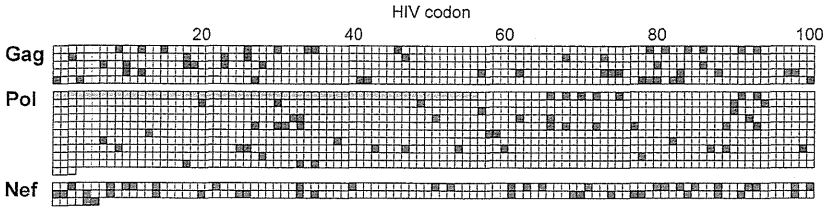

FIG 4 Locations of HLA-associated sites common to HIV-1 clade B-infected Japanese and Caucasian cohorts and those unique to Japan. The locations of all HLA-APs in Gag (500 codons), Pol (1,003 codons), and Nef (206 codons) are illustrated. The residues in the Pol TF protein were not analyzed in the IHAC 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 3.26×10^{-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

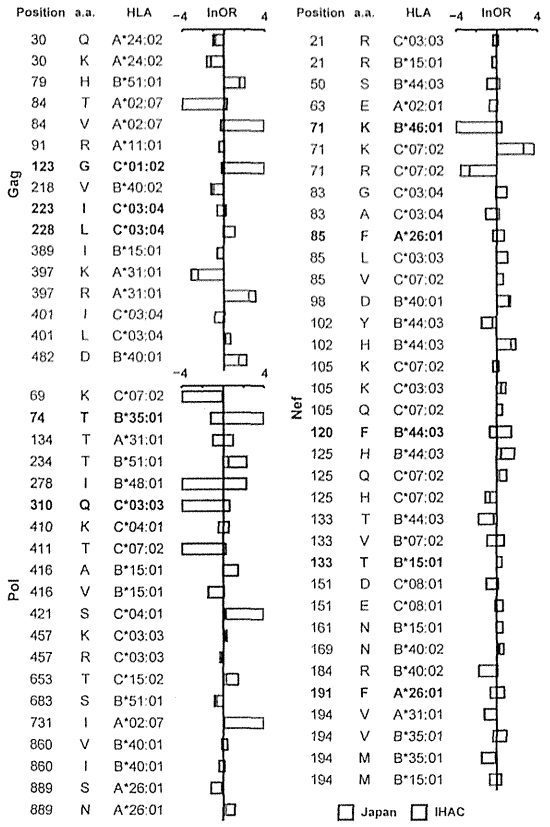

FIG 5 HLA-APs displaying significantly different strengths of selection between Japanese and IHAC cohorts. A phylogenetically corrected interaction test was used to compare the lnOR of selection of HLA-APs in the Japanese cohort versus the IHAC cohort. Comparisons with a P value of <0.01 and a q value of <0.05 are shown. The bars represent the lnORs. Infinite lnORs are set to values of ±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.

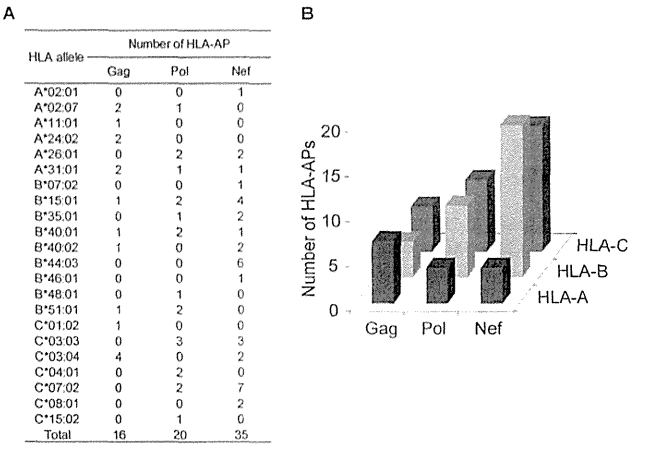

FIG 6 HLA-APs identified as being under differential strengths of selection in Japanese and IHAC cohorts. At a P value of <0.01 and a q value of <0.05, a total of 71 HLA-APs were identified as being under significantly different strengths of selection in the Japanese and IHAC cohorts. (A) Restricting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

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 7.92×10^{-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 $q = 4.48 \times 10^{-3}$ [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 2.03×10^{-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 9.50×10^{-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 correlations 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$^-$ but not in HLA-B*52:01$^+$ 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 nonadapted 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 intercohort 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, intraclade 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 population-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.

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Short communication

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

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Abstract

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

Keywords: Integrase inhibitor; CTL; HIV

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Samples of HIV-1-infected individuals

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

2.2. Synthetic peptides

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

2.3. Cells

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

2.4. Generation of CTL clones

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

2.5. HIV-1 clones

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

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

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

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

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

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

2.8. ELISpot assay

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

2.9. Sequencing of plasma RNA

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

3. Results

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

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

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

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

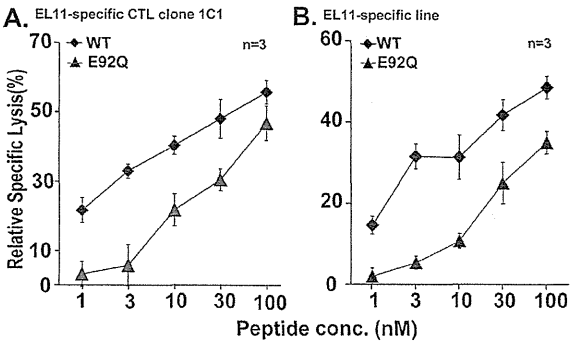

Fig. 1. Relative cytolytic activity of EL11-specific CTLs against target cells pulsed with the WT (EL11) or mutant (EL11–E92Q) peptide. An EL11-specific CTL clone and cell line were generated from PBMCs by stimulating them with WT peptide from a HLA-B*40:02 patient, KI-400, infected with WT virus. The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

