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Degeneracy and Repertoire of the Human HIV-1 Gag p1777–85 CTL Response

June Kan-Mitchell,2,* Melissa Bajcz,* Keri L. Schaubert,* David A. Price,†
Jason M. Brenchley, ‡ Tedi E. Asher, ‡ Daniel C. Douek, ‡ Hwee L. Ng, ‡ Otto O. Yang,‡
Charles R. Rinaldo, Jr.§ Jose Miguel Benito,¶ Brygida Bisikirska,∗ Ramakrishna Hegde,*
Franco M. Marincola, || César Boggiano, # Dianne Wilson, # Judith Abrams,* Sylvie E. Blondelle,#
and Darcy B. Wilson**

CD8+ CTL responses are important for the control of HIV-1 infection. The immunodominant HLA-A2-restricted Gag epitope, SLYNTVATL (SL9), is considered to be a poor immunogen because reactivity to it is rare in acute infection despite its paradoxical dominance in patients with chronic infection. We have previously reported SL9 to be a help-independent epitope in that it primes highly activated CTLs ex vivo from CD8+ T cells of seronegative healthy donors. These CTLs produce sufficient cytokines for extended autocrine proliferation but are sensitive to activation-induced cell death, which may cause them to be eliminated by a proinflammatory cytokine storm. Here we identified an agonist variant of the SL9 peptide, p41 (SLYNTVAAL), by screening a large synthetic combinatorial nonapeptide library with ex vivo-primed SL9-specific T cells. p41 invariably immunized SL9-cross-reactive CTLs from other donors ex vivo and H-2Dbβ2m double knockout mice expressing a chimeric HLA-A*0201/H2-Dd MHC class I molecule. Parallel human T cell cultures showed p41-specific CTLs to be less fastidious than SL9-CTLs in the level of costimulation required from APCs and the need for exogenous IL-2 to proliferate (help dependent). TCR sequencing revealed that the same clonotype can develop into either help-independent or help-dependent CTLs depending on the peptide used to activate the precursor CD8+ T cells. Although Ag-experienced SL9-T cells from two patients were also sensitive to IL-2-mediated cell death upon restimulation in vitro, the loss of SL9 T cells was minimized with p41. This study suggests that agonist sequences can replace aberrantly immunogenic native epitopes for the rational design of vaccines targeting HIV-1.


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side chains in the formation of the peptide-MHC-TCR complex (27) may account for this broad repertoire. In patients, SL9-CTLs have been detected in GALT (21, 29), suggesting that these cells may also be active in mucosal responses. That such an immunogenic epitope is conserved across circulating HIV-1 strains worldwide (31) suggests that it confers a survival benefit upon the virus. In fact, the high frequency of virus-specific CD8^+ T cells in patients who are unable to control the infection raises the possibility that the incidence of immunologically deviant HIV-1 epitopes may be underreported (32, 33).

Human immune responses during active HIV-1 infection are strongly affected by complex virus-host interactions, including issues of epitope dominance, and may not accurately represent the true immunogenicity of component epitopes. We contend that it is important to study HIV-1-specific CTLs generated in vitro from healthy donors as well, particularly because these responses are clearly relevant to a preventive vaccine. We found ex vivo-primed SL9-CTLs to require a lower threshold for activation than CD8^+ T cells specific for cancer-associated or viral peptides studied (24). Moreover, only SL9-CTLs produce sufficient cytokines to support autocrine proliferation ex vivo for months. This unusual characteristic may explain the predominance of this reactivity in CD4^-diminished chronic infection.

Recognition by TCRs is degenerate, in that a single TCR can recognize many epitopes presented by HLA molecules (34). Flexibility of recognition of the SL9 epitope has been demonstrated in CTLs cloned from infected patients (9) and CTL cultures primed from healthy donors (24). A few HIV-1 CTL epitopes have been made more immunogenic in HLA transgenic mice by the deliberate substitution of anchor positions (35) or through the use of algorithms that predict key peptides (36). In this study, we investigated the possibility of identifying SL9 agonists by a high throughput method: a positional scanning synthetic combinatorial nonapeptide library (PS-SCL) with ex vivo-primed CTLs. The ability of a prototypic agonist (p41, SLNYTVAAL) to immunize SL9-cross-reactive CTLs from CD8^+ T cells from healthy donors and HHD mice was assessed. Parallel cultures to p41 and SL9 were established from individuals to compare requirements for activation and proliferation. Clonotype analysis was performed to determine whether reprogramming of SL9-cross-reactive T cells is possible with the agonist and to determine the ability of p41 to restimulate infection-primed SL9-T cells from patients. Our results show that p41 elicits a help-dependent CTL response and may thus be a better immunogen.

