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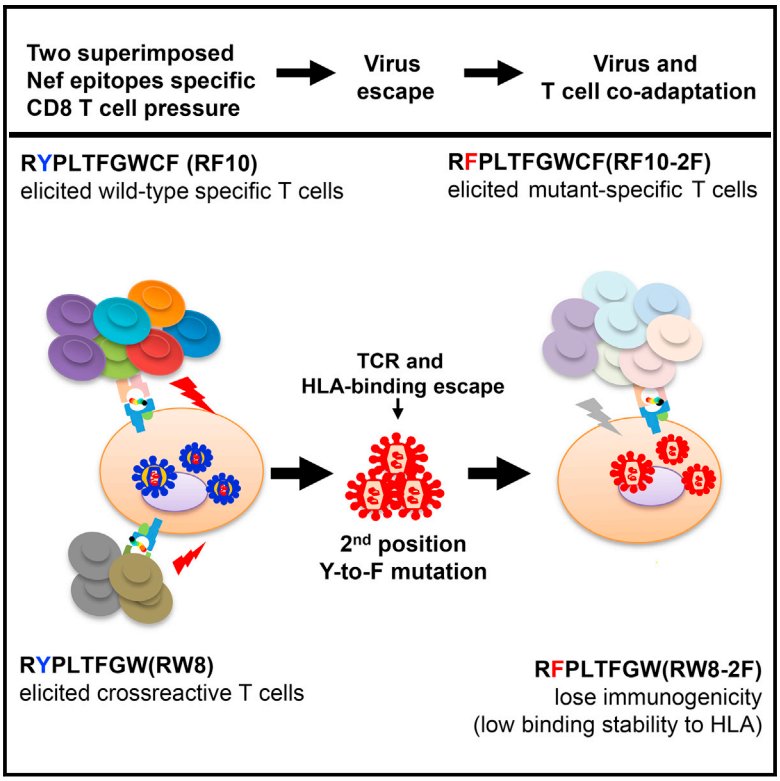
# Effects of a Single Escape Mutation on T Cell and HIV-1 Co-adaptation

## Graphical Abstract




## Highlights

- A single mutation in two superimposed epitopes induces distinct immune responses
- The mutation induces a new T cell repertoire for one epitope but not the other
- The immune variant affects T cell recognition by reducing pMHC-complex stability
- HLA-binding stability and antigen structural features determine immune outcomes

## Authors

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## In Brief

Rational design of T cell vaccines requires understanding of T cell and HIV-1 co-evolution. Sun et al. find that a single immune escape mutation has differential impacts on two HIV epitope-specific responses. The mutation alters the ability of the virus to trigger immune responses.

## Accession Numbers

5HGH
5HGD
5HGB
5HGA

Sun et al., 2016, Cell Reports *15*, 2279–2291
June 7, 2016 
http://dx.doi.org/10.1016/j.celrep.2016.05.017

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# Effects of a Single Escape Mutation on T Cell and HIV-1 Co-adaptation

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http://dx.doi.org/10.1016/j.celrep.2016.05.017

## SUMMARY

**The mechanistic basis for the progressive accumulation of $Y^{135}F$ Nef mutant viruses in the HIV-1-infected population remains poorly understood. $Y^{135}F$ viruses carry the 2F mutation within RW8 and RF10, which are two HLA-A*24:02-restricted superimposed Nef epitopes recognized by distinct and adaptable $CD8^+$ T cell responses. We combined comprehensive analysis of the T cell receptor repertoire and cross-reactive potential of wild-type or 2F RW8- and RF10-specific $CD8^+$ T cells with peptide-MHC complex stability and crystal structure studies. We find that, by affecting direct and water-mediated hydrogen bond networks within the peptide-MHC complex, the 2F mutation reduces both TCR and HLA binding. This suggests an advantage underlying the evolution of the 2F variant with decreased $CD8^+$ T cell efficacy. Our study provides a refined understanding of HIV-1 and $CD8^+$ T cell co-adaptation at the population level.**

## INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTLs) play a critical role in the control of HIV-1 replication during acute and chronic phases of infection (Appay et al., 2008; Borrow et al., 1997; Deng et al., 2015; Koup et al., 1994; Schmitz et al., 1999). However, HIV-1 can escape from the host immune system through Nef-mediated downregulation of cell-surface human leukocyte antigen (HLA) class I molecules (Collins et al., 1998; Tomiyama et al., 2002, 2005), as well as by the selection of escape mutations in various HIV-1 proteins (Goulder and Watkins, 2004; Kawashima et al., 2009; Kuse et al., 2014). Amino acid substitutions, indeed, occur at critical sites within HIV-1 CTL epitopes, and their flanking regions and can affect antigen presentation as well as reduce epitope-HLA binding and weaken T cell receptor (TCR) recognition (Altman and Feinberg, 2004; Bailey et al., 2004; Goulder and Watkins, 2004). Earlier studies at the population level revealed that some of these amino substitutions are HLA-adapted mutations, highlighting the fundamental role of HLA-restricted immune responses in driving and shaping HIV-1 evolution in vivo (Carlson et al., 2015; Chikata et al., 2014; Kawashima et al., 2009; Moore et al., 2002). Although CTLs specific for these escape mutations are not evoked in most cases, the emergence of some HIV-1 mutants has been associated with the rise of new T cell repertoires (Akahoshi et al., 2012; Allen et al., 2005; Feeney et al., 2005; Melhem et al., 2014; Ueno et al., 2007).

The dynamic interaction between CTL response and HIV-1 evolution has been investigated in B27-restricted, HIV-1 Gag-derived KK10-specific CTLs. Highly effective wild-type (WT)-specific CTLs carrying the public TCR clonotype TRBV4-3/TRBJ1-3 can control HIV-1 replication at a relatively early phase of the infection (Almeida et al., 2007), whereas an escape mutation at position 6 (L286M) in KK10 can evade this CTL recognition (Iglesias et al., 2011). An emerging T cell repertoire, in particular, one carrying TRBV6-5/TRBJ1-1, which recognizes both WT and the L268M mutant—can be recruited and control the L286M mutant virus (Ladell et al., 2013; Lichterfeld et al., 2007). However, in some individuals, this cross-reactive CTL can drive a second mutation at position 2 (R264K) in KK10, resulting in total abrogation of the binding of the KK10 epitope to the HLA-B27 molecule, loss of control of the R264K mutant virus, and TCR clonal extinction. Although these studies exemplify the co-adaptation between HIV-1 and the T cell response, little is known about the adaptation of the $CD8^+$ T cell repertoire to escape mutation epitopes that can still be processed and presented to T cells.

A Y-to-F substitution at the second position (2F) of RW8 (RYPLTFGW) and RF10 (RYPLTFGWCF) superimposed with epitopes derived from HIV-1 Nef protein from Japanese HIV-1-infected individuals and in Caucasians. This substitution is