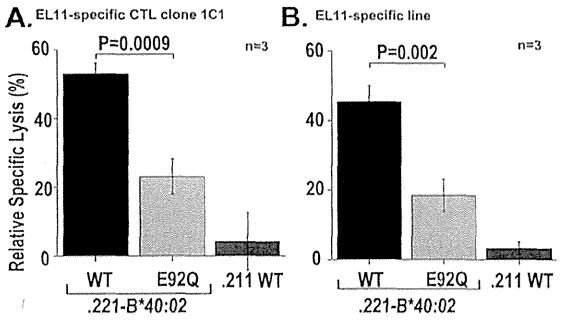

Fig. 2. Relative cytolytic activity of EL11-specific CTLs against target cells infected with WT (NL-432) or mutant virus (NL432-E92Q). Relative cytotoxic activity against 721.221-CD4-B*40:02 cells infected with NL-432 (WT virus) or NL-432-E92Q (E92Q virus) was assessed. (A) WT virus-infected (43.7% of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

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

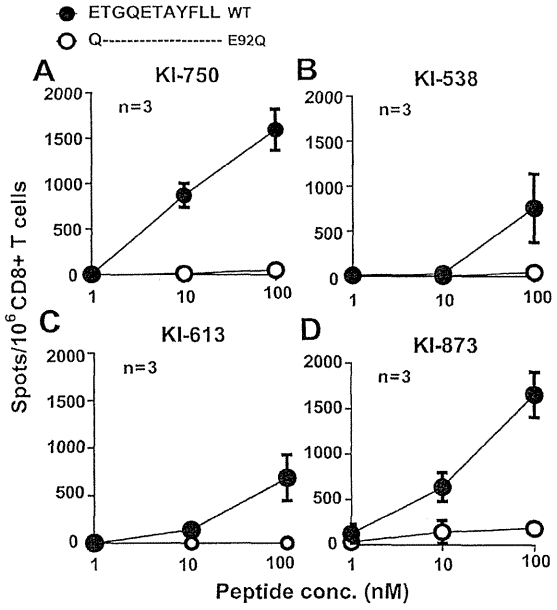

Fig. 3. *Ex vivo* $CD8^+$ T-cells response specific for WT (EL11) or mutant (EL11–E92Q) peptides in chronically HIV-1-infected HLA-B*40:02^+ individuals. $CD8^+$ T cell responses to EL11 or EL11–E92Q peptides by PBMCs from 5 HLA-B*40:02^+ chronically HIV-1-infected individuals were analyzed by performing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

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

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

4. Discussion

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

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

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

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

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

Conflict of interest

The authors have no conflicting financial interests.

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Naturally Selected Rilpivirine-Resistant HIV-1 Variants by Host Cellular Immunity

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Background. Rilpivirine is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines. E138G/A/K in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) are rilpivirine resistance-associated mutations and can be identified in a few ART-naive patients, although at low frequency. The 138th position in HIV-1 RT is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18–restricted cytotoxic T lymphocytes (CTLs). CTL-mediated immune pressure selects escape mutations within the CTL epitope. Here we tested whether E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Methods. The amino acid variation at the 138th position was compared between ART-naive HIV-1–infected patients with and without HLA-B*18. The optimal epitope containing the 138th position was determined and the impact of E138G/A/K on CTL response was analyzed by epitope-specific CTLs. The effect of E138G/A/K on drug susceptibility was determined by constructing recombinant HIV-1 variants.

Results. The prevalence of E138G/A/K was 21% and 0.37% in 19 and 1088 patients with and without HLA-B*18, respectively (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$). The CTL response was completely abolished by the substitution of E138G/A/K in the epitope peptide. E138G/A/K conferred 5.1-, 7.1-, and 2.7-fold resistance to rilpivirine, respectively.

Conclusions. E138G/A/K can be selected by HLA-B*18-restricted CTLs and confer significant rilpivirine resistance. We recommend drug resistance testing before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Keywords. rilpivirine; E138G/A/K; HLA-B*18; CTL.

Rilpivirine is a new-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), with noninferior clinical efficacy demonstrated in large clinical trials, compared with efavirenz [1, 2], and is listed as an alternative key drug in current antiretroviral therapy (ART) guidelines [3, 4]. In those clinical trials, rilpivirine showed more-favorable safety and tolerance profiles compared with efavirenz, although it was also associated with a higher virological failure rate. The most commonly observed NNRTI resistance-associated mutation in rilpivirine-treated patients with virological failure has so far been E138 K [1, 2]. Not only E138 K, but also other substitutions at the 138th position in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), might confer significant rilpivirine resistance [5–7]. The glutamic acid at the 138th position (E138) is well conserved among HIV-1 strains and clinical isolates throughout clades [8]. However, some ART-naive patients are infected with HIV-1 variants harboring other amino acids at the 138th position (E138X), although the proportion of such patients is low [9]. The 138th position is located in one of the putative epitopes of human leukocyte antigen (HLA)–B*18-restricted cytotoxic T lymphocytes (CTLs) [10, 11]. Because CTL immune pressure often selects escape mutations within the epitope [11], E138X may be selected by HLA-B*18-restricted CTLs. In this study, we analyzed the frequency of amino acid variations at the 138th position in ART-naive patients with or without

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Table 1. Amino Acid Variations at the 138th Position of HIV-1 Reverse Transcriptase and Human Leukocyte Antigen–B*18

Amino Acid	HLA-B*18(+)	HLA-B*18(−)
E138 (wild-type)	15	1084
E138G	2	1
E138A	1	2
E138K	1	1

Abbreviation: HLA, human leukocyte antigen.

HLA-B*18, determined the impact of E138X on CTL response, and analyzed the drug susceptibility of recombinant HIV-1 variants harboring E138X.

METHODS

Sequences of HIV-1 Reverse Transcriptase

HIV-1 RT sequences were analyzed using viral RNA extracted from plasma samples [12], and HLA type was determined by standard sequence-based genotyping in 1107 ART-naive infected individuals who visited the Outpatient Clinic of the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, between 2003 and 2012. The amino acid variation at the 138th position of HIV-1 RT was compared between individuals with and those without HLA-B*18, and the statistical significance of the difference was analyzed by Fisher exact test using the Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, Illinois). This study was approved by the institutional ethical committee of the National Center for Global Health and Medicine, and written informed consent was obtained from all the participants according to the Declaration of Helsinki.

