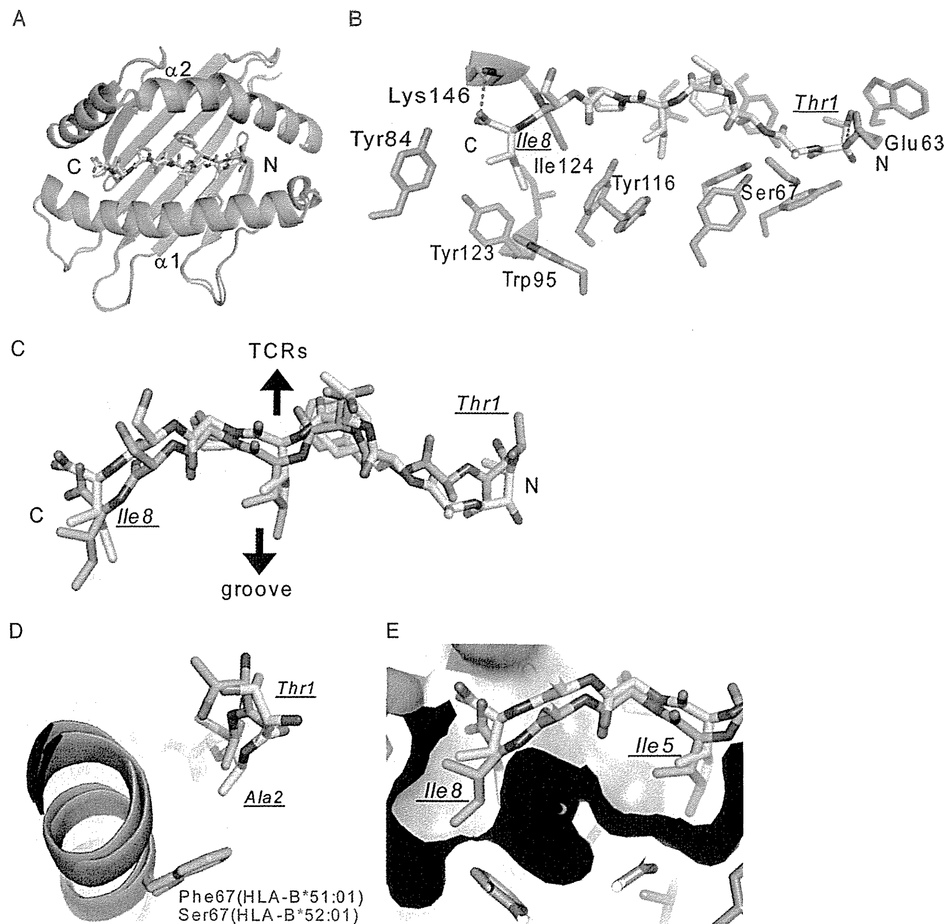

FIG 6 Structural comparison of HLA-B*52:01 and HLA-B*51:01 molecules complexed with the Pol283-8 peptide. (A) Crystal structures of HLA α1-α2 domains complexed with the Pol283-8 peptide (stick model) on the HLA-B*52:01 (green, yellow) and HLA-B*51:01 (cyan, cyan) complexes. This same coloring also applies to panels B to E. (B) Pol283-8 peptide and interacting side chains on the HLA-B*52:01 complex. Hydrogen bonds are shown as blue dotted lines. (C) Comparison of the Pol283-8 peptide conformations of HLA-B*52:01 and HLA-B*51:01 complexes. (D) N-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. (E) C-terminal side of HLA-B*52:01 and HLA-B*51:01 complexes. Surface presentation for the α1-α2 domains is shown in gray.

we had previously reported (45). This finding explains the cross presentation of this peptide by both HLA alleles. On the other hand, there was a notable conformational difference in the N-terminal region of the peptide between the two alleles (Fig. 6C and D). The replacement of Phe67 of HLA-B*51:01 with Ser in HLA-B*52:01 makes a local space, causing the N-terminal region of the peptide (T1 and A2) to reside deeper in the peptide-binding groove. Furthermore, the Gln63Glu mutation in HLA-B*52:01 affords a new interaction with the T1 residue of the peptide. These changes would, to some extent, have hidden the side chains of T1 and A2 (flat surface) from the TCRs, which may have reduced their interactions with TCRs on the HLA-B*52:01-restricted CTLs. On the other hand, the conformation of the C-terminal region of the peptide complexed with HLA-B*51:01 or HLA-B*52:01 was similar, even though C-terminal Ile8 of the peptide exhibited shallower penetration of the hydrophobic groove in the case of HLA-B*52:01 than in that of HLA-B*51:01 (Fig. 6C and E). These results may indicate that the relatively flat surface of the N-terminal side of the peptide contributed to the lower affinity for TCRs in the case of HLA-B*52:01.

DISCUSSION

HLA-B*52:01 and HLA-B*51:01 differ by only two residues, at positions 63 and 67 (44). Substitutions at these residues affect the formation of the B pocket in the peptide-binding pocket (45), suggesting the possibility that HLA-B*52:01 has a peptide motif different from that of HLA-B*51:01. Indeed, HLA-B*52:01-binding peptides have P2 primary anchors that are different from HLA-B*51:01-binding ones (30, 46). Since the Pol283-8 epitope carries Ala at its second position and Ile at the C terminus of the peptide, it is likely that this peptide would effectively bind to HLA-B*51:01 but not to HLA-B*52:01. However, the results of the HLA stabilization assay demonstrated that the Pol283-8 peptide did effectively bind to HLA-B*52:01. Since the HLA-B*52:01-binding peptide is known to have Pro as its preferred P2 anchor residue, this peptide carrying Ala at position 2 may be capable of binding to HLA-B*52:01. A previous study showed cross-recognition of allo-reactive T cells between HLA-B*51:01 and HLA-B*52:01 (47, 48), indicating that some self-peptides can be presented by both of these HLA class I molecules. The findings on the crystal structure

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TABLE 3 Numbers and frequencies of individuals having I135X mutations in a Japanese cohort and a predominantly Caucasian cohort

	No./total no. (%) of individuals				
Cohort	$B^*51{:}01^+$ $B^*52{:}01^-$	$B^*51{:}01^-$ $B^*52{:}01^+$	$B^*51{:}01^+$ $B^*52{:}01^+$	$B^*51{:}01^-$ $B^*52{:}01^-$	Total
Japanese	51/51 (100)	42/49 (85.7)	5/5 (100)	88/151 (58.3)	186/256 (72.6)
Caucasian	125/131 (95.4)	17/26 (65.4)	0/0	331/1,198 (27.6)	473/1,355 (34.9)

of the HLA-B*52:01 molecule complexed with the Pol283-8 peptide clarified that HLA-B*52:01 could bind to the peptide in a fashion similar to but slightly different from that of HLA-B*51:01. These findings support the presentation of the Pol283-8 peptide by both HLA-B*52:01 and HLA-B*51:01.

