Distinct CD8+ T Cell Repertoires Primed with Agonist and Native Peptides Derived from a Tumor-Associated Antigen

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Heteroclitic peptides are used to enhance the immunogenicity of tumor-associated Ags to break T cell tolerance to these self-proteins. One such altered peptide ligand (Cap1–6D) has been derived from an epitope in human carcinoembryonic Ag, CEA_{605–613} (Cap1). Clinical responses have been seen in colon cancer patients receiving a tumor vaccine comprised of this altered peptide. Whether Cap1–6D serves as a T cell agonist for Cap1-specific T cells or induces different T cells is unknown. We, therefore, examined the T cell repertoires elicited by Cap1–6D and Cap1. Human CTL lines and clones were generated with either Cap1–6D peptide (6D-CTLs) or Cap1 peptide (Cap1-CTLs). The TCR Vβ usage and functional avidity of the T cells induced in parallel against these target peptides were assessed. The predominant CTL repertoire induced by agonist Cap1–6D is limited to TCR Vβ1-J2 with homogenous CDR3 lengths. In contrast, the majority of Cap1-CTLs use different Vβ gene and also had diverse CDR3 lengths. 6D-CTLs produce IFN-γ in response to Cap1–6D peptide with high avidity, but respond with lower avidity to the native Cap1 peptide when compared with the Cap1-CTLs. Nevertheless, 6D-CTLs could still lyse targets bearing the native epitope. Consistent with these functional results, 6D-CTLs possess TCRs that bind Cap-1 peptide/MHC tetramer with higher intensity than Cap1-CTLs but form less stable interactions with peptide/MHC as measured by tetramer decay. These results demonstrate that priming with this CEA-derived altered peptide ligand can induce distinct carcinoembryonic Ag-reactive T cells with different functional capacities.


Effective tumor immunotherapy requires the in vivo generation of a sufficient number of tumor Ag-specific CTL, that are capable of sustaining immunity to destroy established tumors while being tolerant to normal tissues. Two major strategies involve adoptive transfer of tumor-reactive T cells (1, 2) or cancer vaccines to activate tumor-reactive T cells in vivo (3, 4). Alternatives involve use of tumor-specific monoclonal antibodies (5), use of TCR transgenic T cells (1–3), and use of altered peptides that can cross-react with the tumor-specific epitope.

Clinical responses have been seen in colon cancer patients receiving a tumor vaccine comprised of this altered peptide. Whether Cap1–6D serves as a T cell agonist for Cap1-specific T cells or induces different T cells is unknown. We, therefore, examined the T cell repertoires elicited by Cap1–6D and Cap1. Human CTL lines and clones were generated with either Cap1–6D peptide (6D-CTLs) or Cap1 peptide (Cap1-CTLs). The TCR Vβ usage and functional avidity of the T cells induced in parallel against these target peptides were assessed. The predominant CTL repertoire induced by agonist Cap1–6D is limited to TCR Vβ1-J2 with homogenous CDR3 lengths. In contrast, the majority of Cap1-CTLs use different Vβ gene and also had diverse CDR3 lengths. 6D-CTLs produce IFN-γ in response to Cap1–6D peptide with high avidity, but respond with lower avidity to the native Cap1 peptide when compared with the Cap1-CTLs. Nevertheless, 6D-CTLs could still lyse targets bearing the native epitope. Consistent with these functional results, 6D-CTLs possess TCRs that bind Cap-1 peptide/MHC tetramer with higher intensity than Cap1-CTLs but form less stable interactions with peptide/MHC as measured by tetramer decay. These results demonstrate that priming with this CEA-derived altered peptide ligand can induce distinct carcinoembryonic Ag-reactive T cells with different functional capacities.


