 25,000 copies/ml (IQR, 6,800 to 98,000 copies/ml), respectively.

HLA-associated polymorphisms derived from the International HIV Adaptation Collaborative (IHAC) cohort, comprising 1,888 treatment-naive individuals with chronic clade B infection from Canada, the United States, and Western Australia (16), identified by identical methods, were used for comparison. The IHAC cohort comprises predominantly Caucasian individuals, with Asians making up less than 5% of the total. The median CD4 count in the IHAC cohort was 260 cells/μl (IQR, 110 to 418 cells/μl).

RT-PCR and sequencing of plasma HIV RNA. HIV-1 RNA was extracted from plasma samples using either a QIAamp MinElute virus spin kit (Qiagen, Valencia, CA) or an EZ1 Virus Mini Kit v2.0 (Qiagen, Valencia, CA). Reverse transcription (RT) was performed using random hexamers with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). HIV-1 Gag, Pol, and Nef genes were amplified from cDNA by nested PCR using Taq DNA polymerase (Promega, Fitchburg, WI) and 10 primer pairs that were designed based on the clade B strain. For subjects with a viral load below 1,000 copies/ml, RT-PCR was performed with region-specific primers using the SuperScript III One-Step RT-PCR System with Platinum Taq kit (Invitrogen, Carlsbad, CA). The 1st-round PCR product was then used in the 2nd-round PCR amplification using Taq DNA polymerase (Promega, Fitchburg, WI) and the 10 primer pairs. The 2nd-round PCR product was purified by using the ExoSap-It reagent containing exonuclease I and alkaline phosphatase (GE Healthcare, Buckinghamshire, United Kingdom). Gag, Pol, and Nef sequences were determined by using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems, Carlsbad, CA) and an ABI 3500 genetic analyzer (Applied Biosystems, Carlsbad, CA). Sequencing reactions were performed in both the 5' and 3' directions to yield a minimum of bidirectional coverage of all regions. The sequence data were then aligned by using SeqScape software (Applied Biosystems, Carlsbad, CA) based on the HXB2 reference sequence (K03455).

Identification of HLA-associated polymorphisms. HLA-APs can be identified in large cross-sectional linked data sets of host (HLA) and HIV genotypes using statistical-association approaches that identify viral polymorphisms significantly over- or underrepresented in individuals harboring a specific HLA class I allele (1, 2, 4, 16–18, 21). HLA-APs that are overrepresented in individuals harboring the relevant HLA are commonly referred to as "adapted" forms, while those underrepresented in individuals harboring the relevant HLA are referred to as "nonadapted" forms (2, 18). As such, "nonadapted" and "adapted" forms can be conceptualized to represent the "immunologically susceptible" and "escape mutant" forms, respectively, for the specific HLA allele in question at that HIV codon position. Statistical association approaches for the identification of HLA-

APs also correct for the confounding influences of viral phylogeny, HIV codon covariation, and linkage disequilibrium (LD) between HLA class I alleles (2, 16, 17, 21).

Associations between HLA class I alleles and HIV-1 amino acid polymorphisms in the Japanese and IHAC data sets were identified using a published phylogenetically corrected logistical-regression model that corrects for HLA LD, HIV phylogeny, and HIV codon covariation as potential confounders (17, 20). Briefly, maximum-likelihood phylogenetic trees were constructed using Gag, Pol, and Nef sequences (one tree per gene), and a model of conditional adaptation was inferred for each observed amino acid at each codon. Amino acids are assumed to evolve independently along the phylogeny to the tree tips (representing the present host). In each host, HLA-mediated selection and HIV amino acid covariation are directly modeled using weighted logistical regression, in which the individual's HLA repertoire and covarying HIV amino acids are used as binary predictors and the bias is determined by the possible transmitted sequences as inferred from the phylogeny (17). To identify which factors (HLA and/or HIV covariation) contribute to selection pressure, we employ a forward-selection procedure where the most significant association is iteratively added to the model, with P values computed using the likelihood ratio test. We performed *post hoc* filtering of the resulting HLA-associated-polymorphism list, restricting our output to instances in which at least 10 individuals carried the allele or polymorphism and at least 10 individuals did not carry the allele or polymorphism. Multiple tests were accounted for using q values, the P value analog of the false-discovery rate (FDR) (22). The FDR is the expected proportion of false positives among results deemed significant at a given threshold; for example, at a q value of <0.2, we expect 20% of identified associations to be false positives. In the analyses identifying HLA-APs, a significance threshold of a q value of <0.2 was employed.

