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# Superimposed Epitopes Restricted by the Same HLA Molecule Drive Distinct HIV-Specific CD8+ T Cell Repertoires

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Superimposed epitopes, in which a shorter epitope is embedded within a longer one, can be presented by the same HLA class I molecule. CD8+ CTL responses against such epitopes and the contribution of this phenomenon to immune control are poorly characterized. In this study, we examined HLA-A*24:02–restricted CTLs specific for the superimposed HIV Nef epitopes RYPLTFGWCF (RF10) and RYPLTFGW (RW8). Unexpectedly, RF10-specific and RW8-specific CTLs from HIV-1–infected HLA-A*24:02+ individuals had no overlapping Ag reactivity or clonotypic compositions. Single-cell TCR sequence analyses demonstrated that RF10-specific T cells had a more diverse TCR repertoire than did RW8-specific T cells. Furthermore, RF10-specific CTLs presented a higher Ag sensitivity and HIV suppressive capacity compared with RW8-specific CTLs. Crystallographic analyses revealed important structural differences between RF10– and RW8–HLA-A*24:02 complexes as well, with featured and featureless conformations, respectively, providing an explanation for the induction of distinct T cell responses against these epitopes. The present study shows that a single viral sequence containing superimposed epitopes restricted by the same HLA molecule could elicit distinct CD8+ T cell responses, therefore enhancing the control of HIV replication. This study also showed that a featured epitope (e.g., RF10) could drive the induction of T cells with high TCR diversity and affinity. *The Journal of Immunology*, 2014, 193: 77–84.

C ytotoxic CD8+ T lymphocytes recognize target cells through the recognition of peptides 8–11 aa long that are presented by MHC class I (MHCI) molecules (1–3). Of note, two epitopes in which a shorter one is embedded within a longer one are defined as superimposed epitopes, and they have been shown to be presented by the same MHCI molecule (4–6). A number of studies have reported CTL responses against such superimposed peptides in the context of an HIV-1 infection (7–10). For instance, these responses include CTLs specific for HLA-B57–restricted p24 Gag-derived peptides, for example, KI8 (residues 162–169, KAFSPEVI) and KF11 (residues 162–172, KAFSPEVIPMF), as well as for HLA-B*35:01–restricted Nef-derived peptides, for example, VY8 (residues 74–81, VPLRPMTY) and RY11 (residues 71–81, RPQVPLRPMTY). However, the biological relevance of this phenomenon remains unclear. Indeed, although it is speculated that CTLs can show cross-reactivity toward superimposed

epitopes and work together effectively against HIV-infected targets, the functional synergism of these cells has not been studied in detail.

HLA-A*24:02 is the most frequent HLA class I allele in Japan, being found in ∼70% of Japanese individuals (11) and of those infected with HIV-1 (12, 13). This allele also occurs in the range from ∼25–64% in other Asian countries and in 18% in white populations (14, 15). Therefore, the study of immune responses to epitopes restricted by this allele is important for our understanding of HIV pathogenesis and vaccine development. We previously reported that Nef138-10 (RYPLTFGWCF, RF10) is an immunodominant CTL epitope in HLA-A*24:02+ Japanese individuals chronically infected with HIV-1 (16, 17). Of interest, Nef138-8 (RYPLTFGW, RW8) is also defined as an optimal epitope presented by HLA-A*24:02 in white individuals (18). Although these superimposed epitopes elicit effective specific CTL responses important for the control of HIV-1 replication in HLA-A*24:02+ individuals, the overlap in terms of reactivity and antiviral ability between RF10- and RW8-specific CTLs remains unknown.

In the present study, we performed a comprehensive analysis of CTL responses specific for RW8- and RF10-superimposed epitopes by using multiple approaches. We used RF10 and RW8 tetramers to identify and isolate cells from chronically HIV-1–infected HLA-A*24:02+ Japanese individuals, α- and β-chain TCR repertoire analyses at the single-cell level to assess the degree of overlap between responses, and crystallographic approaches to reveal the structural basis of RF10– and RW8–HLA-A*24:02 interactions. We report unanticipated differences between RF10- and RW8-specific CTLs, that is, the elicitation of totally distinct CTL responses against superimposed HIV-1 epitopes restricted by the same HLA molecule, as well as distinct TCR repertoires between the featured (RF10) and featureless (RW8) epitope-specific CTLs.

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The online version of this article contains supplemental material.

Abbreviations used in this article: MFI, mean fluorescence intensity; MHCI, MHC class I; tet, tetramer.

## Materials and Methods

*Patient samples*

The study was approved by the Ethics Committees of Kumamoto University and the National Center for Global Health and Medicine. Informed consent

was obtained from all individuals according to the Declaration of Helsinki. Twenty-three HLA-A*24:02[+] treatment-naive individuals chronically infected with HIV-1 and eight HLA-A*24:02[+] HIV-1 seronegative individuals were recruited (Supplemental Table I). Their plasma and PBMCs were separated from whole blood. HLA types were determined by standard sequence-based genotyping.

### HIV-1–specific CTL clones

Ag-specific CTL clones were generated as previously described (17). Briefly, RW8- and RF10-specific CTL cell lines were first obtained by stimulating PBMCs from patient KI-158 with cognate peptides. Peptide-specific CTL clones were then generated from the cell lines by limiting dilution in 96-well U-shaped plates cocultured with $1 \times 10^6$ irradiated feeder PBMCs from healthy donors and $1 \times 10^5$ irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide at 1 μM concentration. All CTL clones were cultured in 200 μl cloning medium (RPMI 1640 containing 10% FBS, 200 U/ml rIL-2, and 2.5% PHA soup) and stimulated weekly with irradiated C1R-A*2402 cells prepulsed with RW8 or RF10 peptide.

### Tetramer staining

HLA-A*24:02 tetrameric complexes were synthesized as previously described (19). For tetramer-binding assays, CTL clones were stained with PE-conjugated RW8 or RF10 tetramers at various concentrations (0–1000 nM) at 37°C for 30 min before staining with FITC-conjugated anti-CD8 mAb at 4°C for 30 min. The cells were analyzed by using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star). For TCR avidity measurements, the tetramer concentration that yielded the $EC_{50}$ mean fluorescence intensity (MFI) was calculated by probit analysis.

### Cell line

C1R-A*2402 and RMA-S-A*2402 cells were previously generated by transfecting HLA-A*24:02 genes into C1R cells and RMA-S cells, respectively (5, 20, 21). The C1R cell line is a human B cell lymphoblastoid line lacking surface expression of HLA-A and partially HLA-B molecules. It was derived from a normal B cell line, Hmy2, through three rounds of mutagenesis and selection with anti-HLA mAb (22). RMA-S cells are a TAP2 deficiency cell line derived from RMA cells. They express high levels of empty MHC molecules (i.e., not carrying endogenous peptides on the cell surface) when cultured at 26°C and very low levels when cultured at 37°C (23). RMA-S-A*2402 and C1R-A*2402 cells were cultured in RPMI 1640 medium containing 10% FCS and 0.2 mg/ml hygromycin B.

### Peptide-binding assay

The binding of peptides to HLA-A*24:02 molecules was tested as previously described (24). Briefly, RMA-S-A*2402 cells were precultured at 26°C for 14–18 h and then incubated at the same temperature for 1 h with either RW8 or RF10 peptide at various concentrations (0–100 nM). Thereafter, they were incubated at 37°C for 3 h. After incubation, the peptide-pulsed cells were stained with anti-HLA class I α3 domain mAb TP25.99 (19) and subsequently with FITC-conjugated sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The MFI was measured by flow cytometry (FACSCanto II).

### Replication suppression assay

Two HIV-1 virus laboratory strains, NL-432-10F and NL-M20A-10F, were used in these assays. They were generated from NL-43 or NL-M20A by site-directed mutagenesis to carry the RYPLTFGWCF sequence (17). The ability of HIV-1–specific CTLs to suppress HIV-1 replication was examined as previously described (17). Briefly, primary CD4^{+} T cells were infected with NL-432-10F and NL-M20A-10F, respectively, for 6 h before being washed with R10 medium. The cells were then cocultured with HIV-1–specific CTL clones at various E:T ratios. Ten microliters of culture supernatant was collected at day 6, and the concentration of p24 Ag in it was determined by performing p24 ELISA (ZeptMetrix, Buffalo, NY).

### ^{51}Cr-release assay

The cytotoxic potential of CTL clones against C1R-A*2402 prepulsed with appropriate peptide at various concentrations (0–100 nM) was determined as previously described (25). Briefly, C1R-A*2402 cells were labeled with 100 μl ^{51}Cr for 1 h before washing and then pulsed for 1 h with peptides. Effector cells were cocultured for 4 h at 37°C with target cells ($2 \times 10^3$/well) at an E:T ratio of 2:1. After centrifugation, 100 μl supernatant was collected and analyzed with a gamma counter. The specific lysis was calculated as [(cpm experiment − cpm supernatant)/(cpm maximum − cpm supernatant)] × 100.

### Ex vivo single-cell TCR repertoire analysis and assessment of TCR diversity

Cryopreserved PBMC samples from patients were thawed, divided, and immediately stained with RW8 or RF10 tetramers, followed by staining with anti-CD3 mAb (Pacific Blue), anti-CD8 mAb (FITC), and 7-amino-actinomycin D. RW8 and RF10 tetramer^{+}CD3^{+}CD8^{+}7-aminoactinomycin D^{−} cells were sorted into a 96-well plates (Bio-Rad) by using a FACSAria I (BD Biosciences). Unbiased identification of TCR α- and β-chain usage was assessed as previously described (26). An Illustra ExoStar (GE Healthcare, Little Chalfont, U.K.), which contains alkaline phosphatase and exonuclease 1, was used to remove unincorporated primers and nucleotides from amplification reaction for the subsequent tailing reaction. The names of all identified TCR genes were given based on the international ImMunoGeneTics information system nomenclature (27). The diversity of TCR clonotypes was calculated by using both the number of different clonotypes and Simpson's diversity index for both α- and β-chains and the formula $D_s = 1 - \Sigma\{[n_i(n_i - 1)]/[N(N - 1)]\}$, where $n_i$ is the TCR clone size of the $i$th clonotype and $N$ is the total number of TCR sequences sampled. This index uses the relative frequency of each clonotype to calculate a diversity index ranging between 0 and 1, with 0 being minimal and 1 being maximal diversity (28).

### Crystallization, data collection, and processing

Soluble peptide–HLA-A*24:02 complexes were prepared as previously described (29). HLA-A*24:02 molecules were purified by Superdex 200 10/300 GL gel-filtration chromatography (GE Healthcare). All crystallization attempts were performed by the hanging drop vapor diffusion method at 18°C with a protein/reservoir drop ratio of 1:1. Crystals were seen after 3–5 d in 0.1 M MES (pH 6.5) and 12% (w/v) polyethylene glycol at 20,000 g/mol. The crystals were briefly soaked in reservoir solution containing 17% (v/v) glycerol, mounted on an x-ray machine with a nylon loop, and then flash-cooled in a stream of gaseous nitrogen. Diffraction data were collected by using beamline NE3A in the KEK Synchrotron Facility (Tsukuba, Japan) and an ADSC Q270 imaging-plate detector at a wavelength of 1.0 Å. Data were indexed, integrated, and scaled by using HKL2000. The data collection statistics are shown in Table I. Data were analyzed by molecular replacement by use of Phaser in CCP4. We used the A24VYG molecule as the search model (Protein Data Bank accession no. 2BCK, http://www.rcsb.org/pdb/home/home.do). All of the structures were further refined by several rounds of refinement made by using the PHENIX program. The refinement statistics are given in Table I.

