Induction of Tumor-Reactive CTL by C-Side Chain Variants of the CTL Epitope HER-2/neu Protooncogene (369-377) Selected by Molecular Modeling of the Peptide: HLA-A2 Complex


*J Immunol* 2002; 169:3545-3554; doi: 10.4049/jimmunol.169.7.3545

http://www.jimmunol.org/content/169/7/3545
Induction of Tumor-Reactive CTL by C-Side Chain Variants of the CTL Epitope HER-2/neu Protooncogene (369-377) Selected by Molecular Modeling of the Peptide: HLA-A2 Complex

Agapito Castilleja,* Darrick Carter,† Clay L. Efferson,* Nancy E. Ward,‡ Kouichiro Kawano,* Bryan Fisk,** Andrzej P. Kudelka,‡ David M. Gershenson,* James L. Murray,¶ Catherine A. O’Brien,† and Constantin G. Ioannides*‡§

To design side chain variants for modulation of immunogenicity, we modeled the complex of the HLA-A2 molecule with an immunodominant peptide, E75, from the HER-2/neu protooncogene protein recognized by CTL. We identified the side chain orientation of E75. We modified E75 at the central Ser5 (E75 wild-type), which points upward, by removing successively the HO (variant SSA) and the CH2-OH (variant S5G). Replacement of the OH with an aminopropyl (CH2)3-NH3 (variant S5K) maintained a similar upward orientation of the side chain. SSA and SSG were stronger stimulators while SSK was a weaker stimulator than E75 for induction of lytic function, indicating that the OH group and its extension hindered TCR activation. SSK-CTL survived longer than did CTL induced by E75 and the variants SSA and SSG, which became apoptotic after restimulation with the inducer. SSK-CTL also recognized E75 endogenously presented by the tumor by IFN-γ production and specific cytolysis. SSK-CTL expanded at stimulation with E75 or with E75 plus agonistic anti-Fas mAb. Compared with SSK-CTL that had been restimulated with the inducer SSK, S5K-CTL stimulated with wild-type E75 expressed higher levels of E75* TCR and BCL-2 Activation of human tumor-reactive CTL by weaker agonists than the nominal Ag, followed by expansion with the nominal Ag, is a novel approach to antitumor CTL development. Fine tuning of activation of tumor-reactive CTL by weak agonists, designed by molecular modeling, may circumvent cell death or tolerization induced by tumor Ag, and thus, may provide a novel approach to the rational design of human cancer vaccines. The Journal of Immunology, 2002, 169: 3545–3554.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Copyright © 2002 by The American Association of Immunologists, Inc.
their biological potency. The pMHC-1 structure can be modeled by using as a “search model” the crystal structure of another peptide that has structural similarities with the pMHC-1 (13, 14). Identification of the positioning of the side chains of the residues in the central area at amino acid positions 4–7 allows changes to be focused in the area that complements the TCR combining site. This area was recently identified as the functional “hot spot” that allows TCR to finely discriminate among similar ligands (10). A side chain in the central area pointing upwards (toward the TCR) can achieve more extensive contact with the TCR than others. This contact is provided by an increase in van der Waals forces from the hydrophobic side chains, or by an increase/decrease in hydrogen bonds by OH groups, or by an increase/decrease in charged interactions. Whether the side chain extension correlates with increased immunogenicity remains unknown.

To address these questions, we examined the binding of the HER-2/neu protooncoprotein (HER-2), CTL epitope E75 (369-377) to HLA-A2 at the atomic level. Molecular models of the E75-HLA-A2 complex indicated that the side chain of the central Ser2 (S373) points upward. Thus, the OH group can either enhance binding at the TCR via a hydrogen bond, or sterically hinder the interaction with the TCR by decreasing the affinity of the TCR for the pMHC-1. If the first hypothesis is true, then removal of the OH group should decrease the affinity of binding by the TCR and decrease signaling, hence variants in which the central Ser is replaced by Ala or Gly should be less immunogenic than wild-type E75. If the second hypothesis is true, then Ala/Gly variants should be more immunogenic than the wild-type E75. To address the requirement that variant-induced CTLs survive their encounter with the wild-type Ag, we created another variant reasoning that stimulation with that variant should protect responding cells from death by overstimulation. This variant should stimulate some of the effector functions weaker than E75, and E75 should activate the variant-induced effectors. The only alternatives that would not disturb the peptide bond were positively and negatively charged side chains. Because the negatively charged amino acids Glu and Asp have bulky carboxyl groups, we replaced Ser5 with the positively charged Lys5 variant. The aminopropyl group of Lys extends farther and has a greater flexibility than the acetyl group of the Glu.