Pol283-8-specific $CD8^+$ T cells were detected in 7 of 14 HLA-$B^*52{:}01^+$ HLA-$B^*51{:}01^-$ individuals chronically infected with HIV-1. A previous analysis showed that $CD8^+$ T cells specific for this epitope are frequently detected in HLA-$B^*51{:}01^+$ individuals chronically infected with HIV-1 (49). These results, taken together, indicate that this epitope is immunodominant in both HLA-$B^*51{:}01^+$ and HLA-$B^*52{:}01^+$ individuals. The analysis of 257 Japanese individuals revealed an association between HLA-B*52:01 and a variety of nonconsensus residues at RT codon 135 (I135X). Specifically, variants 8T, 8L, 8R, and 8V predominated in HLA-$B^*52{:}01^+$ individuals, suggesting that these mutations had been selected by HLA-B*52:01-restricted CTLs. The viral suppression assay revealed that the HLA-B*52:01-restricted CTLs failed to suppress the replication of these mutant viruses. These results support the idea that the I135X mutation can be selected by immune pressure via Pol283-8-specific CTLs in HLA-$B^*52{:}01^+$ individuals. Our previous studies showed that the 8L, 8T, and 8R mutations affected the recognition by Pol283-8-specific, HLA-B*51:01-restricted CTL clones (15, 28). These studies, together with the present study, indicate that accumulation of 8L, 8T, and 8R mutations in the HIV-infected Japanese population may be due to immune pressure by both HLA-B*52:01-restricted and HLA-B*51:01-restricted CTLs. Our analysis of the crystal structure of the HLA-B*52:01-peptide complex demonstrated that position 8 of the Pol283-8 peptide was deeply packed into the hydrophobic groove. Whereas the 8L, 8T, and 8R substitutions likely had a relatively large effect on the structure of the complex, the 8V mutation, resulting in only the deletion of the small methyl group, caused only very limited changes. Thus, the structural analysis supports the idea that the 8L, 8T, and 8R mutations affected the TCR recognition of the peptide and/or its binding to HLA-B*52:01.

The present study confirmed previous studies of nine worldwide cohorts (15) and a Chinese cohort (50) that showed a strong association of I135X with HLA-B*51:01. The I135X mutation was found in 58.3 and 27.6% of HLA-$B^*51{:}01^-$ HLA-$B^*52{:}01^-$ Japanese and predominantly Caucasian individuals, respectively (Table 3), supporting greater population level accumulation of this mutation in Japanese than in other cohorts. Since the Japanese cohort included twice as many HLA-$B^*51{:}01^+$ individuals as the IHAC cohort (21.9% of Japanese and 9.4% of Caucasians in IHAC), the difference in the I135X variant frequency between these two cohorts would be driven, to a large extent, by the higher HLA-B*51:01 prevalence in the former than in the latter. The association of HLA-B*52:01 with this mutation was much weaker than that of HLA-B*51:01 in both cohorts but still highly statistically significant (an lnOR of 11.7 [$P = 8.77 \times 10^{-4}$] versus an lnOR of 40.0 [$P = 5.78 \times 10^{-12}$] in the Japanese cohort and an lnOR of 3.06 [$P = 2.95 \times 10^{-5}$] versus an lnOR of 5.71 [$P = 1.58 \times 10^{-51}$] in IHAC). Because of the relatively low $B^*52{:}01^+$ frequency (~2%) in IHAC, the effect of HLA-B*52:01 on the overall prevalence of I135X was relatively low in this cohort. In contrast, in the Japanese cohort, where the HLA-$B^*52{:}01^+$ prevalence was relatively high (>20%), this allele represents a major driving force behind 135X selection in this cohort. Thus, selection pressure from both HLA-B*51:01 and HLA-B*52:01 likely contributed to the observed population level accumulation of I135X mutations in the Japanese population.

Previous studies showed that HLA-B*51:01-restricted, Pol283-8-specific CTLs have a strong ability to suppress HIV-1 replication *in vitro* (28) and that they suppressed the replication of the 8V mutant virus but failed to suppress that of the 8T, 8L, and 8R mutant viruses (15). The frequency of the Pol283-8-specific CTLs was inversely correlated with the plasma viral load in HLA-B*51:01^+ hemophiliacs infected with HIV-1 approximately 30 years ago (28). The 8T, 8L, and 8R mutations did not affect replication capacity, whereas the 8V mutation conferred a modest fitness cost (15). These findings support the suppression of the wild-type or 8V mutant virus by Pol283-8-specific CTLs as a major mechanism of slow progression to AIDS in Japanese hemophiliacs. This CTL response was also elicited in Chinese HLA-$B^*51{:}01^+$ individuals infected with the 8V mutant virus; furthermore, a low viral load and a high CD4 count were significantly associated with the presence of at least one of three HLA-B*51:01-restricted CTL responses, including a Pol283-8-specific one (50). Thus, these findings support the idea that Pol283-8-specific CTLs play an important role in the control of HIV-1 infection.

The present study demonstrated that HLA-B*52:01-restricted, Pol283-8-specific CTLs also had a strong ability to suppress HIV-1 replication *in vitro* (80% suppression at an E/T cell ratio of 0.3:1). However, the ability of HLA-B*52:01-restricted CTLs to suppress the replication of HIV-1 was weaker than that of HLA-B*51:01-restricted CTLs (Fig. 5B). Inspection of the crystal structures of both HLA molecules complexed with the Pol283-8 peptide suggests that the relatively shallow penetration of the hydrophobic groove of HLA-B*52:01 by the C-terminal side of the peptide, in contrast to the tightly packed binding with HLA-B*51:01, may have resulted in an unstable conformation of the complex. Furthermore, Ser67 of HLA-B*52:01 would have provided more space and loose interactions with the peptide than in the case of the Phe of HLA-B*51:01. Interestingly, the Pol283-8 peptide would have displayed only side chains of Thr1 and Ser7, and some part of the main chains, to CTLs. Therefore, these results suggest that the unstable backbone conformation and side chain positions in the case of HLA-B*52:01 largely contributed to the lower TCR affinity than that afforded by HLA-B*51:01. These results support that selection pressure *in vivo* via the HLA-B*52:01-restricted CTLs would be weaker than that via the HLA-B*51:01-restricted CTLs. Indeed, the prevalence of I135X mutations in HLA-B*51:

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01⁺ individuals was higher than that in HLA-B*52:01⁺ individuals. The difference in the pattern of escape mutant selection by these CTLs between the HLA-B*51:01⁺ and HLA-B*52:01⁺ individuals might also have been due to the difference in their abilities to suppress HIV-1 replication. However, it still remains unclear why the 8T mutant was predominantly selected in the HLA-B*51:01⁺ but not in the HLA-B*52:01⁺ individuals. Further studies are expected to clarify the mechanism to explain how these CTLs selected different patterns of mutations at RT135.

