

### Ⅲ. 研究成果の刊行に関する一覧表

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雑誌

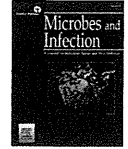
発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
Rahman MA, Kuse N, Murakoshi H, Chikata T, Gatanaga H, Oka S, <u>Takiguchi M.</u>	Raltegravir and elvitegravir-resistance mutation E92Q affects HLA-B*40:02-restricted HIV-1-specific CTL recognition.	Microbes Infect.			In press
Chikata T, Carlson JM, Tamura Y, Borghan MA, Naruto T, Hashimoto M, Murakoshi H, Le AQ, Mallal S, John M, Gatanaga H, Oka S, Brumme ZL, <u>Takiguchi M.</u>	Host-specific adaptation of HIV-1 subtype B in the Japanese population.	J Virol.	88	4764-4775	2014
Ladell K*, Hashimoto M*, Iglesias MC*, Wilmann PG*, McLaren JE, Gras S, Chikata T, Kuse N, Fastenackels S, Gostick E, Bridgeman JS, Venturi V, Arkoub ZA, Agut H, van Bockel DJ, Almeida JR, Douek DC, Meyer L, Venet A, <u>Takiguchi M**</u> , Rossjohn J**, Price DA** and Appay V** (* **Equal contribution).	A molecular basis for the control of preimmune escape variants by HIV-specific CD8+ T cells.	Immunity	38	425-436.	2013
Yagita Y, Kuse N, Kuroki K, Gatanaga H, Carlson JM, Chikata T, Brumme ZL, Murakoshi H, Akahoshi T, Pfeifer N, Mallal S, John M, Ose T, Matsubara H, Kanda R, Fukunaga Y, Honda K, Kawashima Y, Ariumi Y, Oka S, Maenaka K, <u>Takiguchi M.</u>	Distinct HIV-1 Escape Patterns Selected by CTLs with Identical Epitope Specificity	J. Virol.	87	2253-2263	2013
<u>Gatanaga H</u> , Murakoshi H, Hachiya A, Hayashida T, Chikata T, Ode H, Tsuchiya K, Sugiura W, Takiguchi M, Oka S.	Naturally selected rilpivirine-resistant HIV-1 variants by host cellular immunity.	Clin Infect Dis.	57(7)	1051-5	2013

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Okamoto M, Chono H, Kawano Y, Saito N, Tsuda H, Inoue K, Kato I, Mineno J, <u>Baba M</u>	Sustained inhibition of HIV-1 replication by conditional expression of the <i>E. coli</i> -derived endoribonuclease MazF in CD4 <sup>+</sup> T cells	Hum. Gene. Ther. Methods	24	94-103	2013
Mizuhara T, Kato T, Hirai A, Kurihara H, Shimada Y, Taniguchi M, Maeta H, Togami H, Shimura K, <u>Matsuoka M</u> , Okazaki S, Takeuchi T, Ohno H, Oishi S, Fujii N.	Structure-activity relationship study of phenylpyrazole derivatives as a novel class of anti-HIV agents.	Bioorg Med Chem Lett.	23 (16)	4557-4561	2013
Harada S., Yoshimura K., Yamaguchi A., Yusa K., <u>Matsushita S.</u>	Impact of antiretroviral pressure on selection of primary HIV-1 envelope sequences in vitro.	J. Gen. Virol.	94	933-943	2013
Maeda K, Desai D, Aoki M, <u>Nakata H</u> , Kodama E, Mitsuya H.	Delayed emergence of HIV-1 variants resistant to 4'-ethynyl-2-fluoro-2-deoxyadeno-sine: comparative sequential passage study with lamivudine, tenofovir, emtricitabine and BMS-986001	Antivir Ther	19	179-89	2014
Furukawa A, Kamishikiryo J, Mori D, Toyonaga K, Okabe Y, Toji A, Kanda R, Miyake Y, Ose T, Yamasaki S, <u>Maenaka K.</u>	Structural analysis for glycolipid recognition by the C-type lectins Mincle and MCL.	Proc Natl Acad Sci U S A.	110	17438-43	2013

## IV. 研究成果の刊行物・別刷



Microbes and Infection 16 (2014) 434–438



www.elsevier.com/locate/micinf

Short communication

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

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Received 24 February 2014; accepted 11 March 2014

Available online 20 March 2014

### 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<sup>+</sup> 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<sup>+</sup> 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  $\mu$ l of cloning mixture (RPMI 1640 medium containing 10% FCS, 200 U/ml human recombinant interleukin-2,  $5 \times 10^5$  irradiated allogeneic PBMCs from a healthy donor, and  $1 \times 10^5$  irradiated C1R–B\*40:02 cells pulsed with a  $1 \mu$ M 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 <sup>51</sup>Cr-release assay, as previously described [6]. Target cells ( $2 \times 10^5$ ) were incubated for 1 h with 100  $\mu$ l of <sup>51</sup>Cr Na<sub>2</sub>CrO<sub>4</sub> 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 <sup>51</sup>Cr release was determined by measuring the

counts per minute (cpm) in supernatants from wells containing only target cells (cpm spn). Maximum  $^{51}\text{Cr}$  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  $(\text{cpm exp} - \text{cpm spn})/(\text{cpm max} - \text{cpm spn}) \times 100$ , 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  $(\text{specific lysis of exp} - \text{specific lysis of cells without peptide})$ .

### 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  $^{51}\text{Cr}$  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-1 was defined as  $(\text{specific lysis of exp} - \text{specific lysis of uninfected cells})/(\text{percentage of infected cell used})$ .