Priming with variants S5A and S5G enhanced the induction of IFN-γ and E75-specific cytolyis of CTL from two donors known to respond to E75, but the responders died faster than did the cells that had been stimulated by E75. In contrast, variant S5K induced higher levels of IFN-γ, but not of CTL activity against E75 than the E75-induced CTL (E75-CTL). In “weak responder” to E75, S5K-induced CTL (S5K-CTL) recognized E75 with lower affinity than did E75-induced CTL. S5K-CTL survived longer than the E75-CTL, which became apoptotic at restimulation with E75. Of interest, restimulation with E75 resulted in better protection from apoptosis in the S5K-CTL than did restimulation with S5K. This protection was paralleled by higher Bcl-xL to Bad ratios and higher Bcl-2 levels than the ones induced by S5K. Thus, the side chain variants that were less activating than the wild-type Ag induced specific CTL for the E75 expressed on tumors. Such CTL were then expanded by E75, indicating that the nominal Ag or stronger agonistic variants can use priming with weak agonists to bypass induction of apoptosis.

**Materials and Methods**

**Cells, Abs, and cytokines**

HLA-A2 and PBMC were obtained from completely HLA-typed healthy volunteers. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (15–17). mAb to CD3, CD4, CD8 (Ortho Diagnostics, Rantomy, NJ), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), and HLA-A2 (clone BB7.2, American Type Culture Collection, Manassas, VA) were either unconjugated or conjugated with FITC or PE. Ag expression by dendritic cells (DCs) and T cells was determined by FACS analysis using a flow cytometer. EPICS Profile Analyzer, Coulter Electronics, Hialeah, FL). GM-CSF of specific activity (1.25 × 10^7 CFU/250 mg) was from Immunex, Seattle, WA; TNF-α of specific activity (2.5 × 10^12 U/mg) was from Cetus (Emeryville, CA); IL-4 of specific activity (5 × 10^6 IU/mg) was from Biosource International (Camarillo, CA); IL-2 of specific activity (18 × 10^6 IU/mg) was from Cetus; IL-12 of specific activity (5 × 10^7 IU/mg) was a kind gift from Dr. S. Wolf (Department of Immunology, Genetics Institute, Cambridge, MA). The anti-human Fas mAb CH11 was purchased from Upstate Biotechnology (Lake Placid, NY), mAb to actin, Bcl-2, Bcl-xL, and Bad were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All specific mAb and isotype controls were obtained from BD PharMingen (San Diego, CA).

**Synthetic peptides**

Peptides used were E75 (HER-2: 369-377) and its mutated analogs (Table 1). To facilitate presentation, E75 variants mutated at Ser5 are abbreviated based on the position and the substitution. For example, the variant in which serine was replaced by alanine is S5A; the variant in which serine was replaced with glycine is S5G. A7.3 in which the alanine side chain was extended with two methylene groups was obtained by replacement of Ala with glycine (linear side chain). F8-1 was obtained by replacing of Phex with isophenylalanine (1 CH2) deletion. All peptides were prepared by the Synthentic Antigen Laboratory of M. D. Anderson Cancer Center (Houston, TX) and purified by HPLC. The purity of the peptides ranged from 95–97%. Peptides were dissolved in PBS and stored frozen at −20°C in aliquots of 2 mg/ml.

**Molecular modeling of the peptide: HLA-A2 complex**

The coordinates of the native HLA-A2 structure (14, 18, 19) were downloaded from the Brookhaven protein database (ID number: 3HLA). This file was used as a template for manipulations with the Swiss Model (20) program available through the Swiss web site. The Tax peptide bound to the HLA-A2 (21) was mutated manually to yield the bound E75 peptide and the Ala5, Gly5, and Lys5 variants. Each new structure was submitted for energy minimization with the GROMOS96 implementation of the Swiss-PdbViewer. Solvent-accessible surface area was calculated with the GETAREA1.1 online program with the default probe radius, set at 1.4 Å.