Materials and Methods

**HLA class I tissue typing and HLA-A2 subtyping**
Heparinized blood (100 ml) was obtained from healthy HLA-A*0201 volunteers at weekly intervals for 5 wk. The study was approved by the Human Investigation Committee of Wayne State University School of Medicine, and all subjects gave written informed consent. HLA class I typing and A2 subtyping were performed by sequence-specific primer PCR by the Immunogenetics (HLA) Laboratory at the Detroit Medical Center (Detroit, MI) and at the National Cancer Institute.

**Ex vivo immunization of human CD8 T cells with monocyte-derived dendritic cells (DCs)**
The procedures for ex vivo stimulation and characterization of peptide-specific CTLs have been previously described (24). In brief, immature DCs were derived from peripheral blood monocytes cultured for 7 days in RPMI 1640 medium containing 10% autologous serum (complete medium). GM-CSF (1000 U/ml; Genzyme) and IL-4 (500 U/ml; R&D Systems) were added at the initiation of the culture and every third day. Maturation was achieved with an overnight exposure to 1 µg/ml LPS (Escherichia coli serotype 026:B6; Sigma-Aldrich). Positively selected CD8^+ T cells (Dynabeads; Dynal) were primed with irradiated (4000 cGy) peptide-pulsed DCs at a T cell-DC ratio of 5:1 in 48-well or 96-well cluster plates. Cells were cultured in complete medium containing 10 ng/ml IL-7 (Genzyme) and restimulated every 7–10 days with autologous monocytes pulsed with peptides. The index SL9-CTL culture from Donor 1 was derived from sorted SL9-tetrameric HLA-A*0201 peptide complex (tetramer) ^+ cells that were expanded twice by stimulating with 30 ng/ml anti-CD3 mAb (Orthoclone OKT3; Ortho Biotech) in the presence of irradiated PBMC used as feeder cells. IL-2 (20 U/ml; Chiron) was added the next day and every 3 days thereafter. The sorted culture was polyclonal, containing Vβ5.1^+, Vβ5.2^+, Vβ11^+, Vβ12^+, Vβ13.1^+, and Vβ17^+ T cells.

**Assays to assess the specificity of CTLs**

Peptide-specific T cells were enumerated with tetrameric HLA-A*0201 peptide complexes (tetramers) purchased from Beckman Coulter (SL9-tetramer) or prepared by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Tetramer Facility (Bethesda, MD). Cells were stained with 1 µg/ml tetramer for 30 min on ice. For cytotoxicity assays, T2 targets were labeled with 100 µCl of ^51Cr (Amersham Biosciences) for 1 h and washed. Target cells and nonamer peptides at 1 µg/ml were plated at 1–2 x 10^5 labeled cells per well with effector cells at indicated E:T ratios in 96-well U-bottom plates for 4 h at 37°C. The amount of ^51Cr released was determined by scintillation counting in a MicroBeta counter (PerkinElmer-Wallace). Percent lysis was calculated using the formula 

\[
\frac{cpm_{experimental} - cpm_{spontaneous}}{cpm_{total} - cpm_{spontaneous}} \times 100
\]

OptEIA Sets (BD Pharmingen) were used to measure the concentrations of IFN-γ in supernatants of CTLs stimulated for 24 h with peptide-pulsed T2 cells. T2 were pulsed with peptides at concentrations ranging from 10^{-12} to 10^{-5} M and used at the T cell-T2 cell ratio of 1:10. The range of sensitivity for IFN-γ is between 5 and 300 pg/ml, respectively. EC50 values were calculated with GraphPad Prism. IFN-γ secretion at the single cell level was analyzed with the Miltenyi IFN-γ secretion detection kit according to the manufacturer’s specifications (Miltenyi Biotec).

**Nonapeptide library and assay for cytotoxicity with library mixtures**

An SL9-CTL culture was used to scan an L-amino acid nonapeptide PS-SCL as described previously to identify candidate agonist sequences (37). All nonapeptides present in the library have a free N terminus and an amidated C terminus. This library consists of 180 different peptide mixtures, each having one of the 20 natural proteogenic L-amino acids in a defined position and a near equimolar mixture (represented by X) of 19 L-amino acids (L-cysteine omitted), in each of the remaining positions. For example, the first mixture is defined with alanine at position 1 (XXXXXXXXX-NH2), i.e., containing all possible nonapeptide sequences having an alanine at position 1, and mixture 180 is defined with tyrosine at position 9 (XXXXXXXXX-NH2), i.e., containing all possible nonapeptide sequences having a tyrosine at position 9. Each mixture contains 19^9 (1.7 x 10^{18}) different peptides in approximately equimolar concentration; and the entire nonameric library contains 20 x 19^9 (3.4 x 10^{18}) different peptides. For stimulation of the index SL9-CTLs, T cells 7 days after stimulation with anti-CD3 mAb were washed and resuspended at 1 x 10^5 cells/ml in complete medium. Then, 100 µl of this cell suspension was added to triplicate wells of 96-well U-bottom plates containing 2 x 10^4 ^51Cr-labeled T2 cells and the various peptide library mixtures (100 µg/ml). Cells were cultured for 4 h at 37°C and percent lysis determined as above. Each mixture was assayed in triplicate, and scanning was repeated five times.