### 2.8. ELISpot assay

The appropriate amount of EL11 or EL11–E92Q peptides and PBMCs from HLA-B\*40:02<sup>+</sup> 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- $\gamma$  mAb 1-D1K (Mabtech, Stockholm, Sweden). The plates were incubated for 16 h at 37 °C in 5% CO<sub>2</sub> and then washed with PBS before the addition of biotinylated anti-IFN- $\gamma$  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-indolyl phosphate and nitro blue tetrazolium by using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA, USA). The spot number was counted by using an Eliphoto-Counter (Minerva Teck, Tokyo, Japan). The CD8<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T-cells fail to recognize EL11–E92Q peptide

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

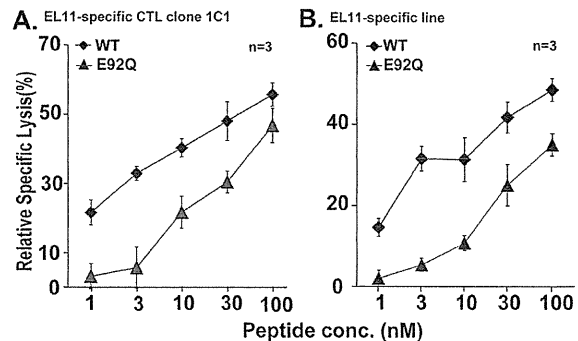


Fig. 1. Relative cytolytic activity of EL11-specific CTLs against target cells pulsed with the WT (EL11) or mutant (EL11–E92Q) peptide. An EL11-specific CTL clone and cell line were generated from PBMCs by stimulating them with WT peptide from a HLA-B\*40:02 patient, KI-400, infected with WT virus. The 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.

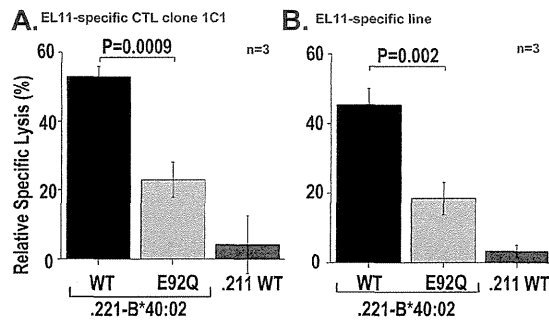


Fig. 2. Relative cytolysis of EL11-specific CTLs against target cells infected with WT (NL-432) or mutant virus (NL432-E92Q). Relative cytotoxic activity against 721.221-CD4-B\*40:02 cells infected with NL-432 (WT virus) or NL-432-E92Q (E92Q virus) was assessed. (A) WT virus-infected (43.7% of total cells were p24 Ag<sup>+</sup>) and E92Q virus-infected (59.1% of total cells were p24 Ag<sup>+</sup>) cells were used as target cells. (B) WT virus-infected (59.2% of total cells were p24 Ag<sup>+</sup>) and E92Q virus-infected (44.5% of total cells were p24 Ag<sup>+</sup>) cells were used as target cells. NL-432 virus-infected 721.221-CD4<sup>+</sup> (.211 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<sup>+</sup> individuals failed to recognize the INI-resistance E92Q mutation *ex vivo*.

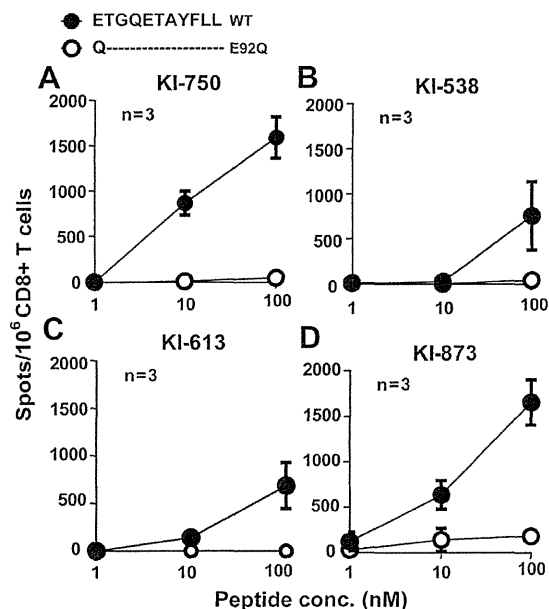


Fig. 3. *Ex vivo* CD8<sup>+</sup> T-cells response specific for WT (EL11) or mutant (EL11–E92Q) peptides in chronically HIV-1-infected HLA-B\*40:02<sup>+</sup> individuals. CD8<sup>+</sup> T cell responses to EL11 or EL11–E92Q peptides by PBMCs from 5 HLA-B\*40:02<sup>+</sup> chronically HIV-1-infected 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 EL11 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<sup>+</sup> 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<sup>+</sup> T cells specific for the EL11 epitope were detected in 4 of the 5 HLA-B\*40:02<sup>+</sup> individuals examined, indicating EL11 to be an immunodominant epitope. However, since the INI-resistance EL11–E92Q epitope may not be recognized by the CD8<sup>+</sup> 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<sup>+</sup> T cell recognition both *in vitro* and *ex vivo* in the HLA-B\*40:02<sup>+</sup> 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<sup>+</sup> 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

The authors thank Sachiko Sakai for her secretarial assistance. This research was supported by the Global COE program “Global Education and Research Center Aiming at the Control of AIDS,” launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture of Japan. It was also funded in part by grants-in-aid for AIDS research from the Ministry of Health, Japan and by Junior Research Associate Supporting Grants (recipient M.A.R) by Takuetsu (Grants for Excellent Graduate Schools, MEXT).

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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-naïve 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.

HIV 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-

Received 20 January 2014 Accepted 5 February 2014

Published ahead of print 12 February 2014

Editor: G. Silvestri

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.00147-14>.