## Results

### Effective induction of RW8- or RF10-specific CTL responses in HIV-1–infected HLA-A*24:02[+] patients

To assess the degree of overlap between CTL responses specific for two superimposed Nef epitopes (RW8 and RF10), we first generated HLA-A*24:02 tetramers with RW8 or RF10 peptides (RW8-tet and RF10-tet, respectively) and compared ex vivo frequencies of 8-mer– or 10-mer–specific CD8^{+} T cells in 23 treatment-naive HLA-A*24:02^{+} individuals with chronic HIV-1 infection. In eight HIV-1 seronegative HLA-A*2402^{+} donors, the frequencies of RW8 and RF10 tetramer^{+} CD8^{+} T cells were 0.080 ± 0.009 and 0.045 ± 0.022% (mean ± SD), respectively (Fig. 1A). We evaluated the mean ± 3 SD as positive staining and therefore considered 0.10 and 0.11% of tetramer[+] CD8+ cells as positive values for RW8-specific and RF10-specific T cells, respectively (Fig. 1B, dashed line). Among the 23 HIV-1–infected individuals studied, 14 and 19 were positive for RW8-specific CTLs and RF10-specific CTLs, respectively (Fig. 1A). Thirteen of the 23 individuals analyzed (56.5%) presented both RW8- and RF10-specific T cells (Fig. 1B). The magnitude of RW8-specific and RF10-specific T cells correlated with one another across individuals (Fig. 1B). However, this correlation was modest, indicating that these populations did not overlap entirely. In fact, the frequency of RF10 tetramer^{+} CD8^{+} cells was significantly higher than that of RW8 tetramer^{+} CD8^{+} cells (Fig. 1A). Taken together, these results indicate that both RW8-specific and RF10-specific CTLs could be effectively elicited in HLA-A*24:02[+] individuals with a chronic HIV-1 infection; however,
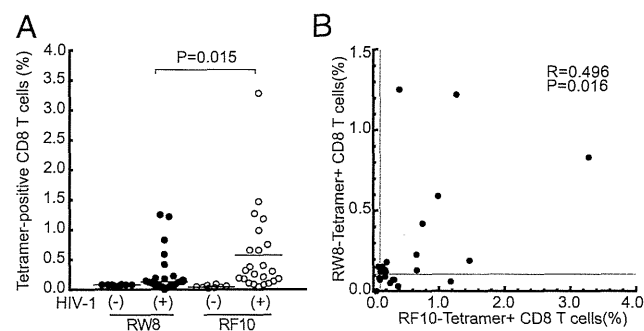
**FIGURE 1.** Frequencies of RW8- and RF10-specific CD8^{+} T cells in individuals with chronic HIV-1 infection. (**A**) Frequencies of total RW8 and RF10 tetramer^{+} CD8^{+} T cells in 23 chronically HIV-1–infected treatment-naive HLA-A*24:02^{+} individuals and in 8 HLA-A*24:02^{+} uninfected controls. Statistical analyses were conducted by using the nonparametric Mann–Whitney $U$ test. (**B**) Correlations between RW8 and RF10 tetramer^{+} CD8^{+} T cell frequencies in HIV-1–infected HLA-A*24:02^{+} individuals. Of tetramer^{+} CD8^{+} cells, 0.10 and 0.11% were considered as positive values for RW8-specific and RF10-specific T cells, respectively (the dashed line indicates the threshold), as described in the text. The correlation was determined by using the Spearman rank test.

they do not appear to be equivalent, which begs the question of their cross-reactive potential.

*Distinct reactivity of RW8- and RF10-specific CTLs*

To investigate whether RW8- or RF10-specific CTLs could cross-recognize the superimposed epitopes, we first performed concurrent RW8 and RF10 tetramer (RW8-tet and RF10-tet, respectively) staining of PBMCs from HIV-1–infected donors. In patients presenting both RF10- and RW8-specific CD8^{+} T cells, these cells did not seem to be RW8 and RF10 cross-reactive, as they failed to stain for both tetramers simultaneously. A representative case (patient KI-

158) is shown in Fig. 2A. To analyze further the fine reactivity toward these epitopes, we next established CTL clones from patient KI-158 presenting both RF10- and RW8-specific CD8^{+} T cells upon initial selection and stimulation with RW8 or RF10 peptides. RF10- and RW8-specific clones were clearly discriminated by using both tetramers together at the same concentration (Fig. 2B). We performed staining using different concentrations of the specific tetramers to measure the TCR avidity of representative RF10- or RW8-specific clones. CTL52 clone (RW8-specific) exhibited a strong affinity for RW8-tet but not for RF10-tet, whereas the CTL173 (RF10-specific) clone exhibited a strong affinity for RF10-tet but not RW8-tet (Fig. 2C), indicating that CTL52 and CTL173 clones had TCRs with high affinity for RW8 peptide–HLA-A*24:02 and RF10 peptide–HLA-A*24:02 complexes, respectively.

Next, we tested the functional avidity of RF10- and RW8-specific clones. RF10-specific clones (CTL170 and CTL173) effectively killed RF10 peptide–pulsed cells but failed to kill RW8 peptide–pulsed targets even at a high concentration of RW8 peptide (Fig. 2D), indicating that these RF10-specific clones did not cross-recognize the 8-mer peptide. RW8-specific clones (CTL52 and CTL72) recognized both RW8 peptide– and RF10 peptide–pulsed targets, but the cytotoxic activity of these clones against RW8 peptide–pulsed target cells was 10- to 50-fold higher than that against the RF10 peptide–pulsed ones (Fig. 2E). Although RW8 clones presented some cross-reactivity toward RF10, they recognized the RW8 peptide with greater efficiency than RF10. Altogether, these results indicate that RW8-specific and RF10-specific CTLs displayed no or poor cross-reactivity for their respective superimposed epitopes.

*Different TCR usage between RW8- and RF10-specific CD8^{+} T cells*

The lack of cross-reactivity between RW8-specific and RF10-specific CD8^{+} T cells implies that distinct clonotypes should

**FIGURE 2.** Recognition of RW8 and RF10 peptides by RW8- or RF10-specific CTL clones. (**A**) Simultaneous RW8 and RF10 tetramer staining of PBMCs from a representative HIV-1–infected donor and an HIV-1–uninfected healthy individual. The percentage of tetramer^{+} cells among CD8^{+} cells is shown. (**B**) Simultaneous RW8 and RF10 tetramer staining of CTL52 and CTL173 clones. The percentage of tetramer^{+} cells among CD8^{+} cells is shown. (**C**) RW8 and RF10 tetramer staining of CTL52 and CTL173 clones at various concentrations. Tetramer binding to CTL52 and CTL173 clones was analyzed by flow cytometry, and the data are shown as MFI. (**D** and **E**) Cytolytic activities of HLA-A*24:02–restricted RF10 (CTL170/CTL173)-specific (D) and RW8 (CTL52/CTL72)-specific (E) CTL clones toward HLA-A*24:02^{+} target cells (C1R-A*2402) prepulsed with the indicated peptide at various concentrations.
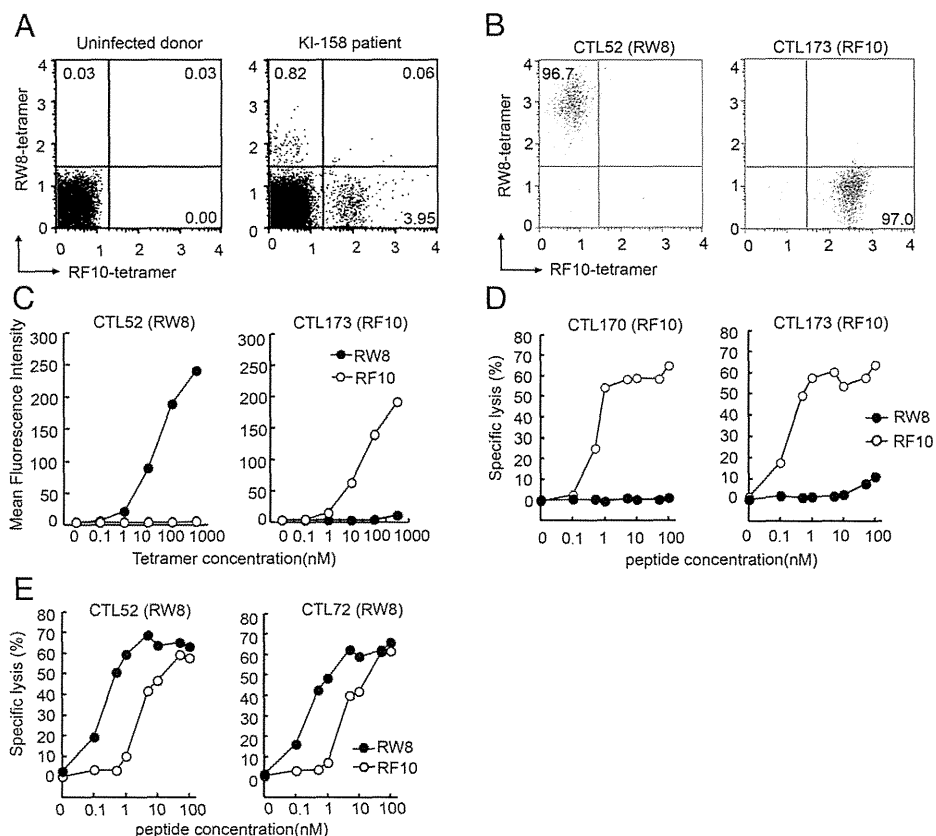
comprise these populations. To verify this point, we performed TCR repertoire analysis at the single-cell level to expose the degree of overlap between the two responses at the clonotypic level. Most previous studies on TCR repertoires in virus infection or tumor studies focused mainly on characterization of the TRB gene (30–32). However, analysis of TRB genes provides only a partial account of the TCR repertoire, because the same TRB gene can pair with different TRA genes (26, 33). In the present study, we sequenced both α- and β-chains of the TCR on RW8-specific and RF10-specific single CD8^{+} T cells sorted by FACS from three patients presenting both RW8- and RF10-specific CD8^{+} T cells, as well as from two individuals with either one (Supplemental Table II). This analysis showed that RW8- and RF10-specific CD8^{+} T cells consisted indeed of entirely distinct sets of clonotypes (Fig. 3, Supplemental Fig. 1). Of note, TRBV7-9 clonotypes were often detected among RW8-specific T cells, whereas TRBV28-1 was frequently found among RF10-specific T cells. Additionally, we observed that the number of distinct clonotypes as well as clonotypic diversity (based on both α- and β-chains) among RW8-

specific CD8^{+} T cells was significantly lower than for RF10-specific ones (Fig. 4). Overall, the differences in α- and β-chain TCR gene usage and overall clonotypic diversity between RW8- and RF10-specific CD8^{+} T cells supported significantly distinct modes of TCR recognition of the RW8 peptide– and RF10 peptide–HLA-A*24:02 molecule complexes.

### Structure of RW8 peptide– and RF10 peptide–HLA-A*24:02 molecular complexes

Considering the potential impact of peptide–MHCI structural constraints on TCR repertoire composition, we next aimed at investigating the molecular basis of the interaction between the HLA-A*24:02 molecule and the RW8 or RF10 peptide to elucidate the determining factor for the lack of overlap between RW8- and RF10-specific CD8^{+} T cell populations. We therefore determined the crystal structure of HLA-A*24:02 in complex with RW8 (HLA-A*24:02-RW8) and HLA-A*24:02-RF10 complex (HLA-A*24:02-RF10) (Table I). The two superimposed epitopes, RF10 and RW8, showed dramatically different conformations when bound to HLA-

## A  Paired T cell receptor usage of RW8-specific CTLs

| Sample | TRAV | TRAJ | CDR3α | TRBV | TRBD | TRBJ | CDR3β | Frequency |
|--------|------|------|-------|------|------|------|-------|-----------|
| KI-026 | TRAV12-2*03 | TRAJ40*01 | CAVPRTGTYKYIF | TRBV7-9*03 | TRBD2*01 | TRBJ2-2*01 | CASSLTSGANTGELFF | 10/18 |
| | TRAV12-2*03 | TRAJ23*01 | CAVSFYNQGGKLIF | TRBV7-9*03 | TRBD1*01 | TRBJ1-2*01 | CASSPRDKPNYGYTF | 8/18 |
| KI-158 | TRDV1*01 | TRAJ40*01 | CALGELGAPGTYKYIF | TRBV20-1*01 | TRBD2*01 | TRBJ2-7*01 | CSARDPVSTYEQYF | 27/31 |
| | TRAV12-2*02 | TRAJ50*01 | CAAFKTSYDKVIF | TRBV20-1*02 | TRBD2*01 | TRBJ2-7*01 | CSARDPIRLISYEQYF | 4/31 |
| KI-654 | TRAV8-4*03 | TRAJ30*01 | CAVSDEVIF | TRBV7-9*07 | TRBD1*01 | TRBJ2-5*01 | CASSIRDRVPETQYF | 13/43 |
| | TRAV9-2*01 | TRAJ16*01 | CALFLDGQKLLF | TRBV7-9*07 | TRBD2*01 | TRBJ2-2*01 | CASSDTSGANTGELFF | 7/43 |
| | TRAV12-2*02 | TRAJ40*01 | CAVPVPGTYKYIF | TRBV7-9*07 | TRBD2*01 | TRBJ2-2*01 | CASSDTSGANTGELFF | 23/43 |
| KI-102 | TRAV8-1*01 | TRAJ10*01 | CAVIFTGGGNKLTF | TRBV7-9*03 | TRBD1*01 | TRBJ2-5*01 | CASSQRDSQETQYF | 65/65 |