Intracellular Cytokine Staining Assay

HIV-1-derived peptides and mutant peptides were synthesized using an automated multiple peptide synthesizer and purified by high-performance liquid chromatography. Peripheral blood mononuclear cells (PBMCs) from chronically HIV-1–infected HLA-B*18-positive patients were stimulated with the peptide (100 nM) in culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum and 200 U/mL recombinant human interleukin 2). After 14 days in culture, the cells were assessed for interferon (IFN)–γ production activity using a FACSCanto II (BD Biosciences, San Jose, California) [13, 14].

Drug Susceptibility Assay

The desired mutations were introduced into the *Xma*I-*Nhe*I region of pTZNX, which encodes the 15th–267th positions of HIV-1 RT (strain BH10) [15, 16]. The *Xma*I-*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. Each molecular clone was transfected into COS-7 cells, and the obtained virions were harvested 48 hours after transfection and stored at −80°C until use. Efavirenz and nevirapine were generously provided by Merck Co, Inc (Rahway, New Jersey) and Boehringer Ingelheim Pharmaceutics Inc (Ridgefield, Connecticut), respectively. Etravirine and rilpivirine were purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). The susceptibility of recombinant HIV-1 variants to efavirenz, nevirapine, etravirine, and rilpivirine was determined in triplicate and repeated 3 times [16]. Fold resistance was calculated by comparing the viral 50% inhibitory concentration (IC_{50}) with that of monoclonal wild-type HIV-1.

Structural Modeling

We constructed structural models of the HIV-1 RT and rilpivirine complex by computational analysis, as described in our

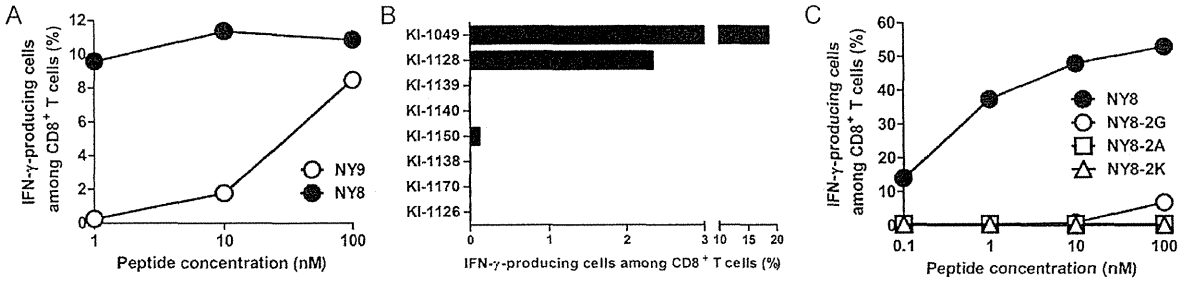

Figure 1. Recognition of human leukocyte antigen (HLA)–B*18-restricted $CD8^+$ T cells. *A*, Identification of the optimal epitope of HLA-B*18-restricted $CD8^+$ T cells. Peripheral blood mononuclear cells (PBMCs) from an HLA-B*18-positive individual chronically infected with human immunodeficiency virus type 1 (HIV-1) were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward each peptide was measured by the intracellular cytokine staining (ICS) assay. *B*, Induction of NY8-specific $CD8^+$ T cells in HLA-B*18-positive individuals chronically infected with HIV-1. PBMCs from 8 chronically HIV-1-infected HLA-B*18-positive individuals were stimulated with NY9 peptide and cultured for 2 weeks. Recognition of the bulk $CD8^+$ T cells toward NY8 peptide were measured by the ICS assay. *C*, Effects of E138G/A/K substitutions on the recognition of HLA-B*18-restricted $CD8^+$ T cells. Recognition of the bulk $CD8^+$ T cells toward each wild-type or mutant peptide was measured by the ICS assay. Abbreviations: IFN-γ, interferon gamma; NY8, NETPGIRY; NY8-2G, NGTPGIRY; NY8-2A, NATPGIRY; NY8-2 K, NKTPGIRY; NY9, NNETPGIRY.

Table 2. Susceptibility of Recombinant HIV-1 Variants to 4 Nonnucleoside Reverse Transcriptase Inhibitors

	IC_{50} (nM), Fold Resistance[a]			
Amino Acid	EFV	NVP	ETR	RPV
E138 (wild-type)	1.2 ± 0.2 (1)	31 ± 3 (1)	1.1 ± 0.1 (1)	0.16 ± 0.04 (1)
E138G	1.6 ± 0.2 (1.3)	30 ± 10 (0.97)	2.4 ± 0.3 (2.2)	0.82 ± 0.09 (5.1)
E138A	2.1 ± 0.3 (1.8)	30 ± 2 (0.97)	2.6 ± 0.2 (2.4)	1.13 ± 0.20 (7.1)
E138K	2.4 ± 0.4 (2.0)	50 ± 10 (1.6)	2.4 ± 0.1 (2.2)	0.43 ± 0.10 (2.7)

Data are presented as mean ± standard deviation.

Abbreviations: EFV, efavirenz; ETR, etravirine; IC_{50}, viral 50% inhibitory concentration; HIV-1, human immunodeficiency virus type 1; NVP, nevirapine; RPV, rilpivirine.

[a] Fold resistance was calculated by comparing viral IC_{50} with that of monoclonal wild-type HIV-1.

previous reports [15, 16]. In brief, the initial models of wild-type RT with rilpivirine were first constructed by homology modelling. The crystal structures of RT with NNRTI (PDB code: 2ZD1 [17]) was used for template structure. We also constructed the respective mutant RTs with rilpivirine by considering every possible conformer of the respective mutant models. The possible conformers were generated from the wild-type homology models using PyMOL software (http://www.pymol.org). Among the conformers, we selected those with the lowest energy as each mutant model.