Statistical analysis. Correlations between the total number of HLA-associated substitutions in each individual and clinical parameters (pVL and CD4 count) were performed using Spearman's correlation. To determine the total number of HLA-associated substitutions within a given HIV-1 sequence, we first identified all HIV-1 sites within that sequence known to be associated with any HLA allele. The specific residue at each site was counted as "HLA associated" if it matched any HLA-associated adapted form or any residue other than a nonadapted form identified at that position. The HLA alleles expressed by the individual were not considered (unless specifically stated); rather, our goal was to enumerate the HLA-APs associated with any HLA allele in each viral sequence. In analyses where host HLA alleles were not considered, HIV sites harboring residues that simultaneously represented a nonadapted and an adapted form associated with different HLA alleles were excluded from consideration.

Detection of differential escape between closely related HLA alleles and between cohorts. Two types of differential escape were investigated. First, we investigated differential escape between closely related HLA class I alleles, defined here as (four-digit) HLA subtype members belonging to the same (two-digit) allele group in the Japanese cohort. Specifically, seven HLA allele groups (A*02, A*26, B*15, B*40, C*03, C*08, and C*14) for which a minimum of two subtype members were represented in the Japanese cohort were investigated. For example, the HLA-A*02 allele group featured subtypes A*02:01, A*02:06, and A*02:07, while the A*26 allele group featured subtypes A*26:01 and A*26:03. For each allele group, we took the union of all HLA-APs identified for all subtype members of the group. Then, in a pairwise manner, we compared their strengths of selection between all HLA subtype members using a previously described phylogenetically corrected interaction test (17). In this analysis, thresholds of a P value of <0.05 and a q value of <0.2 were used to define significance.

Second, we investigated differential HLA-driven escape pathways between Japanese and IHAC cohorts. As outlined in the introduction, HLA-APs identified in human populations differ to some extent due to the presence (or enrichment) of certain HLA alleles in one population versus another. However, in this analysis, we were specifically interested in identifying cases where the same HLA allele drove significantly different escape pathways in the two cohorts. To do this, we took the union of all HLA-APs identified in the Japan and IHAC cohorts that were restricted by HLA subtypes observed a minimum of 10 times in both cohorts. We then compared the strength of selection of each HLA-AP in a pairwise manner between cohorts. The statistical methods used to investigate differential escape between the Japanese and IHAC cohorts are similar to those used to investigate differential escape between HLA subtype members (17), with some modifications, as follows. Briefly, a phylogenetically corrected logistical-regression model was constructed using a single HLA allele as a predictor. Using a likelihood ratio test, we then compared this model to a more expressive one that included an additional interaction term that was 1 if the individual expressed the HLA allele and was in the IHAC cohort or 0 otherwise. In this way, we could obtain a P value, testing the hypothesis that selection is the same in both cohorts (the null hypothesis) or whether selection differs across cohorts (alternative hypothesis). In contrast to the HLA-AP analyses described thus far, the present one does not feature corrections for HLA LD or HIV codon covariation and therefore yields odds ratios of association and P values that differ slightly from the original cohort-specific values. In the intercohort differential-escape analysis, significance was defined as a P value of <0.01 and a q value of <0.05.

Nucleotide sequence accession numbers. The accession numbers for the sequences determined in this study are AB873205 to AB873601 (Gag), AB873908 to AB874270 (Pol), and AB873602 to AB873907 (Nef).

RESULTS

Identification of HLA-associated polymorphisms in chronically HIV-1 clade B-infected Japanese individuals. The first objective of our study was to identify and characterize HLA-APs in Japan, a unique population in terms of its HLA class I distribution and predominantly HIV clade B epidemic. Toward this end, we analyzed linked HIV-HLA genotypes from 430 antiretroviral-therapy-naive Japanese individuals chronically infected with HIV-1 clade B. A total of 78 unique HLA class I alleles, defined at subtype level (four-digit) resolution, were observed in our cohort (see Fig. S1 in the supplemental material) at frequencies consistent with those in the published literature (23). Of these, 37 (including 9 HLA-A, 17 HLA-B, and 11 HLA-C alleles) were observed in at least 10 individuals and thus were included in the statistical analysis of HLA-APs (see Materials and Methods). Amplification and sequencing of HIV-1 Gag, Pol without the transframe (TF) protein, and Nef was successful for 397 (92.3%), 363 (84.4%), and 306 (71.2%) individuals, respectively. As described in Materials and Methods, HLA-APs within these three genes were identified using a phylogenetically corrected logistical-regression model that corrects for the confounding effects of viral phylogeny, HIV-1 codon covariation, and linkage disequilibrium between host HLA class I alleles (16, 17, 20). A false-discovery rate (q value) approach was employed to address multiple tests.