Previous studies showed that the T242N mutant was selected by HLA-B*58:01-restricted and HLA-B*57-restricted CTLs specific for TW10 epitope in HIV-1 clade B-infected and clade C-infected individuals (25–27). Herein we also showed that I135X was selected by Pol283-8-specific CTLs restricted by two different HLA class I molecules. However, the strength and the pattern of the selection of I135X was different between HLA-B*51:01 and HLA-B*52:01. The present study suggests that this difference in the selection pattern was associated with that between the HLA-B*51:01⁺ and HLA-B*52:01⁺ individuals in terms of the ability of Pol283-specific CTLs to suppress HIV-1 replication. Thus, we characterized and experimentally validated distinct HIV-1 escape patterns of CTLs with the same epitope specificity and provided evidence that the extremely high prevalence of 135X in circulating Japanese sequences is likely driven not by one but by two HLA-B alleles.

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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 *XmaI-NheI* region of pTZNX, which encodes the 15th–267th positions of HIV-1 RT (strain BH10) [15, 16]. The *XmaI-NheI* 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

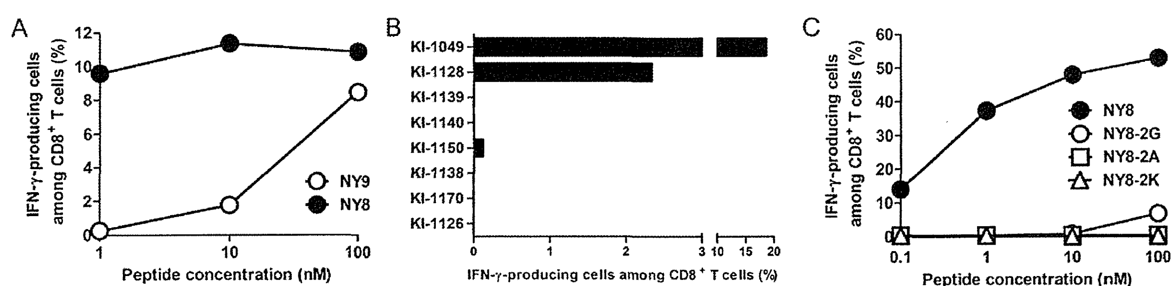

Figure 1. Recognition of human leukocyte antigen (HLA)–B*18-restricted CD8+ T cells. *A*, Identification of the optimal epitope of HLA-B*18-restricted CD8+ T cells. Peripheral blood mononuclear cells (PBMCs) from an HLA-B*18-positive individual chronically infected with human immunodeficiency virus type 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.

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

Amino Acid	IC_{50} (nM), Fold Resistance[a]			
	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

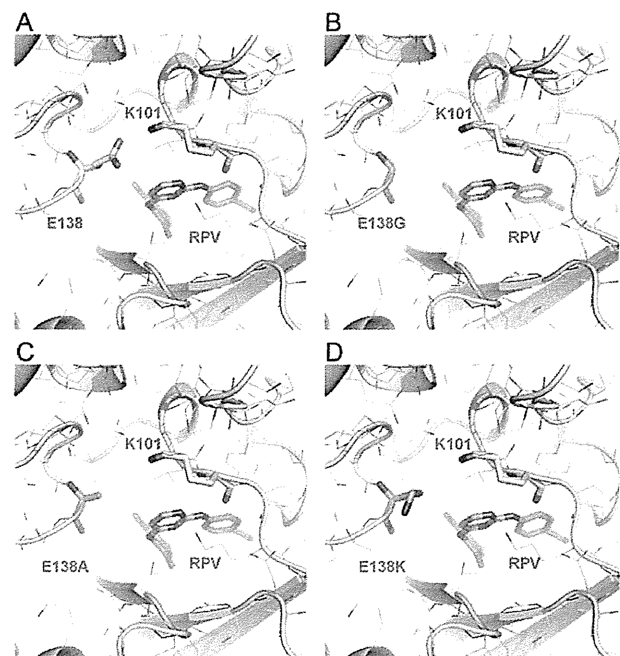

Figure 2. Structural models of human immunodeficiency virus type 1 reverse transcriptase (RT) and rilpivirine. The binding clefts of 4 complexes are shown: $RT_{E138(wild-type)}$ (*A*), RT_{E138G} (*B*), RT_{E138A} (*C*), and RT_{E138K} (*D*). Sticks indicate the amino acids at positions 101 and 138 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.

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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 1C). 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.

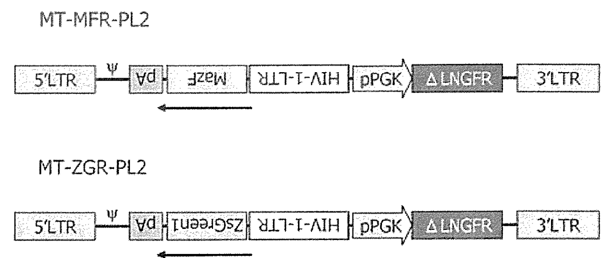

FIG. 1. Construction of retroviral vectors under the control of HIV-1-LTR promoter. The *MazF* gene derived from *Escherichia coli* was inserted directly into the downstream of HIV-1-LTR sequence. The HIV-1-LTR-MazF-polyA cassette was introduced in the opposite direction of the MoMLV-LTR. A truncated 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 (ΔLNGFR+) or MT-ZGR-PL2 (ΔLNGFR+) 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.