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doi:10.1128/JVI.00147-14

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<sup>+</sup> 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/ $\mu$ l (interquartile range [IQR], 190 to 440 cells/ $\mu$ l) 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/ $\mu$ l (IQR, 110 to 418 cells/ $\mu$ l).

**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

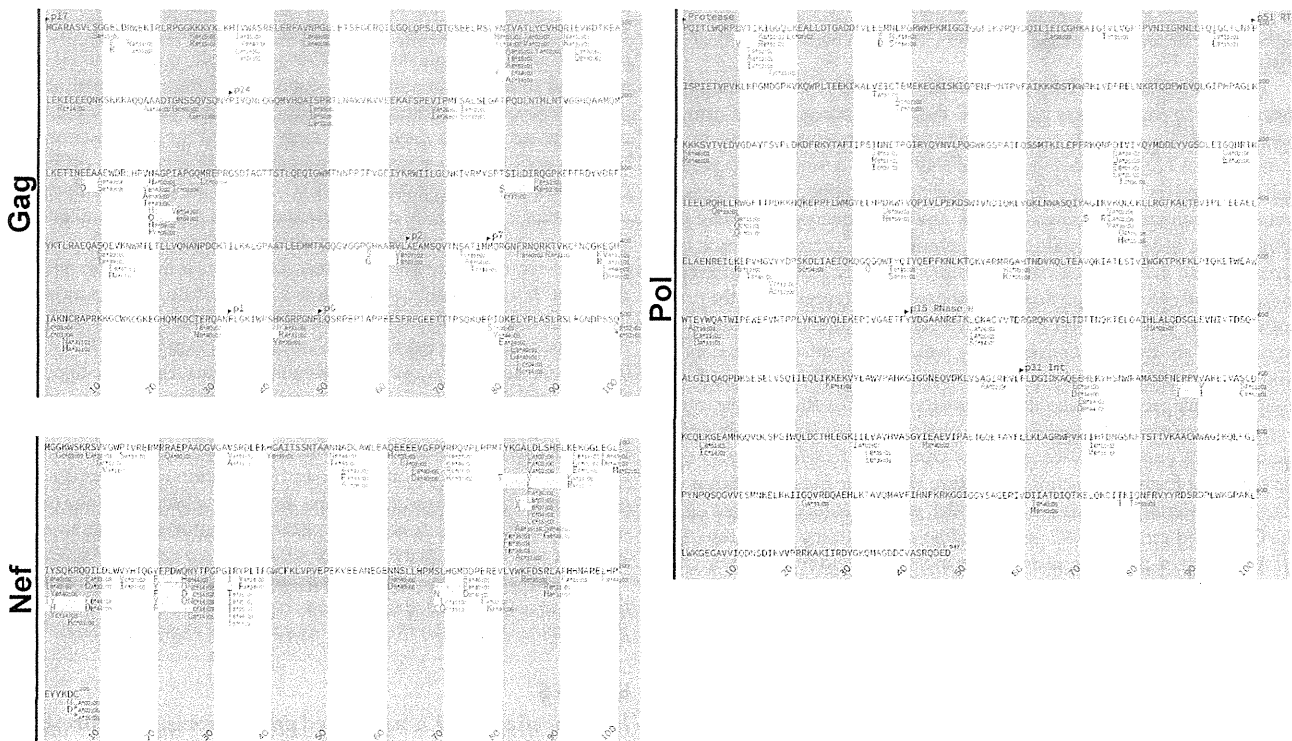


FIG 1 Escape map of HLA-APs for Gag, Pol, and Nef in the Japanese cohort. The escape maps indicate the locations, specific residues, and HLA restrictions of HLA-APs (all  $q < 0.2$ ). The global HIV-1 clade B consensus amino acid sequence is used as a reference. The shaded vertical bars separate blocks 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.

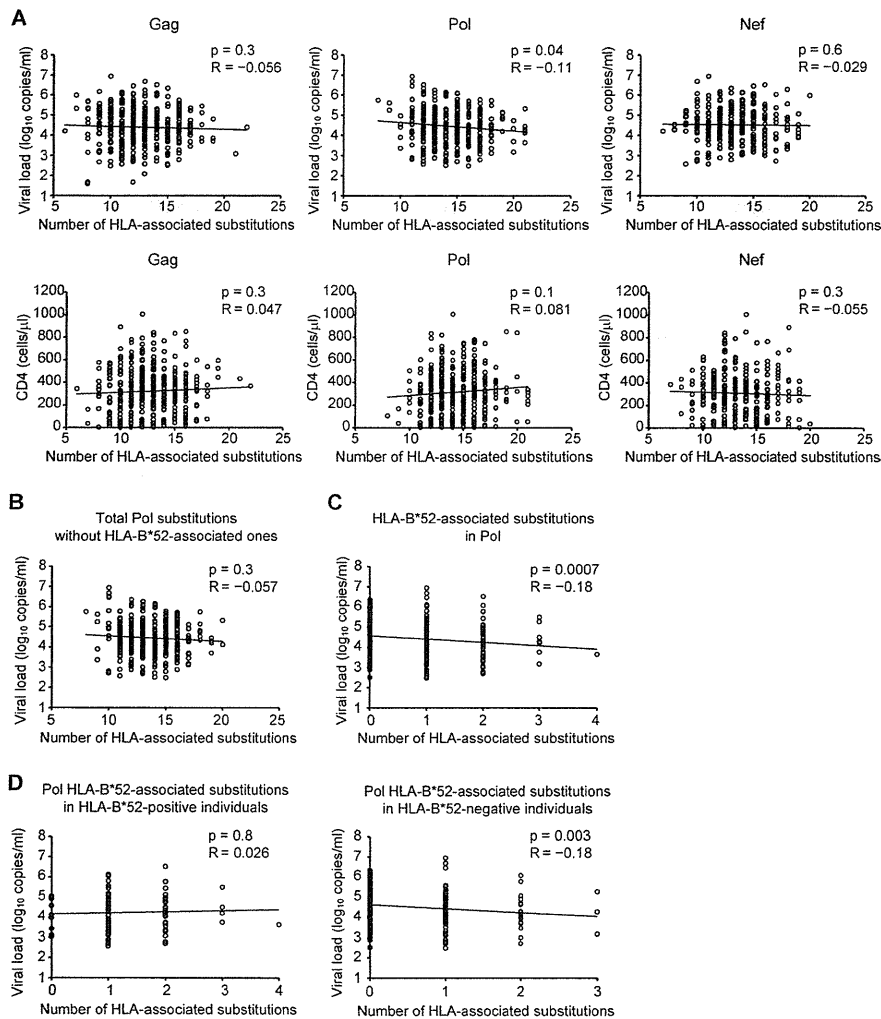
polymorphisms were 78, 140, and 66, respectively, numbers that were also consistent with previous reports from Caucasian and African cohorts that HLA-B alleles restrict more associations than HLA-A or HLA-C alleles (1, 6, 18).

