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HIV-1–Specific CD8 T Cells Exhibit Limited Cross-Reactivity during Acute Infection

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Prior work has demonstrated that HIV-1–specific CD8 T cells can cross-recognize variant epitopes. However, most of these studies were performed in the context of chronic infection, where the presence of viral quasispecies makes it difficult to ascertain the true nature of the original antigenic stimulus. To overcome this limitation, we evaluated the extent of CD8 T cell cross-reactivity in patients with acute HIV-1 clade B infection. In each case, we determined the transmitted founder virus sequence to identify the autologous epitopes restricted by individual HLA class I molecules. Our data show that cross-reactive CD8 T cells are infrequent during the acute phase of HIV-1 infection. Moreover, in the uncommon instances where cross-reactive responses were detected, the variant epitopes were poorly recognized in cytotoxicity assays. Molecular analysis revealed that similar antigenic structures could be cross-recognized by identical CD8 T cell clonotypes mobilized in vivo, yet even subtle differences in a single TCR-accessible peptide residue were sufficient to disrupt variant-specific reactivity. These findings demonstrate that CD8 T cells are highly specific for autologous epitopes during acute HIV-1 infection. Polyvalent vaccines may therefore be required to provide optimal immune cover against this genetically labile pathogen.

Although antiretroviral therapy (ART) effectively controls HIV-1 replication and reduces AIDS-associated mortality (1), the infection remains a major threat to public health. This statement is exemplified by the fact that the number of new cases has remained largely unchanged in the United States despite the availability of ART for nearly two decades (2).

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The coordinates presented in this article have been submitted to the Protein Data Bank under accession numbers 5E00 (for HLA-B*07:02:RFL9) and 5EO1 (for HLA-B*07:02:RFL9).

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; ART, antiretroviral therapy; HLA-I, HLA class I; LANL, Los Alamos National Laboratory; pHLa, peptide-HLA; SFU, spot-forming unit; TFV, transmitted founder virus.

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Moreover, although the emergence of pre-exposure prophylaxis has been shown to reduce the risk of infection, it involves issues related to consistent adherence (3). An effective HIV-1 vaccine is therefore required to gain better control of the epidemic through the prevention of transmission and to provide assistance in the treatment of infected individuals who currently require daily ART.

It is well documented that CD8 T cells are necessary components of an effective immune response against HIV-1 (4–8). However, efforts aimed at developing protective T cell–based vaccines have not been successful in terms of either preventing infection or impacting disease progression in vaccinees who become infected (9–11). One possible reason for these failures is the ability of HIV-1 to mutate rapidly and evade CD8 T cell recognition (8, 12–17). Indeed, this process accounts for much of the genetic diversity observed within the circulating viral population (8, 14). Given the frequency and predictability of these mutations (8, 12–16, 18, 19), it is conceivable that CD8 T cells capable of targeting multiple variant forms of an epitope would enhance immune control of HIV-1 replication.

In the presence of a limited TCR repertoire (20), cross-reactivity against variant Ags may be a critical feature of effective virus-specific CD8 T cell populations (21–23). Several studies have therefore attempted to correlate the degree of cross-reactivity with HIV-1 control (23–29). However, this assessment is complicated in the setting of chronic infection, where heterogeneous viral quasispecies obscure the true identity of the infecting epitope (23–25, 28). In contrast, CD8 T cell cross-reactivity has not been studied extensively in acute HIV-1 infection. Prior works in this area have focused primarily on cross-clade recognition in the context of overlapping peptides (30–32), which still confounds the identification of autologous versus cross-reactive responses to specific epitopes. Thus, the extent and frequency of CD8 T cell cross-reactivity during the acute phase of infection is not yet known.

In this study, we examined the ability of virus-specific CD8 T cells to cross-recognize naturally occurring epitope variants in a
cohort of patients with acute HIV-1 clade B infection. The transmitted founder virus (TFV) sequence was determined in each case to define the true autologous epitopes for each individual HLA class I (HLA-I) molecule. Our data show that cross-reactive CD8 T cells are both uncommon and functionally impaired during acute HIV-1 infection. These findings suggest that monovalent vaccines may not induce optimal immune cover against this genetically diverse virus.