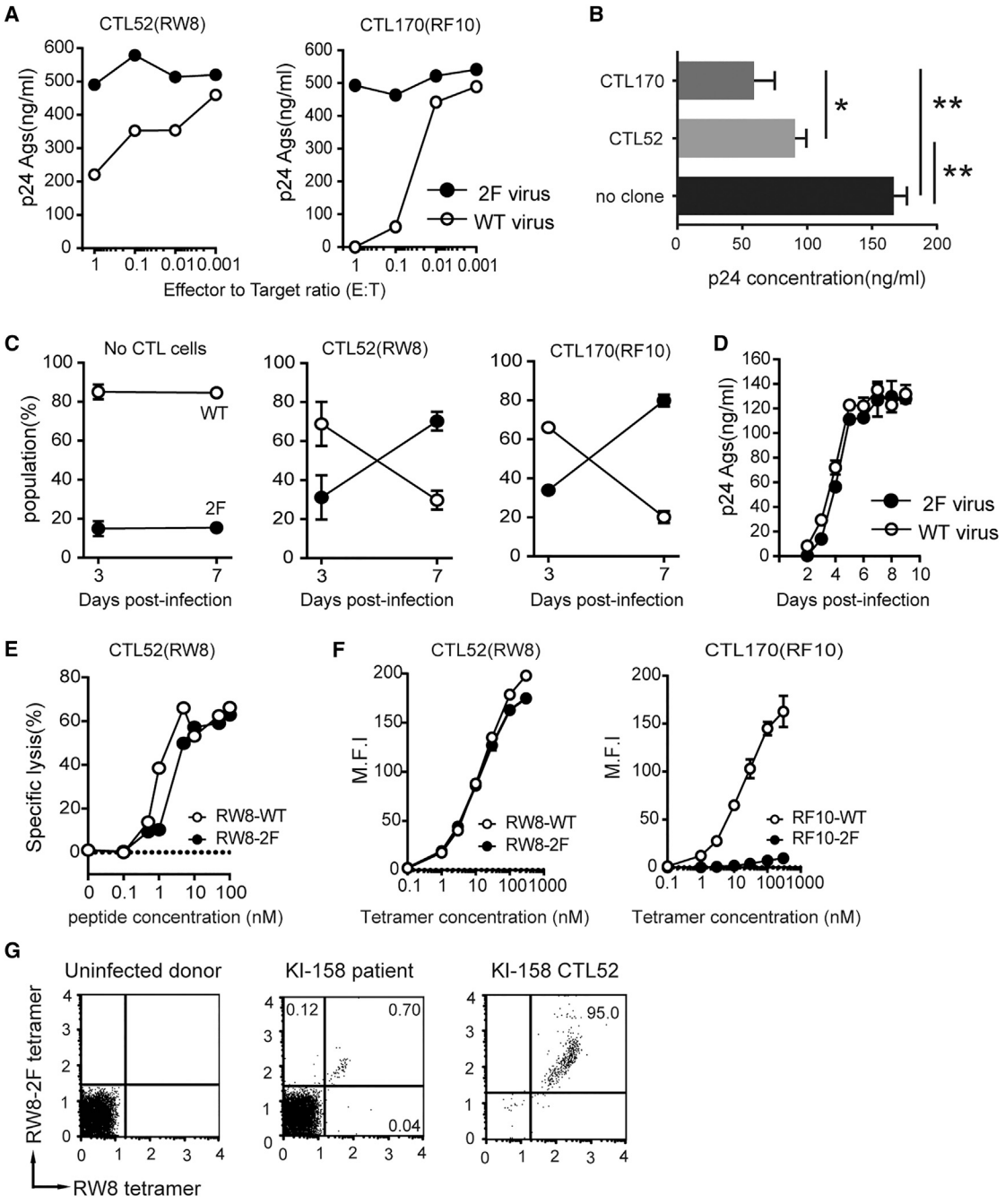


**Figure 1. In Vitro Selection of 2F Mutant Virus by RW8- and RF10-Specific CTLs**

(A) The ability of clone CTL-52 to suppress WT (open circle) and mutant (close circle) virus replication was determined by performing a viral suppression assay. Cultured $CD4^+$ T cells derived from a $HLA\text{-}A^*24{:}02^+$ healthy donor were infected with WT or 2F virus and then co-cultured with the CTL clone at various E:T ratios. (B and C) In vitro selection of viral mutant by RW8- and RF10-specific $CD8^+$ CTL clones. Mixtures of paired virus (WT and the 2F virus at a ratio of 9:1) were used to infect cultured primary CD4 T cells. The infected cells were then co-cultured with an RW8-specific CTL clone (CTL 52) or an RF10-specific one (CTL 170) at an E:T ratio of 1:1. The production of p24 in the culture supernatant was measured by performing p24 ELISA at day 7 post-infection (B). Results are presented as the mean ± SD of data from triplicate assays ($^*p < 0.05$; $^{**}p < 0.01$). The population change in the viral mixture was determined by the relative peak height on the sequencing electrogram (C).

(D) Replication kinetics of the WT and 2F mutant virus in primary CD4 T cells. Profiles of replication kinetics of WT (open circles) and 2F (closed circles) are shown.

(E) The cytolytic activities of a representative $HLA\text{-}A^*24{:}02$-restricted RW8-specific CTL clone (CTL-52) toward C1R-A2402 cells pulsed with the indicated peptide at various concentrations were measured. They were tested at an E:T ratio of 2:1.

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associated with the presence of HLA-A*24:02 (Carlson et al., 2012; Chikata et al., 2014). HLA-A*24:02 is the most frequent HLA class I allele in Japan, being found in approximately 70% of Japanese individuals (Saito et al., 2000). Interestingly, RW8 and RF10 immunodominant CTL epitopes elicit entirely distinct CTL responses (Sun et al., 2014). RF10-specific CTLs display higher TCR diversity and functional avidity than RW8-specific CTLs, making them the main driver for the selection of the 2F mutant in vivo. However, RF10-2F mutant-specific CTLs can be elicited after the emergence of the 2F mutant HIV-1 in HIV-1-infected HLA-A*24:02$^+$ individuals (Fujiwara et al., 2008). The exact process leading to the accumulation of the 2F mutant in HIV-1-infected Japanese individuals remains unclear. Several questions pertaining to this complex interaction between the virus and the CTL responses need to be addressed. For example, do CTLs specific for these two epitopes select the same escape mutation through different mechanism? What is the effect of this mutation on the induction of 2F-specific CTLs de novo and on the recruitment of new clonotypes? It remains unclear why the 2F mutant accumulates in Japanese HIV-1-infected individuals rather than reverting to the WT in the presence of mutant-specific CTLs.

To address these questions, we compared the pressure exerted by RW8- or RF10-specific CTLs on the selection of the 2F mutant, investigated the effects of this mutation on the elicitation of new CTL repertoires by using TCR analysis at a single-cell level, and analyzed peptide-HLA-A*24:02 crystal structures and the stability of peptide binding. Our combined data provide refined mechanistic insight into the co-adaptation of HIV-1 and the CD8$^+$ T cell response at the TCR-peptide binding HLA (p-HLA) level.

## RESULTS

### In Vitro Selection of 2F Mutant by RW8- or RF10-Specific CTLs

To study the ability of RW8- or RF10-specific CTLs to select the 2F mutant, we performed a competitive replication suppression assay using WT and 2F mutant viruses. First, we assessed the ability of RW8- or RF10-specific CTL clone established from patient KI-158 to suppress WT and 2F mutant viruses. In line with the higher functional avidity of RF10-specific CTLs (Fujiwara et al., 2008; Sun et al., 2014), the ability of a representative dominant RF10-specific CTL clone CTL170 to suppress HIV-1 was stronger than that of a representative dominant RW8-specific CTL clone CTL52 (Figure 1A), suggesting that RF10-specific CTLs may have effectively selected the 2F virus. Nonetheless, in a competitive HIV-1 suppression assay with target cells infected with a mixture of WT and mutant virus at a ratio of 9:1, respectively (Figure 1B), we did not observe any significant difference in the selection of the 2F mutant between RW8- and RF10-specific CTL clones (Figure 1C), indicating that both CTLs could effectively select 2F viruses. Also, the kinetics of virus replication was not different between WT and 2F viruses (Figure 1D). These results support the idea that, like RF10-specific CTLs, RW8-specific CTLs also could contribute to the selection of the 2F mutant in vivo.

### Cross-Recognition of WT and 2F Mutant Epitopes by RW8-Specific CTLs

Although the RF10-specific CTL clone CTL170 shows a stronger specificity for the WT epitope than for the 2F mutant (Fujiwara et al., 2008), the specificity of RW8-specific CTLs for WT and the 2F mutant remains unknown. To address this point, we tested RW8-specific CTL clone CTL52 reactivity toward the RW8-WT and RW8-2F peptides. This clone killed both RW8-WT and RW8-2F peptide loaded-HLA-A*24:02$^+$ C1R cells with similar efficacy (Figure 1E). The same result was found for another RW8-specfic CTL clone established from KI-158 (Figures S1A–S1C). In addition, we performed direct assessment of TCR binding avidity for HLA-A*24:02-WT or -2F peptide complexes by using tetramer binding assay on clones CTL52 and CTL170. The RW8 cross-reactive T cell clone (CTL52) TCR had the same avidity for both HLA-A*24:02-WT and HLA-A*24:02-2F peptide complexes (Figure 1F). In contrast, the TCR of an RF10-specific T cell clone had a stronger avidity for the HLA-A*24:02-WT peptide complex than for the HLA-A*24:02-2F peptide complex (Figure 1F). The cross-reactivity of RW8-specfic CTLs was validated by staining ex vivo peripheral blood mononuclear cells (PBMCs) from the same individual (KI-158) by using the same tetramers (Figure 1G). Taken together, these results indicated that RW8-specific CTLs could cross-recognize both RW8-WT and RW8-2F epitopes and that the selection of the 2F mutant by RW8-specific CTLs may have resulted from another mechanism, such as reduced antigen presentation of the 2F mutant in 2F virus-infected cells.

### Induction of RW8/RF10 2F-Specific CTLs in HLA-A*24:02$^+$ HIV-1-Infected Individuals

To address the impact of the 2F mutant on RW8/RF10-specific CTLs, we performed longitudinal analysis of four HLA-A*24:02-positive individuals (KI-092, KI-102, KI-158, and KI-161) who were initially infected with the WT virus and later selected the 2F variant virus. We analyzed PBMCs at early (WT) and chronic (2F) phases of their HIV-1 infection by using four tetramers (RW8-, RW8-2F-, RF10-, and RF10-2F tetramers). All four individuals displayed RF10-specific CTLs at the early (WT) phase and RF10-2F-specific CTLs after the emergence of the 2F virus (Figure 2), in line with our previous observations (Fujiwara et al., 2008). Notably, RW8 cross-reactive CTLs were detected in KI-102 and KI-158 before and after the emergence of the mutant virus, although the frequency of the CTLs went from 1.6p% to 0.04% in KI-102 after 2.5 years (Figure 2). KI-161 had a high frequency (2.94%) of RW8-specific CTLs at the WT phase but did not have RW8 cross-reactive CTLs at both WT and 2F phases. Taken together, these results indicate that

(F) HLA-A*24:02-restricted clones CTL 52 and CTL 170 were stained with RW8 or RW8-2F tetramers at 0.1 nM to 300 nM concentrations of each tetramer. MFI (mean fluorescence intensity) of the CTL clones is presented.

(G) HLA-A*24:02-restricted CTL 52 clone and PBMCs from individual KI-158 were competitively stained with RW8 and RW8-2F tetramers at a 300 nM concentration of each tetramer. The percentage of tetramer-positive cells among CD8$^+$ cells was measured.