## B  Paired T cell receptor usage of RF10-specific CTLs

| Sample | TRAV | TRAJ | CDR3α | TRBV | TRBD | TRBJ | CDR3β | Frequency |
|--------|------|------|-------|------|------|------|-------|-----------|
| KI-026 | TRAV9-2*01 | TRAJ21*01 | CALGVDFNKFYF | TRBV19*01 | TRBD1*01 | TRBJ2-2*01 | CASKGTVTGELFF | 7/27 |
| | TRDV1*01 | TRAJ9*01 | CALGELTNTGGFKTIF | TRBV11-2*03 | TRBD2*01 | TRBJ2-7*01 | CASSYDRGYEQYF | 6/27 |
| | TRDV1*01 | TRAJ9*01 | CALGELSRTGGFKTIF | TRBV28*01 | TRBD2*02 | TRBJ2-7*01 | CASLPSVGKGAYEQYF | 6/27 |
| | TRDV1*01 | TRAJ24*02 | CALWIMTTDSWGKLQF | TRBV4-1*01 | TRBD1*01 | TRBJ2-1*01 | CASSQSPGQGVGEQFF | 4/27 |
| | TRDV1*01 | TRAJ13*02 | CALGELSSGGYQKVTF | TRBV6-1*01 | TRBD1*01 | TRBJ1-2*01 | CASSDVGQGSSNYGYTF | 2/27 |
| | TRDV1*01 | TRAJ36*01 | CALGVLDQTGANNLFF | TRBV28*01 | TRBD1*01 | TRBJ1-2*01 | CASSSPGQGYGYTF | 1/27 |
| | TRAV21*02 | TRAJ7*01 | CAVWYYGNNRLAF | TRBV28*01 | TRBD2*02 | TRBJ2-2*01 | CASSLMGLAGVPGELF | 1/27 |
| KI-158 | TRDV1*01 | TRAJ9*01 | CALGELSGTGGFKTIF | TRBV6-1*01 | TRBD1*01 | TRBJ2-1*01 | CASSEGFGQGGIEQFF | 19/32 |
| | TRDV1*01 | TRAJ53*01 | CALGELLRGGSNYKLTF | TRBV6-2*01 | TRBD2*01 | TRBJ2-7*01 | CASSYSHRLGLHEQYF | 11/32 |
| | TRAV8-6*02 | TRAJ48*01 | CAVLSLISNFGNEKLTF | TRBV19*01 | TRBD2*01 | TRBJ2-7*01 | CASSISAGEGVPEQYF | 2/32 |
| KI-118 | TRDV1*01 | TRAJ9*01 | CALGELSSTGGFKTIF | TRBV28*01 | TRBD1*01 | TRBJ2-1*01 | CASTSFGQGTNEQFF | 12/14 |
| | TRAV13-1*02 | TRAJ20*01 | CAALNDYKLSF | TRBV19*01 | TRBD2*01 | TRBJ2-1*01 | CASSIDPPGLADNEQFF | 2/14 |
| KI-102 | TRDV1*01 | TRAJ9*01 | CALGELSHTGGFKTIF | TRBV11-2*01 | TRBD1*01 | TRBJ2-7*01 | CASSYDRSYEQYF | 4/30 |
| | TRDV1*01 | TRAJ9*01 | CALGELTNTGGFKTIF | TRBV6-6*01 | TRBD1*01 | TRBJ1-2*01 | CASSYSIGTGVNNYGYTF | 3/30 |
| | TRDV1*01 | TRAJ36*01 | CALGVLDQTGANNLFF | TRBV28*01 | TRBD1*01 | TRBJ1-2*01 | CASSSPGQGYGYTF | 7/30 |
| | TRDV1*01 | TRAJ54*01 | CALGVIGIQGAQKLVF | TRBV28*01 | TRBD1*01 | TRBJ1-5*01 | CASSPSTGKGNQPQHF | 7/30 |
| | TRAV25*01 | TRAJ23*01 | CPFYNQGGKLIF | TRBV19*01 | TRBD1*01 | TRBJ1-2*01 | CASSTALRTGNYGYTF | 4/30 |
| | TRAV26-1*01 | TRAJ28*01 | CVVNSGAGSYQLTF | TRBV6-1*01 | TRBD1*01 | TRBJ2-7*01 | CASSETGGTYEQYF | 3/30 |
| | TRAV8-3*02 | TRAJ37*01 | CAVDEGKLIF | TRBV4-1*01 | TRBD1*01 | TRBJ1-1*01 | CASSQRDRGTDTEAFF | 1/30 |
| | TRAV14/DV4*01 | TRAJ33*01 | CAMQDSNYQLIW | TRBV7-9*03 | TRBD2*02 | TRBJ2-1*01 | CASSLVSGRGNEQFF | 1/30 |

**FIGURE 3.**  Clonotypic analysis of RW8- and RF10-specific CD8^{+} T cells. Single RW8- or RF10-specific CD8^{+} T cells from five chronically HIV-1–infected HLA-A*24:02^{+} individuals were FACS sorted, and TCR α- and β-chain sequencing was performed. Paired TCR α- and β-chain usage, CDR3 amino acid sequences, and individual clonotype frequencies are shown. (**A**) Paired T cell receptor usage of RW8-specific CTLs. (**B**) Paired T cell receptor usage of RF10-specific CTLs.
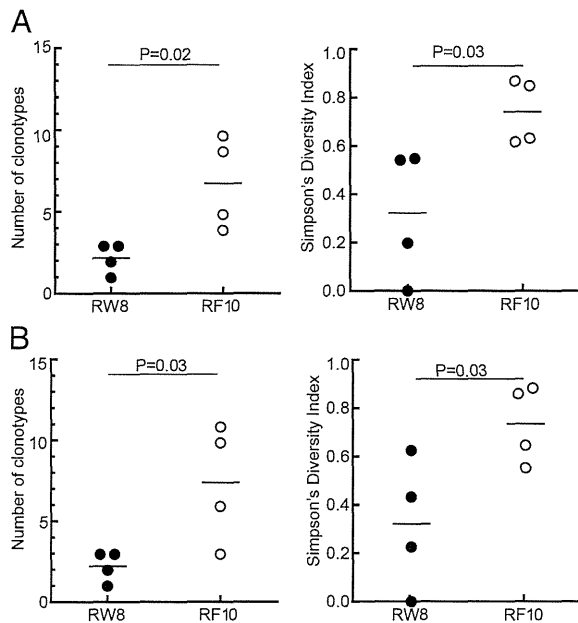
A



B



**FIGURE 4.** TCR repertoire diversity of RW8- and RF10-specific CD8⁺ T cells. TCR repertoire diversity was assessed by using both the number of clonotypes and Simpson's diversity index for α- (**A**) and β-chain (**B**). Statistical analysis was conducted by use of the unpaired $t$ test.

A*24:02 (Fig. 5A). The RW8 peptide was buried in the binding groove. In contrast, the two extra amino acids at the C terminus of the RF10 peptide caused a switch of the Pc anchor residues from Trp[8] in RW8 to Phe[10] in RF10, so that the central region (P4–P7) of RF10 protruded out of the groove. Therefore, the solvent-accessible surface area of the central region of RF10 significantly diverged from that of RW8. These dramatic conformational differences explain the lack of cross-reactivity between RW8- and RF10-specific CTLs, as well as

the necessity to elicit different TCR repertoires to recognize these HLA-A*24:02 superimposed epitopes.

The total hydrogen bonds and van der Waal's interactions between HLA-A*24:02 and the RW8 or RF10 peptide were analyzed in detail (Fig. 5B, Supplemental Table III). The first 3 aa (P1–P3 residues) of both RW8 and RF10 peptides displayed almost identical main-chain conformations. However, the N-terminal anchor residue (Pn) tyrosine (Y) at position P2 formed a hydrogen bond with H70 in the B pocket of HLA-A*24:02. One additional hydrogen bond between P2 residue and Lys[66] and another hydrogen bond between the P1 residue and Arg[170] were also observed in the RF10 peptide. Moreover, whereas the Pc residue Trp[8] of the RW8 peptide formed four hydrogen bonds with the HLA molecule, the subanchor residue Trp[8] and Pc residue Phe[10] of the RF10 peptide formed one more hydrogen bond. This additional hydrogen bonding likely impacted the binding affinity of RF10 for HLA-A*24:02, making it greater than that of RW8.

*Superior HIV-suppressive capacity of RF10-specific CD8⁺ CTL clones*

Measurements of the binding affinity of RF10 and RW8 peptides for HLA-A*24:02 molecules indeed revealed that the RF10 affinity was ~10-fold higher than the RW8 affinity (Fig. 6A). Such differences in peptide–MHCI binding affinity may eventually have affected the efficiency of T cells to recognize their specific targets. We thus investigated the ability of RW8- and RF10-specific CD8⁺ CTL clones to suppress HIV-1 replication in cultures of virus-infected CD4⁺ T lymphocytes. To this end, we used two viruses, NL-432-10F and NL-M20A-10F, both carrying the RYPLTFGWCF sequence. In contrast to NL-432-10F, NL-M20A-10F does not downregulate cell-surface expression of HLA class I molecules (34). A previous study demonstrated the epitope-dependent effect of Nef-mediated HLA class I downregulation on the capacity of HIV-1 specific CTLs to suppress HIV-1 replication. The capacity may be dependent on the expression level of HLAI molecules

Table I.   Statistics for crystallographic data collection and structure refinement

|  | A24, 8-Mer | A24, 10-Mer |
| --- | --- | --- |
| Data collection |  |  |
| Space group | P2₁ | C2 |
| Cell dimensions |  |  |
| a, b, c (Å) | 71.95, 152.52, 90.17 | 161.74, 65.06, 50.42 |
| α, β, γ (°) | 90.00, 90.12, 90.00 | 90.00, 90.23, 90.00 |
| Resolution (Å) | 50.0–2.4 | 50.0–2.4 |
| $R_{merge}$ | 0.087 (0.554)ᵃ | 0.102 (0.469) |
| I/σI | 13.0 (2.2) | 20.0 (2.6) |
| Completeness (%) | 99.3 (99.3) | 97.2 (92.6) |
| Redundancy | 3.1 (3.1) | 4.1 (3.6) |
| Refinement |  |  |
| Resolution (Å) | 36.0–2.4 | 42.7–2.4 |
| No. of reflections | 70,383 | 20,284 |
| $R_{work}/R_{free}$ | 0.1952/0.2185 | 0.1882/0.2223 |
| No. of atoms |  |  |
| Protein | 12,528 | 3,148 |
| Water | 609 | 238 |
| B factors |  |  |
| Protein | 46.5 | 38.4 |
| Water | 38.0 | 40.0 |
| R.m.s.d. |  |  |
| Bond lengths (Å) | 0.004 | 0.003 |
| Bond angles (°) | 0.857 | 0.689 |
| Ramachandran plot (%) |  |  |
| Most favored regions | 87.9 | 96.3 |
| Allowed regions | 12.1 | 3.7 |
| Disallowed regions | 0 | 0 |

ᵃValues in parentheses are for the highest resolution shell.
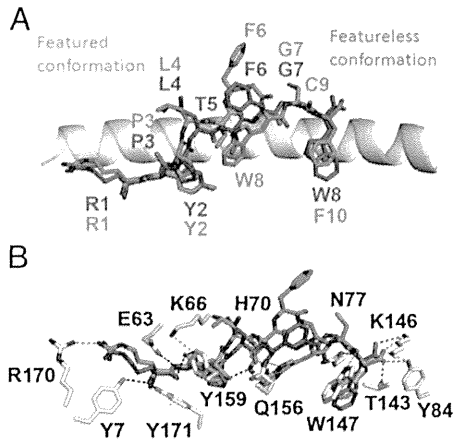R.m.s.d., root mean square deviations.