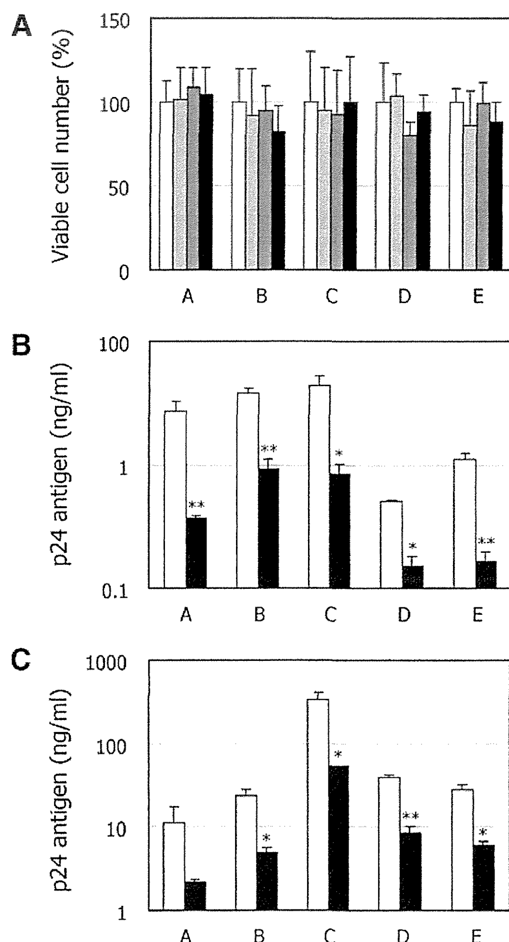

FIG. 2. HIV-1 replication in MazF-PBMCs from different donors. PBMCs and MazF-PBMCs from five different donors (A–E) were infected with HIV-1 (III_B strain) at a multiplicity of infection (MOI) of 0.01 and incubated for 6 days. The culture supernatants on days 3 and 6 after infection were collected, 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.

(MOI = 0.01) compared to those of $CD4^+$ T cells on days 3 and 6, respectively.

The inhibition of HIV-1 replication in MazF-T cells was much more prominent when the cells were infected with clinical isolates. The viruses used for this experiment were isolated from patients with treatment failure and found to be resistant to several nucleoside and nonnucleoside RT inhibitors and protease inhibitors currently available in clinic. Except for the HTK-infected MazF-T cells from donor E, 10-fold or more reduction of p24 antigen levels in the culture supernatants was achieved by MazF-T cells compared to $CD4^+$ T cells (Table 2). In particular, the p24 level in the culture supernatant of the HKW-infected MazF-T cells was < 0.01 ng/ml. These results indicate that the HIV-1-LTR-regulated *mazF* gene confers resistance to a broad-spectrum of HIV-1 strains including multidrug-resistant clinical isolates on $CD4^+$ T cells. Furthermore, the levels of cytokines, such as IL-2, IL-5, IL-10, and IFN-γ, secreted from the III_B-infected MazF-T cells were not altered significantly compared to those from uninfected $CD4^+$ T cells and MazF-T cells (data not shown).

Reduced infectivity of HIV-1 produced from infected MazF-T cells

When $CD4^+$ T cells and ΔLNGFR-selected MazF-T cells from the same donor were infected with HIV-1 (III_B strain) at an MOI of 0.1 and cultured for 6 days, significant reduction of cell viability was observed for the infected $CD4^+$ T cells compared to the mock-infected and HIV-1-infected MazF-T cells due to the cytopathic effect of the replicating virus (Fig. 3A). Like the previous results in PBMCs (Fig. 2C), HIV-1 replication was highly suppressed in MazF-T cells (Fig. 3A). The p24 level in the culture supernatant of MazF-T cells was only 13.1% of that of $CD4^+$ T cells. Then, fresh $CD4^+$ T cells were infected with the viruses obtained from the infected $CD4^+$ T cells (control virus) or the infected MazF-T cells (MazF virus). The amount of viruses for infection was adjusted equally by p24 antigen (10 ng). After incubation for 6 days, the p24 antigen level was found to be significantly higher ($p = 0.022$) in the control virus-infected cells than in the MazF virus-infected cells (Fig. 3B). These results suggest that the infectivity and/or replication efficiency of the MazF virus is lower than the control virus. To confirm this hypothesis, $CD4^+$ T and MazF-T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01. After incubation for 6 days, the amount of p24 antigen and infectivity of the culture supernatants were directly measured. As shown in Table 3, the ratios of virus infectivity (titer) to p24 antigen were 461 and 188 for the control virus and MazF virus, respectively, indicating the reduced infectivity of HIV-1 produced from the infected MazF-T cells.

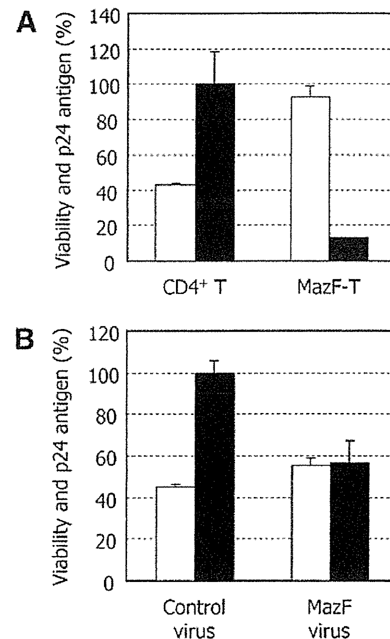

FIG. 3. Replication of HIV-1 produced from $CD4^+$ T and MazF-T cells. **(A)** $CD4^+$ T cells and MazF-T cells from the same donor were infected with HIV-1 (III_B strain) at an MOI of 0.1. After incubation for 6 days, the cell viability (white columns) and p24 antigen levels in the culture supernatants (black columns) were determined. **(B)** $CD4^+$ T cells were infected with the viruses obtained from the infected $CD4^+$ T cells (control virus) or the infected MazF-T cells (MazF virus) in the experiment of panel **(A)**. The amount of viruses for infection was adjusted equally by p24 antigen (10 ng). After incubation for 6 days, the cell viability (white columns) and p24 antigen levels in the culture supernatants (black columns) were determined. In both panels, the cell viability is expressed as a percentage of that in mock-infected $CD4^+$ T cells. The p24 antigen level is expressed a percentage of that in infected $CD4^+$ T cells for panel **(A)** and that in control virus-infected $CD4^+$ T cells for panel **(B)**.

TABLE 2. REPLICATION OF MULTIDRUG-RESISTANT HIV-1 CLINICAL ISOLATES IN MAZF-T CELLS

Cell	*Virus*	*Donor*	*p24 (ng/ml)*
$CD4^+$ T	HTK	C	105.3 ± 7.5
		E	45.8 ± 10.2
		F	35.8 ± 11.2
	HKW	E	0.87 ± 0.52
	HNK	E	16.9 ± 8.1
		F	4.1 ± 2.9
MazF-T	HTK	C	11.0 ± 4.8**
		E	17.9 ± 6.7*
		F	1.1 ± 0.9*
	HKW	E	< 0.01
	HNK	E	0.36 ± 0.16
		F	0.29 ± 0.22

$CD4^+$ T cells and MazF-T cells from three different donors were infected with three multidrug-resistant clinical isolates at an MOI of 0.01 or an equivalent amount of p24 antigen (5 ng) and incubated. The culture supernatants on day 6 after infection were collected and determined for their p24 antigen levels by HIV-1 p24 ELISA. 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 (*$p < 0.05$; **$p < 0.01$).