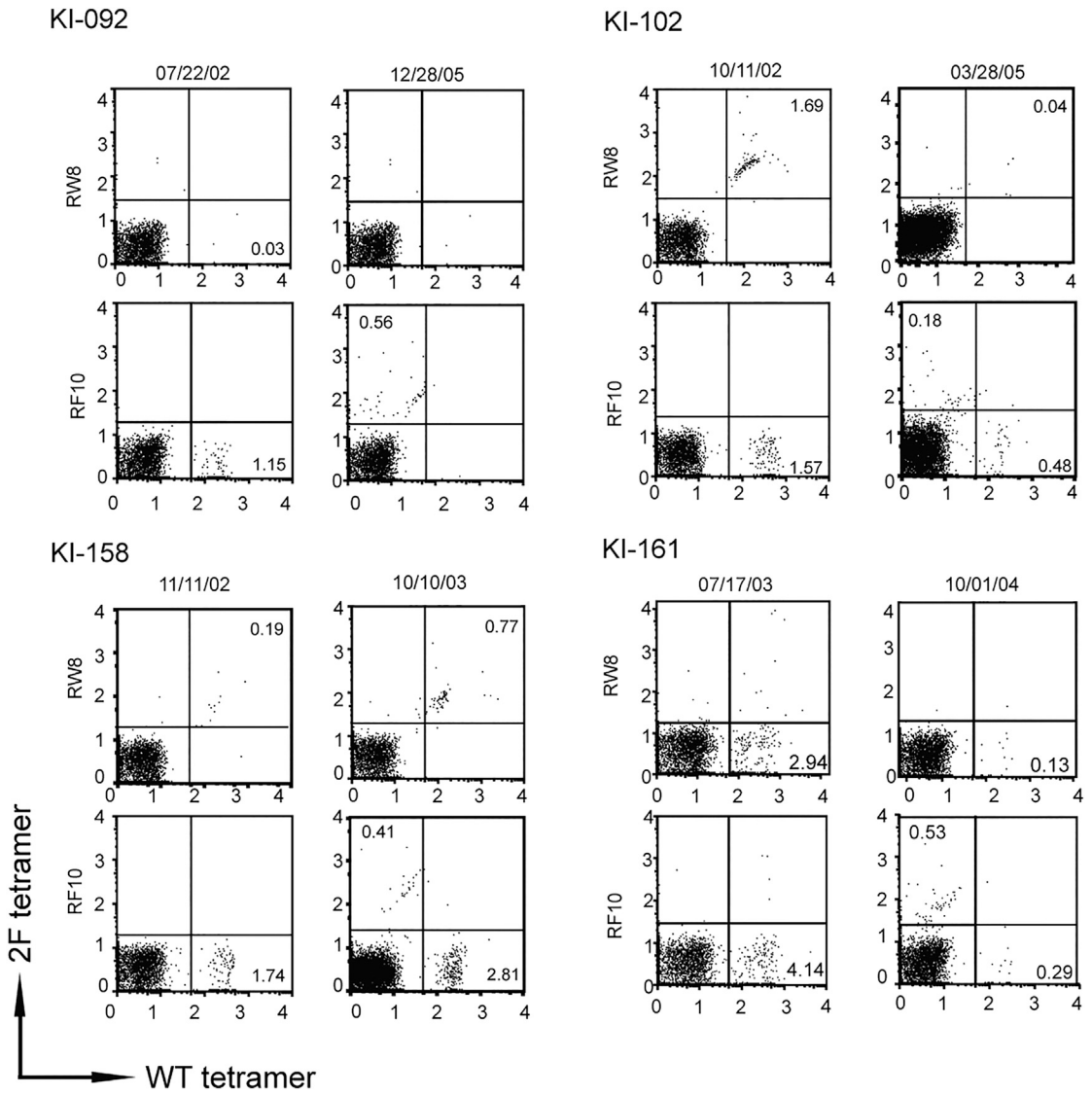


**Figure 2. Longitudinal Analysis of RW8- and RF10-Specific CTLs before and after Emergence of Mutant Viruses**

PBMCs were collected at two different time points (before and after emergence of the mutant virus) from four HIV-1-infected individuals (KI-092, KI-102, KI-158, and KI-161) and competitively stained with the combination of RW8 and RW8-2F tetramers or RF10 and RF10-2F ones at a 300 nM concentration of each tetramer. The percentage of tetramer-positive cells among $CD3^+CD8^+$ 7-$AAD^-$ T cells was measured. Background levels of the tetramer-positive $CD3^+CD8^+$ cells were calculated from the frequencies (mean + 2 SD) of tetramer-positive $CD3^+CD8^+$ cells among PBMCs from five HIV-1-naive individuals. See also Figure S1F.

the emergence of the 2F virus was associated with the differential induction of 2F-specific T cells between RW8 and RF10.

## Distinct Clonotypic Patterns between RF10 and RF10-2F-Specific T Cells

Previous studies showed that RF10-2F mutant-specific CTLs are elicited in HLA-A*24:02$^+$ individuals who were primarily infected with the 2F virus or after emergence of this virus in those primarily infected with the WT virus, suggesting that the mutant epitope virus induced new T cell repertoires (Fujiwara et al., 2008; Tanuma et al., 2008). To clarify the change in T cell repertoires pre-versus post-emergence of the 2F virus, we performed clonotypic analysis of RF10- and RF10-2F-specific CTLs to compare the TCR clonotype between the two CTLs. We stained PBMCs from individuals having the 2F mutant virus with both WT and 2F tetramers and sorted tetramer-positive cells at the single-cell level for TCR analysis. When we sequenced the TCRs of RF10-WT- or RF10-2F-specific CTLs from seven individuals (Figure S1E; Table S1), we found that the clonotype of RF10-2F-specific CTLs was completely different from that of RF10-specific CTLs (Figures 3A and S2). In addition, we performed a longitudinal analysis on three additional individuals (KI-092, KI-158, and KI-161) who had RF10-WT-specific CTLs at an earlier phase of their infection and then shifted toward a RF10-2F-specific CTL response after the emergence of the 2F virus (Figure 2). RF10 WT-specific and 2F-specific CTLs showed no overlapping

clonotypes in these individuals (Figures 3D and S4). This finding indicated that the 2F was a mutant epitope escaped from TCR recognition and that its emergence resulted in the induction of a new RF10-2F-specific T cell repertoire in HLA-A*24:02$^{+}$ individuals primarily infected with the WT virus. There were no differences between RF10-WT-specific and RF10-2F-specific CTLs in terms of $\alpha$ (Figure 3B) and $\beta$ TCR diversity (Figure 3C). Altogether, these results support the notion that the induction of T cells with high TCR diversity can be driven by a featured epitope in the case of RF10-WT/RF10-2F-specific CTLs (Sun et al., 2014).

## Minimal Impact of 2F Virus Emergence on RW8-Specific T Cell Repertoire

Next, we analyzed the clonotypic composition of RW8-specific T cells from five individuals with the 2F virus and compared them with those from the four previously analyzed individuals having the WT virus (Sun et al., 2014). Flow cytometry analysis of PBMCs stained with RW8 and RW8-2F tetramers together showed that RW8-specific CTLs were stained with both tetramers, suggesting that these cells were cross-reactive for RW8-WT and RW8-2F epitopes (Figures 4 and S1D). Therefore, we sorted these RW8 cross-reactive T cells to analyze their TCR at the single-cell level and compared them with the cross-reactive T cells at the early (WT) phase (Figure 4A). In line with RW8 cross-reactive T cells at this phase, the cross-sectional analysis demonstrated a few clonotypes with a dominant one among each individual in RW8 cross-reactive T cells at the 2F phase (Figure 4A). Although the individuals used different $\alpha$ chains, a high prevalence of public TRBV7-9 gene usage with similar CDR3 regions (V7-9*01/03-D1*01-J1/J2-5*01) in individuals infected with WT or 2F viruses was found (Figure S3). This finding implies two possibilities that new cross-reactive CTLs were induced by the RW8-2F variant and that cross-reactive memory CTLs from the WT phase remained after the emergence of the 2F virus. To clarify these possibilities, we performed a longitudinal analysis of the KI-158 individual at both the WT and 2F phases. We observed the same dominant clonotype in RW8-specific CTLs at both phases, indicating that cross-reactive memory T cells had been primed at the WT phase rather than that the recruitment of new T cells by the emergence of the 2F mutant had occurred (Figure 4B and S4). To further clarify the ability of RW8-2F to recruit new CTLs, we analyzed three individuals who had first been infected with a 2F virus. RF10-2F-specific CTLs were detected in all three individuals, whereas RW8-WT/RW8-2F cross-reactive T cells were not (Figure 4C), suggesting that the RW8-2F epitope seemingly had no ability to elicit new CTL repertoires.

## Conformational Differences among Peptide-HLA-A*24:02 Structures

To obtain molecular insights into the impact of the 2F mutation, we determined the crystal structures of HLA-A*24:02-RF10-2F and HLA-A*24:02-RW8-2F (Table S2) and compared them with previously published HLA-A*24:02-RF10 and HLA-A*24:02-RW8 structures (Sun et al., 2014). Overall, WT and 2F mutant peptides/HLA-A*24:02 structures shared similar conformations (Figure 5A), except for minor differences at the position 1 of RF10 and RF10-2F (Figure S5A). However, major differences between HLA-A*24:02 WT and 2F peptide structures were observed in terms of hydrogen bond and water molecule levels. The 8-mer and 10-mer 2F mutant peptides had lost one critical hydrogen bond contact between the P2 residue and the HLA-A*24:02 H70 residue (Figures 5B and 5C) that was present in the 8-mer and 10-mer WT peptides bound to HLA-A*24:02. The sum of the observed differences may account for a decreased affinity of HLA-A*24:02 for mutant 2F peptides. Another interesting result of the analysis concerned the distribution of water molecules in the peptide-binding grooves of different HLA-A*24:02-peptide complexes. The unambiguous electron density of water molecules clearly showed their distinct distribution among four peptide/MHCs (major histocompatibility complexes), which might have contributed to changes in TCR recognition (Figures S5B and S5C). The number of water molecules in the vicinity of HLA-A*24:02-bound 8-mer WT and 2F peptides was identical, but the molecules differed in their positions (Figures 6A and 6B). In contrast, HLA-A*24:02-RF10-WT showed six water-molecule-mediated hydrogen bonds; whereas HLA-A*24:02-RF10-2F harbored eight of them. The position of the water molecules differed particularly around peptide residue P5 (Figures 6C and 6D). As the 10-mer peptide displayed a conformation with an exposed central region (P5–P7), this differential distribution of water molecules around peptide residue P5 might have affected the TCR recognition.

## Effect of the 2F Mutation on Peptide-HLA Binding Stabilization and T Cell Recognition

The structural analysis suggested that the 2F mutation affected the stability of the peptide-HLA-A*24:02 complex. To address this point, we performed a peptide-MHC dissociation assay using RMA-S-HLA-A*24:02 cells (Figure 7A). The stability of peptide binding was presented as the time required for 50% decay of the HLA molecules ($DT_{50}$). The results indicated that the stability of the RF10-WT peptide-HLA-A*24:02 complex was higher than that of the RF10-2F one ($DT_{50}$: 6.22 $\pm$ 0.09 hr versus 3.35 $\pm$ 0.70 hr), indicating that the 2F mutation indeed affected the stability of peptide-HLA binding. Similarly, the stability of RW8 peptide binding to HLA-A*24:02 was higher than that of the RW8-2F one ($DT_{50}$: 4.37 $\pm$ 0.75 hr versus 0.85 $\pm$ 0.31 hr). Next, we investigated the effect of the reduced peptide stability on the recognition of RW8-specific or RF10-specific CTL clones. The RW8-specific CTL clone CTL 52, which showed a similar specific lysis activity toward target cells prepulsed with RW8-WT or RW8-2F peptides (Figure 1E), recognized the RW8-WT peptide better than the RW8-2F one during 1–6 hr of incubation (Figure 7B, left). Similarly, RF10-specific clone CTL189, which cross-recognizes RF10-WT and RF10-2F at a high concentration of exogenous peptides (Fujiwara et al., 2008), showed reduced ability to recognize the RF10-2F peptide as compared with the ability to recognize the RF10-WT one (Figure 7B, right). Taken together, these results indicate that the 2F mutation affected specific CTL recognition since it reduced the stability of peptide-HLA binding. In addition to being a TCR-binding escape mutant (in the case of RF10, but not RW8), the 2F variant was also an HLA-binding escape mutant, thus explaining the irreversible nature of this mutant and accumulation in a population comprising HLA-A*24:02 individuals.