**Killing efficiency assays**

The killing efficiency of CTL clones or primary cultures was tested against HIV-1-infected cells as previously described (38). Briefly, T1 cells (HLA-A2^+) were infected with NLA-3 HIV-1 virus at excess multiplicity (MOI ≥ 3 tissue culture infectious doses per target cell) and used after 4 days as target cells in chromium release assays (as above), including peptide and no peptide controls. The percentage of infected target cells was determined by flow cytometry after intracellular staining for p24 Ag. The efficiency of killing of infected cells (corrected for efficiency of infection) was calculated by:

\[
\frac{\{\text{specific lysis of infected cells/(specific lysis of excess peptide-} \text{treated cells - specific lysis of control cells)}\} \times 100}{\text{fraction of cells expressing p24 Ag at successive time points}}
\]

**Mice, peptide immunization, spleen cell cultures, and cytotoxicity assay**

HDD mice are H-2Db β2m double knockout mice expressing a chimeric HLA-A*0201/H2-Db MHHC class I molecule on a C57BL/6 background (40). The mice were injected s.c. at the base of the tail with 100 µg of SL9
or p41 peptide admixed with 140 μg of the IAβ-restricted HBVc128 Th peptide (TPAYRPPNAPIL) emulsified in IFA. Mice were sacrificed 7 days later, and the spleen cells were restimulated with irradiated peptide-loaded (10 μM) LPS-stimulated HHD lymphoblasts for 6 days. Cytotoxicity of the cultured splenocytes was assessed using HHD-transfected, TAP-negative RMA-S cells loaded with relevant or negative control peptides (40). Studies in mice, including methods of euthanasia, were in compliance with federal guidelines and institutional policies and approved by the Animal Investigation Committee at Wayne State University School of Medicine.

### Statistical methods

Random effects models (41) were used to assess the association of cytotoxicity with E:T ratios and to assess differences in cytotoxicity associated with different peptides used to immunize the animals and the peptides with which the T cells were assessed. E:T ratios were parameterized using indicator variables, making it unnecessary to assume a functional form for the relationship between that ratio and cytotoxicity. Holm’s step-down procedure was used to adjust type I errors for multiple comparisons. An analysis of model residuals was performed to identify animals that may have been disproportionately influential on the results of the analyses. The significance of the correlation between percent cytotoxicity and percent tetramer binding was measured using the Spearman rank order correlation (41).

### Flow cytometry

Cells were labeled with mAbs (BD Biosciences, BD Pharmingen) and analyzed on a FACSCalibur with CellQuest software (BD Biosciences). FITC-labeled KC57 mAb specific for the core Ags of HIV-1 was purchased from Coulter. All mAb reagents were used according to manufacturers’ recommendations. HLA-A*0201-SL9 tetramers labeled with allophycocyanin were purchased from Beckman Coulter Immunomics. Allophycocyanin-labeled p41-tetramers were prepared by the National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA). Stock solutions contained 2 mg of monomer per ml, and the peptides were dissolved in DMSO (10 mg/ml), aliquoted, and stored at −80°C.

### Results

#### Prediction of SL9 agonists from library screening

The 180 library mixtures described in Materials and Methods were assayed with an SL9-CTL culture from Donor 1. Fig. 1A shows representative data of one of five library scans. Significant and reproducible differences in activity were observed between mixtures. With the exception of position 1, few mixtures exhibited significant stimulatory activity, a result that indicates the presence of a limited number of active sequences within the library. Notably, the mixtures defined with the native residues exhibited activity above average at all positions (Fig. 1A, □). Five of these mixtures

![FIGURE 1](http://www.jimmunol.org/)

A. Cytotoxicity of the index SL9-CTL culture to the 180 mixtures of a nonapeptide PS-SCL. Each graph, designated P1–P9, represents a set of 20 mixtures having the defined amino acid listed in the x-axis at a given position. Horizontal lines, average cytotoxicity for the 20 mixtures of this position. B. Frequency of most active library mixtures. The amino acids that defined mixtures ranked among the three most active ones for each position from five different library scans is used to predict candidate peptides.
(positions 1, 3, and 8) were among the three most active mixtures at the corresponding position. At positions 3 and 8, conservative replacements were found among the three most active mixtures: 3Y→W and 8T→S or A.

Based on these results, active mixtures were selected to prepare a set of 32 candidate peptides (Fig. 1B). This selection was based on the following criteria: 1) the most active mixture (except for position 9 where the mixture defined with the native residue), the third most active, was selected over two conservative substitutions; and 2) an additional active mixture with a defined amino acid of different physicochemical character (i.e., at positions 1, 2, 5, 6, and 8). In addition, to address specific substitutions, 9 single- and 33 double-substitution analogs were also synthesized.