Sustained inhibition of HIV-1 replication in MazF-T cells

Since HIV-1 is a highly variable virus during its replication, it is possible that MazF-T cells become no longer inhibitory to HIV-1 replication through the mutations of viral genes. To exclude this possibility, a long-term culture

TABLE 3. RELATIVE INFECTIVITY OF HIV-1 PRODUCED FROM $CD4^+$ T AND MAZF-T CELLS

Cells	*p24 (ng/ml)*	*Titer ($CCID_{50}$/ml)*	*Ratio (titer/p24)*
$CD4^+$ T	42.1±5.6	19,400±1,500	461
MazF-T	7.3±0.5	1,370±580	188

$CD4^+$ T and MazF-T cells were infected with HIV-1 (III_B strain) at an MOI of 0.01. After incubation for 6 days, the amount of p24 antigen and infectivity of the culture supernatants were determined. $CCID_{50}$: 50% cell culture infectious dose determined by Reed-Muench method (Reed and Muench, 1938). Experiments were conducted in triplicate, and all data represent mean±SD.

experiment of HIV-1-infected MazF-T cells and control cells was conducted. In this experiment, ΔLNGFR-selected MazF-T cells and ZsG-T cells were used. Since the primary $CD4^+$ T cells, including MazF-T and ZsG-T cells, cannot survive for a long period of time *in vitro*, the infected cells were replenished with fresh uninfected cells every 7 days. Before replenishment of the cells, the culture supernatants were collected and determined for their p24 antigen levels. As shown in Figure 4, cultures were successfully continued up to day 203 after infection, and the p24 levels of the culture supernatants in MazF-T cells were always below the levels in ZsG-T cells and $CD4^+$ T cells. Except for day 0, 29 samples in each culture group were collected. The average p24 levels of the MazF-T, ZsG-T, and $CD4^+$ T cell groups were 235±146, 569±423, and 815±692 ng/ml, respectively. Statistical evaluation revealed that the difference in p24 antigen levels was highly significant between MazF-T and $CD4^+$ T cell groups ($p=0.00011$), between MazF-T and ZsG-T cell groups ($p=0.00029$), and not between ZsG-T and $CD4^+$ T cell groups ($p=0.11$). Thus, the emergence of escape mutants that replicate well in MazF-T cells was not allowed by the long-term culture of HIV-1-infected cells at least for 200 days.

Sequence analysis of proviral DNA for the infected $CD4^+$ T cells on days 3 and 199, MazF-T cells on day 199, and ZsG-T cells on day 199 revealed that there was no substantial increase or decrease in ACA content of the proviral DNA among the 4 groups (Table 4). The highest number of nucleotide mutations was identified in the proviral DNA of MazF-T cells followed by ZsG-T cells and $CD4^+$ T cells. However, there seemed to be no solid rules of mutation. The virus isolated from MazF-T cells on day 199 was inoculated into $CD4^+$ T cells and MazF-T cells and examined for its replication in these cells. Like the original III_B strain, the replication of this isolate was also significantly restricted in MazF-T cells compared to that in $CD4^+$ T cells (data not shown).

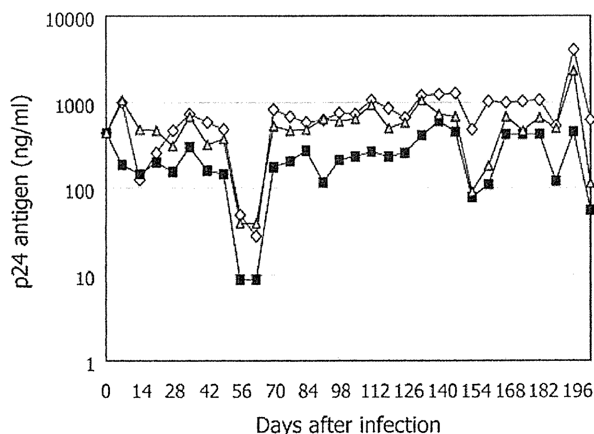

FIG. 4. Long-term culture of infected cells. $CD4^+$ T cells infected with HIV-1 (III_B strain) were cocultured with uninfected $CD4^+$ T cells (white diamonds), ZsG-T cells (gray triangles), or MazF-T cells (black squares) at a ratio of 1:5. On day 3 after coculture, the cells were subcultured with 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 cells were cocultured again with the appropriate cells (either $CD4^+$ T cells, ZsG-T cells, or MazF-T cells) at a ratio of 1:5, and this process was repeated every 7 days up to day 203.

Intracellular localization of MazF

Proteins were extracted from uninfected and HIV-1-infected MazF-T cells and fractionated to the cytoplasm, membrane, nucleus, and cytoskeleton samples. To ensure that the samples were prepared properly, each sample was subjected to Western blot analysis to detect calpine (a cytoplasmic marker) and lamin (a nuclear marker). As shown in Figure 5, MazF was not expressed in uninfected MazF-T cells. However, it was conditionally expressed upon HIV-1 infection and mainly localized in the cytoplasmic fraction.

Expression of Tat and MazF after HIV-1 infection

The expression of MazF and Tat was determined in HIV-1-infected $CD4^+$ T cells and ΔLNGFR-selected MazF-T cells on days 2, 3, 4, and 6 after viral infection. We arbitrarily defined the signal intensity of Tat and MazF in the infected $CD4^+$ T cells on day 2 as one and calculated the relative intensity of each sample. The expression level of Tat in MazF-T cells was similar to that of $CD4^+$ T cells on day 2 (Fig. 6A). The expression of Tat increased significantly in $CD4^+$ T cells in a time-dependent fashion, while the expression of Tat decreased rather than increased in MazF-T cells during the course of infection (Fig. 6A). The expression of MazF was also induced upon HIV-1 infection. However, unlike Tat, the expression level of MazF was sustained throughout the experimental period (Fig. 6B). The expression of MazF was undetectable in uninfected MazF-T cells.