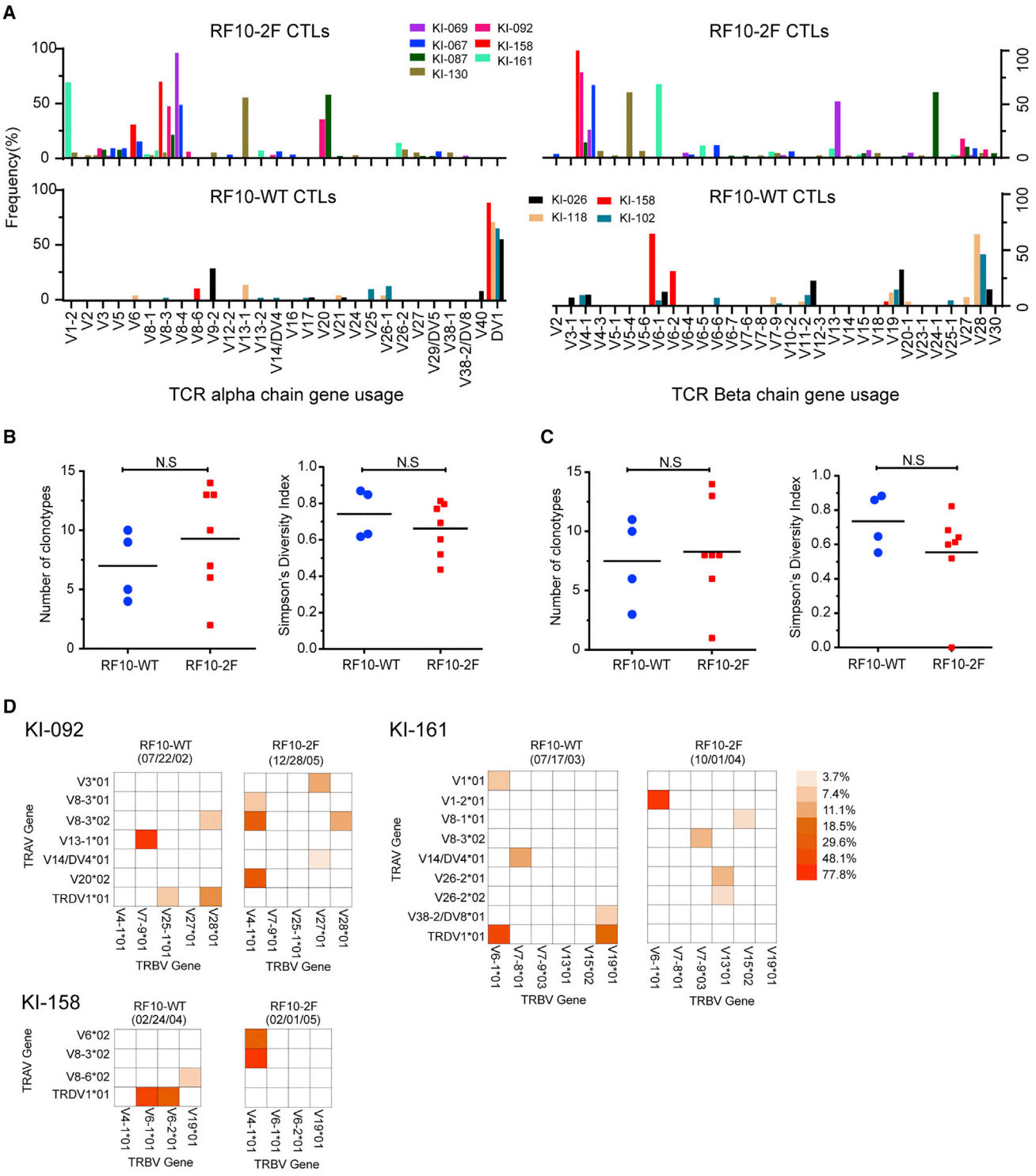


**Figure 3. Different Patterns of TCR Clonotypes between RF10-WT- and RF10-2F-Specific T Cells**

(A) RF10-2F tetramer-positive CTLs were sorted from patients who carried RF10-2F-specific CTLs (n = 7), and the TCRs were analyzed at the single-cell level. The usage of the TRAV gene (left) and TRBV gene (right) was compared for the patients (n = 4) carrying RF10-WT-specific CTLs. TCRs were analyzed by use of the IMGT tool, and the designation of each gene followed IMGT TCR gene nomenclature.

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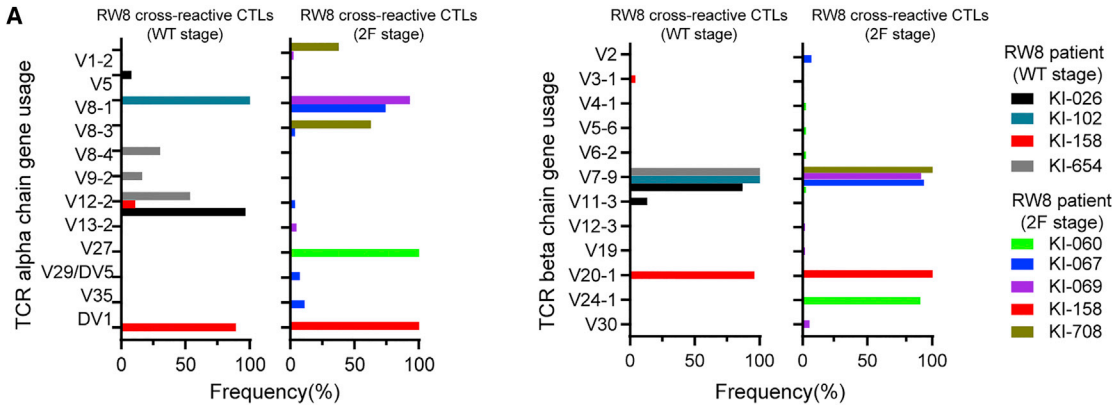


B

| | TRAV | TRAJ | CDR3 | TRBV | TRBD | TRBJ | CDR3 | Fre. |
|---|---|---|---|---|---|---|---|---|
| RW8 (WT-stage) | DV1*01 | J40*01 | CALGELGAPGTYKYIF | V20-1*01 | D2*01 | J2-7*01 | CSARDPVSTYEQYF | 27/31 |
| | AV12-2*02 | J50*01 | CAAFKTSYDKVIF | V20-1*01 | D2*01 | J2-7*01 | CSARDPIRLISYEQYF | 4/31 |
| RW8 (2F stage ) | DV1*01 | J40*01 | CALGELGAPGTYKYIF | V20-1*01 | D2*01 | J2-7*01 | CSARDPVSTYEQYF | 20/20 |

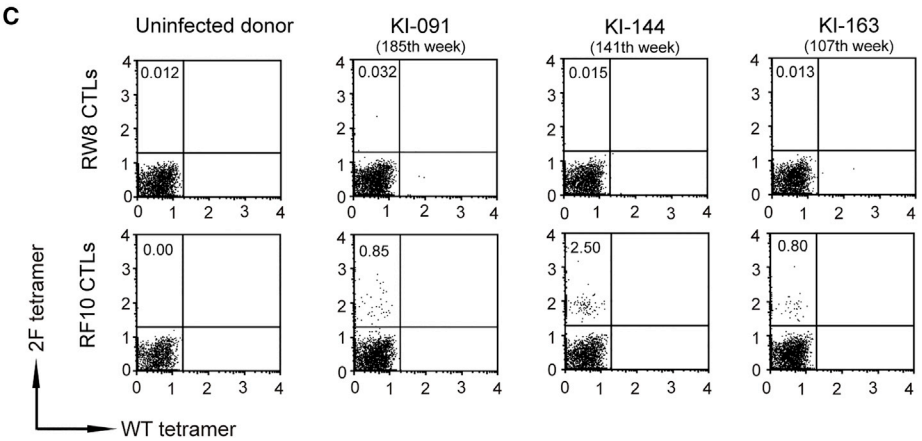


**Figure 4. Effect of the Emergence of the 2F Virus on Induction of RW8-Specific T Cell Repertoire**

(A) RW8/RW8-2F tetramer dual-positive $CD8^+$ T cells were sorted from individuals (n = 5) who carried RW8 CTLs, and then the TCRs were analyzed at the single-cell level. The usage of the TRAV gene (left) and TRBV gene (right) was compared for the patients (n = 4) carrying cross-reactive RW8 CTLs.

(B) Each tetramer-positive CTL was sorted from longitudinal PBMC samples from individual KI-158 for TCR repertoire analysis at the single-cell level. Paired TCR α- and β-chain usages are shown.

(C) Staining of PBMCs from three patients and one healthy donor with the combination of RW8 and RW8-2F tetramers or RF10 and RF10-2F ones at a 300 nM concentration of each tetramer in individuals in whom 2F virus was detected at the early phase of HIV-1 infection. The percentage of tetramer-positive cells among $CD3^+CD8^+$ 7-$AAD^-$ T cells was calculated.

## DISCUSSION

Although it is widely accepted that HIV-1-specific $CD8^+$ T cells can select virus escape mutations, the relationship between virus evolution and the adaptation of the T cell response at the clonotypic level has remained poorly studied. Previous studies on B*2705-KK10-specific T cells has shown that two steps are needed for the virus to escape highly effective WT-specific and mutant-cross-reactive CTL responses. This process is initiated by the selection of the L268M mutant virus by WT-specific CTLs. Nonetheless, the mutant virus can still be controlled by cross-reactive CTLs with high sensitivity for both WT and mutant

(B and C) Comparison of TCR repertoire diversity between RF10- and RF10-2F-specific CTLs. TCR repertoire diversity was assessed by using both the number of clonotypes and Simpson's diversity index for α- (B) and β-chain (C). Statistical analysis was conducted by use of the unpaired t test.