Materials and Methods

HIV-1 patient cohort

PBMCs and plasma samples were obtained from 11 patients acutely infected with HIV-1 clade B. Acute infection was diagnosed at the first screening visit by detectable HIV-1 viral RNA in plasma and a lack of HIV-specific HIV-1 RNA on blot (33). TFV sequences were inferred from the plasma of these 11 patients at Fiebig stage III or earlier using a single genome amplification method, as described previously (34). Another patient with acute infection, contracted after immunization with an experimental canarypox-vectored HIV-1 vaccine (35), was diagnosed at Fiebig stage V, thereby precluding accurate determination of the TFV by single genome amplification analysis. Population-based viral sequencing was performed to determine the autologous HIV-1 sequence in this patient (35). Immunogenicity studies were conducted using PBMCs obtained at a median of 31 d (range, 16–60 d) after the estimated date of infection (Table I). All patients were recruited from the University of Alabama at Birmingham HIV Infection Clinic after obtaining written informed consent and approval from the University of Alabama at Birmingham Institutional Review Board for Human Use.

Peptide selection

Autologous peptides were designed for each acutely infected patient based on HLA-I genotype and the TFV sequence, with reference to optimal HLA-I–restricted epitopes described in the HIV Los Alamos National Laboratory (LANL) Database (http://www.hiv.lanl.gov/content/immunology/pdf/2009/optimal CTL article.pdf). All peptides relevant to each individual HLA-I allele and TFV sequence were determined. For each immunogenic autologous epitope, defined by IFN-γ ELISPOT, a prior study (Carlson et al., manuscript in preparation), two to eight common variants (QuickAlign from the LANL) were selected for further analysis. These variants represented the top 5–10 most commonly occurring epitope mutations in relationships to the autologous form (data available at QuickAlign from the LANL). A list of all autologous epitopes and variants evaluated for cross-reactivity is shown in Supplemental Table I. Autologous or cross-reactive variant epitopes were defined as non-escaped when they represented the most common sequence found in the circulating HIV-1 clade B population (QuickAlign from the LANL) in the absence of predicted HLA-I–associated polymorphisms (13). All other epitopes were classified as escaped.

**Table I. Clinical and HLA-I data**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Fiebig</th>
<th>DPT</th>
<th>Log VL</th>
<th>CD4</th>
<th>HLA-I Alleles</th>
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<td>PHI-1</td>
<td>II</td>
<td>31</td>
<td>5.53</td>
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<td>31</td>
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<td>51</td>
<td>5.40</td>
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<td>42</td>
<td>4.74</td>
<td>932</td>
<td>03:01, 66:01, 42:01, 58:02, 06:02, 17:01</td>
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<td>I</td>
<td>33</td>
<td>7.26</td>
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<td>32</td>
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<td>237</td>
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<td>5.23</td>
<td>286</td>
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<td>I</td>
<td>16</td>
<td>6.45</td>
<td>719</td>
<td>68:01, 68:01, 15:03, 58:02, 02:10, 06:02</td>
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<tr>
<td>PHI-10</td>
<td>I</td>
<td>21</td>
<td>6.00</td>
<td>472</td>
<td>02:01, 03:01, 07:02, 14:02, 07:02, 08:01</td>
</tr>
<tr>
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<td>I</td>
<td>20</td>
<td>6.68</td>
<td>164</td>
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<td>60</td>
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<td>321</td>
<td>02, 02, 27, 44, 02, 05</td>
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</tbody>
</table>
cells were stained with a Live/Dead dye (Invitrogen), anti-CD3-Alexa Fluor 780 (eBioscience), and anti-CD8-PE (BD Biosciences). The cells were then permeabilized and labeled with anti-IFN-γ-Alexa Fluor 700, anti-IL-2-allophycocyanin, anti-TNF-α-PE-Cy7, and anti-granzyme B-V450 (all from BD Biosciences). At least 100,000 CD8+ events were acquired on an LSR II flow cytometer (BD Immunocytometry Systems), and data were analyzed using FlowJo software (version 9.6.4; Tree Star). Polyfunctional analysis was performed using Boolean gating with SFICE and PESTLE software (version 5.1; National Institute of Allergy and Infectious Diseases).