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the native peptide to increase the immunogenicity, substitution of asparagine 610, a TCR contact residue, with an aspartic acid enhanced the induction of CTLs against CEA in vitro, and elicited CTLs that can recognize CEA on tumor cells (30). Furthermore, in a previous study, we observed clinical responses in colorectal cancer patients that received this CEA agonist peptide vaccination with Flt3 ligand-expanded dendritic cells (DCs; Ref. 23). The clinical responses correlated with the expansion of CEA-specific CD8\(^+\) T cells detected by MHC/peptide tetramer staining, confirming the role of the agonist peptide specific CTLs in this treatment strategy. Vaccine-induced CTL can also cross-react to the native epitope in a subset of patients (23, 31). Because the CTL repertoires elicited by the CEA agonist peptide vs the native peptide to determine whether there is overlap or whether these different peptides can induce distinct T cells. To this end, we generated CTL lines and clones from HLA-A2-+ PBMCs in vitro using either the altered agonist CEA peptide or its native peptide for priming. By comparing the TCR usage and functional avidity of the two CTL repertoires in parallel, we demonstrate that the predominant CTLs elicited by agonist peptide have a restricted TCR usage and lower avidity for the native peptide. These findings have implications for optimizing tumor vaccination using the agonist TAA peptide.

Materials and Methods

**Human PBMCs and cell lines**

Blood was obtained from consenting healthy volunteers or patients with metastatic, chemotheraphy-refractory colorectal cancer who also had abnormal serum levels of CEA. This blood was collected in heparinized tubes and PBMCs were purified by ficoll density gradient centrifugation. Determination of HLA-A2/2 phenotype was assessed by staining with an anti-HELA-A2 Ab (clone PA2:2:1; Ref. 32) and flow cytometry. The T2 cell line is a B and T cell hybrid cell line that expresses HLA-A2 (gift of P. Cresswell, Yale University, New Haven, CT). The cells were cultured in completed RPMI 1640 medium (BioWhittaker) supplemented with 5% human serum (BioWhittaker), glutamine, and penicillin/streptomycin (Sigma-Aldrich).

**Peptides and reagents**

Peptides were purchased from Synpep. Peptide sequences were as follows: Cap1 (YLSGANCYLL, Cap1-6D (YLHADDNL), and CEAA691 (MY GLHLVGV). Reconstituent human IL-2 was purchased from Chiron. Reconstituent human GM-CSF was purchased from Bayer. Reconstituent human IL-4, IL-7, and IL-15 were purchased from PeproTech. Abs for flow cytometry were anti-CD107a-FITC, IFN-\(\gamma\)-allophycocyanin (BD Pharmingen), CD8-PerCP (BD Biosciences), and TCR V\(\beta\)-FITC (Beckman Coulter). HLA-A2 tetramers modified for CD8-independent binding, labeled with PE, and specific for Cap1, Cap1-6D, CEAA691, and CMV pp65 were purchased from Immunometrics. The cell lines SW403 and SW1417 were obtained from the American Type Culture Collection.

**Flow cytometry**

Stained cells were analyzed with a four-color FACS Calibur (BD Biosciences). FACS was performed on a MoFlo cell sorter (Dako Cytomation). Flow cytometry data were evaluated with FlowJo software (Tree Star).

**Generation of human Cap1 and Cap1–6D CTL lines and clones**

In all instances, companion T cell lines (that is Cap1-CTLs and 6D-CTLs) were generated from bulk PBMC of individual HLA-A2-+ donors in separate cultures. DCs were generated from PBMC according to published methods (33). Anti-CD107a Ab and DCs were pulsed with 20 \(\mu\)g/ml peptide and incubated with autologous PBMC following by addition of IL-2 (20 U/ml), IL-7 (5 U/ml), and IL-15 (5 ng/ml) on day 3. Subsequent restimulations occurred at weekly intervals by the addition of peptide-pulsed, irradiated PBMC to the cultures followed by cytokine treatment 24 h later. The first restimulation following in vitro priming was performed with autologous PBMC, and all subsequent restimulations were performed with HLA-A2-+ allogeneic PBMC. For generating CTL clones, Cap1-CTLs or Cap1–6D CTLs were stained with Cap1 or Cap1–6D tetramer, respectively, and the tetramer-positive CD8\(^+\) single cells were sorted into individual wells of 96-well plates that were precoated with anti-CD3 and -CD28 Ab. Peptide restimulations for the clones were performed according to the procedures as described for generating the CTL lines.