(D) Longitudinal PBMC samples from three HIV-1 infected HLA-A*24:$02^+$ individuals (KI-092, KI-158, and KI-161) were collected, and each tetramer-positive CTL was sorted for TCR repertoire analysis at the single-cell level. Paired TCR α- and β-chain usages are shown. The heatmap shows the frequency of each clonotype.

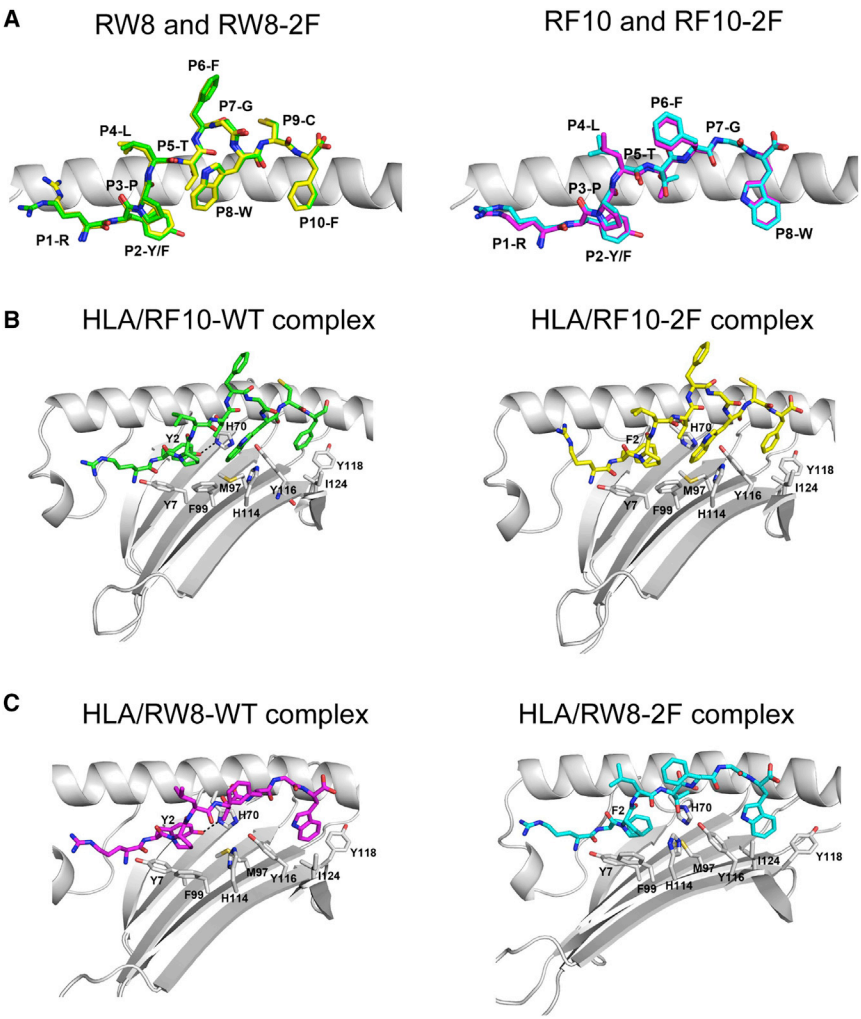


**Figure 5. Comparison of Four HLA-A*24:02-Peptide Structures**

(A) Overview of the structures of HLA-A*24:02 in complex with the four indicated peptides. RF10, RF10-2F, RW8, and RW8-2F are exhibited in green, yellow, magenta, and cyan, respectively. Both RF10 and RF10-2F display featured conformations, whereas both RW8 and RW8-2F display featureless ones. The peptides are shown as ribbon models.

(B and C) Structural comparison between two paired peptides: RF10 and RF10-2F (B); and RW8 and RW8-2F (C). Hydrogen bonds are shown as black dashes.

antigens. In some individuals, these CTLs drive the occurrence of a second mutation, R264K in KK10, which abrogates the binding of the KK10 epitope to the HLA-B*2705 molecule so that no new CTLs can be induced by this mutant epitope. However, the additional compensatory mutation S173A in p24 is needed to rescue virus replicative capacity, which is directly impacted by the R264K mutation. Our present study using $CD8^+$ T cells specific for a HLA-A*24:02-restricted Nef epitope has provided new insights into the HIV-1 escape and T cell clonotype dynamics. We showed that the virus, through a single step of F mutation at position 2, could generate a variant with both TCR- and HLA-binding escape properties, differentially affecting T cells specific for the superimposed epitopes RF10 and RW8. Our results indicate that RF10-WT-specific CTLs, together with RW8 cross-reactive CTLs, could drive the selection of the same escape mutation, 2F. However, after emergence of this mutation, different effects of this variant on the T cell evolution were observed. The appearance of the RF10-2F epitope was associated with the de novo induction of mutant-specific CTLs with entirely different clonotypes. In contrast, the RW8-2F epitope failed to elicit a new T cell repertoire. In sum, the present work describes an original double escape in TCR recognition and HLA-binding affinity, associated with a single mutation in the epitope, which had differential impacts on superimposed HIV epitopes. This highlights the high diversity of scenarios related to the molecular arms race between a virus and the immune response, and it provides refined mechanistic insights into the evolution of the virus at the population level.

Molecular and mechanistic support to explain the differences in TCR repertoire was derived from our X-ray crystallographic data. Both mutant peptides (RF10-2F and RW8-2F) lacked a critical hydrogen bond interaction between the primary anchor residue P2 of the peptide and the H70 residue in HLA-A*24:02. The weaker binding of the mutant peptides to HLA-A*24:02 was most likely caused by changes in the water-molecule-mediated hydrogen bond network, although we cannot formally exclude the possibility that the altered water network was the result, rather than the cause, of binding and stability changes. In the absence of enthalpy-entropy compensation data, we interpret the altered TCR recognition as being similar to the stability-enhancing effect of water-mediated hydrogen bonding in collagen and collagen-binding adhesin interactions (Vitagliano et al., 2011). The binding stability of RF10-2F was similar to that of the RW8-WT epitope and greater than that of RW8-2F, which was unable to induce new T cell responses. The RF10-2F peptide could still be effectively presented at the surface of HIV-1-infected cells and recognized by TCRs. In line with these results, previous studies showed that the stability of the pMHC is critical in the presentation of epitopes to T cells, because an unstable pMHC will be present at lower concentrations or absent on the surface of antigen-presenting cells (Ahlers and Belyakov, 2010; Busch and Pamer, 1998; Harndahl et al., 2012; Sijts and Pamer, 1997; van der Burg et al., 1996). Our results support the concept that the stabilization of escape mutant epitopes appears to be the main driver for the induction of a new T cell repertoire toward escape epitopes.

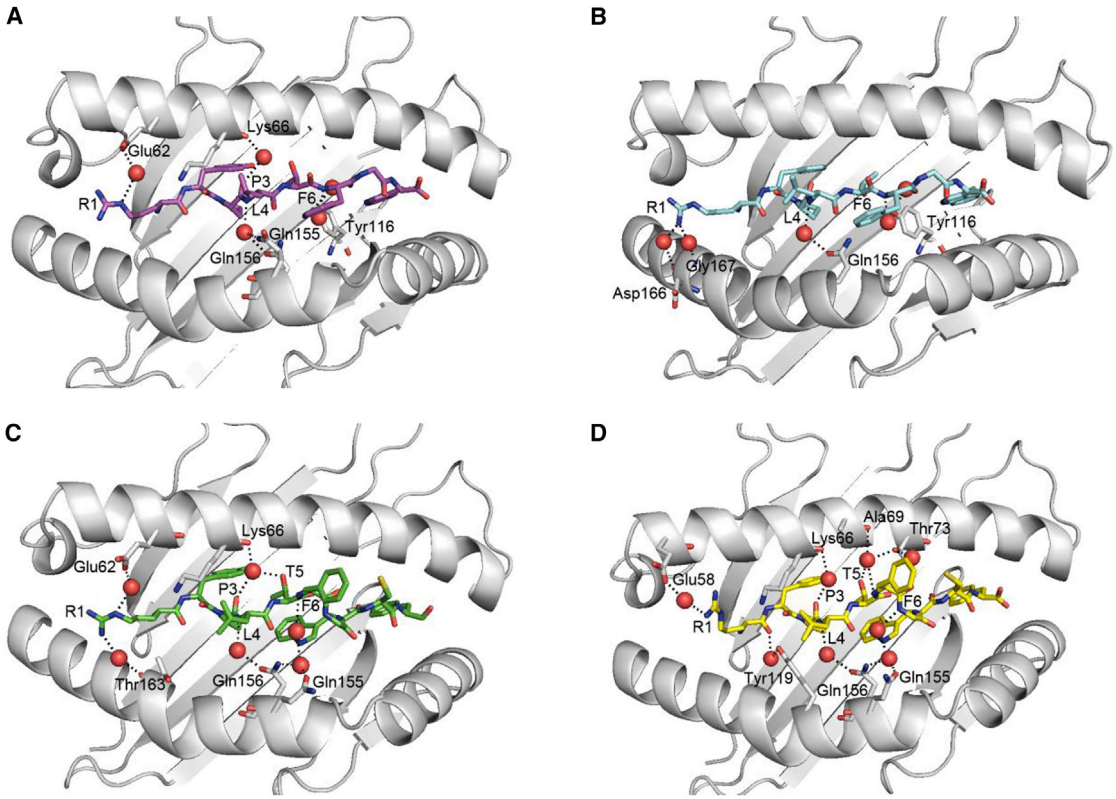


**Figure 6. Detailed Comparison of Water Molecules in the Four Structures of HLA-A*24:02 Molecules**

(A–D) Water-mediated hydrogen bonds between HLA and peptide residues are shown as dotted lines in black. The red spheres represent water molecules. (A) RW8 (magenta), (B) RW8-2F (cyan), (C) RF10 (green), and (D) RF10-2F (yellow).