RESULTS

First, we analyzed the frequency of amino acid variations at the 138th position of HIV-1 RT in 1107 ART-naive individuals. As expected, E138 was found in the majority (1099 cases [99%]) of the analyzed patients. However, 8 cases showed amino acid substitutions, including 3 cases of substitution with glycine (E138G), 3 cases with alanine (E138A), and 2 cases with lysine (E138 K). The frequency of E138G/A/K substitutions was 21% and 0.37% in 19 and 1088 individuals with and without HLA-B*18, respectively (Table 1). There was a significant difference in the frequency of the substitutions (odds ratio, 72.3; $P = 4.9 \times 10^{-25}$), suggesting that E138G/A/K could be selected by HLA-B*18-restricted CTLs.

Next, we delineated the impact of E138G/A/K on the response of HLA-B*18-restricted CTLs. The putative HLA-B*18-restricted CTL epitopes containing the 138th position of HIV-1 RT were NETPGIRYQY (NY10; position 137–146), NETPGIRYQ (NQ9; position 137–145), and NNETPGIRY (NY9; position 136–144) [10, 11]. These 3 peptides were used to stimulate PBMCs of 8 ART-treated HLA-B*18-positive patients chronically infected with HIV-1. IFN-γ production activity was detected in PBMCs from 1 of the 8 patients when stimulated with NY9. To determine the optimal epitope, the bulk $CD8^+$ T cells were further analyzed for NY9 and NETPGIRY (NY8; position 137–144). The bulk $CD8^+$ T cells more efficiently recognized NY8 than NY9 at 1-nM, 10-nM, and 100-nM concentrations (Figure 1*A*). These findings indicate that NY8 was the optimal epitope of HLA-B*18-restricted CTLs. Indeed, NY8-specific $CD8^+$ T cells were induced in 3 of the 8 patients (Figure 1*B*). A

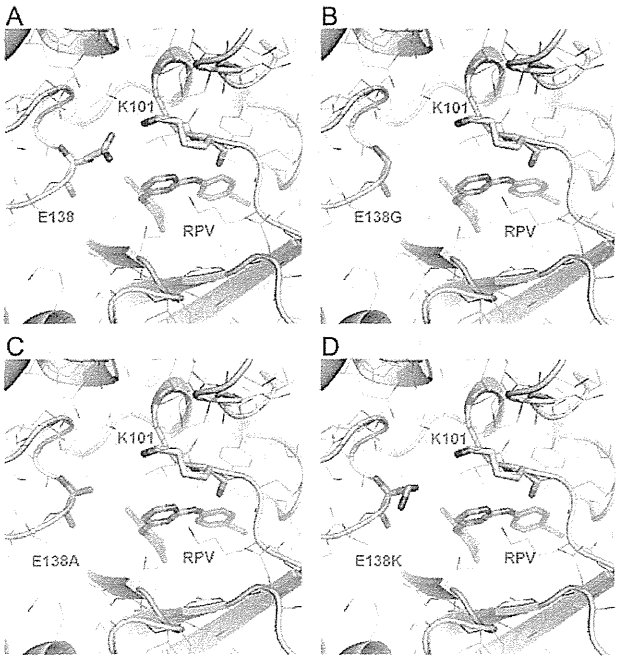

Figure 2. Structural models of human immunodeficiency virus type 1 reverse transcriptase (RT) and rilpivirine. The binding clefts of 4 complexes are shown: $RT_{E138(wild\text{-}type)}$ (*A*), RT_{E138G} (*B*), RT_{E138A} (*C*), and RT_{E138K} (*D*). Sticks indicate the amino acids at positions 101 and 138 of RT, and the atoms of rilpivirine. The mutated residues (E138G, E138A, and E138 K) and rilpivirine atoms are represented by orange and greenish-blue sticks, respectively. Abbreviation: RPV, rilpivirine.

previous study showed that HLA-B*18-binding peptides have 2 anchor residues, E at position 2 and Y/F at the C-terminus [18]. NY8 also had these 2 anchor residues, supporting that this peptide is a HLA-B*18-restricted CTL epitope. To analyze the effect of E138G/A/K on the CTL response, 3 mutant peptides, NGTPGIRY (NY8-2G), NATPGIRY (NY8-2A), and NKTPGIRY (NY8-2 K), were synthesized, and the recognition of the bulk CTLs for these mutant peptides was compared with that for NY8. The bulk CTLs failed to recognize these peptides at 0.1-nM, 1-nM, 10-nM, and 100-nM concentrations, although it effectively recognized NY8 (Figure 1*C*). These substitutions at the 138th position may affect peptide binding to the HLA-B*18 molecule because the second position of HLA-B*18-binding peptides is an anchor for HLA-B*18 [18]. These findings indicate that each of the E138G/A/K affected CTL recognition and allow escape from the HLA-B*18-restricted CTLs.

Finally, we analyzed the effect of E138G/A/K on viral susceptibility to NNRTIs by constructing recombinant HIV-1 variants. Each HIV-1 variant harboring one of E138G/A/K showed comparable replication fitness with wild-type HIV-1. Although the substitutions of E138G/A/K did not confer >2-fold resistance to efavirenz and nevirapine, they conferred mild resistance (2.2- to 2.4-fold) to etravirine. With regard to rilpivirine, E138 K, which was commonly observed in patients with virological failure under rilpivirine-based ART [1, 2], conferred mild resistance, whereas E138G and E138A conferred >5-fold resistance (Table 2). These findings indicate that in addition to E138 K, E138G and E138A can also reduce the clinical response to rilpivirine. The structural modeling suggests that substitution of E138 changes interactions around the rilpivirine-binding cleft (Figure 2). The side chain of E138 in the wild-type RT forms a salt bridge with the lysine at the 101th position (K101) at the edge of the cleft and establishes direct interactions with the pyrimidine moiety of rilpivirine, as seen in the crystal structure of RT with rilpivirine [17]. Meanwhile, mutant RTs with E138G/A/K substitutions could not create such a salt bridge, resulting in changes in the morphology of the binding cleft. In particular, RTs with E138G or E138A can reduce interactions with rilpivirine by creating large gaps between rilpivirine and the substituted 138th residues with small side chains, which seems to cause significant resistance to rilpivirine.