**TCR V\(\beta\) identification by PCR-sequence-specific primers (SSP)**

TCR V\(\beta\) PCR-SSP assay was performed with the previously described methods (34). PCR amplification was performed using the Hot start Taq DNA polymerase kit (Fermentas) in a thermocycler (Eppendorf) with the program as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min. Human aldolase A primers were included in the PCR as an internal control.

The PCR products were run on a 2% agarose gel, and the images were acquired on a UV transilluminator.

**Sequencing of TCR V\(\beta\) genes**

Total RNA was isolated from each TCR clone, and reverse-transcribed into cDNA using the SMART PCR cDNA Synthesis kit (BD Clontech). The TCR \(\beta\)-chains were amplified with the 5‘ anchor primer and the 3‘ primer of V\(\beta\) gene-specific primer described previously. The V\(\beta\) genes were cloned into pCR2.1 TOPO vector (Invitrogen Life Technologies), and DNA sequencing was performed using TCR V\(\beta\) constant primer.

**Chromium release assay**

Functional cytotoxic activity was assessed by a standard chromium release assay. Briefly, Cap1-CTLs or 6D-CTLs were incubated with chromium-labeled T2 target cells pulsed with the indicated concentration of Cap1 or Cap1–6D peptide at a 20:1 E:T ratio. Chromium release was measured in the supernatant after 4 h of incubation at 37°C with 5% CO\(_2\). The percentage of specific lysis was calculated as 100 \(\times\) (experimental release - spontaneous release)/spontaneous release.

**CD107a mobilization/IFN-\(\gamma\) production assay**

As an alternative to the bulk chromium release assay, functional cytotoxic activity was also determined at the single-cell level by assessing the cell surface mobilization of CD107a and intracellular IFN-\(\gamma\) production by flow cytometry according to the procedure previously reported (35). Briefly, Cap1-CTLs or 6D-CTLs were treated with a mixture of brefeldin A, monensin, and a FITC-conjugated Ab to CD107a (BD Pharmingen) in culture medium. The effector T cell lines were incubated with T2 target cells pulsed with the indicated concentration of Cap1 or Cap1–6D peptide at a 1:1 E:T ratio for 5 h at 37°C/5% CO\(_2\). The cells were immediately washed with PBS plus 2% FBS plus 2 mM EDTA and were surface-stained with CD8 and Cap1–6D tetramer. After fixation and permeabilization, the cells were stained intracellularly for IFN-\(\gamma\). Percentage of CD107a+ cells or IFN-\(\gamma\)+ cells among the tetramer-positive populations was assessed by flow cytometry.

**Cytokine release assay by ELISA**

CTL clones were tested for IFN-\(\gamma\) or GM-CSF release using an ELISA kit (BD Biosciences). Tumor cell lines or peptide-pulsed T2 cells were cocultured with the effectors at 20:1 or 10:1 E:T ratio depending on available CTLs. After incubation for 24 h, the supernatants were collected for IFN-\(\gamma\) ELISA following the manufacturer’s instruction.

**Tetramer decay assay**

Tetramer decay assays were performed as previously described (36). Briefly, T cell clones were stained with MHC class I tetramers and CDS for 20 min on ice. The cells were washed two times to remove unbound tetramer. An aliquot corresponding to \(t_0\) was removed just before the addition of blocking anti-HLA-A2 Ab (clone BB7.2; Ref. 37) at 1 mg/ml. Aliquots of cells at the indicated time points were immediately fixed in 2% formaldehyde. Data were acquired in batch by flow cytometry after all time points had been obtained. Decay curves were plotted as the log of normalized mean fluorescence intensity of gated CD8\(^+\)/tetramer-positive cells against time. The slope was calculated as \(\ln (F/F_0)/t\) where \(F_0\) was the total fluorescence at the beginning of the interval, \(F_0\) was the total fluorescence at the end of the interval, and \(t\) was the length of the interval in minutes. \(1/t_0\) was calculated as \(h^2\) mean slope.