The 74 peptides were tested for recognition by CTLs when pulsed onto T2 cells in chromium release assays. Cytotoxicity was used because it seems to be more sensitive than cytokine release in virus-specific CD8+ T cells (43). Lysis was assessed at four peptide dilutions to show relative responsiveness. A peptide was considered cross-reactive if it induced at least 50% of the lysis achieved by an equivalent concentration of SL9. Twenty-two of the 74 peptides were tested for recognition by CTLs when pulsed onto T2 cells in chromium release assays. Cytotoxicity for SL9 is boldface. A peptide is considered cross-reactive (italics) if it induced at least 50% of the lysis achieved by an equivalent concentration of SL9. Data are presented as the means of triplicate experiments with four peptide dilutions.

To assess potential cross-recognition of the peptide agonists by different individuals, we tested the 74 peptides with SL9-CTLs from seven other HLA-A2+ donors. SL9-CTLs from these individuals reacted with 5–9 of the 22 peptides recognized by the index culture, indicating idiotypic specificities (Table I, values in italics). Interestingly, CTLs from five donors recognized at least one peptide with five substitutions. The monosubstituted peptide, p41, was recognized by eight of the nine donors and was selected for further studies. Incidentally, peptides not recognized by index CTLs did not elicit responses from other CTL cultures (data not shown). Our results show that the TCR repertoire for SL9 of healthy donors appear to be broad but overlapping.

Cross-recognition of SL9 agonists by other SL9-CTL cultures

Table I. Recognition of predicted candidate peptide analogs by SL9-CTL cultures from HLA-A2+ donors determined by cytotoxicity

<table>
<thead>
<tr>
<th>Peptide Concentrations (ng/ml)</th>
<th>% Vβ+ cells in tetramer+ population</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Vβ23 (%)</td>
</tr>
<tr>
<td>Donor 1 (index)</td>
<td></td>
</tr>
<tr>
<td>SL9</td>
<td>0</td>
</tr>
<tr>
<td>p34 L</td>
<td>8.9</td>
</tr>
<tr>
<td>p42 E</td>
<td>4.24</td>
</tr>
<tr>
<td>p41 A</td>
<td>0.39</td>
</tr>
<tr>
<td>p49 A</td>
<td>9.10</td>
</tr>
<tr>
<td>p50 E</td>
<td>1.25</td>
</tr>
<tr>
<td>p48 L</td>
<td>12.31</td>
</tr>
<tr>
<td>p60 A</td>
<td>5.12</td>
</tr>
<tr>
<td>p47 L</td>
<td>11.35</td>
</tr>
<tr>
<td>p72 A</td>
<td>27.46</td>
</tr>
<tr>
<td>p73 E</td>
<td>18.18</td>
</tr>
<tr>
<td>p9 A</td>
<td>13.24</td>
</tr>
<tr>
<td>p3 A</td>
<td>8.21</td>
</tr>
<tr>
<td>p4 A</td>
<td>14.19</td>
</tr>
<tr>
<td>p11 A</td>
<td>27.32</td>
</tr>
<tr>
<td>p12 A</td>
<td>0.24</td>
</tr>
<tr>
<td>p11 L</td>
<td>27.32</td>
</tr>
<tr>
<td>p14 A</td>
<td>24.54</td>
</tr>
<tr>
<td>p17 L</td>
<td>12.40</td>
</tr>
<tr>
<td>p18 L</td>
<td>3.28</td>
</tr>
<tr>
<td>p19 L</td>
<td>11.31</td>
</tr>
<tr>
<td>p24 L</td>
<td>25.48</td>
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<tr>
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primed ex vivo from circulating precursor T cells only in the absence of exogenous IL-2 (24). This observation is confirmed in Fig. 2A. SL9-tetramer-binding cells (29%) were detected when IL-2 was omitted (upper left panel), whereas no SL9-tetramer-binding cells (<0.2%) were found in a parallel culture to which IL-2 had been added (upper right panel). With p41 as the immunizing epitope, cross-reactive SL9-tetramer-binding cells (61%) were detected when exogenous IL-2 had been added (Fig. 2A, lower right panel). Requirement for IL-2 at priming and for expansion of p41-specific, SL9-cross-reactive T cells was confirmed with seven donors. Fig. 2B shows the progressive increase in tetramer$^+$ cells with restimulation in a representative culture of SL9- or p41-CTLs. The same percent of p41-CTLs was stained positive with either SL9- or p41-tetramers. Fig. 2C shows that structurally cross-reactive (tetramer$^+$) p41-primed T cells from three donors were cytotoxic to T2 cells pulsed with SL9 or p41. Cytotoxicity was specific, because lysis was minimal against T2 cells alone. The population of tetramer-stained cells in Donor 1 was more discrete and stained at higher intensity than those from Donors 2 and 7. Fig. 3A shows in parallel cultures that p41-induced cross-reactive CTLs were primed by immature or mature DCS. SL9-CTLs, however, required a lower level of costimulation afforded by immature DCS (24) (Fig. 3B). Because exogenous IL-2 appears to serve as a surrogate for helper T cells in CD8$^+$ T cell cultures (45) and helper-dependent CTL responses are more likely to generate memory T cell responses (46), our findings suggest that p41 may be a better immunogen than SL9. Incidentally, p41 cultures appeared to be more consistently expanded (six of six donors) as compared with SL9-CTLs, which were obtained from only ~30% of cultures attempted.