**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.

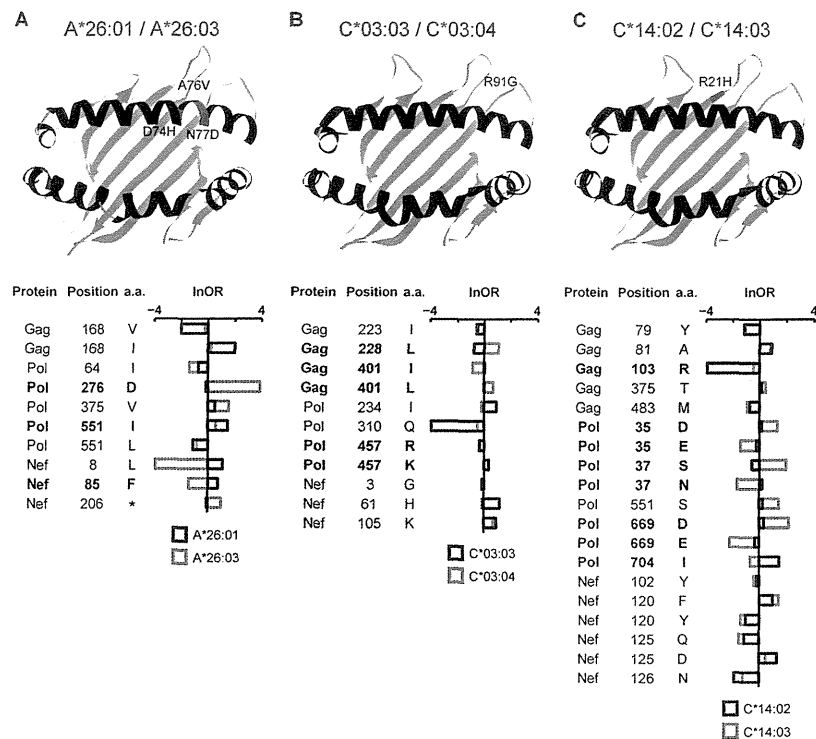
reasoned that B\*52:01-restricted substitutions were likely to be critical mediators of the observed pVL effect.

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<sup>-</sup> individuals (Spearman's  $R = -0.18$ ;  $P = 0.003$ ), but not in HLA-B\*52:01<sup>+</sup> 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<sup>-</sup> individuals. In contrast, no such pVL effects are detectable in B\*52:01<sup>+</sup> 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





**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 InORs, with colors indicating the restricting allele. Infinite InORs are set to values of  $\pm 4$ . 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 (InORs) 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

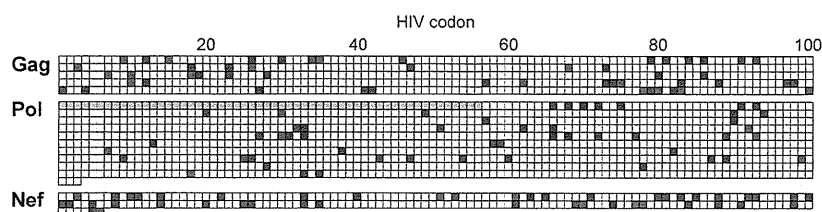


FIG 4 Locations of HLA-associated sites common to HIV-1 clade B-infected Japanese and Caucasian cohorts and those unique to Japan. The locations of all HLA-APs in Gag (500 codons), Pol (1,003 codons), and Nef (206 codons) are illustrated. The residues in the Pol TF protein were not analyzed in the IHAC cohort and are thus excluded (gray bar). The blue squares identify codons that harbored at least one HLA-AP in both Japanese and IHAC cohorts. The red squares indicate codons that harbored HLA-APs in Japan but that were not associated with any HLA alleles in the IHAC cohort.

HLA subtype members varied between HLA allele groups that differed with respect to substitutions within or outside the binding groove, we asked whether the extent of differential escape between subtype members of the former group (comprising A\*02, A\*26, B\*15, B\*40, and C\*08) differed from those of the latter group (comprising HLA-C\*03 and C\*14). Overall, we found no significant differences in the proportions of differential escape between them (34.8% for HLA-C\*03/C\*14 subtypes compared to 36.8% for subtypes of all other HLA alleles;  $P = 0.5$ ) (see Table S2 in the supplemental material). This intriguing result suggests that variations outside the HLA binding groove may contribute as much to differential escape as variations within the binding groove.