**Results**

**Generation of CTL lines primed by either the native or altered epitopes derived from CEA**

To compare the TCR repertoires induced by the native CEA peptide and its agonist, CTLs were generated in parallel from PBMCs of...
of HLA-A2\(^+\) donors with either the Cap1 peptide (Cap1-CTLs) or Cap1–6D peptide (6D-CTLs). Peptide-pulsed DCs were used to prime CTLs in vitro. Following 7 days of culture, peptide-specific CTLs were generated as demonstrated by the positive Cap1 and Cap1–6D tetramer staining of CD8\(^+\) T cells (Fig. 1A). After three cycles of Ag stimulation, the peptide-specific CTLs showed further expansion (Fig. 1B). Consistent with prior reports, Cap1–6D was more efficient in generating Ag-specific T cells in vitro (30). In the CTL lines primed with either peptide, the Cap1 tetramer stained a smaller percentage of CD8 T cells than the Cap1–6D tetramer.
Among the CTL lines induced by Cap1–6D peptide, 77% of CTLs stained with Cap1–6D tetramer, while only 24% stained with Cap1 tetramer, suggesting that only a subset of the CTLs induced by agonist Cap1–6D can bind both agonist Cap1–6D and native Cap1 peptide (Fig. 1B). Interestingly, within the Cap1-primed cultures, a high proportion of T cells (58.5%) were also stained with the Cap1–6D tetramer. These results indicate that Cap1 may induce a pool of CTLs that can recognize Cap1–6D peptide, but the majority of these Cap1–6D-specific T cells may not bind Cap1 tetramer. In addition, within both CTL cultures the overall staining intensity of the Cap1–6D tetramer increased between one (Fig. 1C) and three (Fig. 1D) cycles of Ag stimulation. Regardless of the tetramer used, the expression of CD3 on the CD8+ tetramer-positive population was similar (data not shown). The CTL cultures did not stain with irrelevant tetramers to CEA691 and CMV pp65 (Fig. 1E).

These results were consistent for T cell lines generated from three healthy individuals.

FIGURE 2. Functional capacity of the Cap1-CTL and 6D-CTL lines. A, Cytotoxicity of the Cap1-CTL line (circles) and the 6D-CTL line (triangles) shown in Fig. 1B were assessed by using a 4-h chromium release assay. CTL lines were coincubated with chromium-labeled T2 cells pulsed with the indicated concentration of either Cap1 peptide (filled) or Cap1–6D peptide (open) at an E:T ratio of 20:1. B, Surface CD107a mobilization and IFN-γ production by the Cap1-CTLs (upper panels) and 6D-CTLs (lower panels) were also assessed by flow cytometry after 5 h of incubation with Cap1-pulsed T2 cells. The T2 cells were loaded with increasing peptide concentrations as indicated. CD8+ tetramer-positive T cells were gated and the percentage of CD107a+ cells or intracellular IFN-γ+ cells was assessed. C, The normalized percentage of CD107a+ cells or intracellular IFN-γ+ cells of CD8+ tetramer-positive Cap1-CTLs (circles) and 6D-CTLs (triangles) is shown at indicated Cap1 peptide concentrations. D, CTLs were also generated from PBMC from a patient with metastatic colon cancer with Cap1 (circles) and Cap1–6D peptide (triangles). Production of IFN-γ was accessed by ELISA after stimulation with the indicated peptide.
Cytotoxic activity of Cap1-CTLs and 6D-CTLs was assessed in a 
$^{51}$Cr-release assay against peptide-pulsed targets. Curiously, simi-
lar levels of killing were seen with both CTL lines to either Cap1 or 
Cap1–6D peptide-pulsed targets (Fig. 2A) despite there being a 
higher percentage of tetramer-positive CTLs in the 6D-CTL line 
than in the Cap1-CTL line (Fig. 1B). We further analyzed the 
activities of Cap1 and 6D CTL lines at the single-cell level by 
assessing the cell surface mobilization of CD107a and intracellular 
IFN-γ production by Cap-1 tetramer-positive CD8$^+$ T cells in 
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marker of degranulation, can correlate with cytotoxicity as de-
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within both CTL lines, only a subset of Cap1-6D tetramer-posi-
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to Cap1 peptide than 6D-CTLs: CD107 surface mobilization and 
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percentage of Cap1 tetramer-positive CTL in the 6D-CTL culture 
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CTLs can kill targets with low concentrations of peptide, these 
CTL require higher levels of peptide to trigger IFN-γ production. 
This hierarchy of thresholds for cytotoxicity vs cytokine secretion 
is consistent with prior reports (38).