Given that the side chains of 10-mer peptide residues at P4 and P6 protrude and can be recognized by TCRs, the differences in the number of water molecules around RF10-WT and RF10-2F P5 residues would affect the hydrogen bond network around the TCR recognition site (Shimizu et al., 2013). The RF10-2F peptide was surrounded by a greater number of water molecules than the RF10-WT peptide. A recent crystallographic study suggested that the differences in the water-mediated hydrogen bond network between WT and mutant 10-mer peptides may affect TCR recognition (Shimizu et al., 2013). In addition, we also observed two additional hydrogen bonds at the P1 residue of the RF10-2F peptide, suggesting that conformational changes within RF10-2F may have played a minor role in the escape from RF10-specific CTLs, although we cannot exclude the possibility that the changes were simply caused by the flexibility of the arginine residue (Liu et al., 2011).

Despite the instability of the RW8-2F/HLA-A*24:02 complex, the RW8-2F peptide could still maintain exogenous binding to the HLA molecule, as was exemplified by our finding that RW8-specific CTLs could be cross-reactive and recognize similarly both exogenously presented RW8 and RW8-2F peptides. The conformational features of the peptides emerge here as key factors to determine the diversity and cross-reactive nature of TCRs (Nikolich-Zugich et al., 2004). The featureless conformations of the RW8-WT and RW8-2F epitopes within the HLA-A*24:02 molecule likely allowed RW8-specific TCRs to accommodate both peptides without difficulty and explain why RW8-specific CTLs could be cross-reactive, even before the emergence of the 2F variant. In contrast, like the RF10-WT epitope, RF10-2F still retained a featured conformation within the HLA-A*24:02 molecule. This implies the recruitment of a new clonotypic repertoire specific for the 2F variant, one with TCR characteristics equivalent to those of the WT-specific repertoire, as highlighted by the similar TCR diversity between RF10- and RF10-2F-specific T cells.

Although several epitopes restricted by other HLA alleles have been identified in this region, only HLA-B*35:01 and HLA-A*02 molecules, found in the Japanese population, may contribute to the selection of the 2F mutant. HLA-B*35:01-restricted YF9 and HLA-A*02-restricted PL10 epitopes are superimposed in RF10 (Choppin et al., 2001). Our recent study of HLA-associated polymorphism (HLA-AP) showed no HLA-AP with HLA-B*35:01 and HLA-A*02 within this epitope, indicating that there is no accumulation of mutant epitopes selected by other HLA alleles in this region (Chikata et al., 2014). The F mutation at position 1 might affect YF9-specific CTL recognition although there is no evidence so far for this possibility.

In the present study, we elucidated the molecular mechanism of 2F mutant accumulation in the Japanese population and highlighted the relationship between clonotypic changes in T cells and mutant virus evolution. We showed that one mutation within two superimposed peptides yielded two different types of

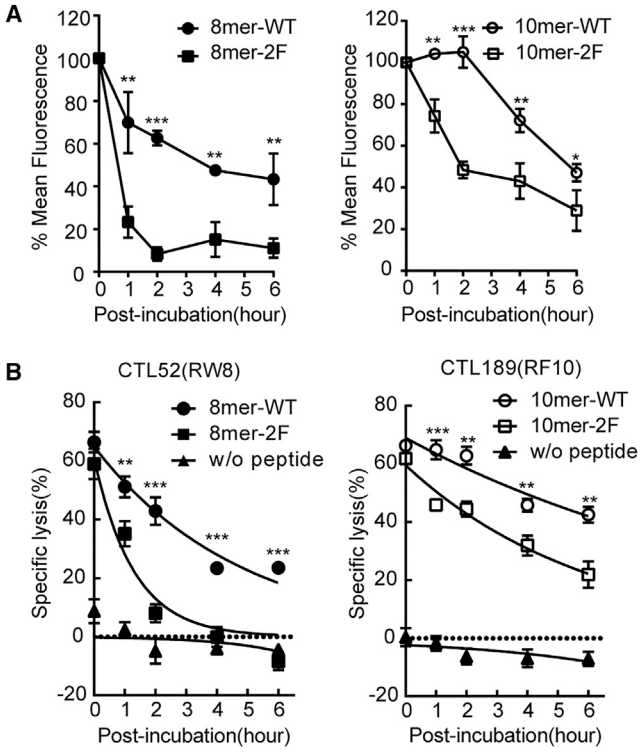


**Figure 7. Effects of the 2F Mutants on Peptide-MHC Binding Stability and T Cell Recognition**

(A) The stability of peptide binding to HLA-A*24:02 molecules was determined by using RMA-S-A*24:02 cells. The cells were incubated at 26°C with a 250-μM concentration of each of the four indicated peptides for 1 hr, and then the cells were incubated at 37°C for 3 hr. After the free, unbound peptide had been washed out, cells were collected at each indicated time point. The relative mean fluorescence (MFI) was measured and plotted as percentage of maximal fluorescence, as described in Experimental Procedures.

(B) Effects of peptide-MHC stability on CTL recognition. C1R-A2402 cells, as target cells, were pre-pulsed with each of the four indicated peptides at a concentration of 100 nM for 1 hr and then washed. A portion of the resultant peptide-loaded cells was taken at each indicated time point and then co-cultured with the CTL52 or CTL189 clone at an E:T ratio of 1:1 for the chromium release assay. Results are given as the mean ± SD of triplicate assays. The statistics were calculated by performing the unpaired t test.

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

outcome, on the one hand inducing a new T cell repertoire and, on the other, preventing the induction of mutant-specific $CD8^+$ T cells. HLA binding stability and the conformational feature of the mutant antigens determined this difference. Thus, the same escape mutations in two different superimposed epitopes changed the ability of HIV-1 to elicit immune responses in HIV-1-infected individuals during co-evolution of HIV-1 and CTLs.

Given that recent population-based studies indicate that viral evolution in response to HLA-mediated immune pressure follows predictable patterns, the study of immune-driven HIV-1 escape would help us to develop strategies for an effective vaccine (Brumme et al., 2009; Chikata et al., 2014; Huang et al., 2011; Rousseau et al., 2008). Taking into consideration that the evolution of HIV-1 is central for the design of vaccines in order to be able to control mutant viruses or their emergence, the design of vaccines that include apparently immunogenic escape variants needs to be carefully considered. The present work illustrates this point well. Initial observations may argue in favor of using 2F as the immunogen. The 2F variant is increasingly prevalent in the population, and it appears to be immunological since 2F RF10-specific $CD8^+$ T cells are detected in HIV-1-infected patients. However, in reality, using such an epitope in an immunogen may actually be disadvantageous. Due to its reduced HLA binding stability, the 2F variant is likely to be poor at inducing $CD8^+$ T cell responses—in particular, those specific to RW8. Moreover, most effective RF10-2F specific responses will not be cross-reactive and, therefore, would provide no control of WT viruses. Overall, the 2F variant is likely to be poorly immunogenic, and its use in vaccines would result overall in weak efficacy. This observation might not be an isolated case, as it has been estimated that approximately 20% of HLA-associated polymorphisms occur at anchor positions and, moreover, that the mutations were predicted to confer an average 10-fold reduction in pMHC binding affinity (Bronke et al., 2013; Carlson et al., 2015). Therefore, a rational design of effective T cell vaccines and immunogens requires a very deep understanding of the molecular mechanisms underlying the induction of specific T cell responses against HIV, certainly to levels of depth not anticipated initially.

## EXPERIMENTAL PROCEDURES

### Ethics Statement

All 13 HLA-A*24:02$^+$ treatment-naive HIV-1-infected individuals were recruited from the National Center for Global Health and Medicine (Table S1). 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 in accordance with the Declaration of Helsinki. HLA types were determined by standard sequence-based genotyping at the HLA Laboratory, Kyoto, Japan.

### HIV-1-Specific CTL Clones

Antigen-specific CTL clones were generated as previously described (Fujiwara et al., 2008). Briefly, RW8 bulk CTLs were obtained by stimulating PBMCs from KI-158 with RW8 peptides; then, CTL clones were generated from the cell lines by the limiting dilution method.

### Tetramer Staining

HLA-A*24:02-tetrameric complexes were synthesized as previously described (Altman et al., 1996). For tetramer staining, CTL clones or individual PBMCs were stained with WT or 2F tetramers at various concentrations or with a combination of WT and 2F tetramers at 300 nM for RW8 and RF10 CTLs at 37°C for 30 min. The cells were subsequently stained with CD3 (fluorescein isothiocyanate; FITC), CD8 (Pacific blue), and 7AAD at 4°C for 30 min.

### Cell Line

C1R-A*2402 and RMA-S-A*2402 cells were generated as previously described (Ikeda-Moore et al., 1997; Karaki et al., 1993; Sun et al., 2014; Takamiya et al., 1994). RMA-S cells are a TAP2-deficiency cell line derived from RMA cells. They stably express high levels of empty MHC molecules when cultured at 26°C but very low levels at 37°C. These cell lines were cultured in R5 medium with 0.2 mg/ml hygromycin B.

### $^{51}$Cr Release Assay

The cytotoxic potential of CTL clones against C1R-A*24:02 prepulsed with appropriate peptides at various concentrations (0–100 nM) was determined as previously described (Fujiwara and Takiguchi, 2007). Effector cells were co-cultured for 4 hr at 37°C with target cells at an effector:target (E:T) ratio of 2:1.

### Viral Suppression Assay

Two modified HIV-1 virus lab strains, NL-432-10F (WT) and NL-432-2F10F (2F), were used in these assays (Fujiwara et al., 2008). The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (Fujiwara et al., 2008). Briefly, activated primary $CD4^+$ T cells were infected with WT or 2F virus for 6 hr before being washed with R10 medium. The cells were then co-cultured with HIV-1-specific CTL clones at various E:T ratios. We collected 10 μl of culture supernatant at day 6, and then the p24 level in it was determined by ELISA.

### Replication Kinetics Assay

The replication kinetics of the WT and 2F viruses were examined as previously described (Akahoshi et al., 2012). In brief, $CD4^+$ T cells ($2 \times 10^6$) were infected with the 500 blue-cell-forming units (BFU) of either WT virus or 2F virus; the culture supernatant was then collected from day 2 to day 10 post-infection. The concentration of supernatant p24 antigen was measured by using ELISA.