Cytotoxicity assay

A killing assay based on 7-aminoactinomycin D (7-AAD) staining was performed according to a modified protocol derived from prior studies (29, 41). This assay directly measures target cell apoptosis (29, 42) as an indicator of CD8 T cell cytotoxicity. To avoid repeated exposure to cytokine stimulation, PBMCs from HIV-1–seronegative donors were used as targets. The HIV-1–seronegative donors were matched at the relevant HLA-I allele restricting the peptide of interest. CD4 T cells were enriched from these PBMCs by magnetic depletion of CD8 T cells (Dynabeads CD8; Invitrogen) and activated for 2 d with PHA (5 μg/ml) in the presence of IL-2 (100 U/ml). Activated CD4 T cell targets (1 × 10^5) were then pulsed for 1 h with the relevant autologous HIV-1–peptide or variants thereof (10 μM). An irrelevant peptide pool representing epitopes derived from CMV, EBV, and influenza virus (2 μg/ml per peptide; National Institutes of Health AIDS Reagents Program) was used as a negative control. Peptide-pulsed target cells were cocultured with the appropriate epitope-specific CD8 T cell lines for 24 h at different E:T ratios as indicated and subsequently stained with anti–CD3-Pacific Blue (BD Biosciences) and anti–CD4-Alexa Fluor 780 (eBioscience). The cells were then washed, stained with 7-AAD (0.25 μg/ml; BD Biosciences) for 20 min at 4°C, and analyzed by flow cytometry. Target cell killing by epitope-specific CD8 T cell lines was determined using the following formula adapted from a previous description (41): % cell death = [(100 × % 7AAD+ target CD4 with effector − % 7AAD+ target CD4 without effector)/100].

Crystalization, data collection, and structure determination

Soluble complexes of HLA-B*07:02 with the Nef RL9 peptide (RPMTFKY K G) or the single-substituted variant RFL9 (RPMTFKY KAL) were prepared as described previously (43). Briefly, the H chain of HLA-B*07:02 and β2-microglobulin were expressed separately in Escherichia coli and purified from inclusion bodies. After refolding in vitro, peptide–HLA (pHLA) complexes were purified using ion exchange chromatography. Crystals of pHLA (5 mg/ml) in 10 mM Tris-HCl (pH 8) and 150 mM NaCl were grown by the hanging-drop vapor diffusion method at 20°C with a protein/reservoir drop ratio of 1:1. Crystals were formed in 18% polyethylene glycol 4000, 0.1 M ammonium acetate, and 0.1 M sodium cacodylate at pH 6.5. Crystals were soaked in a cryoprotectant solution containing mother liquor with the polyethylene glycol 4000 concentration increased to 30% (w/v) and flash frozen in liquid nitrogen. Crystallographic data were collected on the microfocus beamline MX2 at the Australian Synchrotron (Clayton, VIC, Australia) using the ADSC Quantum 315r detector (at 100K). Data were processed as described previously (44, 45). The final models were validated using the Protein Data Bank validation site (http://www.rcsb.org/pdb), and the final refinement statistics are summarized in Table II. Coordinates were submitted to the Protein Data Bank with codes 5EO0 for HLA-B*07:02-RFL9 and 5EO1 for HLA-B*07:02-RFL9. Molecular graphics were created using PyMol software.

Thermal stability assay

The stability of pHLA complexes was determined using a thermal shift assay performed in a real-time detection system (Rotor-Gene 3000; Corbett Life Science). Protein unfolding was monitored with the fluorescent dye Sypro Orange (Sigma-Aldrich). Two concentrations (5 and 10 μM) of each pHLA complex in 10 mM Tris-HCl (pH 8), 150 mM NaCl were heated from 30 to 95°C at a rate of 1°C/min. Fluorescence intensity was measured with excitation at 530 nm and emission at 555 nm. The thermal melting point represents the temperature at which 50% of the protein is unfolded. Experiments were carried out in quadruplicate.

Molecular analysis of TCR usage

Clonotypic analysis of epitope-specific CD8 T cell populations was performed as described previously with minor modifications (46). Briefly, viable tetramer+ CD8 T cell populations were sorted directly ex vivo by flow cytometry at >98% purity, and all expressed TBB gene products were amplified without bias using a template-switch anchored RT-PCR (47). Amplicons were then subcloned, sequenced, and analyzed in accordance with IMGT nomenclature (48).

Statistical analysis

Statistical evaluations were conducted using a Fisher exact test, an area-under-the-curve–based paired t test, the Wilcoxon matched-pairs signed-rank test, and a Spearman rank correlation. All p values were calculated using GraphPad Prism software (version 6.0). Significance was assigned at p values <0.05.