**T cell stimulation by peptide-pulsed DC**

DCs generated from peripheral blood were plated at 1.2 × 10^5 cell/well in 24-well culture plates and pulsed with peptides at 50 µg/ml in serum-free medium for 2 h before the addition of responders, as described (15, 16). E75-induced and S5K-induced CTL lines were maintained by periodic stimulation with peptide pulsed on DCs, followed by expansion in the presence of irradiated feeder cells and PHA. The number of cells expressing a TCR that was specific for HLA-A2 bound to the E75 peptide (E75-TCR+ cells) was performed using E75 dimers (dE75) prepared as described in the manufacturer’s instructions. Empty HLA-A2-IgG dimers were obtained from BD PharMingen. Control without peptide dimers not pulsed with peptide (NP) were prepared in parallel and tested in the same experiment. Positive control influenza matrix peptide M1 (58–66 dimers (dM1) were prepared simultaneously and used in the same experiment. For analysis, cells were incubated in parallel with dNP, and dE75 followed by PE-conjugated anti-mouse IgG1. Intracellular expression of Bcl-2 was determined, following manufacturer’s instructions using FITC-conjugated Bcl-2, Ab, and a matched FITC-conjugated isotype control.

**CTL and cytokine assays**

Recognition by CTL of peptides used as immunogens was performed as described (17). Recognition of E75 and of its variants was considered specific when the percent specific lysis of T2 cells pulsed with E75 minus the SD was higher by at least 5% than the percentage of specific lysis of T2 cells that had been pulsed with peptide plus the SD, as described (22). A significant increase/decrease in CTL activity was defined as an increase/decrease of >20% in the lysis of T2 cells pulsed with peptide by variant-induced CTL compared with wild-type E75-induced CTL. Similarly, a significant increase in IFN-γ induction was defined as an increase of >20% in IFN-γ levels after stimulation with the variant vs after stimulation with the wild-type E75. The 20% value was chosen as a cut-off for significant increase based on the assumption that if a 2-fold increase of the minimum
Table 1. HLA-A2 binding stability by E75 and its variants

<table>
<thead>
<tr>
<th>Code</th>
<th>Sequence</th>
<th>Binding Stability</th>
<th>Ligation Strength</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>E75</td>
<td>KIFGSLAFL</td>
<td>482 28</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>K1G</td>
<td>GIGFSLAFL</td>
<td>138 28</td>
<td>Positive charge →neutral</td>
<td></td>
</tr>
<tr>
<td>S5A</td>
<td>KIF GALAFL</td>
<td>482 28</td>
<td>OH → nonpolar aliphatic</td>
<td></td>
</tr>
<tr>
<td>S5G</td>
<td>KIFGGLAFL</td>
<td>483 30</td>
<td>OH → neutral</td>
<td></td>
</tr>
<tr>
<td>S5K</td>
<td>KIFGKLAFL</td>
<td>482 29</td>
<td>OH → positive charge</td>
<td></td>
</tr>
<tr>
<td>F8K</td>
<td>KIFGOSLAKL</td>
<td>88 30</td>
<td>Aromatic to (+) charged</td>
<td></td>
</tr>
<tr>
<td>F8Y</td>
<td>KIFGSLAYL</td>
<td>482 28</td>
<td>OH in aromatic residue</td>
<td></td>
</tr>
<tr>
<td>F8D</td>
<td>KIFGSLAD</td>
<td>236 28</td>
<td>Aromatic to (−) charged</td>
<td></td>
</tr>
<tr>
<td>A7.3</td>
<td>K1F GSL (Nleu)FL</td>
<td>nd nd</td>
<td>2 CH2 extension of Ala2</td>
<td></td>
</tr>
<tr>
<td>F8-1</td>
<td>K1F GSLA (Iso-Phe)L</td>
<td>nd nd</td>
<td>1 CH2 deletion of Phe8</td>
<td></td>
</tr>
</tbody>
</table>

* The binding stability is an estimate of half time of dissociation (in minutes) from HLA-A2 of peptides of the sequence listed above. The theoretical half-life of dissociation was calculated using Parker’s algorithm (27) available at http://bimas.dctr.ig.gov/molbind/hla-bind.

2 The ligation strength was calculated using the SYFPEITHI program (28). The experimentally determined mean channel fluorescence values for HLA-A2 expression on T2 cells after incubation with peptides and staining with MAAb2.1 mAb were: NP had not been pulsed with peptide, or S5K cells that were not stimulated or permissive to replacement that would be substituted without abolishing the objects of the variant peptide to induce CTL responses.