At a threshold of a q value of <0.2, a total of 284 HLA-APs, comprising 143 adapted and 141 nonadapted associations, were identified in Gag ($n = 94$ associations), Pol ($n = 86$ associations), and Nef ($n = 104$ associations) (Fig. 1; see Table S1 in the supplemental material). HLA-APs were more frequently detected in Nef (occurring at 45 of 206 codons [21.8%]) compared to Gag (51 of 500 codons [10.2%]) or Pol (51 of 947 codons [5.1%]). Although HLA class I allele frequencies in Japan are somewhat distinct globally, the distribution of HLA-APs across HIV-1 proteins was consistent with that reported in previous studies of other populations infected with clade B or C (1, 2, 6, 7, 16). Broken down by HLA locus, the numbers of HLA-A-, HLA-B-, and HLA-C-associated
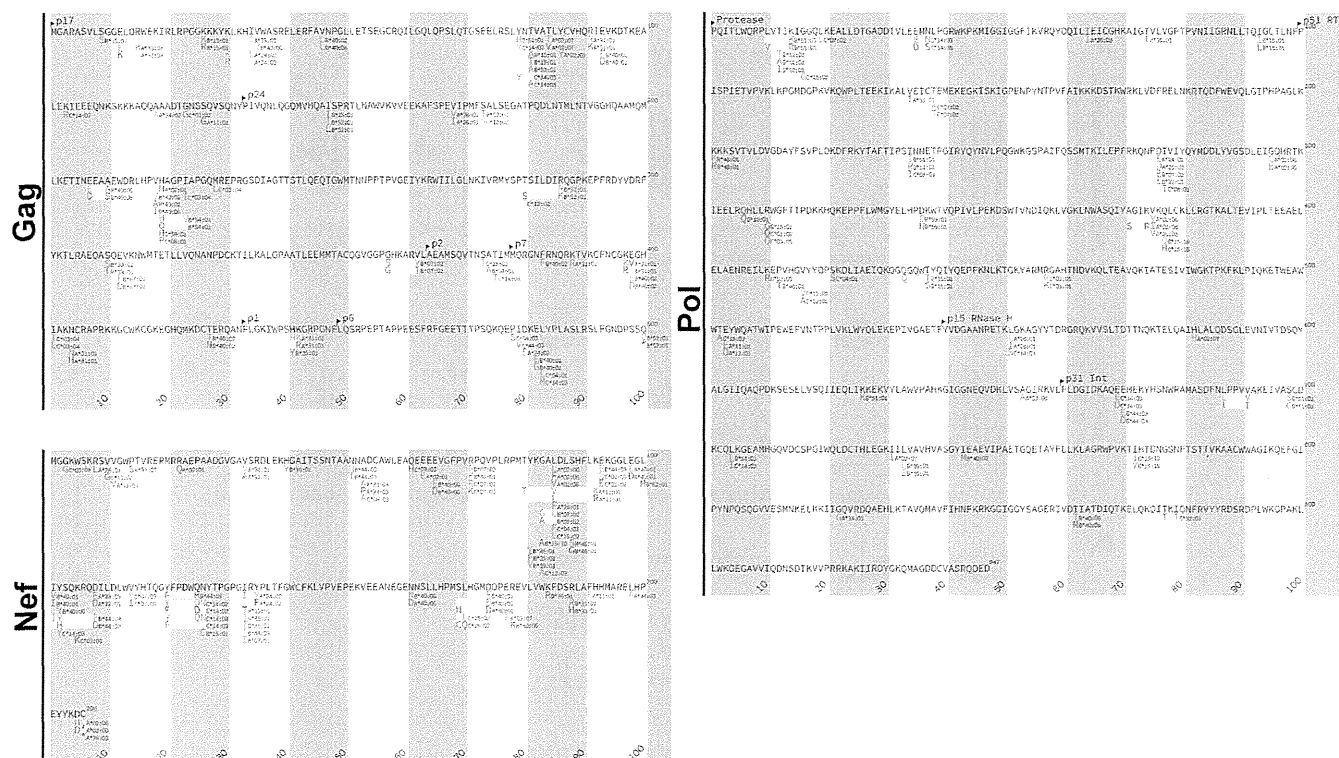
FIG 1 Escape map of HLA-APs for Gag, Pol, and Nef in the Japanese cohort. The escape maps indicate the locations, specific residues, and HLA restrictions of HLA-APs (all $q < 0.2$). The global HIV-1 clade B consensus amino acid sequence is used as a reference. The shaded vertical bars separate blocks of 10 amino acids. Adapted amino acids (those significantly overrepresented in the presence of a given HLA allele) are red. Nonadapted amino acids (those significantly underrepresented in the presence of a given HLA allele) are blue. Polymorphisms associated with the same HLA allele that occur in proximity to one another are grouped together in yellow boxes. A list of all HLA-APs is provided in Table S1 in the supplemental material.