To access whether the function of CEA peptide-specific CTLs 
follow a similar pattern in patients with CEA-expressing mali-
gnancies, we generated CTL lines from colon cancer patients with 
Cap1 peptide and Cap1–6D peptide in vitro. In one of the three 
patients assessed, we could successfully generate CTL to the pep-
tides. Although the 6D-CTLs and Cap1-CTLs from this individual 
had a similar sensitivity to Cap1–6D peptide, Cap1-CTLs were 
again more sensitive than the 6D-CTLs to Cap1 (Fig. 2D). These 
data demonstrate that CTL generated from patients with CEA-
expressing tumors can possess the same hierarchy of functional 
avidity between the Cap1 and Cap1–6D peptides.

**TCR Vβ usage by Cap1- and Cap1–6D-induced CTLs**

To determine whether 6D-CTLs and Cap1-CTLs are derived from 
the same T cells, we analyzed the TCR Vβ gene usage of these two 
CTL lines. We stained the Cap1-6D tetramer-positive 6D-CTLs 
and Cap1 tetramer-positive Cap1-CTLs with different available anti-
TCR Vβ Abs. The majority of tetramer-positive CD8$^+$ T cells 
among both CTL lines were stained by the Ab to Vβ1 (Fig. 3A) but 
not to Vβ2, 3.1, 5, 6.7, 11, 12, 13, or 17 (Fig. 3A). A small pro-
portion of Cap1-CTLs were also stained by anti-Vβ8.1. TCR Vβ 
gene usage of tetramer-sorted CTLs was also evaluated by PCR-
SSP (Fig. 3B). By this approach, Cap1-6D tetramer-positive 6D-
CTLs used three TCR Vβ genes (Vβ1, Vβ9, and Vβ16) while both 
Cap1 tetramer-positive Cap1-CTLs used five TCR Vβ genes (Vβ1, 
Vβ8, Vβ9, Vβ16, and Vβ21). Vβ1, 9, and 16 were used by both 
CTL lines whereas Vβ8 and 21 were only used by the Cap1-CTLs 
but not the 6D-CTLs.

To more precisely analyze the TCR Vβ genes used by Cap1-
CTLs and 6D-CTLs, we generated CTL clones from the CTL 
lines. To reduce biasing of the CTL repertoire that might result 
with multiple Ag stimulations, we performed tetramer-gated FACS 
after only one Ag restimulation. Single Cap1-CTLs or 6D-CTLs 
were sorted into 96-well plates coated with anti-CD3 and -CD28 
Abs for expansion. All the Cap1-CTL clones could bind Cap1 
tetramer, but only three of the five clones could bind Cap1–6D 
tetramer demonstrating that only a subset of the Cap1-CTLs could

**Functional capacity of CTL lines induced by Cap1 and 
Cap1–6D peptide**

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**TCR Vβ gene analysis for CTL lines elicited by Cap1 and 
Cap1–6D peptide**