DISCUSSION

The major findings of the present study were as follows: (1) E138G/A/K substitutions were escape mutations of HLA-B*18-restricted CTLs and they were observed more frequently in HLA-B*18-positive patients than HLA-B*18-negative patients; and (2) we confirmed that these substitutions conferred significant resistance to rilpivirine, demonstrating that drug resistance–associated mutations can be selected naturally by CTL when its epitope is located in the viral protein of antiretroviral targets.

Studies of cellular immunology in HIV-1 have focused mainly on Gag [19, 20]. However, considering that many of the recently identified CTL epitopes are located in Pol [13, 14, 21], analysis of the interaction between CTL and drug susceptibility is warranted. Some escape mutations can persist after viral transmission to other hosts even if the new hosts do not have the corresponding HLAs [22]. Therefore, HIV-1 can adapt to HLA at a population level [23]. In fact, we identified E138G/A/K in ART-naive HLA-B*18-negative patients, although the frequency of such variations was extremely low. However, the same analysis performed in areas with higher prevalence of HLA-B*18, such as Eastern Europe [24], would probably detect higher frequency of E138G/A/K.

HIV drug resistance testing is recommended not only after treatment failure but also before the introduction of the initial treatment, considering the risk that the patient may have acquired drug-resistant viruses from those with treatment failure [3, 25]. The present study may add another reason for drug resistance testing of ART-naive patients: drug resistance–associated mutations may have evolved in the patients selected by their own immunity even if the original transmitted viruses were drug sensitive. At the very least, drug resistance testing should be performed before the introduction of rilpivirine-based ART in HLA-B*18-positive patients.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Sustained Inhibition of HIV-1 Replication by Conditional Expression of the *E. coli*-Derived Endoribonuclease MazF in $CD4^+$ T cells

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Abstract

Gene therapy using a Tat-dependent expression system of MazF, an ACA nucleotide sequence-specific endoribonuclease derived from *Escherichia coli*, in a retroviral vector appears to be an alternative approach to the treatment of human immunodeficiency virus type 1 (HIV-1) infection. MazF can cleave HIV-1 RNA, since it has more than 240 ACA sequences. Significant inhibition of viral replication, irrespective of HIV-1 strains, was observed in $CD4^+$ T cells that had been transduced with the MazF-expressing retroviral vector (MazF-T cells). The growth and viability of MazF-T cells were not affected by HIV-1 infection. Interestingly, the infectivity of HIV-1 produced from MazF-T cells was found to be lower than that from control $CD4^+$ T cells. A long-term culture experiment with HIV-1-infected cells revealed that viral replication was always lower in MazF-T cells than in $CD4^+$ T cells transduced with or without a control vector for more than 200 days. MazF was expressed and mainly localized in the cytoplasm of the infected cells. Unlike in $CD4^+$ T cells, the expression level of Tat gradually decreased rather than increased in MazF-T cells after HIV-1 infection. As a consequence, the expression level of MazF appeared to be well regulated and sustained during HIV-1 infection in MazF-T cells. Furthermore, the levels of cellular mRNA were not affected by HIV-1 infection. Thus, the Tat-dependent MazF expression system has great potential for inhibition of HIV-1 replication *in vivo* without apparent toxicity and may be able to avoid the emergence of resistant strains.

Introduction

ANTIRETROVIRAL THERAPY (ART) BASED on the combination of different classes of inhibitors has dramatically improved the status of human immunodeficiency virus type 1 (HIV-1) infection after its establishment. In fact, more than 20 drugs, targeting reverse transcriptase (RT), integrase, and protease, are available in clinic for treatment of HIV-1 infection (Thompson *et al.*, 2010). ART, with these inhibitors, has brought about a significant decrease in plasma viral load to undetectable levels and has considerably improved the prognosis of infected individuals (Pomerantz and Horn, 2003; Weiss, 2008). However, the benefits of current ART are limited by adverse effects, emergence of drug-resistant mutants, and inability to eradicate latently infected cells (Shibuyama *et al.*, 2006; Hirsch *et al.*, 2008; Coiras *et al.*, 2009). Therefore, it is still mandatory to discover and develop novel antiviral drugs or other treatment options that can inhibit the replication of drug-resistant strains without unacceptable adverse events.

Gene therapy has been proposed as an alternative to ART for HIV-1 infection (Sarver and Rossi, 1993; Dropulić and Jeang, 1994). A number of genetic vectors with antiviral payloads targeting HIV-1 RNA have been investigated. These include antisense RNA, ribozyme, and small interfering RNA (siRNA) (Levine *et al.*, 2006, Morris and Rossi, 2006; Rossi *et al.*, 2007). Payloads targeting HIV-1 entry have also been investigated in both preclinical and clinical studies (Li *et al.*, 2005; van Lunzen *et al.*, 2007). Several clinical trials using HIV-1-resistant $CD4^+$ T cells have been attempted. One trial was to use $CD4^+$ T cells transduced with an adenoviral vector expressing a zinc finger nuclease, which disrupted the *CCR5* locus in the transduced cells and consequently rendered them resistant to HIV-1 infection.

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Another trial was conducted to evaluate the effect of $CD4^+$ T cells modified to conditionally express an antisense RNA (VRX496) against HIV-1 envelope upon HIV-1 infections (Levine *et al.*, 2006). These trials did not show any toxicity to the modified cells. However, the use of antisense RNA and siRNA targeting limited regions of HIV-1 RNA does not seem to be highly effective, since HIV-1 easily circumvented the effect of these RNA inhibitors by rapid mutation of their target sequences (Lee and Rossi, 2004). Therefore, to provide universal gene therapy for HIV-1 infections regardless of HIV-1 subtypes or mutations, we propose a novel gene therapy approach to use the bacterial endoribonuclease MazF as a payload.