The rationale for this approach was to identify amino acids in E75 permissive to replacement that would be substituted without abolishing the objects of the variant peptide to induce CTL responses. Substitutions in side chains that maintain the overall conformation of the peptide backbone in the HLA-A2 are more likely to lead to cross-reactive Ag for wild-type Ag-specific CTL than are substitutions that change the peptide backbone configuration. We modeled the E75-HLA-A2 complex by replacing the human T cell lymphotropic virus-I peptide Tax with E75. The Tax peptide (25, 26) shows the highest structural similarity with E75 of the models available in the databases. The Tax sequence LLFGYPVYV is similar to that of E75:KIFG SL AFL with respect to the position of aromatic residues in P3 and P8 and the aliphatic side chain extensions in the first four and the last three amino acids (only K1 and F8 differ by an NH3 and an OH group extension). The major differences rest in the central area P5 P6:YP vs SL. One Tax analog, P6A, shows even more similarity with E75 YA vs SL, with Ala and Leu differing only in the propyl side chain. This comparison allowed identification of the side chains that point upwards or sideways and will be more likely to contact TCR. The results show that the side chains of Lys1, Ser5, and Phe8 point out of the binding pocket of the MHC (Fig. 1A). The side chains of Phe5, Leu6, and Ala7 point toward the helical “walls” of the pocket (Fig. 1A). The models of the TCR-pMHC-I (HLA-A2) interaction predict that of the side chains pointing away from the MHC, Ser5, Leu6, and Ala7 are most likely to contact the CD83 (Vα + Vβ) region. We focused on Ser5 because the change induced by the removal of the hydroxyl group was likely to have the strongest effects.

Ser was substituted with Ala, Gly, and Lys. These substitutions removed an HO-group (Ala), a HO-CH2-group (Gly), or replaced the OH group with the aminopropyl (CH2-CH2-CH2-NH3) group. The position of the OH suggests that it is less involved in interactions with the HLA-A2 (Fig. 1A). No significant changes of the MHC molecule were necessary to accommodate these modifications (Fig. 1, B–D). Ser5 is preceded by Gly6, which because it does not have a side chain, is very flexible and may allow small accommodations in the model. The positions of Phe7 and Lys7 that precede the Ser8 seem to be unchanged among the four models. These results indicate that Ser5 is in a good structural position to allow side chain replacements in the antigenic peptide that can modify its interactions with TCR. S5A, S5G, and S5K bound to HLA-A2 with similar affinity as did E75 (Table I). In T2-stabilization assays, S5A, S5G, and S5K showed similar stabilizing ability for HLA-A2 as determined with mAb MA2.1 (Table I), and similar scores for times of dissociation and ligation strengths (Table I) with those of E75 as determined using the HLA-peptide binding prediction (27) and SYFPEITHI programs (28).

Increased IFN-γ-inducing and E75-specific CTL-inducing ability of the E75-variants S5A and S5G

To address whether modification of the E75 side chain by deletion or extension would increase or decrease the ability of the modified Ag to stimulate CTL induction and survival, we tested several healthy donors known from previous studies to produce E75-specific CTL at priming (“strong responders”, donors 1 and 2) or exhibit weak CTL activity after several repeated stimulations (weak responders, donor 3). PBMC were stimulated in parallel with autologous DCs pulsed with E75 variants. Donor 1 responded with higher levels of IFN-γ at priming with variants S5K, S5G, and S5A, and lower levels of IFN-γ at priming with control variants F8Y and F8K than at priming with E75 (Fig. 2, A and B). CTL induced by priming with E75 recognized E75 better than did CTL induced by S5K, F8Y, or F8K, whereas CTL induced by S5G and S5A recognized E75 better than CTL induced by E75. S5A and S5G induced both higher levels of IFN-γ and higher cytolytic activity than did E75. Thus, removal of the OH group correlated with higher IFN-γ induction and higher lytic activity against E75. CTL induced by S5K secreted higher levels of IFN-γ, but their recognition of E75 was weaker. Thus, replacement of OH group with aminopropyl group had more selective effect than removal of the OH group. Extension of these results with cells from donor 2 revealed that all at the E75 variants induced higher levels of IFN-γ at priming than did E75: S5K by 36%, S5A by 100%, and S5G by 64% (Fig. 2C). Significantly higher levels of IFN-γ were detected 96 h after stimulation with each variant in response to the highest
yellow, while the other E75 peptide residues are in green. The model is presented rotated with an angle of 180° to facilitate distinction of the side chains pointing upwards. Top, α2 domain; bottom, α1 domain of HLA-A2. HLA-2 is depicted in blue. B, Similar view of the complex for the Ser^5 to Ala^5 model of the peptide-MHC complex. C, The Ser^5 to Gly^5 model. D, The Ser to Lys^5 model. The same orientation is used as in all the molecules above.