**Comparison of HLA-APs between Japanese and non-Asian individuals chronically infected with HIV-1 clade B.** Our second objective was to investigate HLA-APs identified in Japan versus those previously identified in non-Asian cohorts infected with HIV clade B. The comparison cohort in this analysis was the IHAC cohort, comprising 1,888 antiretroviral-naive individuals with chronic clade B infection in Canada, the United States, and Australia (in which <5% of cohort participants were Asian) (16).

HLA-APs differ to some extent between human populations due to the presence (or enrichment) of certain HLA alleles in one population versus another. Indeed, HLA allele frequencies differed markedly between the Japan and IHAC cohorts (see Fig. S1 in the supplemental material). As such, we began with a qualitative comparison of HLA-APs between them, starting with a simple positional analysis. In the Japanese cohort, HLA-APs were observed at a total of 147 codon positions in Gag, Pol, and Nef (Fig. 4). Of these, 117 (79.6%) were also associated with at least one HLA allele in the IHAC cohort. In contrast, the remaining 30 positions (including 16, 7, and 7 in Gag, Pol, and Nef, respectively) that harbored HLA associations in Japan were not associated with any HLA alleles in the IHAC cohort (Fig. 4). That 30/147 (20.4%) HIV codons exhibited evidence of HLA-driven selection in Japan but not in the IHAC cohort already strongly suggests that HIV is evolving under population-specific selection pressures in Japan compared to other regions.

Next, we compared HLA-APs over HIV position and specific HLA restriction. Of the 284 HLA-APs identified in Japan, 188 (66.2%) were not reported in the IHAC cohort. As expected, a substantial portion of these (46 of 188 [24.5%]) were associated with 8 HLA subtypes (A\*26:03, B\*40:06, B\*54:01, B\*55:02, B\*59:01, B\*67:01, C\*08:03, and C\*14:03) common in Japan but essentially absent (<1% frequency) in the IHAC cohort. Others were likely attributable to alleles observed at much higher frequencies in Japan than in the IHAC cohort: for example, an additional 27.1% were associated with HLA alleles present in both cohorts

but whose frequencies were at least 4-fold higher in Japan than in the IHAC cohort. Overall, the results suggest that HLA-APs identified in Japan are quite distinctive, in large part reflecting the unique HLA allele distribution in the Japanese population.

We also wished to investigate the existence of differential HLA-associated escape pathways between the two populations that are not attributable to HLA frequency differences between them—in other words, cases where the same HLA subtypes drive significantly different escape pathways in the Japan and IHAC cohorts. This required the application of statistical tests (see Materials and Methods and below). Specifically, we first identified a list of 551 HLA-APs in HIV Gag, Pol, and Nef, which represented the union of all HLA-APs identified in either the Japan or IHAC cohort for which both the viral polymorphism and the restricting HLA allele were observed in a minimum of 10 individuals per cohort (not shown). The latter criterion was employed in order to achieve some minimal statistical power to compare the strengths of individual associations between cohorts. It is important to emphasize that these criteria would by definition exclude HLA alleles (and/or viral polymorphisms) present in one cohort but essentially absent in the other (as we would have no power, and in fact no rationale, to test whether their strengths of selection were statistically significantly different between cohorts).

For each HLA-AP, we calculated its lnOR of association in each cohort—a measure that can be interpreted as an estimate of the strength of selection exerted by the HLA allele on that particular HIV codon in that cohort. We then applied a phylogenetically corrected interaction test (17) to assess whether these lnORs of selection were significantly different in the Japanese versus the IHAC cohort. In these analyses, statistical significance was defined as a  $P$  value of <0.01 and a  $q$  value of <0.05.

Overall, 71 of 551 (12.8%) HLA-APs originally identified in either the Japan or IHAC cohort exhibited significantly different strengths of selection between the two populations (Fig. 5; see Table S3 in the supplemental material). The HLA-B\*44:03-associated 125H substitution in Nef serves as an example of how to interpret these data. The lnOR of this association is 1.73 in Japan (with a cohort-specific  $P$  value of  $3.26 \times 10^{-6}$ ) versus 0.42 for the IHAC cohort (with a cohort-specific  $P$  value of 0.36). Both lnORs are positive, indicating that 125H is positively associated with B\*44:03 in both cohorts, but the higher lnOR in Japan indicates that the strength of selection of Nef-125H by B\*44:03 is greater in Japan than in the IHAC cohort (indeed, the cohort-specific  $P$  values reveal that this association is significant in Japan but not in the IHAC cohort). Finally, the  $P$  and  $q$  values for the intercohort comparison ( $P = 1.02 \times 10^{-6}$  and  $q = 1.19 \times 10^{-4}$ ) (see Table S3 in the supplemental material) confirm that the strength of selection



