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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.

Cytotoxic 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 MHC class I 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 FY11 (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.

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 × 10^6 irradiated feeder PBMCs from healthy donors and 1 × 10^6 irradiated C1R-A*2402 cells pre pulsed 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 pre pulsed 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 FACS Canto 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 directed mutagenesis to carry the RYPLTFGWCF sequence (17). The C1R cell line is a human B cell lymphoblastoid line lacking surface expression of HLA-A and partially HLA-B molecules.

**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 by using a FACS Canto 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.

**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 γδ3 mAb (Mab22, 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 directed mutagenesis to carry the RYPLTFGWCF sequence (17). The C1R cell line is a human B cell lymphoblastoid line lacking surface expression of HLA-A and partially HLA-B molecules.

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**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–infected HLA-A*24:02+ patients, the frequencies of RW8 and RF10 tetramer+CD3+CD8+ T cells were 0.080 ± 0.009 and 0.045 ± 0.022% (mean ± SD), respectively. We evaluated the mean ± 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, dashed line). 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 elicted in HLA-A*24:02+ individuals with a chronic HIV-1 infection; however,
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- and 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
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 \( \alpha \)- and \( \beta \)-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 \( \alpha \)- and \( \beta \)-chains) among RW8-specific CD8\(^+\) T cells was significantly lower than for RF10-specific ones (Fig. 4). Overall, the differences in \( \alpha \)- and \( \beta \)-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*

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**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 \( \alpha \)- and \( \beta \)-chain sequencing was performed. Paired TCR \( \alpha \)- and \( \beta \)-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*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 Phe10 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 Lys66 and another hydrogen bond between the P1 residue and Arg170 were also observed in the RF10 peptide. Moreover, whereas the Pc residue Trp8 of the RW8 peptide formed four hydrogen bonds with the HLA molecule, the subanchor residue Trp8 and Pc residue Phe10 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 HLA1 molecules.

Table I. Statistics for crystallographic data collection and structure refinement

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

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.
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 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 EC50 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.
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, RYPLTGFWGCF (RF10) presented two epitopes to HLA-A*2402, 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 K18 and KFI0 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 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 different 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 to 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*2402–restricted CTLs specific for superimposed Nef epitopes, RF10 and RW8, by using multiple approaches. We demonstrated that RW8 and RF10 peptides bound to HLA-A*2402, 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.

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Disclosures
The authors have no financial conflicts of interest.

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