Expression of cellular mRNA after HIV-1 infection

When genes related to the T-cell receptor signaling pathways were examined for their expression in $CD4^+$ T and ΔLNGFR-selected MazF-T cells, no perturbation in mRNA expression was observed in both HIV-1-infected $CD4^+$ T and MazF-T cells compared to uninfected $CD4^+$ T cells at 48 and 72 hr after viral infection (data not shown). However, on day 6 after viral infection, the pattern of mRNA expression was perturbed in HIV-1-infected $CD4^+$ T cells (Fig. 7A). The expression of 24 out of the 88 genes in the infected $CD4^+$ T cells was more than two-fold up- or down-regulated compared to that in uninfected $CD4^+$ T cells (Supplementary Table 1; Supplementary Data available online at www.liebertonline.com/hgtb). In contrast, such significant changes of mRNA expression were not

TABLE 4. MUTATION OF HIV-1 AFTER LONG-TERM CULTURE OF INFECTED CELLS

Nucleotide number[a]	*CD4⁺ T (day 3)*	*CD4⁺ T (day 199)*	*MazF-T (day 199)*	*ZsG-T (day 199)*	*Gene*
0258	C	–[b]	T	–	DIS
0520	G	–	A (G→E)[c]	–	*gag*
1004	T	–	C or T	C or T	
1604	G	A (M→I)	G or A (M→I)	A (M→I)	
1713	Duplication (1713–1748)				
2095	A	–	–	G or A	*pol*
2625	C	T (T→I)	–	T (T→I)	
2743	G	–	–	A	
4228	C or T	T	C or T	C	
4689	G	G or A (G→S)	A (G→S)	A (G→S)	*vif*
5252	T (stop codon)	–	C (Stop codon→Q)	–	*vpr*
5638				Deletion (5638–5655)	*vpu*
6735	G	T (Q→H)	T (Q→H)	C or T (Q→H)	*env*
6790	G or A (T or D)	G (T or D→D)	A (T or D→T)	A (T or D→T)	
7035	C	–	A or C	–	
7075	G	G or A (E→K)	–	–	
7701	A	T (K→N)	T (K→N)	–	
7754	T	–	A (M→K)	–	
7765	G	A (E→K)	–	–	
7840	A	–	T (I→L)	–	
7894	C	–	–	C or G (If G, L→V)	
7896				Deletion (7896–7904)	
8752	G or A	G	G	G or A	*nef*
8819	A	–	–	T (I to L)	
8833	T	–	–	C	
8890	T	–	G or T	–	
8964	A	–	C (E→A)	–	
Number of mutations (rate)		10 (0.113%)	16 (0.181%)	12 (0.136%)	
ACA content	271	268	271	270	

[a]Based on HIV-1 clone $pIII_B$ (EU541617).
[b]Identical to the nucleoside in CD4⁺ T cells on day 3 after infection.
[c]Amino acid change.
DIS, dimerzation initiation sequence.

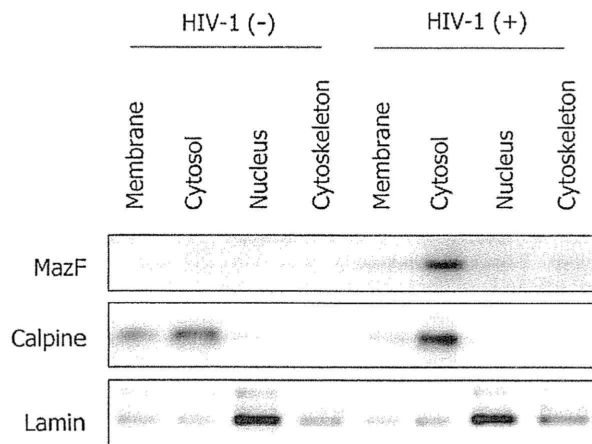

FIG. 5. Expression and intracellular localization of MazF. MazF-T cells were infected with HIV-1 (III_B strain) and incubated for 6 days. The cells were harvested and fractionated to the cytoplasm, membrane, nucleus, and cytoskeleton samples. MazF expression of the samples obtained from uninfected (−) and HIV-1-infected (+) MazF-T cells was determined by Western blot analysis. Calpine and lamin were used as cytoplasmic and nuclear markers, respectively.

observed in MazF-T cells irrespective of HIV-1 infection (Fig. 7B and C), except for 2.8-fold upregulation of the *IL-2* gene in the infected MazF-T cells. However, this upregulation of the *IL-2* gene in the infected MazF-T cells was found to be much lower than that in the infected CD4⁺ T cells (124-fold), suggesting that conditionally expressed MazF suppresses not only the replication of HIV-1 but also the modulation of mRNA expression caused by HIV-1 infection.

Discussion

RNase-based anti-HIV-1 gene therapy is an attractive strategy for destroying HIV-1 RNA in infected cells. In the case of MazF, there are more than 240 target sequences (ACA) in HIV-1 RNA, assuming that the virus has almost no chance to mutate and escape from the attack by MazF. To prove this hypothesis, we conducted a long-term culture experiment of HIV-1-infected CD4⁺ T and MazF-T cells and evaluated viral replication. In general, long-term culture experiments have been conducted with immortalized T-cell lines susceptible to HIV-1 infection. However, our past experiment to induce HIV-1 mutants resistant to the CCR5 antagonist TAK-652 (currently TBR-652 or cenicriviroc) was

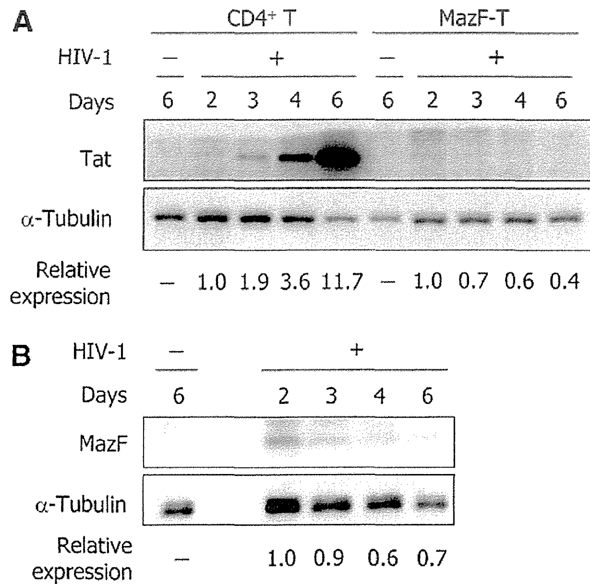

FIG. 6. Expression of Tat and MazF after HIV-1 infection. $CD4^+$ T and MazF-T cells were infected with HIV-1 (III_B strain) and incubated. On days 2, 3, 4, and 6 after viral infection, the cells were subjected to Western blot analysis to determine the expression of **(A)** Tat and **(B)** MazF. The intensity of Tat and MazF signals was normalized by that of α-tubulin, and the intensity of Tat and MazF signals in the infected $CD4^+$ T cells on day 2 was arbitrarily defined as one.