Results

CD8 T cells are poorly cross-reactive during acute HIV-1 infection

In the present study, we evaluated the extent of CD8 T cell cross-reactivity and the qualitative features of cross-reactive responses elicited during acute HIV-1 infection. Immunogenicity data from 11 acutely infected patients showed a total of 18 autologous responses from 87 tested within PBMCs (49). The 18 autologous epitopes are listed in Supplemental Table I. Each of these epitope-specific responses was subsequently analyzed for cross-recognition of the most frequently occurring variants in an IFN-γ ELISpot assay. Only 5 of the 18 CD8 T cell responses were cross-reactive (Fig. 1A, Supplemental Table I). However, these five autologous responses collectively cross-recognized 15 of 29 epitope variants (Fig. 1B, Supplemental Table I). Thus, although only a minority of autologous epitopes induce cross-reactive responses during acute HIV-1 infection, variant cross-recognition is extensive among the resultant CD8 T cell populations.

We also separated the data according to whether CD8 T cell responses were induced in vivo by epitopes without evidence of escape (nonescape epitope) or epitopes with evidence of escape (escaped epitope) prior to infection (see Materials and Methods). Of the five autologous CD8 T cell responses deemed to be cross-reactive, only one was induced by an escaped epitope (Fig. 1A, Supplemental Table I). Furthermore, the four nonescaped epitope-specific CD8 T cell responses cross-recognized significantly greater numbers of variants compared with the single escaped epitope-specific CD8 T cell response (14 of 21 versus 1 of 8, respectively; p = 0.01; Fig. 1B).

Cross-reactive killing of HIV-1–specific targets is compromised during acute infection

A pertinent quality of HIV-1–specific CD8 T cells is the ability to kill infected targets (36, 39). To address whether cross-reactive responses were similarly cytotoxic, we generated CD8 T cell lines specific for three HLA-B*07:02–restricted epitopes: Rev KGAR77–85, Nef RFL9 (RPMTFKY K G AL) were prepared and pu-rified from inclusion bodies. After refolding in vitro, peptide–HLA (pHLA) complexes were purified using ion exchange chromatography. Crystals of pHLA (5 mg/ml) in 10 mM Tris-HCl (pH 8) and 150 mM NaCl were grown by the hanging-drop vapor diffusion method at 20°C with a protein/reservoir drop ratio of 1:1. Crystals were formed in 18% polyethylene glycol 4000, 0.1 M ammonium acetate, and 0.1 M sodium cacodylate at pH 6.5. Crystals were soaked in a cryoprotectant solution containing mother liquor with the polyethylene glycol 4000 concentration increased to 30% (w/v) and flash frozen in liquid nitrogen. Crystallographic data were collected on the microfocus beamline MX2 at the Australian Synchrotron (Clayton, VIC, Australia) using the ADSC Quantum 315r detector (at 100K). Data were processed as described previously (44, 45). The final models were validated using the Protein Data Bank validation site (http://www.rcsb.org/pdb), and the final refinement statistics are summarized in Table II. Coordinates were submitted to the Protein Data Bank with codes 5EO0 for HLA-B*07:02-RFL9 and 5EO1 for HLA-B*07:02-RFL9. Molecular graphics were created using PyMol software.
Autologous and cross-reactive CD8 T cells exhibit similar ex vivo polyfunctionality

Prior studies have implicated a role for CD8 T cell polyfunctionality in the control of HIV-1 replication (50, 51). We therefore examined ex vivo functional responses elicited by paired autologous and cross-reactive epitopes. Representative flow cytometry plots depicting cytotoxic responses (IFN-γ/CD107a and IFN-γ/granzyme B) for an autologous/variant RL9 pair are shown in Fig. 3A (the corresponding 7-AAD data are illustrated in Fig. 2C). The overall polyfunctional profile (IFN-γ, IL-2, TNF-α, CD107a, and granzyme B) was similar in response to the autologous and cross-reactive epitopes (Fig. 3B). Moreover, robust cytotoxic responses were observed for both the autologous and cross-reactive epitopes tested against RL10-specific CD8 T cell lines in vitro, albeit at lower magnitude for one of the variants (RP\textsubscript{EPVPFQL}; Supplemental Fig. 1A). This particular variant was associated with minimal killing activity (Fig. 2B). Taken together, these data reveal a poor correlation between cytotoxic activity and polyfunctionality in response to cross-reactive epitopes.