Growth characteristics, TCR V$\beta$ usage, functional avidities, and pattern of reactivity to natural SL9 variants using parallel SL9 and p41 cultures from the same donors

Figs. 4A and 5A summarize the kinetics of expansion in parallel cultures of CTLs primed with SL9 (Figs. 4Aa and 5Aa) or p41 (Figs. 4Ab and 5Ab) from Donors 6 and 9, respectively. Binding to p41-tetramers was essentially identical with SL9-tetramers in terms of percent staining cells (data not shown). From Donor 6, tetramer-binding cells appeared earlier in the SL9 (day 21) than in the p41 (day 42) culture. However, the number of tetramer$^+$ cells for both was in the same range by day 49 (Fig. 4, Aa and Ab). In the case of Donor 9, tetramer-binding cells appeared around the same time (day 21) after priming with SL9 or p41 (Fig. 5, Aa and Ab). However, the yield of cross-reactive p41-T cells was 10-fold greater than SL9-T cells (~3,400 vs 300 million cells) by day 49, indicating that the magnitude of the response to p41 can be greater than the native epitope in some individuals.

Fig. 4, Ac and Ad, and Fig. 5, Ac and Ad, show an increase in Ag responsiveness of the CD8$^+$ cytotoxic function with maturation of the Ag-specific T cells in culture. Cytotoxicity mediated by early CTLs (<day 36) was invariably lower than by late CTLs (>day 52) at all E:T ratios. This was not likely due to progressive increase in the number of epitope-specific T cells with culture. Early SL9-CTLs from Donor 6 (Fig. 4Ac) and early SL9- and p41-CTLs from Donor 9 (Fig. 5, Ac and Ad) were less efficient killers even though the cultures were >75% tetramer-binding cells. These results suggest dissociation between proliferation and maturation of cytotoxicity in Ag-specific human CD8$^+$ T cells.

To determine whether p41 and SL9 activate different precursors, SL9-tetramer-binding cells in p41- and SL9-CTL cultures from both donors were assessed for TCR V$\beta$ usage, using a panel of mAbs to 21 V$\beta$ chains representing >50% of all V$\beta$ genes. Tetramer$^+$ T cells in SL9 and p41 cultures from Donor 6 were all V$\beta$9 positive (Fig. 4B). This was further analyzed by quantitative sequencing of TCR$\beta$ gene products from sorted tetramer$^+$ cells (17) (Table II). TCR sequencing showed SL9 and p41 cultures to be monoclonal, expressing the identical TCR structure. Thus, activation of the same clonotype with an agonist can result in functional reprogramming of the progenitor cell without altering specificity.

In contrast, tetramer$^+$ T cells stimulated by SL9 or p41 from Donor 9 used different V$\beta$ genes. Tetramer$^+$ cells from SL9-culture stained exclusively for V$\beta$9, while those from p41-T cells were V$\beta$17-positive (Fig. 5B). These findings were validated by TCRB

FIGURE 2. Induction of SL9-cross-reactive CTLs in cultures primed ex vivo with p41. A, Requirement for exogenous IL-2 at priming and subsequent restimulations depends on peptide specificity. SL9-tetramer$^+$ T cells in a representative day 48 culture primed with SL9 (top panels; left, 29% without IL-2; right, 0.2% with IL-2) or with p41 (bottom panels; left, 6% without IL-2; right, 61% with IL-2). B, Progressive increase of tetramer$^+$ cells in cultures primed and expanded with SL9 (top panels) or p41 (bottom panels). The percentages of staining are identical with the SL9- or the p41-tetramer. C, Structurally cross-reactive (SL9-tetramer$^+$) p41-CTLs (day 29) from three donors are cytotoxic to T2 cells pulsed with SL9 (●), p41 (○), or an irrelevant peptide (□).
gene usage (Table II). Thus, SL9-CTLs and p41-CTLs had been expanded from distinct precursor clonotype(s) in this individual. Recruitment of a distinctive pool of precursor CD8^{+}/H11001 cells by p41 should increase the repertoire of the CTL response to the native epitope and may be beneficial in terms of vaccine design.