**FIGURE 5.** Comparison between the structure of the HLA-A24:02 molecule in complex with the RW8 or RF10 epitope peptide. (**A**) Superposition of HLA-A*24:02 in complex with RW8 (pink) or RF10 (green) peptides. The peptides are shown in stick-model form. RF10 displays a featured conformation with the residue F6 exposed to the solvent, whereas RW8 displays a featureless conformation, with the residue F6 hidden in the groove. (**B**) Hydrogen bond interactions between HLA-A*24:02 and RW8 (pink) or RF10 (green) peptide. The hydrogen bonds in common between the two peptides are shown in black.

weaker than that of RF10-specific CTLs, even at an E:T ratio of 1:1, although their respective capacities to suppress NL-M20A-10F were comparable (Fig. 6B). These results indicate that RF10-specific CTLs presented higher Ag sensitivity than did RW8-specific CTLs, implying that the RF10 peptide is more presented on the cell surface than is the RW8 peptide.

In addition to the higher binding affinity of HLA-A*24:02 for the RF10 peptide, we wanted to compare the TCR avidity of RW8- and RF10-specific CD8+ T cell clones. We thus measured TCR avidity of these clones by using the tetramer dilution assay. Twelve clones of each specificity were stained with different concentrations of RW8-tet or RF10-tet (Fig. 6C). The $EC_{50}$ values of the RF10-specific CTL clones were significantly lower than those of the RW8-specific ones (Fig. 6D), indicating that RF10-specific CD8+ T cells had a higher TCR avidity than did the RW8-specific CD8+ T cells.

## Discussion

Screening for optimal CTL epitopes is central for the characterization of antiviral or antitumoral CD8+ T cell responses (36–38). It is not unusual to observe CTL reactivity toward peptides of 8–12 aa in length around an optimal epitope. This observation is thought to reflect the flexibility of TCR–MHC pairing to accommodate peptides close to the optimal one, such that the same CD8+ T cells are able to recognize these peptides (39–41). In the present study, we examined CD8+ T cell responses against two superimposed HIV nef epitopes (RW8 and RF10) restricted by HLA-A*24:02. Using RW8 and RF10 tetramers, we could discriminate between T cells specific for these two peptides and could show that these cells represented two distinct populations with independent reactivity. Furthermore, we applied single-cell TCR analysis to characterize both TCR α- and β-chain repertoires directly
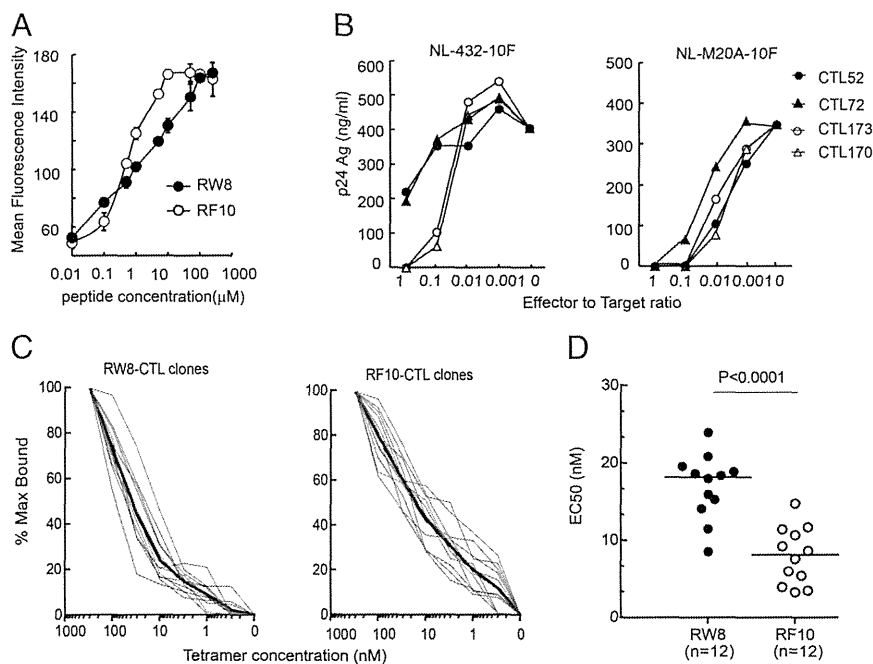
carrying the epitope peptide (35). RF10-specific CTL clones 170 and 173 completely suppressed the replication of both NL-432-10F and NL-M20A-10F at E:T ratios of 1:1 and 0.1:1, respectively, indicating that RF10-specific CTLs could strongly suppress HIV-1 replication regardless of Nef-mediated downregulation of HLA class I molecules (Fig. 6B). In comparison, the ability of RW8-specific CTL clones 52 and 72 to suppress NL-432-10F was



**FIGURE 6.** Efficacy of superimposed epitope-specific CTLs to suppress HIV-1 replication. (**A**) Binding of RW8 and RF10 peptides to HLA-A*24:02 molecules on RMA-S-A*2402 cells quantified by use of the HLA-A*24:02 stabilization assay. (**B**) HIV suppressive capacity of two RW8-specific CTL clones (CTL 52 and 72) and two RF10-specific CTL clones (CTL 170 and 173). Cultured CD4+ T cells derived from an HLA-A*24:02+ donor were infected with NL-432-10F or NL-M20A-10F and then cocultured with the indicated CTL clones at various E:T ratios. HIV-1 p24 Ags in the supernatant were measured on day 6 postinfection by performing an enzyme immunoassay. (**C**) Normalized MFI of staining with specific tetramers at concentrations from 0.3 to 300 nM for RF10- and RW8-specific CTL clones was used to calculate TCR avidity. Individual clones (gray lines) and the mean value of each group (black line) are shown. (**D**) TCR avidity of RF10- and RW8-specific CTL clones. The $EC_{50}$ of tetramer staining (MFI) was calculated for each clone from (C). The statistical comparison was conducted by using the nonparametric Mann–Whitney $U$ test.

from cryopreserved PBMCs and could show that different TCR repertoires were elicited as responses against the two superimposed epitopes. RW8 and RF10 epitopes presented by HIV-1–infected cells are therefore recognized by independent specific T cells. Thus, RYPLTFGWCF (RF10) presented two epitopes to HLA-A*24:02, with each eliciting a distinct CTL response. We analyzed in the present study the ability of CTL clones from a single individual to recognize these epitopes and to suppress HIV-1. Additional analyses using CTL clones from other individuals would be useful to confirm the conclusion of the present study.

Previous studies indicated that HLA-B57–restricted KI8 and KF10 or HLA-B35–restricted VY8 and RY11 superimposed epitopes induce independent CTL responses (7, 10), but that HLA-B54–restricted FV9 and FP10 superimposed epitopes elicit mainly cross-reactive CTLs in HIV-1–infected patients (8). Although the detailed mechanisms remain unclear, these studies suggest that different lengths or conformations of a peptide may determine the nature of the CTL response. Our comprehensive analysis of RW8-specific and RF10-specific CD8$^+$ T cells, showing no overlap or cross-reactivity between these two populations, is in line with a recent report that peptide length determines the outcome of TCR/peptide–MHCI engagement (42). This study shows indeed that a given TCR is predisposed to engage peptides of a defined length so that TCR plasticity and cross-reactivity are strictly restricted to a single MHCI–peptide length.

Emerging evidence also indicates that conformational features of peptides presented in the groove of HLA molecules can partially determine the diversity of the TCR repertoire (43), although consensus is still lacking. It was reported that epitopes with featured conformations are associated with a highly diverse TCR repertoire (44–46) and that a featureless epitope results in the generation a less diverse TCR repertoire (47). However, the opposite result was also reported, with a featureless epitope (HCMV pp65, FPTKDVAL) being associated with diverse TCR usage (48). In the present study, we examined two immunodominant superimposed epitopes, derived from the same antigenic source and restricted through the same MHC allele, and we used unbiased single-cell *TRA* and *TRB* sequence analyses to compare TCR α- and β-chain repertoire diversity in the same individuals. Compared with previous studies, the present one was therefore particularly appropriate for investigating the effect of epitope conformation on TCR repertoire diversity. Our data showed that featured (RF10) and featureless (RW8) epitope conformations were indeed associated with a diverse and restricted TCR repertoire, respectively, in line with the putative availability of clonotypes in the naive T cell pool able to recognize the epitopes.

A diverse TCR repertoire is thought to facilitate the selection of CTLs with high avidity and therefore to influence their functional properties and efficacy against viruses (49–53). We indeed found that the binding affinity of specific tetramers for RF10-specific CTL clones was significantly higher than that for RW8-specific ones, suggesting that the former CTLs had higher TCR avidity than did the latter ones. Moreover, RF10-specific clones presented a stronger ability to suppress HIV-1 in vitro than did RW8-specific clones, and the frequency of RF10-specific CTLs was higher than that of RW8-specific CTLs in HIV-1–infected individuals. Taken together, our data support the idea that the selection of high-avidity TCRs is associated with TCR repertoire diversity and suggest that RF10-specific CTLs exert a superior control of HIV-1 replication in vivo compared with RW8-specific CTLs.

In conclusion, we investigated HLA-A*24:02–restricted CTLs specific for superimposed Nef epitopes, RF10 and RW8, by using multiple approaches. We demonstrated that RW8 and RF10 pep-

tides bound to HLA-A*24:02, resulting in different peptide conformations. This difference was responsible for the induction of totally different CTL responses, that is, no cross-reactivity, distinct TCR repertoires, and different functional avidity. Our study provides a clear demonstration that superimposed epitopes restricted by the same HLA molecule could elicit entirely different CD8$^+$ T cell responses. We show that this difference was linked to featured versus featureless epitope conformations, yielding distinct TCR repertoires for the two CTL populations. The featured RF10 epitope was associated with the induction of T cells carrying TCRs with high diversity and avidity. This finding is directly relevant to our understanding of CD8$^+$ T cell–mediated control of HIV-1, as well as to the choice of immunogens for vaccine design. Our findings indicate that targeting a single viral sequence, for example, RF10, can lead to the induction of two immune responses against HIV and thus enhance the suppression of its replication.

## Acknowledgments

## Disclosures

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Supplemental figure 1

## TCR repertoire usage for RW8-specific CTLs

| Sample | Va | Ja | CDR3 | Frequency | Sample | Vb | Db | Jb | CDR3 | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|
| KI-026 | TRAV12-2*03 | TRAJ40*01 | CAVPRTGTYKYIF | 16/29 | KI-026 | TRBV7-9*03 | TRBD2*01 | TRBJ2-2*01 | CASSLTSGANTGELFF | 14/30 |
| | TRAV12-2*03 | TRAJ23*01 | CAVSFYNQGGKLIF | 12/29 | | TRBV7-9*03 | TRBD1*01 | TRBJ1-2*01 | CASSPRDKPNYGYTF | 12/30 |
| | TRAV5*01 | TRAJ4*01 | CAETLPGGGYNKLIF | 1/29 | | TRBV11-3*01 | TRBD1*01 | TRBJ2-2*01 | CASSLAVRGLSGDPFF | 4/30 |
| KI-158 | TRDV1*01 | TRAJ40*01 | CALGELGAPGTYKYIF | 33/37 | KI-158 | TRBV20-1*01 | TRBD2*01 | TRBJ2-7*01 | CSARDPVSTYEQYF | 43/49 |
| | TRAV12-2*02 | TRAJ50*01 | CAAFKTSYDKVIF | 4/37 | | TRBV20-1*02 | TRBD2*01 | TRBJ2-7*01 | CSARDPIRLISYEQYF | 4/49 |
| KI-654 | TRAV8-4*03 | TRAJ30*01 | CAVSDEVIF | 13/43 | | TRBV3-1*01 | TRBD1*01 | TRBJ2-7*01 | CASSQPTGREQYF | 2/49 |
| | TRAV9-2*01 | TRAJ16*01 | CALFLDGQKLLF | 7/43 | KI-654 | TRBV7-9*07 | TRBD1*01 | TRBJ2-5*01 | CASSIRDRVPETQYF | 20/65 |
| | TRAV12-2*02 | TRAJ40*01 | CAVPVPGTYKYIF | 23/43 | | TRBV7-9*07 | TRBD2*01 | TRBJ2-2*01 | CASSDTSGANTGELFF | 45/65 |
| KI-102 | TRAV8-1*01 | TRAJ10*01 | CAVIFTGGGNKLTF | 65/65 | KI-102 | TRBV7-9*03 | TRBD1*01 | TRBJ2-5*01 | CASSQRDSQETQYF | 86/86 |