Cross-reactive epitopes bind HLA-I with similar predicted affinities

Prior studies have indicated that HLA-I binding correlates directly with immunogenicity and may contribute to the generation of potent antiviral CD8 T cells (52, 53). Using NetMHC software, we assessed the predicted HLA-I binding affinity (peptide concentration required for 50% binding, or IC\textsubscript{50}) for the five founder epitopes with cross-reactive responses and their corresponding variants. We did not detect a statistically significant difference in binding affinity between autologous and variant epitopes that elicited a response by IFN-γ ELISPOT (Fig. 4).
However, nonimmunogenic variants displayed significantly lower predicted binding affinities compared with the corresponding autologous epitopes \((p = 0.02; \text{Fig. 4})\). These data suggest that HLA-I binding is an important prerequisite for variant cross-reactivity but fail to provide an explanation for impaired cytotoxicity.

**Poor cross-reactive killing is associated with low Ag sensitivity**

The ability of CD8 T cells to respond at low Ag concentrations has been associated with enhanced elimination of infected targets and is an important feature of HIV-1 control \((25, 38, 54, 55)\). As previous studies have linked Ag sensitivity to TCR recognition of pHLA \((25, 38, 54, 56)\), we indirectly assessed whether inferior cross-reactive targeting might be associated with poor TCR avidity by measuring Ag sensitivity directly ex vivo (see Materials and Methods). Cross-reactive CD8 T cell responses against most of the epitope variants evaluated for cytotoxic activity required higher peptide concentrations (lower Ag sensitivity) to stimulate a 50% maximal IFN-\(\gamma\) response \((\text{Fig. 5A, 5B})\). The autologous RFL9 epitope-specific response, which exhibited similar target killing to its cross-reactive counterpart \((\text{Fig. 2D})\), displayed comparable Ag sensitivity for the variant \((\text{Fig. 5C})\). Similar patterns emerged when we examined the absolute magnitude of the IFN-\(\gamma\) response at each peptide concentration \((\text{Supplemental Fig. 2A–C})\). By estimating EC\(_{50}\) (peptide concentration stimulating 50% maximal response), we saw a trend toward higher values for the epitope variants \((p = 0.06; \text{Fig. 5D})\). Additionally, we observed a significant negative correlation between EC\(_{50}\) and CD8 T cell–mediated target killing assessed from area under the curve \((r^2 = 0.53, p = 0.04; \text{Fig. 5E})\). These results indicate that the impaired cytotoxic activity of cross-reactive CD8 T cells is associated with lower levels of Ag sensitivity.

**Weakly cross-reactive variants fail to downregulate CD8**

As CD8 downregulation after antigenic stimulation is associated with enhanced Ag recognition and target killing \((29, 57)\), we analyzed CD8 expression by RL10 epitope-specific T cells in vitro following exposure to autologous and variant peptides (the corresponding 7-AAD data are shown in Fig. 2B). Only the variant that elicited the weakest cytotoxic response \((\text{RP}^{\text{EPVPFQL}})\) failed to downregulate CD8 expression \((\text{Supplemental Fig. 1B})\).

**CD8 T cells targeting the HLA-B*27:05–restricted KK10 epitope can exhibit limited cross-reactivity during acute infection**

Prior studies have suggested that protective HLA-I molecules restrict cross-reactive T cell responses that may contribute to HIV-1 control \((23, 24, 26)\). In particular, the HLA-B*27 allele is thought to exert its protective effect via targeting of the immunodominant p24 Gag epitope KK10 \((\text{KRWIILGLNK}_{263–272})\) \((26, 58–60)\). The
The ability of KK10-specific CD8 T cells to cross-recognize common variants has also been implicated in the control of viral replication during chronic infection (26). None of the 11 patients from whom TFV sequences were derived possessed protective HLA-I alleles. We therefore obtained samples from an HLA-B*27+ patient diagnosed with acute infection after receiving an experimental HIV-1 vaccine (35). The TFV sequence could not be resolved in this patient because his infection was identified at Fiebig stage V. However, population-based sequencing predicted that the wild-type KK10 epitope was dominant from the time of HIV-1 acquisition until 32 mo postinfection (35). This patient mounted robust CD8 T cell responses against KK10 (35), akin to observations in other HLA-B*27+ subjects acutely infected with HIV-1 clade B (60). In an IFN-γ ELISPOT assay, we tested KK10-specific CD8 T cells from this individual for cross-reactivity against two epitope variants with stable HLA-I binding (26, 35, 61). Positive responses were detected in each case (data not shown). We then generated a KK10-specific CD8 T cell line to evaluate cytotoxic activity. CD4 T cells pulsed with the variant peptides were killed poorly relative to the autologous epitope (Fig. 6A). Similarly, cross-recognition of both variants was associated with impaired ex vivo Ag sensitivity (Fig. 6B, Supplemental Fig. 2D). These data suggest that even cross-reactive CD8 T cells restricted by a protective HLA-I allele can be compromised in their ability to kill infected targets during primary HIV-1 infection.