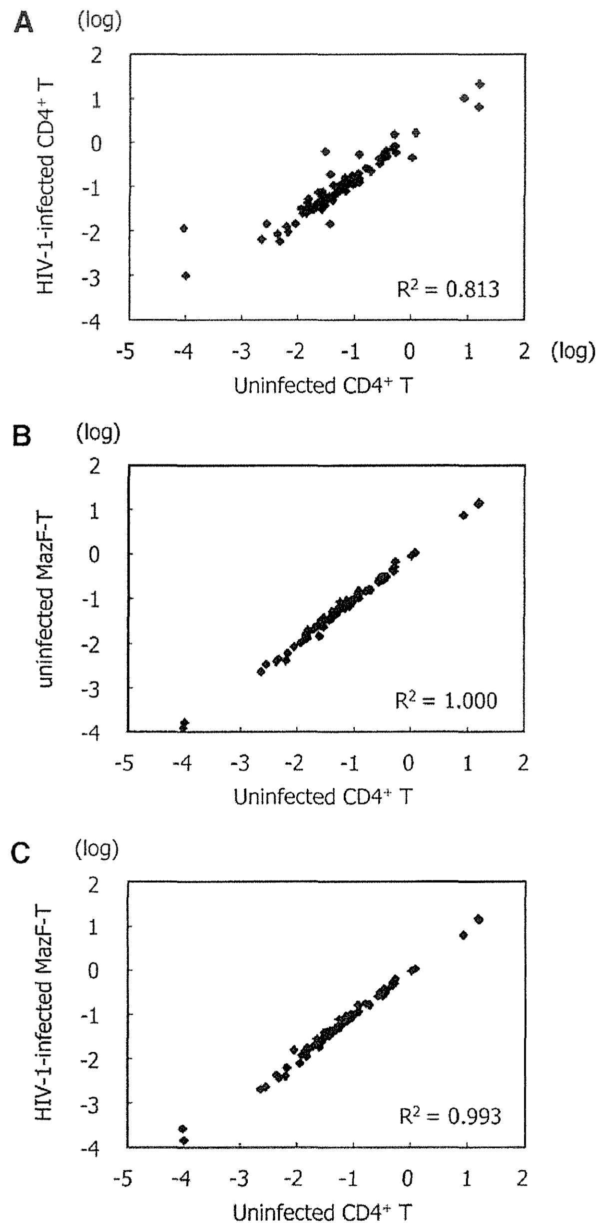

FIG. 7. Expression of cellular mRNA after HIV-1 infection. $CD4^+$ T and MazF-T cells were infected with HIV-1 (III_B strain) and incubated for 6 days. The total RNA was extracted from the cells and subjected to comprehensive mRNA expression analysis for the genes related to the T-cell receptor signaling pathways. The horizontal and vertical axes of all panels represent the relative expression level (logarithmic scale) of genes in the indicated cells. **(A)** HIV-1-infected $CD4^+$ T cells vs. uninfected $CD4^+$ T cells, **(B)** uninfected MazF-T cells vs. uninfected $CD4^+$ T cells, and **(C)** HIV-1-infected MazF-T cells vs. uninfected $CD4^+$ T cells.

conducted with PBMCs, since T-cell lines do not express CCR5 on their surface unless genetically modified (Baba *et al.*, 2000; Baba *et al.*, 2007).

Using PBMCs or primary $CD4^+$ T cells for a long-term culture experiment seems to have several advantages. For instance, the cells are more physiological than T-cell lines, so they are highly susceptible to a variety of HIV-1 strains including clinical isolates. To the contrary, a disadvantage is constant supply of $CD4^+$ T cells of which source must be the same donor. Especially in this study, MazF-T cells had to be prepared from $CD4^+$ T cells by transduction with a MazF-expressing retroviral vector. Another disadvantage is that viral replication is affected more intensively by cell conditions in $CD4^+$ T cells than in T-cell lines due to complex procedures for cell preparation. In fact, the p24 antigen levels in the culture supernatants of MazF-T cells as well as the control cells were considerably low on days 56 and 63 and relatively high on day 196 (Fig. 4). However, even so, the p24 antigen levels in the culture supernatants of MazF-T cells were always lower than those of ZsG-T cells and $CD4^+$ T cells, indicating that MazF did not allow the generation of mutants escaping from its inhibitory effect. This seems to be a great advantage over other known antiviral strategies including small molecule inhibitors and RNA-based gene therapy, such as antisense RNA, ribozyme, and siRNA.

In the sequence analysis of proviral DNA for the infected cells before and after long-term cultures, we could not identify the specific mutations caused by the presence of MazF. There was no decrease in ACA content of the proviral DNA in MazF-T cells on day 199 after infection compared to that in the control cells despite the fact that the number of mutations was the highest (Table 4). There was a point mutation from C to T at nucleotide number 258 of the proviral DNA in MazF-T cells on day 199. The region is known as the dimerization initiation sequence (DIS), which is

believed to be essential for viral RNA packaging (Harrison and Lever, 1992). However, St. Louis and colleagues reported that the mutation did not change the fitness for viral replication (St. Louis *et al.*, 1998). There was a 36-bp insertion (duplication) into the *gag* region (#1713–1748) of the proviral DNA in $CD4^+$ T cells on day 3. However, this insertion was considered not to impair the formation of functional p6 protein. The stop codon (TAG) existed in the *vpr* region (#5252) of the proviral DNA in $CD4^+$ T cells and ZsG-T cells, which produce a truncated form of Vpr. This stop codon was missing in MazF-T cells because of a point mutation from T to C. The influence of the point mutation and production of full-length Vpr on HIV-1 replication is unclear.

Another important issue to be clarified is the safety of MazF in infected and uninfected cells. We demonstrated in this study that MazF-T cells efficiently inhibited HIV-1 replication without affecting cell growth. The previous report also showed that conditional expression of MazF under the control of HIV-1 LTR rendered $CD4^+$ T cells resistant to HIV-1 replication without affecting cell proliferation (Chono *et al.*, 2011a). These results suggest that viral RNA is efficiently and preferentially suppressed without damaging cellular mRNA. In fact, the global gene expression analyses relating to T-cell receptor signaling pathway by PrimerArray™ revealed that HIV-1 infection altered the expression levels of several genes in $CD4^+$ T cells (Fig. 7A). However, the baseline expression of MazF did not affect the gene expression pattern in uninfected MazF-T cells (Fig. 7B). More importantly, the altered gene expression observed in infected $CD4^+$ T cells was completely suppressed in infected MazF-T cells (Fig. 7C). These results clearly indicate that MazF expression does not show any negative impact on the gene expression related to $CD4^+$ T-cell functions. Tat protein expression was not sustained and gradually decreased in infected MazF-T cells, while it accumulated in infected $CD4^+$ T cells (Fig. 6A). These results suggest that Tat RNA is susceptible to cleavage by MazF and that the reduced expression of Tat protein is a limiting factor for sustained MazF expression. The level of intracellular Tat in MazF-T cells upon HIV-1 infection was kept constantly low compared to that in HIV-1 infected $CD4^+$ T cells. The level of MazF expression in the MazF-T cells described in this study is assumed to be lower than that observed in the MazF-expressing cells in the presence of tetracycline (Shimazu *et al.*, 2007). These cells apparently died after exposure to tetracycline.