## TCR repertoire usage for RF10-specific CTLs

| Sample | Va | Ja | CDR3 | Frequency | Sample | Vb | Db | Jb | CDR3 | Frequency |
|---|---|---|---|---|---|---|---|---|---|---|
| KI-026 | TRAV9-2*01 | TRAJ21*01 | CALGVDFNKFYF | 10/34 | KI-026 | TRBV19*01 | TRBD1*01 | TRBJ2-2*01 | CASKGTVTGELFF | 12/44 |
| | TRDV1*01 | TRAJ9*01 | CALGELTNTGGFKTIF | 6/34 | | TRBV11-2*03 | TRBD2*01 | TRBJ2-7*01 | CASSYDRGYEQYF | 9/44 |
| | TRDV1*01 | TRAJ9*01 | CALGELSRTGGFKTIF | 6/34 | | TRBV28*01 | TRBD2*02 | TRBJ2-7*01 | CASLPSVGKGAYEQYF | 6/44 |
| | TRDV1*01 | TRAJ24*02 | CALWIMTTDSWGKLQF | 4/34 | | TRBV4-1*01 | TRBD1*01 | TRBJ2-1*01 | CASSQSPGQGVGEQFF | 4/44 |
| | TRAV40*01 | TRAJ32*02 | CFLGSYGGATTKLIF | 3/34 | | TRBV28*01 | TRBD2*01 | TRBJ2-2*01 | CASSLRPGRANTGELFF | 3/44 |
| | TRDV1*01 | TRAJ13*02 | CALGELSSGGYQKVTF | 2/34 | | TRBV3-1*01 | TRBD1*01 | TRBJ1-1*01 | CASSSLGQGAPEAFF | 3/44 |
| | TRDV1*01 | TRAJ36*01 | CALGVLDQTGANNLFF | 1/34 | | TRBV6-1*01 | TRBD1*01 | TRBJ2-3*01 | CASSDFSKGTDTQYF | 3/44 |
| | TRAV21*02 | TRAJ7*01 | CAVWYYGNNRLAF | 1/34 | | TRBV6-1*01 | TRBD1*01 | TRBJ1-2*01 | CASSDVGQGSSNYGYTF | 2/44 |
| | TRAV17*01 | TRAJ15*01 | CATDAKAGTALIF | 1/34 | | TRBV28*01 | TRBD1*01 | TRBJ1-2*01 | CASSSPGQGYGYTF | 1/44 |
| KI-158 | TRDV1*01 | TRAJ9*01 | CALGELSGTGGFKTIF | 22/37 | | TRBV28*01 | TRBD2*02 | TRBJ2-2*01 | CASSLMGLAGVPGELF | 1/44 |
| | TRDV1*01 | TRAJ53*01 | CALGELLRGGSNYKLTF | 11/37 | KI-158 | TRBV6-1*01 | TRBD1*01 | TRBJ2-1*01 | CASSEGFGQGGIEQFF | 31/48 |
| | TRAV8-6*02 | TRAJ48*01 | CAVLSLISNFGNEKLTF | 2/37 | | TRBV6-2*01 | TRBD2*01 | TRBJ2-7*01 | CASSYSHRLGLHEQYF | 15/48 |
| | TRAV8-6*02 | TRAJ39*01 | CAVSCLRNNAGNMLTF | 2/37 | | TRBV19*01 | TRBD2*01 | TRBJ2-7*01 | CASSISAGEGVPEQYF | 2/48 |
| KI-118 | TRDV1*01 | TRAJ9*01 | CALGELSSTGGFKTIF | 15/21 | KI-118 | TRBV28*01 | TRBD1*01 | TRBJ2-1*01 | CASTSFGQGTNEQFF | 16/25 |
| | TRAV13-1*02 | TRAJ20*01 | CAALNDYKLSF | 3/21 | | TRBV19*01 | TRBD2*01 | TRBJ2-1*01 | CASSIDPPGLADNEQFF | 3/25 |
| | TRAV21*02 | TRAJ37*02 | CAVFSSNTGKLIF | 1/21 | | TRBV7-9*03 | TRBD1*01 | TRBJ2-2*01 | CASSPGSGELGAGELFF | 2/25 |
| | TRAV26-1*01 | TRAJ42*01 | CIVYGGSQGNLIF | 1/21 | | TRBV23-1*01 | TRBD1*01 | TRBJ1-5*01 | CASSQSRQGAQPQHF | 1/25 |
| | TRAV6*02 | TRAJ13*02 | CALLGISGGYQKVTF | 1/21 | | TRBV11-2*01 | TRBD1*01 | TRBJ2-7*01 | CASSLGRDRPTPYEQYF | 1/25 |
| KI-102 | TRDV1*01 | TRAJ54*01 | CALGVIGIQGAQKLVF | 9/38 | | TRBV27*01 | TRBD1*01 | TRBJ2-1*01 | CASTTSNEQFF | 2/25 |
| | TRDV1*01 | TRAJ36*01 | CALGVLDQTGANNLFF | 8/38 | KI-102 | TRBV28*01 | TRBD1*01 | TRBJ1-2*01 | CASSSPGQGYGYTF | 10/41 |
| | TRAV26-1*01 | TRAJ28*01 | CVVNSGAGSYQLTF | 5/38 | | TRBV28*01 | TRBD1*01 | TRBJ1-5*01 | CASSPSTGKGNQPQHF | 7/41 |
| | TRDV1*01 | TRAJ9*01 | CALGELSHTGGFKTIF | 4/38 | | TRBV19*01 | TRBD1*01 | TRBJ1-2*01 | CASSTALRTGNYGYTF | 6/41 |
| | TRDV1*01 | TRAJ9*01 | CALGELTNTGGFKTIF | 4/38 | | TRBV11-2*01 | TRBD1*01 | TRBJ2-7*01 | CASSYDRSYEQYF | 4/41 |
| | TRAV25*01 | TRAJ23*01 | CPFYNQGGKLIF | 4/38 | | TRBV6-6*01 | TRBD1*01 | TRBJ1-2*01 | CASSYSIGTGVNNYGYTF | 3/41 |
| | TRAV8-3*02 | TRAJ37*01 | CAVDEGKLIF | 1/38 | | TRBV4-1*01 | TRBD2*01 | TRBJ2-7*01 | CASSPVAGVYEQYF | 3/41 |
| | TRAV14/DV4*01 | TRAJ33*01 | CAMQDSNYQLIW | 1/38 | | TRBV28*01 | TRBD2*01 | TRBJ2-1*01 | CASSLESLGLASVDEQFF | 2/41 |
| | TRAV13-2*01 | TRAJ10*01 | CAEVSTGGGNKLTF | 1/38 | | TRBV25-1*01 | TRBD1*01 | TRBJ2-5*01 | CASSAPRETQYF | 2/41 |
| | TRAV17*01 | TRAJ15*01 | CATDWQAGTALIF | 1/38 | | TRBV6-1*01 | TRBD1*01 | TRBJ2-7*01 | CASSETGGTYEQYF | 2/41 |
| | | | | | | TRBV4-1*01 | TRBD1*01 | TRBJ1-1*01 | CASSQRDRGTDTEAFF | 1/41 |
| | | | | | | TRBV7-9*03 | TRBD2*02 | TRBJ2-1*01 | CASSLVSGRGNEQFF | 1/41 |

**Figure S1. Clonotypic analysis of RW8- and RF10-specific CD8+ T cells**

Single RW8- or RF10 CTLs from 5 HIV-1-infected HLA-A*24:02+ individuals were FACS sorted, and TCR α and β chain sequencing was performing.

TCR α and β chain usage, CDR3 amino acid sequences, and individual clonotype frequencies are shown.

## Supplemental Table I. Information of participants in this study

| ID | HLA | | | | | | VL (copies/ml) | CD4 (cells/μl) | Date (M/D/Y) |
|---|---|---|---|---|---|---|---|---|---|
| | A allele | | B allele | | C allele | | | | |
| U[a]-4 | 1101 | **2402** | 5201 | 5201 | 1202 | 1402 | | | |
| U-13 | 0201 | **2402** | 5101 | 6701 | 0702 | 1402 | | | |
| U-14 | 0201 | **2402** | 3501 | 5401 | 0102 | 0303 | | | |
| U-15 | **2402** | **2402** | 1501 | 5401 | 0102 | 0801 | | | |
| U-23 | **2402** | 3303 | 4403 | 5101 | nt[b] | | | | |
| U-24 | 0201 | **2402** | 5201 | 5901 | nt | | | | |
| U-35 | 0201 | **2402** | 3501 | 5201 | 0303 | 1202 | | | |
| U-36 | 0201 | **2402** | 3901 | 5201 | nt | | | | |
| KI[c]-021 | **2402** | 2602 | 5101 | 6701 | 0702 | 1402 | <50 | 578 | 11/02/04 |
| KI-026 | 0206 | **2402** | 4006 | 5101 | 0801 | 1402 | 40000 | 526 | 07/07/05 |
| KI-042 | **24** | 31 | 35 | 60 | 03 | 07 | 4800 | 441 | 01/20/00 |
| KI-060 | **2402** | 2601 | 4002 | 5201 | 1202 | 0304 | 13000 | 533 | 08/30/03 |
| KI-067 | **2402** | **2402** | 4801 | 5201 | 1202 | 1202 | 89000 | 234 | 01/25/00 |
| KI-068 | **2402** | 3303 | 0702 | 4403 | nt | | 15000 | 346 | 12/13/01 |
| KI-069 | **2402** | **2402** | 4006 | 5201 | 0304 | 1202 | 19000 | 263 | 11/16/99 |
| KI-071 | **2402** | 3101 | 4006 | 5201 | nt | | 48000 | 292 | 01/25/00 |
| KI-092 | 0206 | **2402** | 4801 | 5101 | 0801 | 1402 | 220 | 971 | 08/14/03 |
| KI-102 | 0206 | **2402** | 0702 | 3501 | 0303 | 0702 | 13000 | 355 | 03/28/05 |
| KI-108 | **2402** | **2402** | 5201 | 5201 | 1202 | 1202 | 2100 | 469 | 12/18/03 |
| KI-113 | **2402** | **2402** | 0701 | 5201 | 0702 | 1202 | 31000 | 192 | 07/24/01 |
| KI-116 | **2402** | **2402** | 1501 | 5201 | 0303 | 1202 | 250000 | 156 | 12/13/01 |
| KI-117 | 0201 | **2402** | 1301 | 4801 | nt | | 180000 | 151 | 07/31/01 |
| KI-118 | **2402** | 3101 | 3902 | 4403 | 0702 | 1403 | 240 | 279 | 07/31/01 |
| KI-123 | **2402** | 2601 | 1501 | 1518 | 0704 | 0801 | 66000 | 406 | 08/20/02 |
| KI-127 | 0206 | **2402** | 4002 | 5101 | nt | | 9300 | 519 | 10/20/05 |
| KI-130 | **2402** | **2402** | 0702 | 5201 | 0702 | 1202 | 14000 | 351 | 10/02/01 |
| KI-133 | **2402** | **2402** | 4006 | 4403 | 0801 | 1403 | 5500 | 664 | 12/18/03 |
| KI-148 | 1102 | **2402** | 2704 | 2711 | 1202 | 1502 | 13000 | 647 | 02/08/02 |
| KI-158 | **2402** | 3303 | 5201 | 4403 | 1202 | 1403 | 200 | 611 | 10/10/03 |
| KI-188 | **2402** | **2402** | 1501 | 4403 | 0303 | 1403 | 630 | 360 | 08/05/04 |
| KI-194 | 0201 | **2402** | 5201 | 5201 | 1202 | 1202 | 210 | 672 | 08/20/05 |

U[a]: HIV-1 seronegative individual    nt[b]: not test    KI[c]: HIV-1 seropositive patients

**Supplemental Table II. Information on patients used for TCR repertoire analysis of RW8- and RF10-specific CTLs**

| Patient ID | HLA-A | Median VL (copies/ml) | Median CD4 (cells/μl) | Sample date (Mo/Day/Yr) | Tetramer frequency in CD8 T cells(%) | |
|---|---|---|---|---|---|---|
| | | | | | RW8 | RF10 |
| KI-026[a] | A0206/A2402 | $9.2 \times 10^4$ | 502 | 01/20/2005 | 1.07 | 1.2 |
| KI-102[b] | A0206/A2402 | 580 | 482 | 08/23/2002 | 0.91 | 0.72 |
| KI-118[a] | A3101/A2402 | 240 | 279 | 07/31/2001 | N.D[c] | 0.15 |
| KI-158[b] | A3303/A2402 | 160 | 321 | 05/24/2004 | 0.62 | 1.42 |
| KI-654[a] | A6802/A2402 | $1.8 \times 10^4$ | 357 | 07/08/2009 | 1.23 | N.D |