CTLs with higher avidity, as measured in vitro, have been reported to exhibit greater in vivo antiviral activity when adoptively transferred (47). Here, the functional avidities of SL9- and p41-CTLs were determined operationally as the negative logarithm of the peptide concentration that resulted in 50% maximal IFN-γ/H9253 release after a 24 h stimulation period with T2 cells (EC_{50}) (Figs. 4C and 5C). For Donor 6, EC_{50} values of SL9-CTLs were 8.9 \times 10^{-9} M (with SL9) and 6.8 \times 10^{-9} M (with p41), and those of p41-CTLs were 5.6 \times 10^{-9} M and 6 \times 10^{-8} M, respectively. Because both cultures were derived from sister cells, this clonotype appeared to be less avid for p41. For Donor 9, EC_{50} values for SL9-CTLs were 7.5 \times 10^{-9} M (with SL9) and 1.2 \times 10^{-7} M (with p41), and 1.1 \times 10^{-8} M and 1.2 \times 10^{-8} M for p41-CTLs, respectively. Therefore, SL9-CTLs were highly avid for SL9 but recognized p41 less efficiently. In contrast, p41-CTLs recognized SL9 as well as p41 peptide. For this person, p41 would mobilize a broader CTL response than the native SL9.

Next, we compared the ability of SL9- and p41-CTLs to recognize seven naturally occurring variants of SL9. Because both cultures from Donor 6 were derived from sister cells, they exhibited the same pattern of cross-reactivity to these variants (Fig. 4D). In contrast, SL9-CTLs from Donor 9 recognized SL9 and variants containing tyrosine in position 3 with further single or double amino acid replacements (6I, 6I8V, 2V and 8V). No reactivity was detected against the other common mutant of clade A and B viruses, 3F, which encodes a phenylalanine in position 3. Variants of 3F with additional single amino acid changes were also not recognized (3F8V and 3F6I8V; Fig. 5D). Although 3F binds 27 times less well than the SL9 peptide (9), this is unlikely to be a major factor for the lack of reactivity because peptides were used at saturating levels (1 μM). These results are consistent with published results showing that the cross-reactivity of T cell responses to SL9 and 3F in infected people is typically \approx 50\% (10). The lack of cross-reactivity may be explained by the striking structural differences between the crystal structures of HLA-A2 with SL9 and 3F (27). It is interesting that only p41-CTLs, but not SL9-CTLs, recognize both peptide-MHC complexes.

**Lysis of HIV-1 acutely infected cells by p41-CTLs**

Because the use of synthetic peptide-pulsed target cells bypasses virologic and cellular factors that contribute to the efficiency of epitope presentation and recognition by CTLs, the ability of p41-CTLs to lyse acutely infected T1 cells was measured (48). Fig. 6 shows that SL9- and p41-CTLs from the same donor lysed T1 cells infected by the NL4-3.1 HIV-1 virus (~75% infected) to a similar degree at the E:T ratio of 10:1 (Fig. 6, top and bottom panels, respectively). T1 cells, T1 cells pulsed with SL9, and infected T1 cells pulsed with SL9 were
also included as negative or positive controls. Both cultures were minimally cytotoxic to uninfected T1 cells, indicating an absence of lymphokine-activated killer activity. Both SL9-CTLs and p41-CTLs were highly cytotoxic in the presence of the SL9 peptide to uninfected and infected T1 cells.

In vivo immunization of HHD mice

To assess immunogenicity in vivo, spleen cells from peptide-immunized mice were restimulated with irradiated peptide-loaded (10 μM) LPS-stimulated HHD lymphoblasts for 6 days. Cytotoxicity was assessed by pulsing HHD-transfected, TAP-negative RMA-S cells with various peptides. Fig. 7A summarizes the results.
Table II. TCRBV usage, CDR3 amino acid sequence, and percent frequency of parallel cultures of SL9- and p41-CTLs from donor 6 (Fig. 4) and donor 9 (Fig. 5)

<table>
<thead>
<tr>
<th>Donor</th>
<th>Specificity</th>
<th>TCR Vβ (IMGT nomenclature)</th>
<th>CDR3</th>
<th>TCRBJ</th>
<th>% Frequency</th>
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<tr>
<td>6</td>
<td>SL9</td>
<td>3.1</td>
<td>CASSPTGGDTQY</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>p41</td>
<td>3.1</td>
<td>CASSPTGGDTQY</td>
<td>2.3</td>
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</tr>
<tr>
<td>9</td>
<td>SL9</td>
<td>3.1</td>
<td>CASSQVKGQGNEKLF</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>p41</td>
<td>19.0</td>
<td>CASSINDLSNQPH</td>
<td>1.5</td>
<td>100</td>
</tr>
</tbody>
</table>

The IMGT nomenclature for TCR Vβ is shown here. These results are consistent with Arden nomenclature used for staining by Vβ-specific mAbs.

directed at the immunizing peptide (self-reaction) and cross-reactivity (cross-reaction) at multiple E:T ratios (1.6:1–100:1) from 19 mice immunized by SL9 (Fig. 7Aa) and 13 mice immunized by p41 (Fig. 7Ab). Fig. 7Ac shows analyses using the mixed effects statistical model to compare self- and cross-reactive cytotoxicity between the two groups of mice at all E:T ratios. Splenocytes from SL9 mice were less able to lyse SL9-pulsed target cells than those from p41 mice. Moreover, they were less cross-reactive to p41. In contrast, p41 induced greater cytotoxicity to both peptides. Similar conclusions were reached when the splenocyte cultures were assayed for IFN-γ-secreting cells by ELISPOT (data not shown).