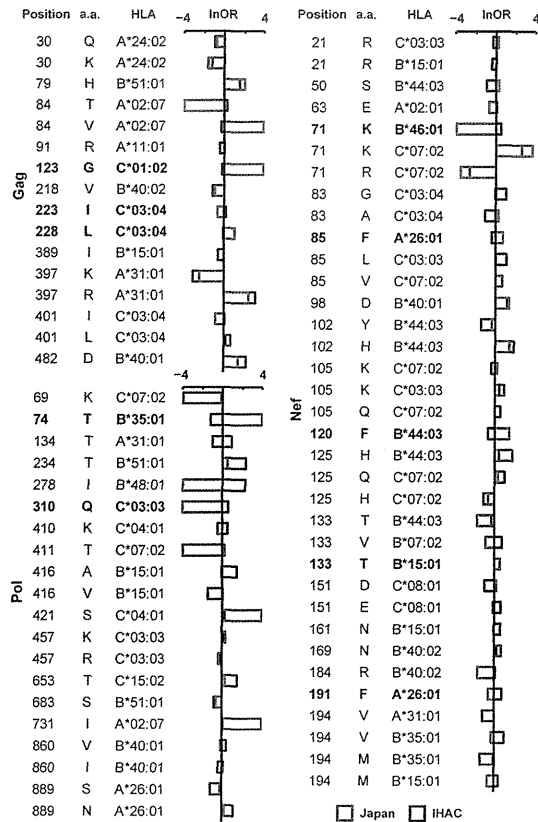


FIG 5 HLA-APs displaying significantly different strengths of selection between Japanese and IHAC cohorts. A phylogenetically corrected interaction test was used to compare the lnOR of selection of HLA-APs in the Japanese cohort versus the IHAC cohort. Comparisons with a  $P$  value of  $<0.01$  and a  $q$  value of  $<0.05$  are shown. The bars represent the lnORs. Infinite lnORs are set to values of  $\pm 4$ . Boldface type indicates HLA-APs that display diametrically opposed directions of selection between the cohorts (defined here as lnORs of association that were positive in one cohort but negative in the other, where the cohort-specific  $P$  values were  $<0.05$  in both cases). A complete list of all comparisons with a  $P$  value of  $<0.05$  is available in Table S3 in the supplemental material.

of Nef-125H by B\*44:03 is significantly greater in Japan than in the IHAC cohort. Importantly, this difference is not simply attributable to intercohort differences in B\*44:03 frequencies (which are comparable between populations [see Fig. S1 in the supplemental material]).

In addition to the HLA-B\*44:03-associated 125H polymorphism in Nef, we identified 21 other HLA-APs whose strengths of selection were significantly greater in Japan than in the IHAC cohort, yielding a total of 22 (out of 71 [31.0%]) HLA-APs in this category. Conversely, 39 of 71 (54.9%) differentially selected HLA-APs exhibited strengths of selection that were greater in the IHAC cohort than in Japan. The HLA-A\*26:01-associated 889S substitution in Pol serves as an example. The lnOR of this association is  $-0.18$  in Japan (with a cohort-specific  $P$  value of 0.3) versus  $-1.17$  for the IHAC cohort (with a cohort-specific  $P$  value of  $7.92 \times 10^{-9}$ ). Both lnORs are negative, indicating that 889S is negatively associated with A\*26:01 in both cohorts, but the more negative value for the IHAC cohort indicates that this association is stronger in the IHAC cohort than in Japan. Finally, the  $P$  and  $q$  values for the intercohort comparison ( $P = 1.15 \times 10^{-4}$  and  $q =$

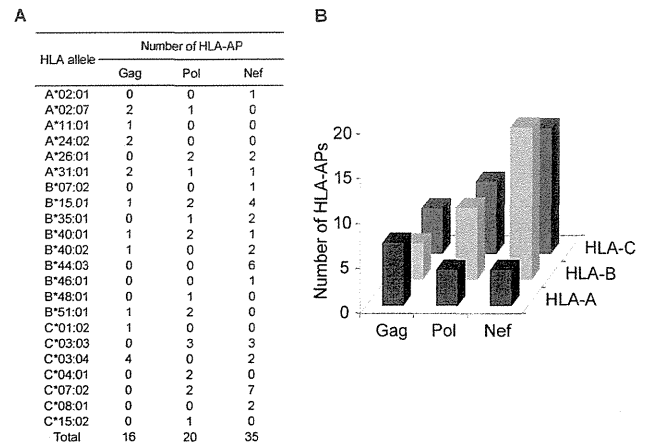


FIG 6 HLA-APs identified as being under differential strengths of selection in Japanese and IHAC cohorts. At a  $P$  value of  $<0.01$  and a  $q$  value of  $<0.05$ , a total of 71 HLA-APs were identified as being under significantly different strengths of selection in the Japanese and IHAC cohorts. (A) Restricting HLA alleles and their HIV-1 protein locations. (B) Numbers of differentially selected HLA-APs, broken down by HLA locus and HIV-1 protein.

$4.48 \times 10^{-3}$  [see Table S3 in the supplemental material]) confirm that the strength of the negative association between Pol-889S by A\*26:01 is significantly greater in the IHAC cohort than in Japan.

Strikingly, the remaining 10 (out of 71 [14.1%]) differentially selected HLA-APs displayed diametrically opposed directions of selection between the cohorts (defined here as lnORs of association that were positive in one cohort but negative in the other, where the cohort-specific  $P$  values were  $<0.05$  in both cases) (Fig. 5). The HLA-B\*44:03-associated 120F substitution in Nef serves as an example. The lnOR of this association is 1.44 in Japan (with a cohort-specific  $P$  value of  $2.03 \times 10^{-4}$ ), indicating that HLA-B\*44:03 is significantly positively associated with 120F in Japan. In contrast, the lnOR of this association is  $-0.69$  in the IHAC cohort (with a cohort-specific  $P$  value of  $9.50 \times 10^{-3}$ ), indicating that HLA-B\*44:03 is significantly negatively associated with 120F in IHAC. The  $P$  and  $q$  values for the intercohort comparison ( $P = 2.15 \times 10^{-8}$  and  $q = 3.75 \times 10^{-6}$  [see Table S3 in the supplemental material]) confirm that the opposing directions of selection of Nef-120F by B\*44:03 between the Japanese and IHAC cohorts is a statistically significant observation.