### In Vitro Competitive Viral Suppression Assay

Primary CD4 T cells from HLA-A*24:02$^+$ HIV-1-negative individuals were co-infected with WT and 2F mutant viruses at a ratio of 9:1. The infected cells were incubated with or without CTL52 or CTL170 at an E:T ratio of 1:1. The culture supernatants were collected from day 3 to day 7, after which the p24 concentration was measured by ELISA (Kawashima et al., 2010). Two microliters of supernatant was directly used as the template for PCR amplification with a Superscript III One-Step RT-PCR kit (Invitrogen), and the same primer sets as described earlier were used (Fujiwara et al., 2008). The ratio of WT and 2F viruses in the supernatant was determined by the relative peak height on the sequencing electrogram obtained with sequence scanner v1.0.

### Ex Vivo Single-Cell TCR Repertoire Analysis and Assessment of TCR Diversity

RW8 cross-reactive and RF10-2F tetramer$^+$CD3$^+$CD8$^+$7-AAD$^-$ cells from cryopreserved PBMC samples were sorted into a 96-well plate by using a BD FACS Aria I. The PCR and sequencing were performed as previously described (Sun et al., 2012, 2014). 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 (Venturi et al., 2007).

### Peptide-MHC Dissociation Assay

The binding stability of four peptides to HLA-A*24:02 molecules was determined by using RMA-S-A*24:02 cells. The cells were cultured at 26°C for 14–18 hr and then continued to be incubated at 26°C for 1 hr in the presence of 250 μM synthetic RW8, RW8-2F, RF10, or RF10-2F and medium only as a negative control. Then they were incubated at 37°C for 3 hr, subsequently washed three times with cold culture medium, and thereafter suspended in culture medium. After 0, 1, 2, 4, or 6 hr, samples were taken, washed twice, and immediately stained with monoclonal antibody (mAb) A11.1 for 30 min, washed, and stained with FITC-conjugated sheep anti-mouse immunoglobulin G (IgG) as the secondary antibody (Ab). Expression of HLA-A*24:02 was determined by flow cytometry. The decay of A*24:02-peptide complexes was determined as the percent median fluorescence intensity (MFI) remaining: $(MFI_{t(+pep)} - MFI_{t(-pep)})/(MFI_{t\,=\,0(+pep)} - MFI_{t\,=\,0(-pep)})$ (Brooks et al., 1998), and the peptide binding stability was presented as the time required for 50% decay of the HLA molecules ($DT_{50}$), as calculated by linear regression analysis (Dong et al., 2004). The $^{51}Cr$ release assay was conducted as described earlier; briefly, after 0, 1, 2, 4, or 6 hr, labeled cells were harvested and washed and then used as target cells in killing assays, with an E:T ratio of 1:1. The specific lysis activity was calculated as described earlier (Fujiwara and Takiguchi, 2007).

### Crystallization, Data Collection, and Processing

Soluble peptide-HLA-A*24:02 complexes were prepared as previously described (Shi et al., 2011; Sun et al., 2014). The individual proteins were then purified by SuperdexTM 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. 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 (Minor, 1997). Data were analyzed by molecular replacement using MolRep in CCP4 (Collaborative Computational Project, Number 4, 1994). We used the A24VYG molecule (PDB: 2BCK) as the search model. All of the structures were further refined by several rounds of refinement using the PHENIX program (Adams et al., 2002). The data collection and refinement statistics are given in Table S2. The detailed interactions between HLA-A*24:02 and peptide were analyzed by using Contact in the CCP4 package (Collaborative Computational Project, Number 4, 1994). The PyMOL Molecular Graphics System (Schrödinger; http://www.pymol.org) was used to prepare figures.

### Statistical Analyses

We calculated the significance of differences for data given in Figures 1B, 3B, 3C, and 7 by using the unpaired t test; and p values of $<0.05$ were considered to be significant. Results were reported as the mean ± SD.

## ACCESSION NUMBERS

The accession numbers for the structures of pMHCs of HLA-A*24:02-RF10, HLA-A*24:02-RF10-2F, HLA-A*24:02-RW8, and the HLA-A*24:02-RW8-2F reported in this paper are PDB: 5HGH, 5HGD, 5HGB, and 5HGA, respectively.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.017.

## AUTHOR CONTRIBUTIONS

M.T. and X.S. conceived and designed the experiments. X.S., Y.S., T.A., M.F., and N.K. performed the experiments. X.S., Y.S., C.S., G.F.G., and M.T. analyzed the data; H.G. and S.O. contributed reagents, materials, and analysis tools. X.S., C.S., V.A., and M.T. contributed to the writing of the manuscript.

## ACKNOWLEDGMENTS

We thank Dr. Masaya Baba for critical reading of the manuscript and Sachiko Sakai for her secretarial assistance. This research was supported by a JSPS KAKENHI grant-in-aid for scientific research (B) (grant no. 26293240), the Ministry of Health, Labor, and Welfare of Japan: Research on HIV/AIDS (grant no. H24-AIDS-007), the Japan Agency for Medical Research and Development (grant no. 15Afk0410019), the International Research Center Aiming at the Control of AIDS Group for Globally Advanced Research at Kumamoto University, and the China Ministry of Science and Technology National 973 Project (grant nos. 2013CB531502 and 2014CB542503).

Received: October 22, 2015
Revised: April 13, 2016
Accepted: May 2, 2016
Published: May 26, 2016

## REFERENCES

Adams, P.D., Grosse-Kunstleve, R.W., Hung, L.W., Ioerger, T.R., McCoy, A.J., Moriarty, N.W., Read, R.J., Sacchettini, J.C., Sauter, N.K., and Terwilliger, T.C. (2002). PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D Biol. Crystallogr. *58*, 1948–1954.

Ahlers, J.D., and Belyakov, I.M. (2010). Lessons learned from natural infection: focusing on the design of protective T cell vaccines for HIV/AIDS. Trends Immunol. *31*, 120–130.

Akahoshi, T., Chikata, T., Tamura, Y., Gatanaga, H., Oka, S., and Takiguchi, M. (2012). Selection and accumulation of an HIV-1 escape mutant by three types of HIV-1-specific cytotoxic T lymphocytes recognizing wild-type and/or escape mutant epitopes. J. Virol. *86*, 1971–1981.

Allen, T.M., Yu, X.G., Kalife, E.T., Reyor, L.L., Lichterfeld, M., John, M., Cheng, M., Allgaier, R.L., Mui, S., Frahm, N., et al. (2005). De novo generation of escape variant-specific CD8+ T-cell responses following cytotoxic T-lymphocyte escape in chronic human immunodeficiency virus type 1 infection. J. Virol. *79*, 12952–12960.

Almeida, J.R., Price, D.A., Papagno, L., Arkoub, Z.A., Sauce, D., Bornstein, E., Asher, T.E., Samri, A., Schnuriger, A., Theodorou, I., et al. (2007). Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J. Exp. Med. *204*, 2473–2485.

Altman, J.D., and Feinberg, M.B. (2004). HIV escape: there and back again. Nat. Med. *10*, 229–230.

Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Appay, V., Douek, D.C., and Price, D.A. (2008). CD8+ T cell efficacy in vaccination and disease. Nat. Med. *14*, 623–628.

Bailey, J., Blankson, J.N., Wind-Rotolo, M., and Siliciano, R.F. (2004). Mechanisms of HIV-1 escape from immune responses and antiretroviral drugs. Curr. Opin. Immunol. *16*, 470–476.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M.S., Peffer, N., Meyers, H., Nelson, J.A., Gairin, J.E., Hahn, B.H., Oldstone, M.B., and Shaw, G.M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. *3*, 205–211.

Bronke, C., Almeida, C.A., McKinnon, E., Roberts, S.G., Keane, N.M., Chopra, A., Carlson, J.M., Heckerman, D., Mallal, S., and John, M. (2013). HIV escape mutations occur preferentially at HLA-binding sites of CD8 T-cell epitopes. AIDS *27*, 899–905.

Brooks, J.M., Colbert, R.A., Mear, J.P., Leese, A.M., and Rickinson, A.B. (1998). HLA-B27 subtype polymorphism and CTL epitope choice: studies with EBV peptides link immunogenicity with stability of the B27:peptide complex. J. Immunol. *161*, 5252–5259.

Brumme, Z.L., John, M., Carlson, J.M., Brumme, C.J., Chan, D., Brockman, M.A., Swenson, L.C., Tao, I., Szeto, S., Rosato, P., et al. (2009). HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. PLoS ONE *4*, e6687.

Busch, D.H., and Pamer, E.G. (1998). MHC class I/peptide stability: implications for immunodominance, in vitro proliferation, and diversity of responding CTL. J. Immunol. *160*, 4441–4448.

Carlson, J.M., Brumme, C.J., Martin, E., Listgarten, J., Brockman, M.A., Le, A.Q., Chui, C.K., Cotton, L.A., Knapp, D.J., Riddler, S.A., et al.; International HIV Adaptation Collaborative (2012). Correlates of protective cellular immunity revealed by analysis of population-level immune escape pathways in HIV-1. J. Virol. *86*, 13202–13216.

Carlson, J.M., Le, A.Q., Shahid, A., and Brumme, Z.L. (2015). HIV-1 adaptation to HLA: a window into virus-host immune interactions. Trends Microbiol. *23*, 212–224.

Chikata, T., Carlson, J.M., Tamura, Y., Borghan, M.A., Naruto, T., Hashimoto, M., Murakoshi, H., Le, A.Q., Mallal, S., John, M., et al. (2014). Host-specific adaptation of HIV-1 subtype B in the Japanese population. J. Virol. *88*, 4764–4775.

Choppin, J., Cohen, W., Bianco, A., Briand, J.P., Connan, F., Dalod, M., and Guillet, J.G. (2001). Characteristics of HIV-1 Nef regions containing multiple CD8+ T cell epitopes: wealth of HLA-binding motifs and sensitivity to proteasome degradation. J. Immunol. *166*, 6164–6169.

Collaborative Computational Project, Number 4 (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. *50*, 760–763.

Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D., and Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature *391*, 397–401.