[a]: treatment naïve    [b]: STI (structured treatment interruption) patients    [c]: N.D: not detected

## Supplemental Table III. Hydrogen bonds and van der Waal's interactions for the HLA-A*2402-peptide complexes

| | Peptide | | Hydrogen bond partner | | |
|---|---|---|---|---|---|
| | Residue | atom | Residue | atom | Contact residue |
| A24-8mer | $R^1$ | N | $Y^7$ | OH | $M^5, Y^7, Y^{59}, E^{63}, Y^{159}, R^{170}, Y^{171}$ (28)[a] |
| | $R^1$ | N | $Y^{171}$ | OH | |
| | $R^1$ | O | $Y^{159}$ | OH | |
| | $Y^2$ | N | $E^{63}$ | OE1 | $Y^7, S^9, F^{22}, A^{24}, V^{27}, M^{45}, E^{63}, K^{66}, H^{70}, M^{97},$ $Y^{159}$ (38) |
| | $Y^2$ | O | $Y^{159}$ | OH | |
| | $Y^2$ | OH | $H^{70}$ | ND1 | |
| | $P^3$ | | | | $Y^7, F^{99}$ (12) |
| | $L^4$ | | | | $K^{66}, H^{114}, Q^{155}, Q^{156}$ (5) |
| | $T^5$ | | | | $H^{70}, T^{73}, M^{97}, Y^{116}$ (11) |
| | $F^6$ | | | | $N^{77}, W^{147}, V^{152}, Q^{156}$ (9) |
| | $G^7$ | | | | $N^{77}, W^{147}$ (7) |
| | $W^8$ | N | $N^{77}$ | OD1 | $N^{77}, I^{80}, Y^{84}, L^{95}, Y^{116}, Y^{123}, T^{143}, K^{146}, W^{147}$ (64) |
| | $W^8$ | O | $Y^{84}$ | OH | |
| | $W^8$ | O | $T^{143}$ | OG1 | |
| | $W^8$ | OXT | $K^{146}$ | NZ | |
| A24-10mer | $R^1$ | N | $Y^7$ | OH | $M^5, Y^7, E^{63}, K^{66}, Y^{159}, G^{167}, R^{170}, Y^{171}$ (28) |
| | $R^1$ | N | $Y^{171}$ | OH | |
| | $R^1$ | NH2 | $R^{170}$ | NH1 | |
| | $R^1$ | O | $Y^{159}$ | OH | |
| | $Y^2$ | N | $E^{63}$ | OE1 | $Y^7, S^9, F^{22}, A^{24}, M^{45}, E^{63}, K^{66}, V^{67}, H^{70}, Y^{159}$ |
| | $Y^2$ | O | $Y^{159}$ | OH | (38) |
| | $Y^2$ | O | $K^{66}$ | NZ | |
| | $Y^2$ | OH | $H^{70}$ | ND1 | |
| | $P^3$ | | | | $F^{99}, Y^{159}$ (12) |
| | $L^4$ | | | | $K^{66}, Q^{156}, Y^{159}$ (5) |
| | $T^5$ | | | | $H^{70}, T^{73}$ (12) |
| | $F^6$ | | | | |
| | $G^7$ | | | | $V^{152}, W^{147}$ (3) |
| | $W^8$ | NE1 | $Q^{156}$ | NE2 | $N^{77}, M^{97}, F^{99}, H^{114}, Y^{116}, W^{147}, Q^{156}$ (32) |
| | $W^8$ | O | $N^{77}$ | ND2 | |
| | $C^9$ | O | $W^{147}$ | NE1 | $N^{77}, W^{147}$ (7) |
| | $F^{10}$ | N | $N^{77}$ | OD1 | $N^{77}, I^{80}, Y^{84}, Y^{116}, Y^{123}, T^{143}, K^{146}$ (42) |
| | $F^{10}$ | O | $Y^{84}$ | OH | |
| | $F^{10}$ | O | $K^{146}$ | NZ | |

[a]: Residues within 4 Å of peptide with total number of contacts in parentheses

## BRIEF REPORT: BASIC AND TRANSLATIONAL SCIENCE

# Low Raltegravir Concentration in Cerebrospinal Fluid in Patients With ABCG2 Genetic Variants

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**Abstract:** Adenosine triphosphate–binding cassette transporter G2 (ABCG2) is expressed on the cerebrospinal fluid (CSF) side of choroid plexus epithelial cells, which form the blood–CSF barrier. Raltegravir was recently identified as a substrate of ABCG2. In the present study, we analyzed the relationship between single-nucleotide polymorphisms of ABCB1 and ABCG2 genes and raltegravir concentrations in 31 plasma and 14 CSF samples of HIV-infected patients treated with raltegravir-containing regimens. The mean CSF raltegravir concentration was significantly lower in CA (25.5 ng/mL) and AA ($<10$ ng/mL) genotypes at position 421 in ABCG2 gene compared with CC (103.6 ng/mL) genotype holders ($P = 0.016$).

**Key Words:** antiretroviral therapy, raltegravir, cerebrospinal fluid concentrations, blood–cerebrospinal fluid barrier, adenosine triphosphate–binding cassette transporter G2

## INTRODUCTION

Anatomical sanctuary sites in HIV-infected patients, where local drug exposure is lower than systemic compartment, are currently under intense investigation because they are suspected of hindering viral elimination by antiretroviral therapy (ART) and acting as sites for the selection of drug-resistant viruses during combination treatment. Especially the

brain, the largest sanctuary site, in which residual viruses may cause chronic encephalitis and neurocognitive disorders, is one of the hottest foci of current HIV researches. Raltegravir, one of the preferred integrase inhibitors in the current ART guidelines, is highly effective in penetrating the central nervous system,[1] although a high interpatient variability has also been reported.[2,3]

Anatomically, the blood–cerebrospinal fluid (CSF) barrier makes tight junction and consists of choroid plexus epithelial cells in the cerebral ventricle. The adenosine triphosphate–binding cassette transporter B1 (ABCB1), also known as P-glycoprotein or multidrug resistance protein 1, and the adenosine triphosphate–binding cassette transporter G2 (ABCG2), also known as breast cancer resistance protein, are expressed on the CSF side of choroid plexus epithelial cells, and both are involved in the active transport of drugs.[4,5] Moreover, ABCB1 and ABCG2 are also expressed in the intestines and contribute to the absorption of the drugs. Recently, raltegravir was found to be a substrate of both ABCB1 and ABCG2.[6] In the present study, we analyzed the relations between raltegravir plasma and CSF concentrations and single-nucleotide polymorphisms (SNPs) of ABCB1 and ABCG2 genomes.

## MATERIALS AND METHODS

HIV-1–infected patients treated with raltegravir-containing regimens (raltegravir 400 mg twice daily with 2 nucleotide/nucleoside reverse transcriptase inhibitors and/or protease inhibitors) were recruited at the AIDS Clinical Center, National Center for Global Health and Medicine, Tokyo, Japan. Blood samples were withdrawn into heparinized tubes 12 hours after raltegravir dosing (trough level), and the plasma was separated and stored at $-80°C$. Stocked residues of CSF samples taken 3–4 hours after raltegravir dosing for clinical purposes were also subjected to analysis. The Ethics Committee for Human Genome Studies at the National Center for Global Health and Medicine approved this study (NCGM-A-000122-02) and allowed us the use of only residues of samples that were originally obtained for clinical purposes. Each patient provided a written informed consent.

Plasma and CSF raltegravir concentrations were measured by the reverse-phase high-performance liquid chromatography (HPLC) method. Briefly, 200 µL of plasma or CSF and 400 µL of ethyl acetate were vortexed in a tube for 10 seconds and centrifuged. The organic phase was transferred to a new tube and evaporated to dryness. Subsequently, the

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residue was reconstituted in 250 μL of mobile phase, and 50 μL was injected into HPLC. Chromatography was performed, using Chromaster HPLC system (Hitachi, Tokyo, Japan) with RF-10A fluorescence detector (Shimadzu, Kyoto, Japan). Inertsil ODS-3 column (150 × 4.6 mm, 5-μm particle size; GL Sciences, Tokyo, Japan) was used as the analytical column. The flow rate was maintained at 1.5 mL per minute with fluorescence detection at 307 nm (excitation) and 415 nm (emission). The mobile phase consisted of acetonitrile/ethanol/phosphoric acid/water (20.8:20.8:0.1:58.3, vol/vol). Raltegravir calibration standards ranged from 10 to 2500 ng/mL. The accuracy of the analysis at 3 concentration levels ranged from $-8.4\%$ to $+4.9\%$. Intraassay and interassay precisions were $<4.8\%$ and $<7.6\%$, respectively. This assay was validated for both plasma and CSF raltegravir concentrations.

Genomic DNA was isolated from peripheral blood mononuclear cell, using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of allelic variants of ABCB1 1236 C>T (rs1128503), 2677 G>T/A (rs2032582), 3435 C>T (rs1045642), 4036 A>G (rs3842), and ABCG2 421 C>A (rs2231142) was carried out using the TaqMan Drug Metabolism Assays by the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA), according to the protocol provided by the manufacturer.

Differences between the groups were analyzed for statistical significance using the Kruskal–Wallis test. $P$ values $<0.05$ denoted the presence of statistically significant difference. Analysis was performed using the SPSS Statistics software version 21 (IBM, Armonk, NY).

## RESULTS

Plasma samples were collected from 31 patients, and stocked CSF samples from another group of 14 patients were used for the measurement of raltegravir concentrations.

All 45 patients (Japanese = 44, Myanmarian = 1) were subjected to SNP analysis of ABCB1 and ABCG2 genomes (Table 1). At position 1236 of ABCB1 gene, CC, CT, and TT genotypes were identified in 7, 21, and 17 patients, respectively. At position 2677, GG, GT, TT, GA, TA, and AA genotypes were identified in 8, 14, 11, 7, 4, and 1 patients, respectively. At position 3435, CC, CT, and TT genotypes were identified in 14, 17, and 14 patients, respectively. At position 4036, AA, AG, and GG genotypes were identified in 25, 18, and 2 patients, respectively. None of the genotypes of these SNPs in ABCB1 genome showed significant correlation with raltegravir concentration in plasma or CSF. At position 421 of ABCG2 gene, CC, CA, and AA genotypes were identified in 26, 14, and 5 patients, respectively. There was no significant correlation between the genotype at position 421 and trough concentration of raltegravir in plasma (Fig. 1A). However, in all 3 AA genotype holders, CSF raltegravir concentration was less than the lower limit of quantification (10 ng/mL) (Fig. 1B). Furthermore, in one of 4 CA genotype holders, CSF raltegravir concentration was below the detection limit, although it was higher than 25 ng/mL in any of the 7 CC genotype holders. The CA and AA genotype holders had significantly lower raltegravir concentrations in the CSF than the CC