Of interest, the 71 HLA-APs identified as being under significantly different selection in the Japan and IHAC cohorts were differentially distributed across HLA loci and HIV proteins (Fig. 6A and B). Specifically, HLA-A-associated polymorphisms that were significantly differentially selected across cohorts were most abundant in Gag, followed by Pol and Nef, whereas differentially selected HLA-B-associated and HLA-C-associated polymorphisms were most numerous in Nef, followed by Pol and Gag. Taken together, the results support the existence of HLA class I alleles that drive significantly different HIV escape pathways in global populations infected with the same viral clade. The uneven distribution of the locations of these differentially selected polymorphisms across HLA loci and HIV regions raises the intriguing hypothesis that Gag and Pol/Nef may differentially evolve under selection pressures dominated by HLA-A versus HLA-B/C allele-restricted immune responses, respectively.

## DISCUSSION

The present study comprised two major objectives, both of which are novel in terms of populations studied and/or analytical methods used. First, we characterized HLA-APs in HIV-1 clade B Gag, Pol, and Nef and their relationship with clinical parameters in a large Japanese cohort. Second, we compared HLA-APs in Japanese versus non-Asian populations infected with HIV clade B to identify population-specific differences in their selection. In particular, we wished to identify HLA-APs that are unique to Japan by virtue of the distinctive HLA distribution in this population, as well as cases where the same HLA allele drives divergent escape pathways in Japan versus non-Asian populations.

This study is the first to identify HLA-APs in HIV-1's structural and functional genes in Japanese populations. Only one previous study investigated HLA-APs in HIV-1 clade B-infected Asians (11): the study comprised 231 Chinese individuals infected during a narrow-source outbreak and identified 141 HLA-associated polymorphisms at two-digit resolution. Our study differs from the previous study with respect to the cohort size, HLA genetics of the host population, HLA-typing resolution, and type of epidemic. Using phylogenetically informed approaches, we identified 284 HLA-APs within HIV-1 Gag, Pol, and Nef in our cohort, supporting a strong influence of population-specific, HLA-driven immune pressures in shaping HIV-1 evolution in Japan. In contrast to a previous study undertaken in a predominantly Caucasian population that observed approximately one-half of the total number of Gag HLA-APs to be located within or flanking reported CTL epitopes (3), the majority of HLA-APs identified in the present study were not located near reported CTL epitopes. This discrepancy may be due to the limited number of Asian-specific HLA-restricted CTL epitopes identified to date, underscoring the need for further epitope discovery in these populations.

This study revealed differential frequencies of HLA-APs across HIV genes in the Japanese population. Consistent with previous studies of HLA-APs in HIV clade B (2, 16, 18), HLA-APs were more frequently detected in Nef than in Gag and Pol. Also consistent with previous observations in Caucasian, African, Chinese, and Mexican populations (1, 6, 11, 15, 18), the number of HLA-B-associated polymorphisms in our cohort was higher than that of HLA-A- or HLA-C-associated polymorphisms, further supporting a dominant role of HLA-B in HIV evolution (32). An interesting feature of the Japanese population is that approximately 70% of individuals carry HLA-A\*24:02 (23). Despite sufficient statistical power to detect HLA-A\*24:02-associated polymorphisms in our cohort, we identified only 9 of them, 6 of which were located in epitopes identified by our group (33–35). A possible explanation for the relatively low number of A\*24:02-associated polymorphisms in Japan is that they have accumulated over time in circulating sequences so that they are no longer significantly enriched among persons expressing HLA-A\*24:02. Further analysis of mutations selected by HLA-A\*24:02-restricted CTLs should clarify the mechanism whereby high-frequency HLA alleles influence the formation of HIV-1 polymorphisms.

Protective HLA alleles, such as HLA-B\*57, -B\*58, and -B\*27, select Gag mutations affecting viral replication in Caucasians and Africans (36–41) that may also provide some clinical benefit if they are transmitted to hosts lacking these alleles (42, 43). HLA-B\*57, -B\*58, and -B\*27 are not present at appreciable frequencies in Japan (23). It is therefore perhaps unsurprising that no corre-

lations between HLA-associated substitutions in Gag and HIV clinical parameters were observed in our cohort. In contrast, we observed a weak but significant inverse correlation between the frequency of HLA-APs in Pol and the plasma viral load, which appeared to be driven by polymorphisms selected by HLA-B\*52:01, an allele identified as protective in Japan (24). Upon further stratification by HLA-B\*52:01 expression, the inverse correlation between VL and the total number of B\*52:01-associated Pol substitutions was maintained in HLA-B\*52:01<sup>-</sup> but not in HLA-B\*52:01<sup>+</sup> individuals. Taken together, these findings suggest that transmitted B\*52:01-associated polymorphisms could reduce viral fitness in a dose-dependent manner, though further studies will be required to assess this. In addition, these substitutions were not located within or near known B\*52:01-restricted epitopes. Thus, further research will be required to identify these epitopes and elucidate their mechanisms of escape.

Many previous studies of HLA-APs were performed at two-digit HLA resolution (1–4, 6). Here, we performed HLA genotyping at four-digit resolution, which allowed us to investigate differential escape between closely related HLA subtypes in the Japanese cohort. Nearly one-half of the HLA-APs identified in Japan were restricted by HLA allele groups containing two or more subtype members (A\*02, A\*26, B\*15, B\*40, C\*03, C\*08, and C\*14). For five of these groups (A\*02, A\*26, B\*15, B\*40, and C\*08), subtype members differed by substitutions within the peptide-binding groove, while for the remaining two groups (HLA-C\*03 and -C\*14), subtype members differed by substitutions located outside the peptide-binding groove. Reasoning that amino acid differences located within the peptide-binding groove could modulate the nature or presentation of CTL epitopes, we hypothesized that the former group would generally exhibit distinct HLA-APs between subtype members, while the latter would generally exhibit similar or identical HLA-APs. However, we were surprised to observe substantial evidence for differential HLA-AP selection between closely related HLA subtypes regardless of whether they differed in sequence within or outside the peptide-binding groove. Significantly differential HLA-AP selection was observed at 3 of 9 HLA-C\*03-associated sites and 5 of 14 HLA-C\*14-associated sites (Fig. 3), proportions that were not significantly lower than the frequency of differential selection between subtypes that differed in their peptide-binding grooves.