A. The Cap1-CTL lines and 6D-CTL line were stained with 
Cap1 tetramer and Cap1–6D tetramer, respectively, along with anti-CD8 and the indicated panel of anti-TCR Vβ Abs. The tetramer-positive T cells were 
gated and analyzed for TCR Vβ staining. B. Cap1–6D tetramer-positive CD8$^+$ CTLs and Cap1 tetramer-positive CD8$^+$ CTLs were respectively purified 
from the 6D-CTL line and Cap1-CTL line through tetramer-guided sorting. The cDNA of the sorted CTLs were used as templates and 24 specific PCR 
primers for TCR Vβ were used for PCR-SSP assay. The internal control is a band for the human aldolase A gene. Reactions showing only the internal 
control band are considered as negative. The reaction that shows an additional band at the right size is considered as a specific Vβ positive. Each well 
represents a PCR with a specific Vβ primer as shown on the top Vβ1–24.
recognize both the Cap1 peptide and the Cap1–6D peptide. All 6D-CTL clones generated were able to bind both Cap1 and Cap1–6D tetramers (Fig. 4). By anti-Vβ1 Ab staining, three Cap1-CTL clones were Vβ1+ (Cap1-D2, E1, and E8) and the other two were Vβ1− (Cap1-B3 and E10) (Table I). Interestingly, only the Vβ1+ Cap1-CTL clones could bind to Cap1–6D tetramer. All 6D-CTL clones were Vβ1− except for the clone Cap1–6D-C6 (Table I). Thus, TCR Vβ appears to be the predominant repertoire used by CTLs that could react to Cap1–6D peptide. Vβ CDR3 sequencing of these clones demonstrate four TCR Vβ genes used by the five Cap1-6D clones, and five TCR Vβ used by the eight Cap1–6D clones. Importantly, there was no overlap in Vβ usage between Cap1–6D-derived clones and Cap1-derived clones. Among the Cap1-CTL clones, Vβ1− CTLs had longer 11-aa CDR3 regions, while Vβ1+ CTLs had CDR3 regions with either 8- or 9-aa lengths. All Vβ1+ 6D-CTL clones had an 8-aa length CDR3 (Table I). Conserved motifs within the CDR3 of the Cap1-CTLs or 6D-CTLs were not apparent. One noticeable difference, however, between the Vβ1+ Cap1-CTL clones and Vβ1+ 6D-CTL clones was the Jβ usage: 6D-CTLs preferably used Jβ2, and all generated Vβ1+ Cap1-CTLs used Jβ1.3. In summary, the 6D-CTL repertoires appear to be less diverse than the repertoire elicited by native Cap1 peptide. The predominant 6D-CTLs used distinctive Vβ1-Jβ2 TCRs with the 8-aa CDR3, while the Cap1-CTLs used different TCR Vβ genes with the 8- to 10-aa CDR3.

Functional capacity of the CTL clones induced by Cap1 or Cap1–6D peptide

Five Cap1 clones and seven Cap1–6D clones expanded sufficiently to perform functional assays. IFN-γ production in response to varying concentrations of peptide was assessed by ELISA. Consistent with the results with the CTL lines, the Cap1-CTL clones were functionally more sensitive than 6D-CTL clones to the native Cap1 peptide (Fig. 5A). The data indicate that these Cap1 clones have a 10–100 times higher avidity for recognizing Cap1 peptide to produce IFN-γ than Cap1–6D clones, while Cap1-CTL clones and 6D-CTL clones had comparable avidities to recognize Cap1–6D peptide (Fig. 5B). This difference was also seen in additional clones evaluated in separate experiment (Fig. 5C). Two Cap1 clones (Cap1-B3 and Cap1-E10) did not respond to Cap1–6D peptide and were also TCR Vβ1− (Table I), and not able to bind Cap1–6D tetramer (Fig. 4). One Cap1–6D clone (6D-E5) could not produce IFN-γ to either Cap1 or Cap1–6D peptide even though this clone had a high binding intensity for the Cap1 and Cap1–6D tetramer (Fig. 4). This clone may have lost the ability to produce IFN-γ during the cell expansion and actually lost the capacity to expand with subsequent restimulations. Indeed, the level of IFN-γ secretion varied between the clones with the identical TCR Vβ (6D-D6 and D7) at the same peptide concentration. To determine whether the T cell clone could produce other cytokines, we assessed available Cap1 and Cap1–6D clones for GM-CSF production as well (Fig. 5D). We saw the same pattern where the Cap1 clone possessed higher functional avidity to the peptides than the Cap1–6D clone. In summary, 6D-CTL clones possess high avidity for Cap1–6D peptide but low avidity for native Cap1 peptide. In contrast, a subset of Cap1-CTL clones (Vβ1+) possess high avidity for the Cap1–6D peptide, but have higher avidity for Cap1 peptide than 6D-CTLs.