FIGURE 1. A, Molecular model of the E75 peptide bound to the MHC. The MHC-peptide complex was modeled as described in Materials and Methods, and rendered using the Pov-Ray implementation of the Swiss PdbViewer. Residues within 6 Å of Ser^5 are displayed with their van der Waals radii in red. Ser^5 is in yellow, while the other E75 peptide residues are in green. The model is presented rotated with an angle of 180° to facilitate distinction of the side chains pointing upwards. Top, α2 domain; bottom, α1 domain of HLA-A2. HLA-2 is depicted in blue. B, Similar view of the complex for the Ser^5 to Ala^5 model of the peptide-MHC complex. C, The Ser^5 to Gly^5 model. D, The Ser to Lys^5 model. The same orientation is used as in all the molecules above.

dose (25 μg) of exogenously pulsed peptide in the presence of IL-2 for 2 days. Significant differences in IFN-γ induction were not observed when E75 or its variants were used at 1.0 or 5.0 μg/ml at 48 or 72 h. The E75-specific lytic activity of CTL induced by S5A was significantly higher than the lytic activity of CTL induced by E75 (Fig. 2D). The increase in lytic activity by S5A paralleled the increase in IFN-γ in response to S5A. Recognition of E75 by S5K-CTL was lower than the recognition by E75-CTL. CTL induced by the E75, S5K-CTL, and S5A-CTL all recognized the indicator SKOV3.A2 tumor. To determine whether E75-specific tumor-lytic CTLs were present in the variant-induced CTL, we performed cold-target inhibition of tumor lysis. Tumor lysis by S5K-CTL was inhibited less by T2-E75 than lysis by E75-CTL (Fig. 2E). This confirmation that S5A can induce both higher IFN-γ and higher lytic activity against E75 suggested that the OH group of Ser^5 hindered the TCR interaction with peptide-HLA-A2 and that removal of the OH group allowed a stronger TCR activation. However, at restimulation, the number of cells stimulated by S5A and S5G dropped faster than the number of cells that had been stimulated by E75. Cells stimulated by S5K survived longer than E75-stimulated cells (Fig. 2F), suggesting that the stimulus from the (CH2)3-NH3 was more effective than stimuli from the CH3 or the CH2-OH in maintaining the survival of responders.

Stimulation with S5K enhanced survival of responding T cells

Cancer patients are weak responders to E75 and require repeated stimulation for CTL induction. To clarify the differences between E75 and S5K in the induction of cytolysis, we tested T cells from donor 3 for whom several stimulations with E75 were required to induce detectable CTL activity, but responded with IFN-γ secretion at priming (16). S5K and E75 induced similar levels of IFN-γ at priming and at restimulation (Fig. 3A). The kinetics of induction of E75-specific CTL in relation to the number of stimulations is shown in Fig. 3B. E75 again induced higher E75-specific lytic activity than did S5K. Like donor 2, E75-stimulated cells from donor 3 declined in number after the third stimulation with Ag more than the S5K-stimulated cells (Fig. 3C). These results showed that S5K induced better survival in responders than E75. These results were confirmed in subsequent stimulation experiments. In parallel experiments, priming with E75 induced lower levels of Bcl-2 in CD8^+ cells than did priming with S5K. There were only small differences in Fas ligand, Fas, and IL-2Ra expression between E75-stimulated and S5K-stimulated donor 3 CD8^+ cells (A. Castilleja and C. G. Ioannides, unpublished observations).