polymorphisms were 78, 140, and 66, respectively, numbers that were also consistent with previous reports from Caucasian and African cohorts that HLA-B alleles restrict more associations than HLA-A or HLA-C alleles (1, 6, 18).

Correlation between the total number of HLA-associated substitutions and clinical parameters in Japanese individuals. We next wished to investigate the relationship between the presence of HLA-associated substitutions in each gene and the patient HIV-1 pVL and CD4 count in the Japanese cohort. As described in Materials and Methods, substitutions within a given HIV-1 sequence were counted as HLA associated if they had been identified as being associated with any HLA class I allele in our study, regardless of the HLA alleles expressed by the patient. For example, Gag-9S is an HLA-B*15:01-associated nonadapted polymorphism (Fig. 1; see Table S1 in the supplemental material); as such, any amino acid other than S at codon 9 was counted as an HLA-associated substitution. Similarly, Gag-123G is an HLA-C*01:02-associated adapted polymorphism (but no specific nonadapted forms, restricted by C*01:02 or others, were identified at this position); as such, any sequence harboring G at codon 123 was counted as having an HLA-associated substitution at this site.

A weak yet statistically significant inverse correlation was observed between pVL and the total number of HLA-associated substitutions in Pol (Spearman's $R = -0.11$; $P = 0.04$) (Fig. 2A). However, no such correlations were observed for Gag (Spearman's $R = -0.056$; $P = 0.3$) or Nef (Spearman's $R = -0.029$; $P = 0.6$) (Fig. 2A). Moreover, no significant correlations were ob-

served between the total number of HLA-associated substitutions in any HIV protein and the CD4 count (Fig. 2A). Though the overall association is weak, the results raise the intriguing hypothesis that selection of certain HLA-driven substitutions in Pol could modulate the pVL in the Japanese population.