MazF is an endoribonuclease encoded by *Escherichia coli* and specifically cleaves ACA sequence of RNA. It has been reported that overexpression of MazF in mammalian cells preferentially cleaves mRNA but not rRNA (Shimazu *et al.*, 2007). A previous study demonstrated that conditional expression of MazF under the control of HIV-1 long terminal repeat (LTR) rendered $CD4^+$ T cells resistant to HIV-1 replication without affecting cell growth (Chono *et al.*, 2011a). Since HIV-1 RNA has more than 240 ACA sequences, it is assumed that viral RNA is highly susceptible to MazF. Indeed, conditional expression of MazF by Tat was shown to suppress the replication of not only HIV-1 but also simian/human immunodeficiency virus (SHIV) without affecting cellular mRNA. Furthermore, autologous transplantation of MazF-modified $CD4^+$ T cells in cynomolgus macaques proved to be safe, and the modified cells showed little or no immunogenicity (Chono *et al.*, 2011b). These results suggest that a conditional expression system of MazF is an attractive candidate for anti-HIV-1 gene therapy.

In this study, we evaluated HIV-1 replication in $CD4^+$ T cells from different donors after transduction with a MazF-expressing retroviral vector. The cells were infected with a variety of HIV-1 strains including multidrug-resistant clinical isolates. We also conducted a long-term culture experiment with HIV-1-infected $CD4^+$ T cells transduced with a MazF-expressing vector and found that viral replication was always lower in the cells than in those transduced with or without a control vector. In addition, the localization and expression of MazF and its effect on cellular mRNA expression were also determined.

Materials and Methods

Retroviral vectors

Preparation of retroviral vectors used in this study has been previously described (Chono *et al.*, 2011a). Briefly, gibbon ape leukemia virus (GaLV)-enveloped γ-retroviral vectors MT-MFR-PL2 and MT-ZGR-PL2 (Fig. 1) were manufactured at Takara Bio (Otsu, Japan). As for MT-MFR-PL2 vector, an HIV-1-LTR-MazF-polyA cassette was introduced into the opposite direction of the Moloney murine leukemia virus (MoMLV)-LTR at the multicloning site of retroviral vector plasmid (pMT) (Lee *et al.*, 2004). A truncated form of the human low affinity nerve growth factor receptor gene (*ΔLNGFR*) (Verzeletti *et al.*, 1998) was also introduced into the retroviral vector as a surface marker. The *ΔLNGFR* gene is under the control of human phosphoglycerate kinase (PGK) promoter. As the control vector MT-ZGR-PL2, the *mazF* gene of MT-MFR-PL2 was replaced by the ZsGreen1 (a fluorescence protein) gene.

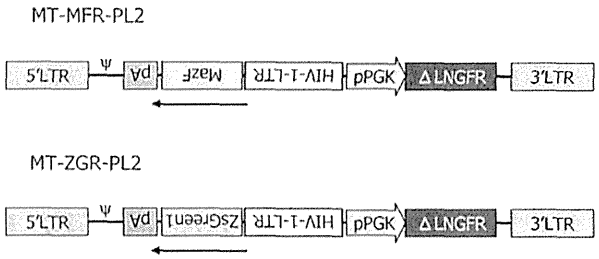

FIG. 1. Construction of retroviral vectors under the control of HIV-1-LTR promoter. The *MazF* gene derived from *Escherichia coli* was inserted directly into the downstream of HIV-1-LTR sequence. The HIV-1-LTR-MazF-polyA cassette was introduced in the opposite direction of the MoMLV-LTR. A truncated form of the human *ΔLNGFR* was also introduced into the retroviral vector as a surface marker. The *ΔLNGFR* gene is under the control of the human PGK promoter. The vector was designated as pMT-MFR-PL2. As a control vector, the *mazF* gene of pMT-MFR-PL2 was replaced by the ZsGreen1 gene (pMT-ZGR-PL2). LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; *ΔLNGFR*, low affinity nerve growth factor receptor gene; PGK, phosphoglycerate kinase.

Cells

$CD4^+$ T cells were prepared from peripheral blood mononuclear cells (PBMCs) from healthy volunteers who gave their written informed consent. Gene transfer study into primary $CD4^+$ T cells for the purpose of this study was approved by the ethics committee of Takara Bio Inc. Peripheral blood samples were collected by leukapheresis and washed with Cytomate (Baxter, Deerfield, IL). PBMCs were isolated by Ficoll-Paque PLUS (Amersham Biosciences, Amersham, United Kingdom) density gradient centrifugation. $CD4^+$ T cells were isolated from PBMCs by positive selection using anti-CD4 monoclonal antibody (mAb)-conjugated beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated cells were activated by anti-CD3 and anti-CD28 mAb-conjugated beads (Dynabeads Human T-Activator CD3/CD28; Life Technologies, Carlsbad, CA) for 3 days at a cell-to-bead ratio of 1:1 in GT-T503 medium (Takara Bio) supplemented with 1% autologous plasma, 0.2% human serum albumin, and 600 IU interleukin (IL)-2. The activated $CD4^+$ T cells were transduced with MT-MFR-PL2 or MT-ZGR-PL2 on a RetroNectin (Takara Bio) coated plate, according to the manufacturer's instructions, and incubated at 37°C. After 24 hr, the transduction was repeated once by the same method, and the cells were further incubated for 4 days. The average transduction efficiency was 60–70%, as determined by ΔLNGFR expression. For long-term culture experiment, the cells transduced with MT-MFR-PL2 ($\Delta LNGFR^+$) or MT-ZGR-PL2 ($\Delta LNGFR^+$) were concentrated with more than 95% purity using anti-CD271 mAb-conjugated beads (Miltenyi Biotec) followed by expansion for 5 days. The cells transduced with MT-MFR-PL2 and MT-ZGR-PL2 were designated as MazF-T and ZsG-T cells, respectively. All cells were stored at −80°C until use.

Viruses

Two X4 HIV-1 (III_B and HTK), three R5 HIV-1 (Ba-L, HKW, and HNK), and one R5X4 HIV-1 (HE) were used for infection

experiments (Nitanda *et al.*, 2005). III_B, Ba-L, and HE are laboratory-adapted HIV-1 strains, while HTK, HKW, and HNK are clinical isolates resistant to several nucleoside and nonnucleoside RT inhibitors and protease inhibitors. III_B and HE strains were propagated and titrated in MT-4 cells (Harada *et al.*, 1985), and the other strains were propagated in PBMCs. The culture supernatants were harvested, and their p24 antigen levels were determined. All samples were stored at −80°C until use.