Molecular similarities enable TCR-mediated cross-reactivity

To understand the molecular determinants of cross-reactivity, we focused on the HLA-B*07:02–restricted Nef epitopes RL9 (RPMTYKGAL77–85) and RFL9 (RPMTFKGAL77–85), both of which were autologous sequences tested for cross-reactivity in two different individuals (Fig. 2C, 2D). RL9-specific CD8 T cells failed to cross-recognize RFL9, whereas RFL9-specific CD8 T cells effectively cross-recognized RL9 (Supplemental Table I). In thermal stability assays, no significant differences were detected between the RL9-HLA-B*07:02 complex (63.8 ± 0.9°C) and the RFL9-HLA-B*07:02 complex (60.9 ± 1.4°C). These findings suggest that neither Tyr nor Phe at peptide position 5 affect pHLA stability.

Next, we solved the crystal structures of RL9–HLA-B*07:02 and RFL9–HLA-B*07:02 at 1.85 and 1.70 Å resolution, respectively (Fig. 7A, 7B, Table II). Superimposition of the HLA-B*07:02 binding clefts showed no movement, with a root mean square distance of 0.12 Å (Fig. 7C). The different peptides also adopted a similar...
conformation (root mean square distance of 0.15 Å) (Fig. 7D). Of note, the distinct residues at position 5 (Tyr and Phe) were solvent exposed and therefore available for TCR-mediated contacts.

The structural similarities between RL9–HLA-B*07:02 and RFL9–HLA-B*07:02 suggest that specific TCRs may be able to cross-recognize both complexes. To test this hypothesis, we conducted a molecular analysis of TCR expression in tetramer+CD8+ T cell populations isolated directly ex vivo from an acutely infected individual with comparable responses to the autologous RFL9 and variant RL9 epitopes (Fig. 2D). The corresponding repertoires were almost identical, dominated in each case by a TRBV7-9/CASSLALGTQVAFF/TRBJ1-1 clonotype (Table III). These data indicate that structurally conserved variants can be cross-recognized by the initially mobilized autologous epitope-specific CD8 T cell population in the context of acute HIV-1 infection.

Enhanced CD8 T cell cross-reactivity during chronic HIV-1 infection

Prior reports have suggested that the CD8 T cell repertoire expands and diversifies during persistent viral infection and may therefore become more cross-reactive (26, 62, 63). All 12 patients in our cohort were placed on ART shortly after acute infection, but 6 individuals still had detectable viremia (median viral load, 29,666 plasma HIV-1 RNA copies/ml; range, 87–132,000), likely due to adherence difficulties with the complicated regimen prescribed in this study (64). To evaluate the possibility that cross-reactivity may develop with time, we quantified variant-specific responses during chronic infection in these six patients. Although we did not detect responses to epitope variants during acute infection, we observed an increase in cross-reactivity at chronic time points in four of six patients (Fig. 8A–F). This increase was significant across all comparisons (p = 0.0003; Fig. 8G). Due to the likely presence of viral quasispecies during chronic infection, however, these responses may represent the de novo emergence of variant-specific CD8 T cells.

Discussion

Cross-reactivity is a potentially desirable attribute of Ag-specific CD8 T cells operating within a finite immune system (21, 22) and has been associated with responses that are likely to control

FIGURE 6. CD8 T cells targeting the HLA-B*27:05–restricted KK10 epitope exhibit impaired cross-reactivity during acute infection. CD8 T cell lines specific for the HLA-B*27:05–restricted KK10 epitope (KRWIILGLNK) were derived from an acutely infected individual and evaluated for cytolytic activity against peptide-pulsed HLA-B*27:05–matched CD4 T cell targets in a 7-AAD staining assay (A). Sequences of the autologous and variant peptides are indicated in the boxes. The corresponding ex vivo Ag sensitivity data are shown in (B).