We next wondered whether the observed correlation between Pol polymorphisms and lower pVL could be attributed to polymorphisms restricted by HLA alleles that are protective in Japanese populations. HLA-B*67:01 and the HLA-B*52:01-HLA-C*12:02 haplotype are examples of such protective alleles (24). As such, we investigated whether they could play a role in the observed pVL correlation. No HLA-B*67:01-associated substitution was identified in Pol, whereas four HLA-B*52:01-associated and one HLA-C*12:02-associated substitutions were detected in the protein (see Table S1 in the supplemental material). Exclusion of the single HLA-C*12:02-associated substitution from analysis did not affect the relationship between the number of HLA-associated substitutions in Pol and pVL (data not shown). In contrast, exclusion of the four HLA-B*52:01-associated Pol substitutions substantially weakened the overall relationship between the number of HLA-associated Pol substitutions and pVL (Spearman's $R = -0.057$; $P = 0.3$) (Fig. 2B). Similarly, specific consideration of only HLA-B*52:01-associated Pol substitutions revealed a highly significant inverse correlation with pVL (Spearman's $R = -0.18$; $P = 0.0007$) (Fig. 2C) that represented the strongest such relationship detected in Pol for common HLA alleles observed in our cohort (see Fig. S2 in the supplemental material). We therefore
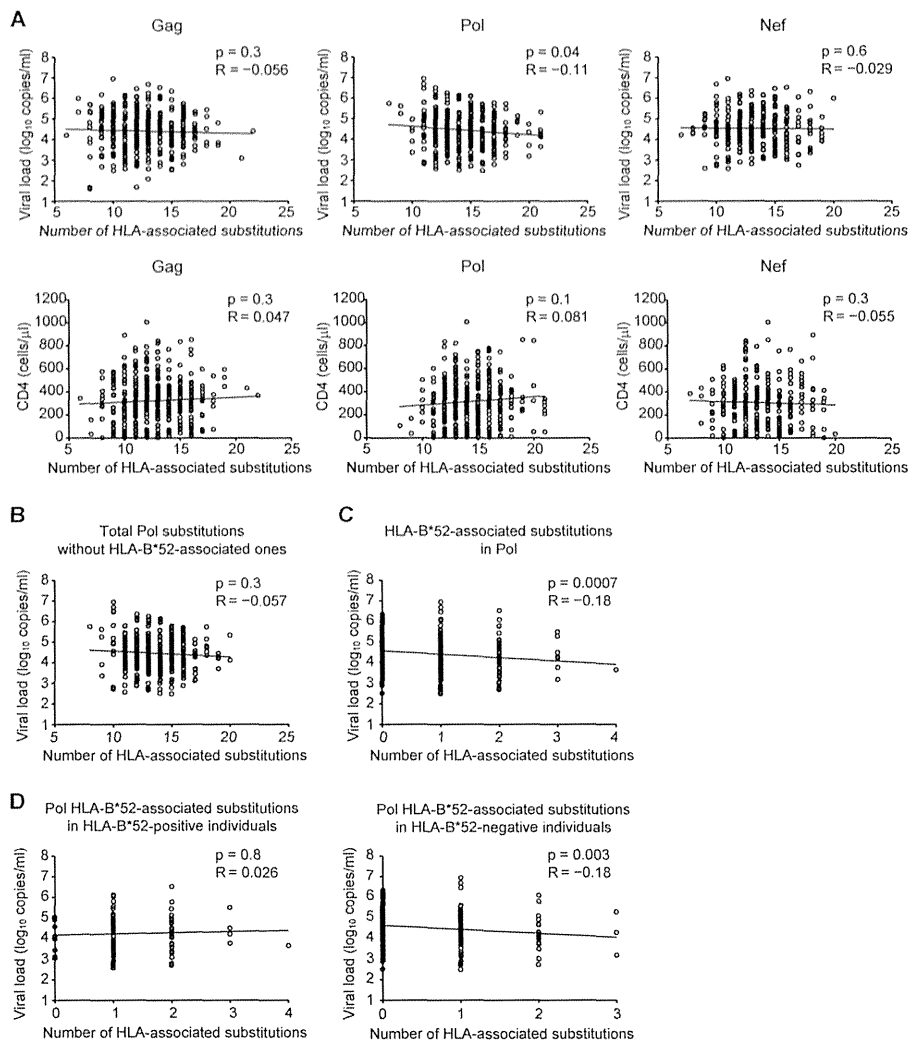
FIG 2 Correlations between HLA-associated substitutions in Gag, Pol, and Nef and viral load or CD4 count. The total number of HLA-associated substitutions in each subject's Gag, Pol, and Nef sequence was determined (see Materials and Methods). (A) Correlation between the number of HLA-associated substitutions in Gag, Pol, or Nef and pVL or CD4 count. (B) Correlation between pVL and the number of HLA-associated substitutions in Pol, with HLA-B*52:01-associated substitutions excluded. (C) Correlation between pVL and the number of HLA-B*52:01-associated substitutions in Pol (all patients). (D) Correlation between the number of HLA-B*52:01-associated substitutions in Pol in HLA-B*52:01-positive individuals (left) and HLA-B*52:01-negative individuals (right). Analyses were performed using Spearman's correlation. Linear regression lines are included in the plots.

reasoned that B*52:01-restricted substitutions were likely to be critical mediators of the observed pVL effect.