Our study also demonstrated that the cytokine levels secreted from the infected MazF-T cells were not altered significantly compared to those from uninfected $CD4^+$ T cells and MazF-T cells. The reason for this discrimination by MazF between viral RNA and cellular mRNA is unknown and remains to be elucidated. It is possible that (1) MazF is expressed at a level that does not affect cellular mRNA but can destroy viral RNA; (2) MazF is expressed for a limited period of time that is not sufficient to destroy cellular mRNA; or (3) cellular mRNA is protected from the deleterious effect of MazF by the formation of polysome structures or other mechanisms. These hypotheses do not exclude each other, and more than one mechanism may play a crucial role in the safety of the current MazF-expressing system.

The ability of MazF to inhibit HIV-1 replication without affecting cell growth may be attributed to its conditional expression in HIV-1-infected MazF-T cells (Figs. 5 and 6). When Tat is expressed, it triggers not only the transcription of the *mazF* gene under the HIV-1 LTR promoter but also the transcription of full-length viral genome (Karn, 1999). The expression of MazF may cause the cleavage of newly synthesized HIV-1 RNA, including *tat*-encoding region, before forming the polysome structures. Consequently, Tat is no longer expressed in the infected MazF-T cells. Thus, the intracellular level of MazF is well controlled and kept below the threshold that impairs cell growth and functions. The influence of different MazF expression levels on HIV-1 inhibition and cell growth are currently under investigation.

At present, it is also unclear why the virus produced from MazF-T cells (MazF virus) is less infectious than that from $CD4^+$ T cells (control virus). Since this phenomenon occurred to the progeny virus after only one passage (Fig. 3A and Table 3), it is unlikely that a critical mutation was introduced into the progeny virus. In fact, Western blot analysis revealed that there was no quantitative difference in structural and nonstructural HIV-1 proteins between the control virus and MazF virus (data not shown). If MazF could be incorporated into virions in the process of assembly, it might be able to destroy viral RNA after their packaging and budding from MazF-T cells. Further studies are needed to determine whether virion-associated MazF exists and can cleave viral RNA.

The ideal target cells for gene modification appear to be $CD34^+$ hematopoietic stem cells in terms of possible viral eradication from HIV-1-infected patients. The permanent antiviral effect could be conferred on $CD34^+$ cells by modification with the MazF system. However, MazF has not been used in humans. Therefore, we would first propose an *ex vivo* gene therapy study with MazF-transduced autologous $CD4^+$ T cells in patients. In the proposed study, we expect that a pool of HIV-1-resistant $CD4^+$ T cells will persist in their bodies without cell death even upon HIV-1 infection and suppress viral replication.

In conclusion, the selective inhibition of HIV-1 replication by intracellular MazF expression was demonstrated in the primary $CD4^+$ T cells infected with clinical isolates as well as various laboratory-adapted strains. The effect was blood donor–independent. In addition, MazF did not allow the generation of escape mutants after long-term culture of the infected cells. Thus, our MazF-expressing system seems to be promising for the treatment of HIV-1 infection.

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Author Disclosure Statement

No competing financial interests exist.

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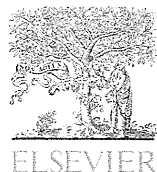

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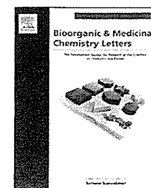

Structure–activity relationship study of phenylpyrazole derivatives as a novel class of anti-HIV agents

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ABSTRACT

The structure–activity relationship of phenylpyrazole derivative **1** was investigated for the development of novel anti-HIV agents. Initial efforts revealed that the diazenyl group can be replaced by an aminomethylene group. In addition, we synthesized various derivatives by the reductive amination of benzaldehydes with 5-aminopyrazoles and carried out parallel structural optimization on the benzyl group and the pyrazole ring. This optimization led to a six-fold more potent derivative **32j** than the lead compound **1**, and this derivative has a 3′,4′-dichloro-(1,1′-biphenyl)-3-yl group.

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Human immunodeficiency virus (HIV) infection remains one of the most serious threats to public health that causes acquired immunodeficiency syndrome,[1] in which progressive failure of the immune system allows life-threatening opportunistic infections. According to estimates in the UNAIDS Report 2012, ~34 million people worldwide are living with HIV and ~2.5 million people are newly infected with HIV.[2] This global health threat has triggered intensive drug discovery efforts for a number of anti-HIV drugs, including nucleoside reverse transcriptase inhibitors (NNRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors and CC chemokine receptor type 5 (CCR5) antagonists.[3] Highly active antiretroviral therapy involving co-administration of NRTIs, NNRTIs and PIs suppresses HIV replication to control disease progression in HIV-infected patients.[4] However, the emergence of drug-resistant HIV variants[5] and drug-related adverse effects such as dyslipidemia[6] hinder treatment to control chronic HIV replication. To overcome these problems, the development of novel effective anti-HIV agents with fewer adverse effects are needed.

During the course of our research project for the development of novel anti-HIV agents by random screening of an in-house small molecule library,[7] we identified a phenyldiazenylpyrazole compound **1** that showed submicromolar anti-HIV activity (Fig. 1). To the best of our knowledge, the biological activities of the phenyldiazenylpyrazole derivatives have not been documented. Herein, we report the structure–activity relationship study from a lead compound **1**, including substitution of the diazo linkage, and the modification of the right benzene and left *N*-phenylpyrazole parts.

The structure–activity relationship study began by improving the stability of the diazo linkage between the amidobenzene and phenylpyrazole groups, because diazo compounds can be extensively metabolized by intra- and extra-cellular enzymatic components of intestinal bacteria.[8] To explore the biomimetic equivalents of the diazo structure, we designed a styrene derivative **2** and an aminomethylene derivative **3** (Fig. 1).

Synthesis of compound **2** is outlined in Scheme 1. Pyrazole derivative **5** was obtained from the condensation of (*Z*)-3-amino-2-(2-chloroacetyl)but-2-enenitrile **4** and phenylhydrazine. Separately, $FeCl_3$-mediated reduction of the nitro group of compound **7** followed by acylation provided propionamidophenol **9**, which was subjected to formylation using hexamethylenetetramine to afford aldehyde **10**.[9] Benzyl-protected aldehyde **11** was obtained by alkylation of **10** with benzyl bromide. Reaction of aldehyde **11** using phosphonium salt **6** gave an olefin **12**. Subsequent exposure of compound **12** to TMSI provided the desired styrene derivative **2**.

Abbreviations: HIV, human immunodeficiency virus; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

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