To examine SL9-specific T cells at the single-cell level, splenocyte cultures were tested for HLA-A2 tetramer+ cells. Because murine CD8 does not bind to human HLA class I Ags, we expected only highly avid HHD CTLs would be stained (49). As shown in Fig. 7B, only two of 19 SL9-splenocyte cultures contained fewer than 10% tetramer+ CD8+ T cells. Moreover, significant staining (14–38%) was detected in eight noncytotoxic cultures (<15% lysis at the E:T ratio of 100:1). The Spearman rank correlation between the frequency of tetramer binding and cytotoxicity confirmed a marginal association between the number of tetramer-stained cells and the percent cytotoxicity in SL9-splenocyte cultures (Spearman’s r = 0.47, p = 0.04; Fig. 7B, right panel). The intensity of staining was the same for tetramer-binding cells from cytotoxic or noncytotoxic cultures. Therefore, immunization of HHD mice with the SL9 peptide induced highly avid tetramer-binding CD8+ T cells that were noncytotoxic. Positive staining was more predictive of cytotoxicity for mice immunized with p41. These results suggest that p41 is a superior immunogen to SL9, with a potential role in an HIV-1 CTL-based vaccine.

p41 peptide stimulates proliferation of SL9-specific T cells from patients

To determine whether SL9-T cells primed by the natural infection can be stimulated by p41 and their requirement for proliferation in vitro, purified CD8+ T cells were isolated from the peripheral blood of two patients and restimulated in vitro by A2-matched allogeneic monocytes pulsed with either SL9 or p41. CD8+ T cells were >98% homogeneous with no contaminating CD4+ T cells. Fig. 8 shows SL9-T cells from an HLA-A2 nonprogressor patient who was diagnosed in 1986 but has not received antiviral treatment since 1999 (50). The autologous viral sequence at the time of sampling was confirmed to be SL9NTVATL. Four hundred thousand purified CD8+ T cells containing ~4,800 tetramer+ cells were plated per well in duplicate for each restimulation condition (Fig. 8A). Without exogenous IL-2, tetramer+ cells increased ~25-fold (~120,000 cells) in 7 days after stimulation with SL9, with little change in the total number of T cells per well (Fig. 8B). However, supplementation with a low dose of exogenous IL-2 led to reduced yield of tetramer+ cells (~40,000 cells) (Fig. 8C). Thus, infection-primed SL9-tetramer+ cells can be sensitive to exogenous IL-2, as with ex vivo-primed SL9-CD8+ T cells from healthy donors. p41 was also capable of stimulating SL9-T cells from this patient to proliferate without exogenous IL-2, albeit to a lesser extent (~90,000 cells) (Fig. 8D). Interestingly, the number of tetramer+ cells was less affected by the addition of IL-2 when stimulated by p41 as when stimulated with SL9 (40,000 vs 108,000 cells). IL-2 also significantly increased the number of nonspecific T cells (~1.2 × 106 cells, Fig. 8E). The scatter plot of PBMCs stimulated with SL9 and IL-2 revealed a skewed and somewhat diffuse population of tetramer+ cells with a reduced staining intensity (mean fluorescence intensity, 182) (Fig. 8C). This was distinct from tetramer+ cells derived under the other conditions (mean fluorescence intensities, 372, 421, and 269 for Fig. 8B, Fig. 8D, and Fig. 8E, respectively). A skewed distribution on the dot plot is often caused by dying cells.

Fig. 9 shows IL-2-independent in vitro expansion of SL9-tetramer+ cells by SL9 or p41 in another nonprogressor patient. Before culture, there was definite tetramer staining but the tetramer+ cells did not form a discrete population (Fig. 9A). Seven days after the first restimulation with either peptides, the number of tetramer+ cells decreased precipitously (Fig. 9B and D). However, the number of tetramer+ cells increased greatly after a second
restimulation and the tetramer\(^+\) cells formed a more discrete population (Fig. 9, C and E). Tetramer\(^+\) T cells expanded by SL9 or p41 were functional, as shown by their ability to secrete IFN-γ after stimulation with either peptide (Fig. 9, F–I). Infection-primed SL9-T cells from both patients did not require exogenous IL-2 to proliferate after stimulation with p41, suggesting that the help independence of infection-primed SL9-T cells can be a stable phenotype.