Recognition of CEA-expressing tumor cell lines

To determine whether the T cell clones could recognize tumor cell-expressing endogenous CEA, we coincubated the clones with the tumor cell lines SW403 (HLA-A2+, CEA−) and SW1417 (HLA-A2−, CEA+) (Fig. 6). Consistent with the functional results with the peptide titration, only Cap1-E8 that had a highest avidity to the native cap-1 peptide generated significant levels of IFN-γ in response to SW403. Clones Cap1-B3 and CapD-C5 produced much lower levels of cytokines to SW403. The remaining clones failed to recognize the tumor cell line.

Half-life of tetramer binding on CTL clones elicited by Cap1 peptide and Cap1–6D peptide

Based upon the intensity of tetramer staining, the binding affinity of 6D-CTLs for the native peptide was generally higher than that of Cap1-CTLs (Figs. 1D and 4). Nevertheless, these 6D-CTLs had lower functional avidity. Uncoupling between intensity of tetramer staining and sensitivity of the T cell to recognize Ag has been

Table I. TCR Vβ usage and CDR3 sequences of each Cap1-CTL and 6D-CTL clones

<table>
<thead>
<tr>
<th>CTL Clones</th>
<th>TCR Vβ</th>
<th>CDR3</th>
<th>Jβ</th>
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<td>Jβ2.6</td>
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The TCR of a Cap1 clone can bind the Cap1 complexes more stably. These tetramer decay results demonstrate that C binding (Fig. 7). Functional assay for the Cap1-CTL and 6D-CTL clones. Cap1 and 6D-CTL clones were generated and incubated with T2 target cells pulsed with the indicated concentration of (A) Cap1 peptide or (B) Cap1–6D peptide at a 10:1 E:T ratio for 24 h. The Cap1-CTL clones Cap1-B3 (○), Cap1-D2 (■), and Cap1-E1 (▲); and 6D-CTL clones 6D-A6 (○), 6D-B7 (■), 6D-D6 (△), 6D-D7 (△), 6D-F5 (▲), and 6D-E5 (□) were assessed for IFN-γ secretion into culture supernatants by cytokine ELISA. C, Additional CTL clones were generated in separate experiments and assessed for responses to peptide-pulsed T2 cells at an E:T ratio of 20:1. T2 cells were pulsed at the indicated peptide concentration. IFN-γ production from Cap1-CTL clones Cap1-E8 (○) and Cap1-E10 (□) and 6D-CTL clone 6D-C5 (▲) were also assessed by ELISA. D, Cap1-E1 and 6D-F5 were also assessed for GM-CSF production by ELISA.

FIGURE 5. Functional assay for the Cap1-CTL and 6D-CTL clones. Cap1 and 6D-CTL clones were generated and incubated with T2 target cells pulsed with the indicated concentration of (A) Cap1 peptide or (B) Cap1–6D peptide at a 10:1 E:T ratio for 24 h. The Cap1-CTL clones Cap1-B3 (○), Cap1-D2 (■), and Cap1-E1 (▲); and 6D-CTL clones 6D-A6 (○), 6D-B7 (■), 6D-D6 (△), 6D-D7 (△), 6D-F5 (▲), and 6D-E5 (□) were assessed for IFN-γ secretion into culture supernatants by cytokine ELISA. C, Additional CTL clones were generated in separate experiments and assessed for responses to peptide-pulsed T2 cells at an E:T ratio of 20:1. T2 cells were pulsed at the indicated peptide concentration. IFN-γ production from Cap1-CTL clones Cap1-E8 (○) and Cap1-E10 (□) and 6D-CTL clone 6D-C5 (▲) were also assessed by ELISA. D, Cap1-E1 and 6D-F5 were also assessed for GM-CSF production by ELISA.