Finally, stratification of B*52:01-associated Pol substitutions by host B*52:01 expression revealed that the inverse correlation with pVL remained strongly detectable in HLA-B*52:01⁻ individuals (Spearman's $R = -0.18$; $P = 0.003$), but not in HLA-B*52: 01⁺ individuals (Spearman's $R = 0.026$; $P = 0.8$) (Fig. 2D). We interpret our observations as suggesting that HLA-B*52:01-restricted Pol substitutions possess fitness costs that manifest themselves in terms of lower pVL upon transmission to, and persistence in, HLA-B*52:01⁻ individuals. In contrast, no such pVL effects are detectable in B*52:01⁺ individuals, likely because the fitness costs of these substitutions are outweighed by the advantages conferred by immune escape.

Differential escape between HLA subtypes in Japanese individuals. Our final goal in characterizing HLA-APs in Japan was to investigate the extent of differential escape between closely related

HLA subtypes. In particular, we hypothesized that HLA subtype members differing with respect to the amino acids located within in the peptide-binding groove of the HLA molecule may differ with respect to the nature (or binding affinity) of the specific HIV epitopes presented (25–28), and therefore, that they may exhibit differential escape pathways. In contrast, we hypothesized that HLA subtype members that differ with respect to amino acids located outside the peptide-binding groove may be more likely to present the same epitopes (29–31) and therefore will generally exhibit less evidence for differential escape between them. Of the 284 HLA-APs identified in our cohort, 128 were restricted by HLA allele groups (A*02, A*26, B*15, B*40, C*03, C*08, and C*14) containing two or more subtype members (see Table S1 in the supplemental material). For five of these allele groups (A*02, A*26, B*15, B*40, and C*08), subtype members differed by substitutions within the peptide-binding groove (see Fig. S3 in the supplemental material), supporting their potential as candidates

A A*26:01 / A*26:03   **B** C*03:03 / C*03:04   **C** C*14:02 / C*14:03

A76V D74H N77D   R91G   R21H

Protein	Position	a.a.	lnOR
Gag	168	V	
Gag	168	I	
Pol	64	I	
Pol	**276**	**D**	
Pol	375	V	
Pol	**551**	**I**	
Pol	551	L	
Nef	8	L	
Nef	**85**	**F**	
Nef	206	*	

☐ A*26:01
☐ A*26:03

Protein	Position	a.a.	lnOR
Gag	223	I	
Gag	228	L	
Gag	401	I	
Gag	401	L	
Pol	234	I	
Pol	310	Q	
Pol	**457**	**R**	
Pol	**457**	**K**	
Nef	3	G	
Nef	61	H	
Nef	105	K	

☐ C*03:03
☐ C*03:04

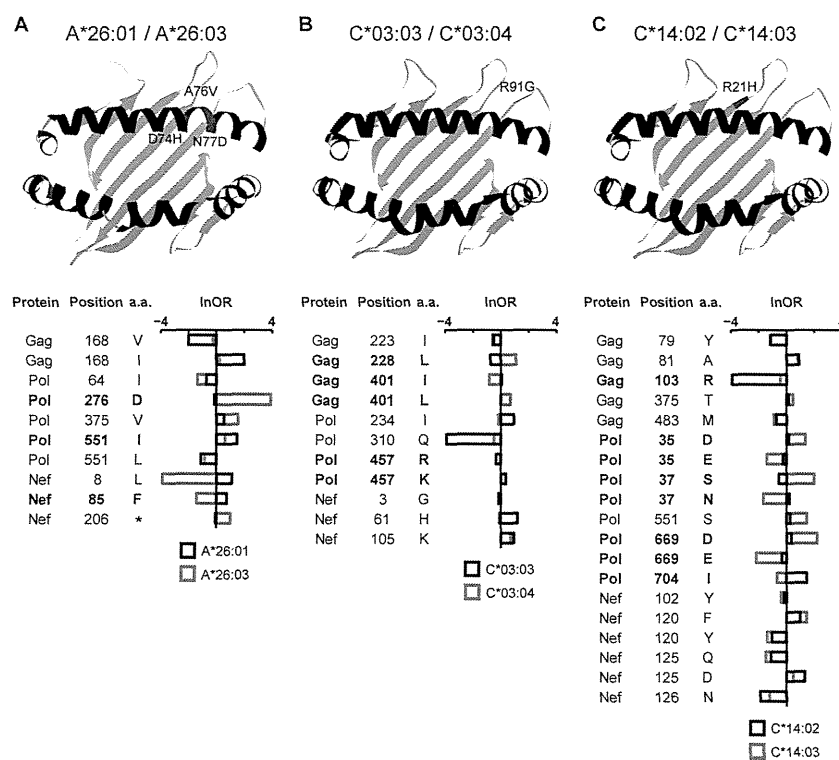| Protein	Position	a.a.	lnOR
Gag	79	Y	
Gag	81	A	
Gag	103	R	
Gag	375	T	
Gag	483	M	
Pol	35	D	
Pol	35	E	
Pol	37	S	
Pol	37	N	
Pol	551	S	
Pol	669	D	
Pol	669	E	
Pol	704	I	
Nef	102	Y	
Nef	120	F	
Nef	120	Y	
Nef	125	Q	
Nef	125	D	
Nef	126	N	