Anti-HIV-1 assay

$CD4^+$ T cells and MazF-T cells were suspended in GT-T503 culture medium at a concentration of 5×10^5 cells/ml and restimulated with Dynabeads Human T-Activator CD3/CD28 at a cell-to-bead ratio of 5:1. After incubation for 3 days, the cells were infected with HIV-1 at a multiplicity of infection (MOI) of 0.1, 0.01, or an equivalent amount of p24 antigen (5 ng), and incubated for an additional 6 days. The culture supernatants on days 3 and 6 after viral infection were collected, and their p24 antigen levels were measured by an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix, Buffalo, NY). The viability of $CD4^+$ T cells and MazF-T cells, either infected or uninfected, were also evaluated on day 6 by a tetrazolium dye method, as previously described (Pauwels *et al.*, 1988).

Cytokine production assay

$CD4^+$ T cells and MazF-T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01, as described above. The culture supernatants on day 2 after infection were collected, and the levels of IL-2, IL-5, IL-10, and interferon (IFN)-γ were measured by cytokine ELISA kits (R&D Systems, Minneapolis, MN).

Long-term culture of infected cells

$CD4^+$ T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01 and incubated at 37°C. On day 7 after viral infection, the infected $CD4^+$ T cells (1×10^5 cells/ml) were cocultured with either uninfected MazF-T cells, ZsG-T cells, or $CD4^+$ T cells at a ratio of 1:5. On day 3 after coculture, the cells were subcultured with fresh culture medium at a ratio of 1:3 and further incubated. On day 7, the culture supernatants were collected and examined for their p24 levels by ELISA. The viable cell number was counted and resuspended in fresh culture medium at a concentration of 1×10^5 cells/ml. The cells were cocultured again with the appropriate cells (either MazF-T cells, ZsG-T cells, or $CD4^+$ T cells) at a ratio of 1:5, and this process was repeated every 7 days.

Sequence analysis

In the long-term culture experiment, genomic DNA was extracted from the infected $CD4^+$ T cells on days 3 and 199, MazF-T cells on day 199, and ZsG-T cells on day 199 by a DNA extraction kit (Wako, Tokyo, Japan). The extracted DNA was subjected to polymerase chain reaction (PCR). The PCR consists of 30 cycles (98°C for 10 sec, 60°C for 30 sec, and 72°C for 10 min) with the forward primer 5′-GAAAGGGAAACCA GAGGAGC-3′ and the reverse primer 5′-GCTGCTTATATGC AGGAATCT-3′, which generated a fragment including nucleotide 209 through 9053 of the gene corresponding to the III_B strain (EU541617). The amplification was repeated once with the same primer pair, and the amplified products were isolated by gel electrophoresis and purified with NucleoSpin Gel and PCR Clean-up kit (Machrey-Nagel, Düren, Germany). The purified DNA was sequenced directly with a cycle sequence kit (BigDye Terminator version 3.1; Applied Biosystems, Foster City, CA), using an automated DNA analyzer (Applied Biosystems).

Western blot analysis

To determine the intracellular localization of MazF, MazF-T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01 and incubated at 37°C. On day 6 after viral infection, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and fractionated by Subcellular Protein Fractionation Kit (Thermo Scientific, Yokohama, Japan), according to the manufacturer's instruction. The fractionated samples were suspended in sodium dodecyl sulfate (SDS) electrophoresis buffer and incubated at 95°C for 10 min. For gel electrophoresis, the sample solutions were loaded into the wells of a 4–20% Tris-Glycine gel (Invitrogen, Carlsbad, CA). After completion of electrophoresis, the gel was transferred to a polyvinylidene fluoride (PVDF) membrane (Invitrogen) in transfer buffer at 25 V (constant voltage) for 120 min at 4°C. The membrane was incubated overnight at 4°C in the blocking buffer (5% slim milk and 0.1% Tween20 in PBS) containing either anti-lamin A/C polyclonal antibody (Cell Signaling, Danvers, MA), anti-calpain monoclonal antibody (Millipore, Billerica, MA), or anti-MazF polyclonal antibody (in-house preparation). Each membrane was washed three times and incubated at room temperature for 1 hr in the blocking buffer containing appropriate peroxidase-conjugated secondary antibody. The membranes were washed five times in the washing buffer and soaked in substrate solution (SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate; Thermo Scientific) for 5 min at room temperature. Protein signals were detected by a CCD camera (LuminoShot 400 Jr., Takara Bio).

To determine the time-dependent expression of Tat and MazF, $CD4^+$ T and MazF-T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01 and incubated at 37°C. On days 2, 3, 4, and 6 after viral infection, the cells were suspended in SDS electrophoresis buffer, incubated at 95°C for 10 min, and subjected to Western blot analysis, as described above. The antibodies used for detection of α-tubulin, HIV-1 Tat, and MazF were anti-α-tubulin polyclonal antibody (Cell Signaling), anti-Tat polyclonal antibody (Abcam, Cambridge, United Kingdom), and the in-house anti-MazF polyclonal antibody, respectively.

Comprehensive mRNA expression analysis

MazF-T and $CD4^+$ T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01 and incubated at 37°C. On day 6 after viral infection, the total RNA was extracted from the cells using RNeasy® Mini Kit (QIAGEN, Tokyo, Japan). The extracted RNA was subjected to comprehensive mRNA expression analysis for the genes related to the T-cell receptor signaling pathways using PrimerArray™ (Takara Bio), according to the manufacturer's instruction.

Data analysis

Data were analyzed for their statistical significance by the Student *t*-test.