S5K-induced CTL recognized E75 with lower affinity than E75-induced CTL

Weaker recognition of E75 by the S5K-CTL raised the question of whether S5K induced smaller numbers of CTL than E75, or whether the CTL induced by S5K had lower affinity for E75 than for S5K. To address the recognition of variant-induced CTL, we tested their ability to recognize E75 and the inducing variant in parallel. S5A-CTL (donor 1) recognized S5K weaker than S5A (24% decrease), suggesting that extension of the CH2 side chain in parallel. S5A-CTL (donor 1) recognized S5K weaker than S5A (24% decrease), suggesting that extension of the CH2 side chain in position 5 with OH and (CH2)3-NH3 groups, respectively, hindered TCR recognition. Similarly, donor 3 S5K-CTL recognized E75 weaker than they recognized S5K (Fig. 4A). To verify that S5K is recognized with lower affinity than E75 by donor 3 E75-CTL, we performed concentration-dependent lysis. E75-CTL recognized S5K with lower affinity than E75 (24% decrease), suggesting that extension of the CH2 side chain in position 5 with OH and (CH2)3-NH3 groups, respectively, hindered TCR recognition. Similarly, donor 3 S5K-CTL recognized E75 weaker than they recognized S5K (Fig. 4A). To verify that S5K is recognized with lower affinity than E75 by donor 3 E75-CTL, we performed concentration-dependent lysis. E75-CTL recognized S5K with lower affinity than E75. S5K recognition was close to recognition of E75 (32 vs 41%) only at high concentrations (50 μg/ml; Fig. 4B). Similarly, S5K-CTL recognized E75 with lower affinity than S5K (Fig. 4C). These results demonstrated that the OH and aminopropyl groups selectively modulated the affinity of recognition. To address whether E75-specific CTL were present in smaller numbers in S5K-CTL, we tested recognition of E75 at the same concentration (10 μg/ml) at four E:T ratios (10,
SSK-CTL recognize endogenously presented E75

Because SSK-CTL survived longer than E75-CTL, this raised the possibility that SSK could be used to induce CTL-recognizing tumors. To determine whether SSK-CTL recognized endogenous E75 in cytolysis assays, we performed cold-target inhibition of tumor lysis. T2-E75 inhibited lysis of freshly isolated ovarian tumor OVA-16 (HLA-A2/H11001, HER-2 high) by 21% in an 8-h CTL assay, and by 45% in a 16-h assay (Fig. 5, A and B). Similar inhibition (38%) was observed against SKOV3.A2 in a 16-h assay (data not shown). These results indicated that SSK-CTL recognized the endogenously presented E75 by ovarian tumor cells overexpressing HER-2. The levels of inhibition of lysis indicative of specific recognition were similar to those levels observed with donor 2, E75-CTL, and SSK-CTL (Fig. 2E). We also tested SSK-CTL ability to secrete IFN-γ at an encounter with the ovarian tumor SKOV3.A2 and its HLA-A2* counterpart SKOV3. This was necessary because the tumor and responding lymphocytes shared HLA-A3. SSK cells secreted high levels of IFN-γ within 20 h, when IL-12 was used as costimulator (Fig. 5C). IFN-γ was induced even in the absence of IL-12, but at lower levels. mAb inhibition experiments indicated that IFN-γ secretion was associated with recognition of HLA-A2. (data not shown). This indicated that present among the SSK-induced CTL was a subpopulation of cells that recognized endogenously presented E75 by cytolyis and IFN-γ secretion.