☐ C*14:02
☐ C*14:03

FIG 3 Polymorphic positions in HLA class I molecules and differential escape between pairs of HLA subtypes. In each ribbon diagram depicting the HLA-peptide-binding groove, the locations of residues differing among subtype members of the HLA-A*26 (A), HLA-C*03 (B), and HLA-C*14 (C) allele groups are highlighted in red and labeled with their locations and amino acids. HLA-AP comparisons between subtype members are shown in the corresponding plot below. The horizontal bars represent the lnORs, with colors indicating the restricting allele. Infinite lnORs are set to values of ±4. Boldface type indicates HLA-APs whose strengths of selection are statistically significantly different between the two subtype members ($P < 0.05$; $q < 0.2$). a.a., amino acid.

for differential HLA-AP selection. In contrast, members of the C*03 and C*14 subtypes differed by substitutions outside the peptide-binding groove (see Fig. S3 in the supplemental material), suggesting that their epitope repertoires (and thus escape pathways) would be more similar to one another.

We began by simply comparing HLA-APs identified in the context of the different HLA subtypes. As expected, viral polymorphisms associated with HLA subtype members differing within their peptide-binding grooves appeared to be quite specific to each HLA subtype (see Fig. S3A to D and F in the supplemental material). Surprisingly, however, viral polymorphisms associated with HLA subtype members differing only with respect to amino acids located outside their peptide-binding grooves also appeared to be quite specific to each HLA subtype (see Fig. S3E and G in the supplemental material). For example, HLA-C*03:03 and C*03:04, which differ only by substitutions at position 91 that have no contact with the groove (29–31), were associated with a total of 11 HLA-APs, none of which appeared to be shared (see Fig. S2E in the supplemental material). Similarly, HLA-C*14:02 and C*14:03, which differ only by a substitution at position 21 located outside the floor of the peptide-binding groove (see Fig. S2G in the supplemental material), shared only 10 of the 24 HLA-APs identified between them.

However, qualitative comparisons of HLA-APs meeting a specific significance threshold, such as those described above, are not statistically robust (since individual associations may fail to meet the threshold and thus not be detected, or variations in allele frequency may limit the power to detect associations). Thus, to

explicitly investigate whether the above-mentioned examples represent statistically significant instances of differential escape between subtype members, we applied a phylogenetically corrected interaction test to compare their strengths of selection between subtypes (17). For each HLA allele group, we took the union of all HLA-APs identified for all subtype members and compared their strengths of selection between all subtype members in a pairwise manner. Representative examples of our results are shown in Fig. 3. For example, HLA-A*26:01 and -A*26:03 differ with respect to substitutions at amino acids 74, 76, and 77, located within the peptide-binding groove of the HLA molecule (see Fig. S3B in the supplemental material). A total of 10 HLA-APs, located at 8 HIV codons, were originally identified as associated with either HLA-A*26:01 or -A*26:03 (see Fig. S3B in the supplemental material). Although qualitatively, all 10 HLA-APs appear to be differentially selected by HLA-A*26:01 or -A*26:03 (see Fig. S3B in the supplemental material), the phylogenetically corrected interaction test revealed only 3 of them (located at Pol residues 276 and 551 and Nef residue 85) to be significantly differentially selected in terms of their natural logarithms of the odds ratios (lnORs) of association ($P < 0.05$; $q < 0.2$) (Fig. 3A). Surprisingly, significant differential escape was also observed between subtype members that differed only with respect to substitutions outside their peptide-binding grooves: 3 of 9 (33.3%) sites restricted by HLA-C*03 allele group members and 5 of 14 (35.7%) sites restricted by C*14 allele group members similarly exhibited statistically significant evidence of differential selection (Fig. 3B and C).

To determine whether the extent of differential escape between