FIGURE 6. Recognition of CEA-expressing tumor cell lines by CTL clones. The indicated Cap1 and 6D-CTL clones were coincubated with SW407 (CEA⁺, HLA-A2⁺; ■) and SW1417 (CEA⁺, HLA-A2⁺; □). IFN-γ production from the clones was assessed by ELISA.
Although Cap1 tetramer could stain the 6D-CTL clones with a higher intensity, they actually possess less stable binding with a faster off-rate as demonstrated in the tetramer decay assay. These results are consistent with the notion of two-step binding of TCR to the peptide/MHC complex where binding affinity and binding stability can result from distinct TCR contact points. Here, we demonstrate that it is the higher stability of binding with the Cap1-CTL clone, and not the intensity of tetramer staining, that correlates with the capacity to produce effector cytokine. Our results also suggest that at least two populations of Cap1-specific CTLs may coexist endogenously: 1) CTLs with moderate binding affinity as shown by tetramer-binding intensity but low functional avidity as determined by IFN-γ production (the majority of 6D-CTLs belong to this category) and 2) CTLs with relatively low binding affinity to the peptide/MHC complexes but high functional avidity, such as CTLs primed by Cap1 peptide. Repetitive immunization with Cap1–6D may induce only the former. Presumably, CTLs with both higher affinity as well as higher avidity to this self-Ag may have been deleted in the thymic development. Future approaches for immunotherapy with peptide agonist will need to focus upon inducing heterogeneous Ag-specific responses comprised of T cells that not only possess high-affinity TCR, but also T cells that possess high functional avidity.

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Disclosures
The authors have no financial conflict of interest.

References

FIGURE 7. Tetramer decay assay for Cap1-CTL clone and 6D-CTL clone. The Cap1 CTL clone Cap1-E1 (●) and the 6D-CTL clone 6D-F5 (▲) were stained with Cap1 tetramer and Cap1–6D tetramer, respectively, along with anti-CD8. After adding anti-HLA class I Ab (clone BB7.2 at 1 mg/ml) to block the rebinding of the tetramer, the log of normalized mean fluorescence intensity of tetramer-positive cells was assessed by flow cytometry at the indicated time points. A, Cap1 tetramer decay for the two clones. B, Cap–6D tetramer decay for the two clones. C, The half-life ($t_{1/2}$) of each clone for tetramer decay was calculated. □, Cap1-E1 clone; ■, Cap1D-F5 clone.

higher avidity for the native epitope as demonstrated by their ability to recognize the low density of endogenous Ag presented by CEA-expressing tumor cell lines.

Functional avidity is thought to reflect the strength of interaction between a T cell and its target Ag and is typically assessed as the sensitivity of a T cell to different peptide concentration. TCR avidity is thought to exert fine control over the response of a T cell by influencing the binding and signaling of the TCR complexes. In this regard, 6D-CTLs are less sensitive than Cap1-CTLs to producing cytokine in response to the native peptide. Cap1-CTLs have a higher functional avidity for the native peptide despite having a lower intensity of binding to Cap-1 tetramer. Importantly, this difference in functional avidity between 6D-CTLs and Cap1-CTL was confirmed when T cells clones were generated and assessed. Nevertheless, CTLs elicited by agonist peptide are able to kill target cells with low Ag expression. These results are consistent with prior findings where the threshold for triggering cytotoxicity is lower than that for IFN-γ production. This result may also partially explain the infrequent and temporary clinical response seen in clinical studies: vaccination with Cap1–6D is more efficient at expanding CTLs that could possess some lytic activity against the tumor, but this vaccination may be inefficient at inducing cytokine responses to the endogenous Ag.