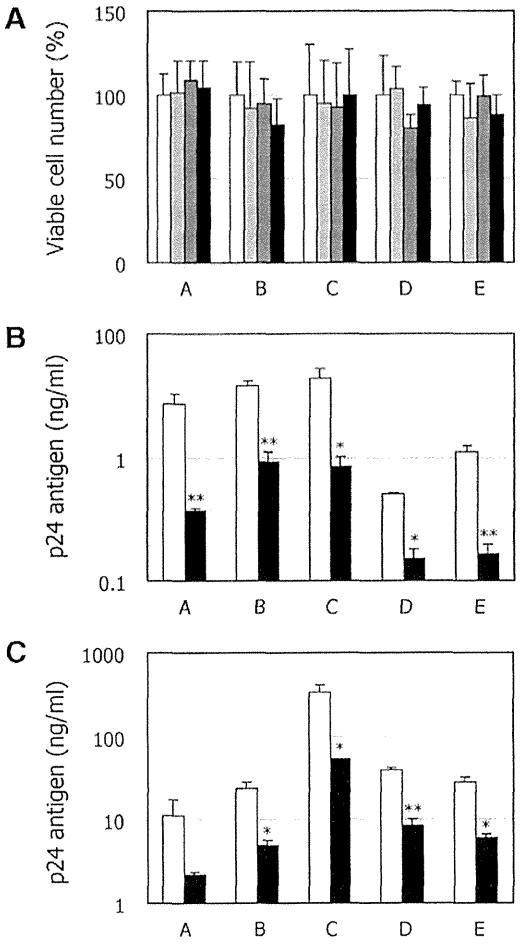

FIG. 2. HIV-1 replication in MazF-PBMCs from different donors. PBMCs and MazF-PBMCs from five different donors (A–E) were infected with HIV-1 (III_B strain) at a multiplicity of infection (MOI) of 0.01 and incubated for 6 days. The culture supernatants on days 3 and 6 after infection were collected, and their p24 antigen levels were measured by HIV-1 p24 ELISA. **(A)** The viability of mock-infected PBMCs (white columns), infected PBMCs (light gray columns), mock-infected MazF-PBMCs (dark gray columns), and infected MazF-PBMCs (black columns) on day 6 was determined by a tetrazolium dye method. **(B, C)** The p24 antigen levels in the culture supernatants of infected PBMCs (white columns) and MazF-PBMCs (black columns) on days 3 and 6 after infection are shown in panels **(B)** and **(C)**, respectively. Experiments were conducted in triplicate, and all data represent mean ± SD. Statistical analysis was performed by *t*-test between PBMCs and corresponding MazF-PBMCs from the same donor (*$p < 0.05$; **$p < 0.01$). PBMC, peripheral blood mononuclear cell; ELISA, enzyme-linked immunosorbent assay.

Results

Inhibition of HIV-1 replication in MazF-transduced PBMCs from different donors

In this experiment, PBMCs instead of $CD4^+$ T cells were used for preparing MazF-transduced cells (MazF-PBMCs). PBMCs were obtained from five different donors. The gene transfer efficiencies were 44–70%, as determined by ΔLNGFR expression. When PBMCs and MazF-PBMCs were infected with HIV-1 (III_B strain) at an MOI of 0.01 and cultured for 6 days, there was no significant difference in cell viability among the mock-infected PBMCs, HIV-1-infected PBMCs, mock-infected MazF-PBMCs, and HIV-1-infected MazF-PBMCs, irrespective of the donors (Fig. 2A). In contrast to the cell viability, HIV-1 replication was significantly suppressed in MazF-PBMCs compared to that in corresponding PBMCs (Fig. 2B and C). Although the p24 antigen levels varied considerably from one donor to another, on average, 5.4- and 5.1-fold reduction of p24 antigen levels was observed in the culture supernatants on days 3 and 6 after infection, respectively. These results suggest that the PBMCs transduced with the HIV-1-LTR-regulated *mazF* gene acquire resistance to HIV-1 replication without affecting their viability.

Inhibition of various HIV-1 strains in MazF-T cells

$CD4^+$ T cells and MazF-T cells from the same donor (donor C in Fig. 2) were infected with two laboratory-adapted HIV-1 strains (Ba-L and HE strains) in addition to III_B. The Ba-L and HE strains are R5 (CCR5-using as a coreceptor for infection) and R5X4 (CCR5- and CXCR4-using) viruses, respectively, while the III_B strain is an X4 (CXCR4-using) virus. The gene transfer efficiency was 63%, as determined by ΔLNGFR expression. As shown in Table 1, the infection of MazF-T cells with Ba-L did not affect cell viability, which is consistent with the results of the MazF-T cells infected with III_B (Fig. 2A). Again, significant inhibition of HIV-1 replication was observed in the MazF-T cells infected with Ba-L and HE (Table 1). For instance, 4.3- and 2.9-fold reduction of p24 antigen levels was observed in the culture supernatants of the MazF-T cells infected with Ba-L

TABLE 1. R5 AND R5X4 HIV-1 REPLICATION IN MAZF-T CELLS

Cell	*Virus*	*MOI*	*Day*	*Viable cells (%)*	*p24 (ng/ml)*
$CD4^+$ T	Mock	–	6	100	–
	Ba-L	0.01	3	N.D.	5.5 ± 0.7
			6	134.3 ± 12.5	712 ± 57
		0.1	3	N.D.	209 ± 41
			6	110.1 ± 5.9	3,168 ± 119
	HE	0.01	3	N.D.	13.8 ± 1.2
			6	N.D.	38.9 ± 2.4
MazF-T	Mock	–	6	100	–
	Ba-L	0.01	3	N.D.	1.3 ± 0.5**
			6	104.5 ± 3.9	248 ± 117**
		0.1	3	N.D.	25.7 ± 8.4*
			6	94.5 ± 6.0	1,619 ± 253**
	HE	0.01	3	N.D.	2.1 ± 0.1**
			6	N.D.	12.3 ± 2.7**

$CD4^+$ T cells and MazF-T cells from one donor were infected with R5 HIV-1 (Ba-L strain) or R5X4 HIV-1 (HE strain) at an MOI of 0.01 or 0.1 and incubated. The culture supernatants on days 3 and 6 after infection were collected and determined for their p24 antigen levels by HIV-1 p24 ELISA. The cell viability on day 6 was determined by a tetrazolium dye method. Experiments were conducted in triplicate, and all data represent mean ± SD. Statistical analysis was performed by *t*-test between $CD4^+$ T cells and corresponding MazF-T cells (*$p < 0.05$; **$p < 0.01$). MOI, multiplicity of infection; ELISA, enzyme-linked immunosorbent assay. N.D., not determined.