FIGURE 7. Structural comparison of cross-recognized Nef-derived peptides bound to HLA-B*07:02. The structures of RL9 (RPMTYKGAL) and RFL9 (RPMTFKGAL) in complex with HLA-B*07:02 were solved by x-ray crystallography (Table II). (A) RFL9 (magenta sticks) or (B) RL9 (blue sticks) are shown in the Ag-binding cleft (gray cartoon) of HLA-B*07:02. Position 1 (P1) arginine, P5 tyrosine or phenylalanine, and P9 leucine are labeled. (C) Overlay of the Ag-binding cleft (residues 1–180) of HLA-B*07:02 (light pink cartoon) bound to RFL9 (magenta sticks) and HLA-B*07:02 (light blue cartoon) bound to RL9 (blue sticks). P1 arginine, P5 tyrosine or phenylalanine, and P9 leucine are labeled. (D) Side view of the overlay showing RFL9 (magenta sticks) and RL9 (blue sticks) bound to HLA-B*07:02 (gray).
HIV-1 (24, 25). However, this phenomenon is not well understood during acute infection, where naturally elicited HIV-1–specific CD8 T cells suppress the initial viremia and dictate long-term outcome (4, 7). To address this knowledge gap, we performed a comprehensive evaluation of CD8 T cell cross-reactivity in patients with acute HIV-1 clade B infection. Importantly, we also determined the TFV sequence in each case. The low frequency of epitope mutations in patients identified at Fiebig stage III or earlier facilitated the accurate identification of immunodominant CD8 T cell responses (65, 66). Our results suggest that cross-reactive CD8 T cells are infrequent during acute HIV-1 infection. Moreover, in the uncommon instances where cross-reactive responses were detected, the variant epitopes were poorly recognized in cytotoxicity assays. These findings applied to both neutral (HLA-B*07) and protective (HLA-B*27) restriction elements.

The inferior cytotoxic capacity of cross-reactive CD8 T cells during acute HIV-1 infection likely reflects the exquisite specificity of the immune system, although differences in epitope processing and HLA-I binding may also play a role (5, 52, 67–70). Although we did not assess Ag processing efficacy, we did find that nonimmunogenic epitope variants bound HLA-I with lower affinities, at least as determined in silico. In contrast, cross-recognized variants exhibited HLA-I binding affinities comparable to those determined for the corresponding autologous epitopes. This finding was confirmed experimentally for the RL9 and RFL9 epitopes in the context of HLA-B*07:02. Nonetheless, variant-specific responses typically displayed attenuated levels of Ag sensitivity. Thus, impaired cross-reactivity during acute HIV-1 infection reflects both diminished HLA-I binding and suboptimal TCR recognition within the initially mobilized CD8 T cell population.

The crystal structures of RL9 and RFL9 in complex with HLA-B*07:02 revealed remarkably similar peptide conformations in the Ag-binding cleft, with the solvent-exposed tyrosine and phenylalanine residues at position 5 likely facilitating TCR cross-recognition. Indeed, autologous RFL9-specific CD8 T cells were highly cross-reactive against the RL9 variant and both epitope forms were targeted by almost identical TCR repertoires. It is less clear, however, why autologous RL9-specific CD8 T cells did not cross-recognize RFL9. One clue may come from a previous study involving the EBV-derived epitope FLRGRAYGL (EBNA3A325–333), in which

### Table II. Data collection for structural analysis

<table>
<thead>
<tr>
<th></th>
<th>HLA-B*07:02 (RPMTYKGAL)</th>
<th>HLA-B*07:02 (RPMTFKGAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temperature</td>
<td>100K</td>
</tr>
<tr>
<td></td>
<td>Space group</td>
<td>P2_12_2_1</td>
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<tr>
<td></td>
<td>Cell dimensions (a, b, c) (Å)</td>
<td>50.66, 81.86, 110.37</td>
</tr>
<tr>
<td></td>
<td>Resolution (Å)</td>
<td>46.04–1.85 (1.95–1.85)</td>
</tr>
<tr>
<td>Total number of observations</td>
<td>292,311 (42,140)</td>
<td>288,139 (41,681)</td>
</tr>
<tr>
<td>Number of unique observations</td>
<td>39,928 (5,704)</td>
<td>50,622 (7,409)</td>
</tr>
<tr>
<td>Multiplicity</td>
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<td>5.7 (5.6)</td>
</tr>
<tr>
<td>Data completeness (%)</td>
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<td>98.2 (99.7)</td>
</tr>
<tr>
<td>Energy (kcal/mol)</td>
<td>9.8 (2.0)</td>
<td>7.7 (2.5)</td>
</tr>
<tr>
<td>R&lt;sub&gt;merge&lt;/sub&gt; (%)</td>
<td>7.3 (43.3)</td>
<td>8.2 (52.2)</td>
</tr>
</tbody>
</table>

R<sub>merge</sub> = Σ(F<sub>i</sub>–|F<sub>i</sub>|)/Σ(F<sub>i</sub>) for all data except w%5%, which were used for R<sub>merge</sub> calculation. Values in parentheses are for the highest resolution shell. RMS, root mean square.