This observation raised several hypotheses. HLA polymorphic sites outside the peptide-binding groove may indirectly influence the binding groove conformation, thus altering HLA-peptide interactions and/or T cell recognition. Another possibility is selection by NK cells, as KIR may recognize sites outside the peptide-binding groove. Indeed, KIR3DL1 binds to the loop including position 91 of HLA-B\*57:01 (44). However, it is not clear whether KIR2DLs, which are receptors for HLA-C, can bind to the loop outside the peptide-binding groove of HLA-C molecules. A recent study showed that HLA-C antigens are expressed at different levels on the cell surface, even among HLA-C subtypes (45). This study also observed a strong positive correlation between the HLA-C expression level and the strength of HLA-C-mediated selection pressure conferred on HIV. Differential expression levels of these HLA-C subtype members in Japanese populations thus provide another potential explanation for this observation for future follow-up.

Our second objective was to investigate differential HLA-APs between Japanese and non-Asian cohorts infected with HIV clade

B. Here, the IHAC cohort (comprising clade B-infected Canadians, Americans, and Australians) was used as a comparison group (16). HLA-APs identified in human populations differ to some extent due to population-specific HLA distributions, yielding population-specific HLA-APs driven by HLA alleles present in one population but not another (15). Indeed, two-thirds of the HLA-APs identified in Japan had not previously been identified due to the presence of the restricting HLA alleles in Japan but their absence (or far lower prevalence) in the IHAC cohort.

What remains unknown however, is the extent to which the same HLA allele may drive significantly different escape pathways in different human populations. To this end, we applied novel phylogenetically corrected statistical approaches to assess the extent to which HLA-APs identified in either Japan or the IHAC cohort restricted by HLA alleles present in both populations exhibited significantly different strengths of selection. Of the 551 HLA-APs investigated, 71 (12.9%) were significantly differentially selected in Japan versus the IHAC cohort at a stringent statistical threshold of a  $q$  value of  $<0.05$ . Of these 71, 31% exhibited significantly greater strengths of selection in Japan than in the IHAC cohort, whereas 55% exhibited greater strengths of selection in the IHAC cohort than in Japan. Surprisingly, the remaining 14% displayed diametrically opposed selection pathways in the two cohorts (where an HIV polymorphism represented the adapted form associated with a given allele in one cohort but the non-adapted form associated with the same allele in the other cohort). It is important to emphasize that these significantly different pathways of HLA-AP selection are not simply attributable to differences in HLA frequency between the cohorts.

We feel that these are intriguing observations that merit further study. Nevertheless, we propose the following potential interpretations. First, these differences could be explained by functional differences in HIV-1-specific T cells elicited between the Japanese and Caucasian cohorts, possibly as a result of differences in host genetics (for example, in the genes that encode the T-cell receptor and/or modulate its expression). Such differences may influence the structure of the T-cell receptor(s) and thus the quality, quantity, and/or makeup of the HIV-1-specific T cell repertoire, thus influencing the specific escape mutations selected in the context of peptide-bound HLA. Further analysis of HIV-1-specific T cells driving the selection of these mutants in both cohorts is therefore warranted. It is also important to note that the inter-cohort HLA-AP comparisons, unlike previous analyses, did not correct for HLA LD or HIV codon covariation. Although both the Japan and IHAC cohorts feature HIV clade B infections, intra-clade differences in the viral backbone could also influence differential escape via epistatic effects. In-depth analyses of intercohort differences in HIV codon covariation relationships are therefore also warranted. Intercohort differences in HLA LD are another possible contributor. Finally, the HLA-APs differentially selected between cohorts appeared to be unevenly distributed by HLA locus; while HLA-A-associated polymorphisms exhibiting differential selection between cohorts were more abundant in Gag than in other proteins, HLA-B- and HLA-C-associated polymorphisms exhibiting differential selection between cohorts tended to be more abundant in Nef. This suggests that intercohort differential HLA-APs across HIV proteins may be arising as a result of cellular immune pressures exerted by distinct HLA class I loci, though this also requires further study.

Nevertheless, the present study confirms the existence of pop-

ulation-specific HIV-1 adaptations that are attributable to the unique HLA allele distributions of the population (15). We additionally provide evidence of population-specific HIV adaptation to HLA-restricted immune responses that cannot be explained by differential HLA frequencies alone, cases where the same HLA allele drives significantly different, sometimes opposing, escape pathways in different host populations. Taken together, the results support differential HIV-1 adaptation to human populations worldwide that might be driven by multiple host and viral mechanisms.

## ACKNOWLEDGMENTS

We thank Madoka Koyanagi and Rie Maruyama for the collection of samples from patients, Mari Hasegawa and Sayaka Nagata for technical assistance, Sachiko Sakai for her secretarial assistance, and Kyle Cobarrubias for assistance in constructing Fig. 1.

T.C. is a JSPS Research Fellow. A.Q.L. is the recipient of a Frederick Banting and Charles Best Masters award from the Canadian Institutes for Health Research (CIHR). Z.L.B. is the recipient of a New Investigator Award from the CIHR and a Scholar Award from the Michael Smith Foundation for Health Research (MSFHR). This research was supported by the Global COE program Global Education and Research Center Aiming at the Control of AIDS, launched as a project commissioned by the Ministry of Education, Science, Sports, and Culture, Japan, and by Grants-in-Aid for AIDS Research from the Ministry of Health, Labor, and Welfare, Japan.

We have no financial conflicts of interest.

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