**TABLE 1.** Genotype Frequencies of ABCB1 and ABCG2 Polymorphisms and Raltegravir Concentrations

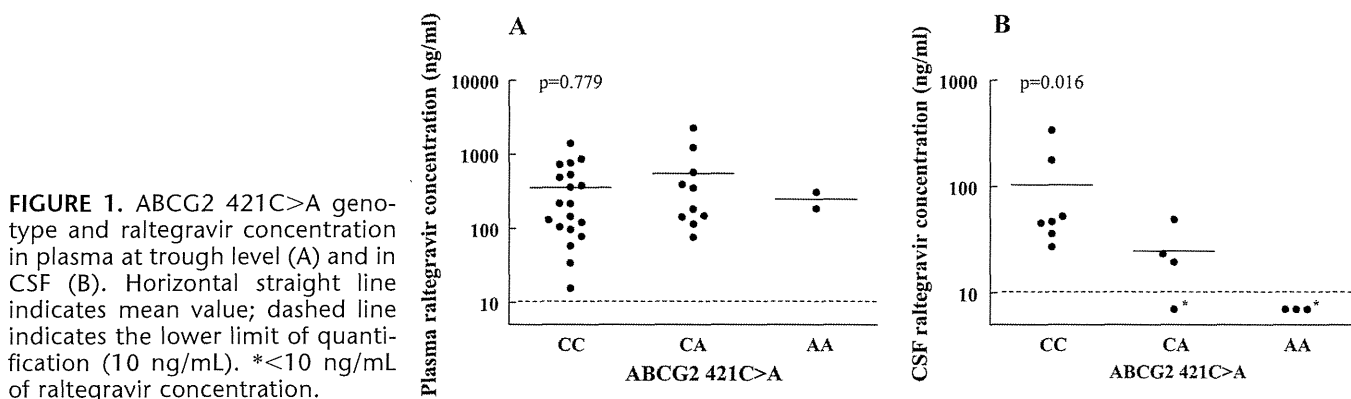| | | n | Raltegravir Concentration (ng/mL)* | P |
|---|---|---|---|---|
| Plasma (n = 31) | | | | |
| ABCB1 | | | | |
| 1236 C>T | rs1128503 | | | |
| CC | | 3 | 480.1 ± 347.7 | |
| CT | | 16 | 489.5 ± 602.4 | 0.485 |
| TT | | 12 | 289.9 ± 324.5 | |
| 2677 G>T/A | rs2032582 | | | |
| GG | | 5 | 254.6 ± 161.2 | |
| GT | | 12 | 569.9 ± 716.6 | |
| TT | | 6 | 197.1 ± 70.5 | 0.254 |
| GA | | 4 | 648.9 ± 208.4 | |
| TA | | 4 | 215.1 ± 219.4 | |
| 3435 C>T | rs1045642 | | | |
| CC | | 9 | 358.2 ± 253.9 | |
| CT | | 13 | 533.5 ± 659.0 | 0.680 |
| TT | | 9 | 287.8 ± 362.0 | |
| 4036 A>G | rs3842 | | | |
| AA | | 17 | 408.5 ± 535.7 | |
| AG | | 13 | 402.5 ± 457.7 | 0.594 |
| GG | | 1 | 572.8 | |
| ABCG2 | | | | |
| 421 C>A | rs2231142 | | | |
| CC | | 19 | 355.5 ± 366.8 | |
| CA | | 10 | 550.1 ± 699.0 | 0.779 |
| AA | | 2 | 247.8 ± 88.7 | |
| CSF (n = 14) | | | | |
| ABCB1 | | | | |
| 1236 C>T | rs1128503 | | | |
| CC | | 4 | 140.8 ± 151.5 | |
| CT | | 5 | 35.6 ± 19.5 | 0.330 |
| TT | | 5 | 23.2 ± 14.7 | |
| 2677 G>T/A | rs2032582 | | | |
| GG | | 3 | 31.7 ± 20.0 | |
| GT | | 2 | 36.1 ± 23.6 | |
| TT | | 5 | 23.2 ± 14.7 | 0.137 |
| GA | | 3 | 188.0 ± 146.5 | |
| AA | | 1 | <10 | |
| 3435 C>T | rs1045642 | | | |
| CC | | 5 | 122.5 ± 137.5 | |
| CT | | 4 | 30.4 ± 18.6 | 0.325 |
| TT | | 5 | 24.6 ± 17.5 | |
| 4036 A>G | rs3842 | | | |
| AA | | 8 | 41.5 ± 56.6 | |
| AG | | 5 | 103.0 ± 132.5 | 0.061 |
| GG | | 1 | <10 | |
| ABCG2 | | | | |
| 421 C>A | rs2231142 | | | |
| CC | | 7 | 103.6 ± 116.0 | |
| CA | | 4 | 25.5 ± 16.8 | 0.016 |
| AA | | 3 | <10 | |

*Data are mean ± SD for concentrations ≥10 ng/mL.

**FIGURE 1.** ABCG2 421C>A genotype and raltegravir concentration in plasma at trough level (A) and in CSF (B). Horizontal straight line indicates mean value; dashed line indicates the lower limit of quantification (10 ng/mL). *<10 ng/mL of raltegravir concentration.

genotype holders (*P* = 0.016), when the concentration below the lower limit of quantification was considered 10 ng/mL.

## DISCUSSION

ABCG2 is diffusely expressed, whereas ABCB1 is weakly expressed on the CSF side of choroid plexus epithelial cells,[7,8] suggesting that the contribution of ABCB1 may be minor and that ABCG2 expression level in the choroid plexus is more likely to influence raltegravir concentration in the CSF than ABCB1. Previous studies indicated that genetic polymorphism of ABCG2 altered the protein expression level in plasmid transfection experiments.[9,10] Especially, C to A nucleotide substitution at position 421 significantly reduced the expression. The low expression induced by this nucleotide substitution may impair raltegravir transport from capillary blood to CSF, resulting in low raltegravir concentrations in CSF in holders of the CA/AA genotype at position 421. However, this SNP did not alter plasma raltegravir concentration significantly. Transporters other than ABCG2 may also exist in the intestines and further enhance raltegravir absorption. The presence of any antiretroviral at a concentration lower than that required for viral suppression could select drug-resistant HIV variants. In fact, we reported previously one patient with CSF raltegravir-resistant HIV variant, although the variant was not detected in the plasma.[11] The present study indicate that the genotype of this patient was AA at position 421 and that raltegravir concentration was below the lower limit of quantification in the CSF of this patient. Special attention should be paid to the raltegravir-containing ART of individuals with the CA/AA genotype at position 421 with active viral replication in the CNS, such as patients with HIV encephalitis.

Our study has certain limitations. Raltegravir concentrations were measured in plasma at trough level in 31 patients, and it was measured in stocked CSF samples of another group of 14 patients. First, we could not investigate the correlation between plasma and CSF concentrations because no paired plasma and CSF samples from the same subjects were available. Second, the time of CSF sampling in relation to raltegravir dosing varied among 3–4 hours. However, the population pharmacokinetic modeling of raltegravir concentration in the CSF showed a stable time course regardless of the dosing time.[2,12] Therefore, it is unlikely that the

sampling time had a large impact on CSF concentration of the CA/AA genotype at position 421 in ABCG2 gene. Further analysis of the correlation between ABCG2 genotype and raltegravir CSF concentration is warranted.

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BASIC AND TRANSLATIONAL SCIENCE

# Low Prevalence of Transmitted Drug Resistance of HIV-1 During 2008–2012 Antiretroviral Therapy Scaling up in Southern Vietnam

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**Background:** The recent expansion of antiretroviral therapy (ART) program in resource-limited setting has raised concern about possible transmission of drug resistance (TDR). We assessed the prevalence of TDR over a 5-year period among treatment-naive individuals in Southern Vietnam during rapid ART scale-up.

**Methods:** Drug resistance mutations among antiretroviral-naive HIV-1–infected patients in Ho Chi Minh City were evaluated prospectively from 2008 to 2012 by HIV-1 pol gene sequencing. TDR was defined according to the World Health Organization list for surveillance of transmitted HIV-1 drug resistance in 2009.

**Results:** Pol sequence was obtained in 1389 individuals (median age: 30 years, males: 52.3%). Risks of HIV-1 infection included heterosexual contact in 60.7%, injection drug use in 22.4% and both 5.2%. The majority was infected with CRF01_AE (97%), whereas 19 were infected with subtype B. Over the 5-year study period, TDR was detected in 58 individuals (4.18%): 28 (2.02%) against nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 (0.29%) against both NRTIs and NNRTIs. The most common TDR was K103N (0.5%) for NNRTI. The annual prevalence of TDR remained low to moderate (2008: 2.4%; 2009: 5.2%; 2010: 5.48%; 2011: 2.72%; 2012: 5.36%), and there was no clear trend over time.

**Conclusions:** There was no increase in TDR prevalence in Southern Vietnam during and after the 2008–2012 rapid scale up of ART.

**Key Words:** HIV, transmitted drug resistance, Vietnam

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## INTRODUCTION

The recent roll-out campaigns in resource-limited settings to scale up antiretroviral therapy (ART) seem to have improved the morbidity and mortality of HIV-infected individuals. In Vietnam, where the HIV epidemic affected 249,660 individuals including 52,325 AIDS-related deaths up to the end of 2011, a national effort to facilitate ART supply has been implemented, and the ART coverage rate has rapidly increased from 18.1% in 2006 to 53% in 2011, saving 18,110 lives from AIDS-related deaths between 2000 and 2009.[1]

The expansion of ART program, however, has been accompanied by concerns on HIV drug resistance and risk of subsequent transmission of drug resistance (TDR) in new cases of HIV infection.[2] The WHO recommends surveillance of TDR where ART is being scaled up[3,4] and the Vietnam Authority of HIV/AIDS Control issued in 2008 a 5-year plan to assess and prevent HIV drug resistance. Because the large part of HIV epidemic in Vietnam has been driven by intravenous drug users (IDUs),[1,5] it is theoretically possible that the transmission of drug-resistant HIV spreads fast by sharing contaminated needles. The recent increase in HIV transmission by sexual intercourse in Vietnam also makes the TDR problem more difficult to control.[5] In addition, the pattern of antiretroviral drug use has been changing according to the global policy on ART recommendations or increased availability of second-line ART.[6–9] It is therefore important to monitor the prevalence of TDR and its pattern in Vietnam on a regular basis. Previous surveys and studies demonstrated low-to-moderate prevalence of TDR in Vietnam.[10–17] However, those studies were conducted using a cross-sectional setting or included monitoring for only a short period of time. To the best of our knowledge, there are no data on long-term monitoring of the prevalence of TDR in Vietnam.

This study was designed to assess the prevalence of TDR over a 5-year period in HIV-infected treatment-naive

individuals from Southern Vietnam during the 2008–2012 rapid ART scale-up.

## METHODS

### Study Population

Antiretroviral-naive individuals who visited the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam, were enrolled in the study from 2008 until 2012. The enrollment of consecutive antiretroviral-naive patients started in October and ended when 300 enrollments had been achieved. In 2009 and 2012, the enrollment was stopped at 250 and 270, respectively, for the operational reasons. After securing written informed consent, plasma samples were collected and stored at −80°C. At the end of the year's sampling, the frozen plasma samples were shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo, Japan, for genotypic resistance testing. Patients with history of exposure to any antiretroviral drug, including mono or dual therapy were excluded. The study protocol was approved by the institutional ethical review boards of both Hospital for Tropical Diseases in Vietnam and NCGM in Japan (NCGM#360).

### Genotypic HIV-1 Resistance Testing and Subtype Determinations

Drug resistance genotyping was performed using in-house protocols at NCGM. Briefly, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription–polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify the pol-reverse transcriptase (RT) and protease (PR) region. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 11.0 (Software Development, Tokyo). The subtypes of HIV-1 were determined by using RT gene with "Genotyping/NCBI" tool using BLAST algorithm (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Drug resistance mutations were identified from the list for surveillance of transmitted drug resistance mutations.[18] All sequences obtained from the study have been deposited in the DNA Data Bank of Japan database (accession no: AB894875 to AB896651).

### Statistical Analysis

Differences between 2 groups were tested for statistical significance by using $\chi^2$ test for categorical data and the Mann–Whitney test for continuous variables. Logistic regression model was used to identify the factors associated with infection by TDR. Differences were considered significant if the $P$ value was less than 0.05. Statistical analyses were performed using IBM SPSS Statistics software version 21J (IBM Japan, Inc, Tokyo, Japan).