### Table III. Clonotypes of representative autologous and cross-reactive CD8 T cells

<table>
<thead>
<tr>
<th>TRBV</th>
<th>CDR3β</th>
<th>TRBJ</th>
<th>Frequency (%)</th>
<th>Count</th>
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<tbody>
<tr>
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<tr>
<td>7-9</td>
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<td>1</td>
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<tr>
<td>7-9</td>
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<td>1-1</td>
<td>1.11</td>
<td>1</td>
</tr>
<tr>
<td>7-3</td>
<td>CASSLNGDQYYF</td>
<td>2-3</td>
<td>1.11</td>
<td>1</td>
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<tr>
<td>Cross-reactive RL9 (RPMTYKGAL)</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Overlapping clonotypes are indicated in bold type and symbol matched.
a Tyr to Phe substitution led to a decrease in TCR binding affinity due to the absence of an exposed hydrogen bond (43). A similar scenario may lead to the mobilization of subtly different CD8 T cell clonotypes in the presence of a TFV incorporating the RL9 epitope, potentially constraining variant cross-reactivity within the cognate TCR repertoire (71, 72).

Several studies have suggested that cross-reactive CD8 T cells are associated with viral control during chronic HIV-1 infection (23–26). For example, work by Ladell et al. (26) elegantly demonstrated the expansion of a KK10-specific clonotype that effectively cross-recognized both the autologous epitope and a common variant during the transition from acute to chronic HIV-1 infection. This may be an exceptional case associated with elite control, however, as our data show that cross-reactive responses in the acute phase of infection are typically suboptimal with respect to variant-specific cytotoxicity. Indeed, the HLA-B*27 individual in our cohort was unable to suppress viral replication effectively and was started on ART soon after diagnosis. Nonetheless, ongoing recruitment of new clonotypes may enable variant recognition via repertoire diversification over time (62). Effective immune control of HIV-1 may therefore depend not only on the initially mobilized CD8 T cell repertoire (73), but also on the availability of specific clonotypes within the naive pool that can reinforce particular epitope-specific responses in the face of emerging variants (63).

Although we did not demonstrate the in vivo relevance of CD8 T cell cross-reactivity, our work suggests that immunization with epitope variants may be required as part of an effective vaccine against HIV-1. Certain polyvalent formulations (e.g., mosaic vaccines) are designed in precisely this way to broaden the initial response (74, 75). However, the present study also shows that immunogenicity (e.g., as measured in IFN-γ ELISPOT assays) does not equate with efficacy (i.e., lysis of HIV-1–infected targets). Detailed functional evaluations will therefore be required to assess the potential utility of vaccines that aim to elicit cross-reactive CD8 T cell responses.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.


Supplemental Figure 1. *In vitro* expanded RL10-specific CD8 T-cell lines elicit polyfunctional autologous and cross-reactive responses with down-regulated CD8 expression. CD8 T-cell lines were stimulated with the RL10 epitope and common variants in an ICS assay. (A) Dual production of IFN-γ/CD107a and IFN-γ/Granzyme B after stimulation with the indicated peptides. (B) Mean fluorescence intensity (MFI) for CD8 expression by CD8 T cells expressing IFN-γ after stimulation with the indicated peptides.
Supplemental Figure 2. Impaired antigen sensitivity of cross-reactive CD8 T cells by absolute magnitude of the IFN-γ response. Ex vivo antigen sensitivity by absolute numbers of IFN-γ-secreting cells is shown for autologous and cross-reactive CD8 T-cell responses to the HLA B*07:02-restricted epitopes RL10 (A), RL9 (B), and RFL9 (C), together with their respective variants. (D) Corresponding data for the HLA B*27:05-restricted KK10 epitope.
<table>
<thead>
<tr>
<th>Epitope number</th>
<th>Name</th>
<th>Sequence classification</th>
<th>Location</th>
<th>Epitope location</th>
<th>Variant sequences tested</th>
<th>Classification</th>
<th>Acute response</th>
<th>Chronic response</th>
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