Deng, K., Pertea, M., Rongvaux, A., Wang, L., Durand, C.M., Ghiaur, G., Lai, J., McHugh, H.L., Hao, H., Zhang, H., et al. (2015). Broad CTL response is required to clear latent HIV-1 due to dominance of escape mutations. Nature *517*, 381–385.

Dong, H.L., Li, Z.S., Ye, J., Qu, P., Huang, Y.Y., Wu, W., Lu, S.Y., Chen, G.S., and Sui, Y.F. (2004). Identification of HLA-A2-restricted CTL epitope encoded by the MAGE-n gene of human hepatocellular carcinoma. Cancer Biol. Ther. *3*, 891–898.

Feeney, M.E., Tang, Y., Pfafferott, K., Roosevelt, K.A., Draenert, R., Trocha, A., Yu, X.G., Verrill, C., Allen, T., Moore, C., et al. (2005). HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. J. Immunol. *174*, 7524–7530.

Fujiwara, M., and Takiguchi, M. (2007). HIV-1-specific CTLs effectively suppress replication of HIV-1 in HIV-1-infected macrophages. Blood *109*, 4832–4838.

Fujiwara, M., Tanuma, J., Koizumi, H., Kawashima, Y., Honda, K., Mastuoka-Aizawa, S., Dohki, S., Oka, S., and Takiguchi, M. (2008). Different abilities of escape mutant-specific cytotoxic T cells to suppress replication of escape mutant and wild-type human immunodeficiency virus type 1 in new hosts. J. Virol. *82*, 138–147.

Goulder, P.J., and Watkins, D.I. (2004). HIV and SIV CTL escape: implications for vaccine design. Nat. Rev. Immunol. *4*, 630–640.

Harndahl, M., Rasmussen, M., Roder, G., Dalgaard Pedersen, I., Sørensen, M., Nielsen, M., and Buus, S. (2012). Peptide-MHC class I stability is a better predictor than peptide affinity of CTL immunogenicity. Eur. J. Immunol. *42*, 1405–1416.

Huang, K.H., Goedhals, D., Carlson, J.M., Brockman, M.A., Mishra, S., Brumme, Z.L., Hickling, S., Tang, C.S., Miura, T., Seebregts, C., et al.; Bloemfontein-Oxford Collaborative Group (2011). Progression to AIDS in South Africa is associated with both reverting and compensatory viral mutations. PLoS ONE *6*, e19018.

Iglesias, M.C., Almeida, J.R., Fastenackels, S., van Bockel, D.J., Hashimoto, M., Venturi, V., Gostick, E., Urrutia, A., Wooldridge, L., Clement, M., et al. (2011). Escape from highly effective public CD8+ T-cell clonotypes by HIV. Blood *118*, 2138–2149.

Ikeda-Moore, Y., Tomiyama, H., Miwa, K., Oka, S., Iwamoto, A., Kaneko, Y., and Takiguchi, M. (1997). Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J. Immunol. *159*, 6242–6252.

Karaki, S., Kariyone, A., Kato, N., Kano, K., Iwakura, Y., and Takiguchi, M. (1993). HLA-B51 transgenic mice as recipients for production of polymorphic HLA-A, B-specific antibodies. Immunogenetics *37*, 139–142.

Kawashima, Y., Pfafferott, K., Frater, J., Matthews, P., Payne, R., Addo, M., Gatanaga, H., Fujiwara, M., Hachiya, A., Koizumi, H., et al. (2009). Adaptation of HIV-1 to human leukocyte antigen class I. Nature *458*, 641–645.

Kawashima, Y., Kuse, N., Gatanaga, H., Naruto, T., Fujiwara, M., Dohki, S., Akahoshi, T., Maenaka, K., Goulder, P., Oka, S., and Takiguchi, M. (2010). Long-term control of HIV-1 in hemophiliacs carrying slow-progressing allele HLA-B*5101. J. Virol. *84*, 7151–7160.

Koup, R.A., Safrit, J.T., Cao, Y., Andrews, C.A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D.D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. *68*, 4650–4655.

Kuse, N., Akahoshi, T., Gatanaga, H., Ueno, T., Oka, S., and Takiguchi, M. (2014). Selection of TI8-8V mutant associated with long-term control of HIV-1 by cross-reactive HLA-B*51:01-restricted cytotoxic T cells. J. Immunol. *193*, 4814–4822.

Ladell, K., Hashimoto, M., Iglesias, M.C., Wilmann, P.G., McLaren, J.E., Gras, S., Chikata, T., Kuse, N., Fastenackels, S., Gostick, E., et al. (2013). A molecular basis for the control of preimmune escape variants by HIV-specific CD8+ T cells. Immunity *38*, 425–436.

Lichterfeld, M., Kavanagh, D.G., Williams, K.L., Moza, B., Mui, S.K., Miura, T., Sivamurthy, R., Allgaier, R., Pereyra, F., Trocha, A., et al. (2007). A viral CTL escape mutation leading to immunoglobulin-like transcript 4-mediated functional inhibition of myelomonocytic cells. J. Exp. Med. *204*, 2813–2824.

Liu, J., Dai, L., Qi, J., Gao, F., Feng, Y., Liu, W., Yan, J., and Gao, G.F. (2011). Diverse peptide presentation of rhesus macaque major histocompatibility complex class I Mamu-A 02 revealed by two peptide complex structures and insights into immune escape of simian immunodeficiency virus. J. Virol. *85*, 7372–7383.

Melhem, N.M., Smith, K.N., Huang, X.L., Colleton, B.A., Jiang, W., Mailliard, R.B., Mullins, J.I., and Rinaldo, C.R. (2014). The impact of viral evolution and frequency of variant epitopes on primary and memory human immunodeficiency virus type 1-specific $CD8^+$ T cell responses. Virology *450-451*, 34–48.

Minor, Z.O.W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. *276*, 307–326.

Moore, C.B., John, M., James, I.R., Christiansen, F.T., Witt, C.S., and Mallal, S.A. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science *296*, 1439–1443.

Nikolich-Zugich, J., Slifka, M.K., and Messaoudi, I. (2004). The many important facets of T-cell repertoire diversity. Nat. Rev. Immunol. *4*, 123–132.

Rousseau, C.M., Daniels, M.G., Carlson, J.M., Kadie, C., Crawford, H., Prendergast, A., Matthews, P., Payne, R., Rolland, M., Raugi, D.N., et al. (2008). HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load. J. Virol. *82*, 6434–6446.

Saito, S., Ota, S., Yamada, E., Inoko, H., and Ota, M. (2000). Allele frequencies and haplotypic associations defined by allelic DNA typing at HLA class I and class II loci in the Japanese population. Tissue Antigens *56*, 522–529.

Schmitz, J.E., Kuroda, M.J., Santra, S., Sasseville, V.G., Simon, M.A., Lifton, M.A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B.J., et al. (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science *283*, 857–860.

Shi, Y., Qi, J., Iwamoto, A., and Gao, G.F. (2011). Plasticity of human $CD8\alpha\alpha$ binding to peptide-HLA-A*2402. Mol. Immunol. *48*, 2198–2202.

Shimizu, A., Kawana-Tachikawa, A., Yamagata, A., Han, C., Zhu, D., Sato, Y., Nakamura, H., Koibuchi, T., Carlson, J., Martin, E., et al. (2013). Structure of TCR and antigen complexes at an immunodominant CTL epitope in HIV-1 infection. Sci. Rep. *3*, 3097.

Sijts, A.J., and Pamer, E.G. (1997). Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes. J. Exp. Med. *185*, 1403–1411.

Sun, X., Saito, M., Sato, Y., Chikata, T., Naruto, T., Ozawa, T., Kobayashi, E., Kishi, H., Muraguchi, A., and Takiguchi, M. (2012). Unbiased analysis of $TCR\alpha/\beta$ chains at the single-cell level in human CD8+ T-cell subsets. PLoS ONE *7*, e40386.

Sun, X., Fujiwara, M., Shi, Y., Kuse, N., Gatanaga, H., Appay, V., Gao, G.F., Oka, S., and Takiguchi, M. (2014). Superimposed epitopes restricted by the same HLA molecule drive distinct HIV-specific CD8+ T cell repertoires. J. Immunol. *193*, 77–84.

Takamiya, Y., Schönbach, C., Nokihara, K., Yamaguchi, M., Ferrone, S., Kano, K., Egawa, K., and Takiguchi, M. (1994). HLA-B*3501-peptide interactions: role of anchor residues of peptides in their binding to HLA-B*3501 molecules. Int. Immunol. *6*, 255–261.

Tanuma, J., Fujiwara, M., Teruya, K., Matsuoka, S., Yamanaka, H., Gatanaga, H., Tachikawa, N., Kikuchi, Y., Takiguchi, M., and Oka, S. (2008). HLA-A*2402-restricted HIV-1-specific cytotoxic T lymphocytes and escape mutation after ART with structured treatment interruptions. Microbes Infect. *10*, 689–698.

Tomiyama, H., Akari, H., Adachi, A., and Takiguchi, M. (2002). Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8(+) T-cell cytolytic activity and cytokine production. J. Virol. *76*, 7535–7543.

Tomiyama, H., Fujiwara, M., Oka, S., and Takiguchi, M. (2005). Cutting Edge: Epitope-dependent effect of Nef-mediated HLA class I down-regulation on ability of HIV-1-specific CTLs to suppress HIV-1 replication. J. Immunol. *174*, 36–40.

Ueno, T., Idegami, Y., Motozono, C., Oka, S., and Takiguchi, M. (2007). Altering effects of antigenic variations in HIV-1 on antiviral effectiveness of HIV-specific CTLs. J. Immunol. *178*, 5513–5523.

van der Burg, S.H., Visseren, M.J., Brandt, R.M., Kast, W.M., and Melief, C.J. (1996). Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J. Immunol. *156*, 3308–3314.

Venturi, V., Kedzierska, K., Turner, S.J., Doherty, P.C., and Davenport, M.P. (2007). Methods for comparing the diversity of samples of the T cell receptor repertoire. J. Immunol. Methods *321*, 182–195.

Vitagliano, L., Berisio, R., and De Simone, A. (2011). Role of hydration in collagen recognition by bacterial adhesins. Biophys. J. *100*, 2253–2261.