**Discussion**

The absence of CTL reactivity to SL9 during the critical early phase of viral clearance in acute infection has cast doubts about the importance of SL9 as a potential immunogen for vaccines. We have reported that ex vivo-primed SL9-CTLs are highly activated and sensitive to cytokine-mediated cell death, which may account for their selective elimination during the proinflammatory innate cytokine storm in early infection (51). Here, we explored whether it was possible to use an agonist of SL9 to generate an effective CTL response. This approach was feasible because SL9-CTL recognition is degenerate (9, 24), and functional reprogramming of CD4\(^+\) T cells by an alternative peptide ligand has been reported in TCR transgenic mice (52). To identify cross-reactive agonists, we screened a large synthetic combinatorial nonapeptide library with ex vivo-primed SL9-CTLs. This systematic and unbiased approach does not make assumptions regarding contact positions for MHC or TCR; therefore it is distinct from approaches taken by others (36, 53). Although agonists that differed from the native SL9 peptide by five to six of the nine amino acid position(s) were recognized by a panel of SL9-CTL cultures, only the monosubstituted p41 with a replacement of T\(\rightarrow\)A in position 8 consistently primed SL9-cross-reactive CTLs. These studies provide a new perspective on the T cell repertoires responding to SL9 and related peptides, which may help to explain why SL9 responses do not control infection.

p41-CTLs primed ex vivo from all healthy seronegative donors and in vivo from HHD mice efficiently recognized SL9. CD8\(^+\) T cell priming is tightly regulated, particularly by the affinity and binding characteristics of the TCR for its homologous MHC-peptide ligands (54), the expression of costimulatory molecules (55), and cytokines in the microenvironment (51, 56). We found productive priming with p41 with both mature and immature DCs, indicating greater tolerance for strong costimulation afforded by mature DCs than with SL9 (24). Moreover, p41-CTLs were generated only if exogenous IL-2 was added immediately postpriming in vitro and their proliferation was dependent on continuous IL-2 supplementation (help dependent). We also found that SL9-CD8\(^+\) T cells primed by natural infection from our chronically infected patients retained the SL9-help-independent phenotype; i.e., proliferation in vitro did not require exogenous IL-2. Moreover, addition of even a low dose of IL-2 in a parallel culture resulted in a significant loss of tetramer-binding T cells in the patient depicted in
Fig. 8. Thus, SL9-T cells from patients can be sensitive to IL-2-mediated activation induced cell death, as with ex vivo-primed CTLs from healthy individuals (24). It is interesting that the deleterious effect of IL-2 on SL9-T cells was ameliorated if p41 was used for restimulation. These findings show that reprogramming of at least this characteristic of Ag-experienced SL9-T cells is possible through the use of a presumably less immunogenic agonist.

Inducible cytokine production by virus-specific CD8 effector T cells in murine models of virus infection is a complex and strictly regulated process (51). IL-2 production is determined by the differentiation state of the T cell population and whether the stimulus is mediated through the TCR or through cytokine receptors, or both (57). Secretion of IL-2 is usually transient and elicited exclusively through TCR recognition. Much less is known about production of IL-2 by human CTLs. Recent studies showed that IL-2 can be induced in HIV-1-specific cytotoxic CD8 T cells from nonprogressor but not progressor patients (58, 59). The ability of SL9-CTLs to produce IL-2 over a prolonged period, however, appears to reflect an aberrant state of activation and may be unique to this specificity. As with murine virus-specific T cells, induction of IL-2 in SL9-T cells is mediated through the TCR, because its secretion was modulated by TCR activation with p41.

Parallel cultures allowed the comparison of SL9- and p41-T cells from the same donor. From Donor 6 (Fig. 4 and Table II), we showed through sequencing of the CDR3 domain of the TCR that SL9 and p41 generated qualitatively distinct CTLs through unequal stimulation of sister progenitor cells. In contrast, p41 recruited different precursor cells from SL9 in Donor 9 (Fig. 5), as shown by different TCR Vβ usage. These SL9- and p41-T cells recognized different SL9 variants, indicating that vaccination with p41 would have broadened the repertoire of the SL9 response. This appears to be an important consideration, as shown in a recent study in SIV-infected macaques (60). Our results showed that the cross-reactivity of the T cell repertoire for SL9 may be different among healthy people. Furthermore, these precursor cells are sensitive to costimulation- and cytokine-mediated activation-induced cell death when stimulated with SL9, consistent with having a memory phenotype (61–63). One intriguing possibility is that SL9 provokes a cross-reactive memory T cell response to one or more common pathogen(s). This would also explain why SL9 reactivity is rare in acute infection, when cross-reactive memory cells are driven into apoptosis (heterologous immunity) (64). From the perspective of vaccine design, it implies that it may be difficult to induce useful SL9 responses with the native Gag protein in HLA-A2+ people.

This study supports previous reports that the SL9-CTL repertoire is complex and sustainable (9, 17, 27). Whether this repertoire poses inherent limitations to the use of agonists as vaccines is uncertain. However, defining the T cell repertoires to SL9 and related peptides may at least help us to understand why these responses do not control infection and may also provide insights into other immunodominant HIV-1 epitopes. Ultimately, the exploitation of TCR degeneracy in the response to SL9 may lead to improvement of the design of HIV-1 vaccines.

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Disclosures
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References
Theoretical Biology and Biophysics, Los Alamos, NM.