## RESULTS

### Characteristics of Study Population

The study enrolled 1426 individuals but 20 were later found to be ineligible after providing written informed consents (previous ART exposure, n = 17, insufficient blood withdrawn, n = 2 had, negative for HIV infection, n = 1). The remaining 1406 participants were assigned to the drug resistance test. The characteristics of these subjects are summarized in Table 1. Approximately 63% of the study participants were men, and the latter were older than females (31 years vs 29 years; $P < 0.001$). The most frequently reported HIV transmission route was heterosexual contact (65.9%), followed by injection drug use (IDU) (29.6%). Very few (0.1%) declared homosexual contact as a risk for HIV infection. The majority of patients with IDU were men, and the percentage of IDUs was greater in men than that in women (men: 42%; women: 3.4%; $P < 0.001$). The proportion of IDUs had decreased over time (35% in 2008, 17.6% in 2012) and the prevalence of hepatitis C infection, which reflects possible multiple needle sharing, had decreased simultaneously. These changes in the study population reflect preponderance of HIV epidemic in male IDUs in the early phase and recent expansion to the general population in Vietnam.[1]

### Prevalence of Transmitted Drug Resistance Mutations

Among the 1406 individuals who underwent HIV drug resistance genotyping, we obtained the complete sequences of both PR and RT in 1389 individuals. The majority were infected with CRF01_AE (98%), whereas 17 were infected with subtype B. Over the 5-year study period, drug resistance mutations were detected in 58 individuals (4.18%): 28 (2.02%) against nucleos(t)ide reverse transcriptase inhibitors (NRTIs), 19 (1.37%) against nonnucleoside reverse transcriptase inhibitors (NNRTIs), and 15 (1.08%) against protease inhibitors (PIs), including 4 against both NRTIs and NNRTIs. Table 2 summarizes the prevalence of the specific drug resistance mutations. The annual prevalence of TDR was persistently low during the study period, ranging from 2.40% to 5.48%, and no clear trend was noted over time. In thymidine analog mutations (TAMs), mutations at codon 215 were the most frequent (0.36%) followed by K219Q (0.22%). In other NRTI-related mutations, V75M and mutations at codon 74 and 184 were relatively frequent, of which V75M was reported previously as frequent d4T-resistance-related mutation among CRF01_AE.[19] We did not identify mutations related to Q151M complex or insertions at codon 69. The most common NNRTI mutation was K103N (0.5%), followed by Y181C (0.43%), G190A and E (0.36%), and mutations at codon 188 (0.22%). The most common PI-associated mutations was M46L (0.43%) and M46I (0.29%) but both were considered polymorphisms.[20,21] All other PI-associated mutations were rare; only 1 among 1389 sample (0.07%) harbored each mutation. Of those, D30N, L76V, and L90M were major mutations, whereas F53Y was not major mutation[20,21] and not clinically significant when it occurred alone without any other PI mutations.

**TABLE 1.** Patients' Characteristics

| | Total | Year of Sampling | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | | 2008 | 2009 | 2010 | 2011 | 2012 |
| Patients, n | 1406 | 298 | 250 | 294 | 297 | 267 |
| Male gender, n (%) | 881 (62.7) | 213 (71.5) | 150 (60) | 184 (62.6) | 154 (51.9) | 180 (67.4) |
| Age, median (range) | 30 (16–66) | 29 (16–58) | 29 (20–60) | 30 (17–55) | 31 (19–66) | 33 (18–65) |
| Living in HCMC, n (%) | 735 (52.3) | 163 (54.7) | 132 (52.8) | 148 (50.3) | 150 (50.5) | 142 (53.2) |
| Time since HIV diagnosis, n (%) | | | | | | |
| <6 mo | 975 (69.3 | 224 (75.2) | 181 (72.4) | 233 (79.3) | 138 (46.5) | 199 (74.5) |
| ≥6 mo | 431 (30.7) | 74 (24.8) | 69 (27.6) | 61 (20.7) | 159 (53.5) | 68 (25.5) |
| Risk of HIV transmission, n (%) | | | | | | |
| Heterosexual contact, alone | 854 (60.7) | 148 (49.7) | 143 (57.2) | 149 (50.7) | 210 (70.7) | 204 (76.4) |
| IDU, alone | 315 (22.4) | 73 (24.5) | 68 (27.2) | 90 (30.6) | 44 (14.8) | 40 (15.0) |
| Heterosexual and IDU | 73 (5.2) | 29 (9.7) | 5 (2) | 3 (1) | 29 (9.8) | 7 (2.6) |
| Homosexual contact | 2 (0.1) | 2 (0.7) | 0 | 0 | 0 | 0 |
| Other/unknown | 162 (11.5) | 46 (15.4) | 34 (13.6) | 52 (17.7) | 14 (4.7) | 16 (6.0) |
| HIV-1 subtype, n (%) | | | | | | |
| CRF01_AE | 1378 (98.0) | 295 (99.0) | 246 (98.4) | 289 (98.3) | 289 (97.3) | 255 (95.5) |
| Subtype B | 19 (1.5) | 1 (0.7) | 4 (1.6) | 2 (1) | 6 (2) | 6 (2.2) |
| Other/unclassified | 9 (0.8) | 0 | 0 | 2 (0.7) | 1 (0.6) | 6 (2.2) |
| HBs antigen positive, n (%) | 217 (15.4) | 42 (14.1) | 43 (17.2) | 49 (16.7) | 47 (15.8) | 36 (13.5) |
| Anti-HCV antibody positive, n (%) | 557 (39.6) | 148 (49.7) | 106 (42.4) | 117 (39.8) | 105 (35.4) | 81 (30.3) |
| CD4 cell count, cells/μL, median (range) | 110 (1–1322) | 70 (1–1042) | 115 (1–753) | 95 (1–1048) | 253 (2–1322) | 47 (1–1211) |
| Plasma HIV-1 RNA levels, log copies/mL, median (range) | 5.01 (1.59–6.90) | 4.81 (1.69–5.70) | 4.38 (1.69–5.70) | 5.23 (1.59–6.61) | 5.02 (2.31–6.90) | 5.38 (1.60–6.83) |

HCMC, Ho Chi Minh City; CRF01_AE, circulating recombinant form01_AE; HBs antigen, hepatitis B virus surface antigen; anti-HCV antibody, anti-hepatitis C virus antibody.

The presence of TDR did not correlate with any specific demographic factor, risk group, or year of study enrollment, although the odds ratio of acquiring TDR was relatively low in heterosexual individuals (Table 3). Annual trends of TDR prevalence in particular HIV risk categories are shown in Table 4. TDR prevalence in heterosexual contact alone, IDU alone, and IDU plus heterosexual contact were 3.33%, 5.41%, and 2.78% respectively, which were not statistically different. Although no significant annual trend was noted over the study period among them, the TDR prevalence in the HIV risk group of IDU alone were higher than the WHO first threshold 5% in the year 2009, 2010, and 2012 (4.10% in 2008, 5.88% in 2009, 6.67% in 2010, 2.27% in 2011%, and 7.69% in 2012). Phylogenetic tree analysis showed no clustering of sequences from the study participants with TDR. Details of the 4 individuals with TDR in more than 1 group of antiretrovirals are listed in Table 5. One individual had very extensive resistance: M41L, M184V, T215Y in NRTI-associated mutations, and Y181C and G190A in NNRTI-associated mutations. Overall, persistently low prevalence of TDR during the last 5 years of ART expansion was noted. However, individuals with multiple-drug resistances were identified during ART expansion. This finding highlights the importance of TDR and undermines the efficacy of currently scaled up ART regimens.

## DISCUSSION

In this study, we traced the prevalence of TDR over a relatively long period of time (from 2008 to 2012) in treatment-naive individuals in Southern Vietnam during rapid ART scaling up program. Our result of 4.18% of overall TDR prevalence was similar to those described previously in Vietnam.[10–17] However, the study covered longer period of time and demonstrated the stability of TDR prevalence over this period. In comparison, all the other previous surveillance studies conducted in Vietnam were shorter in duration. Primary HIV drug resistance is one of the main concerns in any ART program because it can compromise the clinical outcome of ART, especially in countries with limited ART options. Our data of persistently low prevalence of TDR in Southern Vietnam possibly reflect the success of the recent ART scale-up program in this country.

The TDR rate in our study, however, ranged from 2.4% to 5.5%, reaching the threshold of low prevalence according to the WHO definition (<5%) in 2009, 2010, and 2012.[4] Considering lower viral replication fitness of strains harboring drug resistance mutations than that of wild-type strain, the rate of pretreatment resistance in chronic HIV infection could underestimate the real drug resistance transmission with time since HIV infection. In particular, the low-level prevalence of M184V[22] despite widespread use of lamivudine, which is sometimes used for treatment of hepatitis B virus infection, could be related to the lower viral fitness. Of note, the percentage of individuals diagnosed as HIV positive more than 6 months before study enrollment was higher in 2011 (53.5%) than that in other study periods, and the TDR prevalence in 2011 was lower (2.72%) than that in 2009, 2010, and 2012. Most cases had chronic HIV infection at the time of HIV

**TABLE 2.** Prevalence of Transmitted Drug Resistance Mutations

| | Total | 2008 | 2009 | 2010 | 2011 | 2012 |
|---|---|---|---|---|---|---|
| Study population (n) | 1389 | 292 | 250 | 292 | 294 | 261 |
| Any TDR [n (%)] | 58 (4.18) | 7 (2.40) | 13 (5.20) | 16 (5.48) | 8 (2.72) | 14 (5.36) |
| RT in total [n (%)] | 43 (3.10) | 7 (2.40) | 9 (3.60) | 14 (4.79) | 4 (1.36) | 10 (3.83) |
| NRTI [n (%)] | | | | | | |
| Any | 28 (2.02) | 3 (1.03) | 6 (2.40) | 11 (3.76) | 3 (1.02) | 5 (1.92) |
| Thymidine analog mutations | | | | | | |
| M41L | 2 (0.14) | | | 1 | 1 | |
| D67N | 1 (0.07) | | 1 | | | |
| D67E | 1 (0.07) | | | 1 | | |
| K70E | 1 (0.07) | | | 1 | | |
| T215Y | 1 (0.07) | | | | 1 | |
| T215I | 1 (0.07) | | 1 | | | |
| T215S | 1 (0.07) | | | | 1 | |
| T215D | 2 (0.14) | | 2 | | | |
| K219Q | 3 (0.22) | | 1 | 2 | | |
| Others | | | | | | |
| K65R | 2 (0.14) | | | 2 | | |
| L74V | 1 (0.07) | 1 | | | | |
| L74I | 4 (0.29) | 1 | | 2 | | 1 |
| V75M | 6 (0.43) | 1 | | 2 | | 3 |
| M184V | 3 (0.22) | | 1 | | 2 | |
| M184I | 2 (0.14) | | | 1 | | 1 |
| NNRTI [n (%)] | | | | | | |
| Any | 19 (1.37) | 5 (1.71) | 3 (1.20) | 4 (1.37) | 3 (1.02) | 4 (1.53) |
| K101E | 4 (0.29) | 1 | 2 | 1 | | |
| K103N | 7 (0.50) | 1 | 1 | 1 | | 4 |
| Y181C | 6 (0.43) | 1 | | 2 | 1 | 2 |
| Y188L | 1 (0.07) | | | | 1 | |
| Y188H | 1 (0.07) | | | 1 | | |
| Y188C | 1 (0.07) | | | 1 | | |
| G190A | 4 (0.29) | 2 | | 1 | 1 | |
| G190E | 1 (0.07) | | | | 1 | |
| PI [n (%)] | | | | | | |
| Any | 15 (1.08) | 0 | 4 (1.60) | 2 (0.68) | 4 (1.36) | 5 (1.92) |
| D30N | 1 (0.07) | | | | 1 | |
| M46I | 4 (0.29) | | 2 | | | 2 |
| M46L | 6 (0.43) | | 1 | | 3 | 2 |
| M46I/L | 1 (0.07) | | | 1 | | |
| F53Y | 1 (0.07) | | | 1 | | |
| L76V | 1 (0.07) | | | | | 1 |
| L90M | 1 (0.07) | | 1 | | | |

diagnosis, and the exact latency from infection to diagnosis or to study enrollment was unavailable. Thus, the longer duration from diagnosis to study participation allows more frequent reversion from TDR into wild-type virus. This should be taken into account in the interpretation of the results of the study.

Although our study participants did not represent the national HIV-infected population in Vietnam but were rather HIV-infected individuals living in or near Ho Chi Minh City (HCMC), their age, sex, and the distribution of HIV risks were almost comparable with the national HIV-infected population in Vietnam. Notably, HCMC accounts for approximately 50% of the entire population receiving ART in Vietna,[12] and ART had been widely accessible in

HCMC since the early phase of ART scale-up or even before ART scale-up at private clinics. Since previous studies had predicted increased TDR rates after 5–8 years of ART scale-up,[2] HIV-infected individuals in HCMC are considered to be at higher risk of TDR compared with those in other areas of Vietnam. In addition, a previous study conducted in HCMC showed that 73% of patients on ART reported having injected drugs,[1] and the sentinel surveillance in 2009 showed that HCMC had high HIV prevalence among IDUs (46%).[1] Since IDU is considered a risk factor for poor adherence and emergence of drug resistance,[23,24] patients in HCMC are considered the key population for TDR monitoring. Although no statistical relationship was