Antiapoptotic effects of E75 in SSK-activated CD8+ cells

Induction of CTL by the variant SSK raised the question of whether such cells could survive an encounter with E75 since E75 is present in vivo. To address whether E75 can induce CD95-mediated apoptosis, E75-CTL and SSK-CTL were stimulated with E75 and SSK in parallel in the presence of the agonistic Ab CH11. Three days after stimulation with E75, 46% of the E75-CTL had undergone apoptosis, whereas only 15.4% of the SSK-CTL were apoptotic after stimulation with SSK (data not shown). In contrast, when SSK-CTL were stimulated with SSK or E75, cells stimulated with E75 survived longer and may have increased in number as compared with the cells stimulated with SSK. Stimulation of SSK-CTL with 25 or 50 μg/ml E75 for 4 days increased the number of CD8+ cells by 26 and 64%, respectively. Stimulation of the same cells with anti-Fas mAb increased their numbers by 0.93 and 27%, respectively (Fig. 6, A and B), but no increase in cell number.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Induction of effector functions in donor 1 (A and B) and donor 2 (C–E) at priming with the wild-type CTL epitope E75 and its variants. A and C, IFN-γ; B, D, and E, Cytolysis. A and C, IFN-γ was determined from supernatants collected from the same cultures which were used on day 8 for CTL assays. B, D, and E, Equal numbers of effectors from each culture were tested in the same experiment. Results indicate the percentage of E75-specific lysis obtained by subtracting the specific lysis of T2 cells not pulsed with peptide, from the specific lysis of T2 cells pulsed with 25 μg/ml E75 in the same experiment. The E:T was 20:1. Stimulators were autologous DCs pulsed with 25 μg/ml peptide. NPs indicate control effectors that were stimulated only with autologous DCs which were not pulsed with peptide. F, Effectors E75-CTL, SSK-CTL, and S5A-CTL lysed the indicator ovarian tumor SKOV3.A2. Specific cold target inhibition indicates the percentage of inhibition of lysis of SKOV3.2 cells by cold (unlabeled) T2-E75 cells minus inhibition of lysis in the presence of T2-NP cells. S5G-CTL were not used in this experiment because their numbers declined rapidly after restimulation. E:T ratio was 30:1, cold:hot ratio was 10:1. F, Percentage of live cells in donor 2 cultures primed and restimulated with each variant 30 days after priming. Note the decrease in live cells in cultures stimulated with S5A or SSG. *, p < 0.05.
was observed in the absence of CH11. Notably, S5K-CTL continued to respond to S5K with higher levels of IFN-γ, but lower levels of IL-2, than did cells treated with E75 (data not shown).

To address whether E75 and S5K interfered with apoptosis pathways, S5K-CTL were restimulated with E75 or S5K at two different concentrations or remained unstimulated (group 0, DC only) in the presence of CH11. Apoptosis analysis was performed at 24 and 96 h. Both E75 and S5K inhibited the residual Fas-apoptosis within 24 h and this inhibition was peptide concentration-dependent (Fig. 6C). When apoptotic cells were counted on day 4, both peptides were protective, but E75 seemed to be more protective than S5K (Fig. 6C, day 4).

To confirm the antiapoptotic effects of E75 and S5K on S5K-CTL, we performed cell cycle analysis. Analysis of cells in the subG1 phase (Fig. 6D) showed that 46% of the unstimulated S5K cells became apoptotic. E75 and E75 + CH11 inhibited this apoptosis by 83%. S5K had a slightly lower inhibitory effect (63% inhibition). S5K + CH11 reduced apoptosis by only 24% compared with unstimulated S5K-CTL–peptide concentration-dependent (Fig. 6C). When apoptotic cells were gated on day 4, both peptides were protective, but E75 seemed to be more protective than S5K (Fig. 6C, day 4).

FIGURE 3. A, Kinetics of IFN-γ production; B, E75-specific CTL induction; and C, survival of donor 3 CTL stimulated by E75 and S5K. Experimental details as described in the text and the legend to the Fig. 2. A, IFN-γ was determined on day 3 after stimulation with each peptide. The numbers 1, 2, and 3 indicate the number of stimulations. Equal numbers of live cells from E75- and S5K-stimulated cultures were stimulated with autologous DC pulsed with the corresponding peptide. C, The number of live cells recovered was determined 1 wk after the third and the fifth stimulations.

Apoptosis resistance in stimulated T cells at day 4 is mainly due to the intrinsic pathway (29). Because resistance to Fas-induced apoptosis was suggestive of TCR-induced protection, we investigated the effects of E75 and S5K in up-regulation of Bcl-2, Bcl-xL, and Bad. Unstimulated and DC-NP-stimulated CD8+ cells from S5K-CTL were used as negative controls, while S5K-CTL stimulated with the agonists A7.3 and F8-1 were used as positive controls.

FIGURE 4. A, Specificity of S5A-CTL, S5K-CTL, and E75-CTL. A, Donor 1 S5A-CTL recognized S5K less efficiently than S5A. Donor 3 S5K-CTL recognized E75 with lower affinity than S5K. T2 cells were pulsed with E75 and S5K at 10 μg/ml. B, Donor 3 E75-CTL recognized S5K with lower affinity than E75. C, Donor 3 S5K-CTL recognized E75 with lower affinity than S5K-CTL. Concentration-dependent recognition of E75 and S5K in the same experiment. Targets were T2 cells pulsed with the indicated concentrations of peptide. B and C, Results of a 6-h CTL assay. E:T ratio was 10:1. *, p < 0.05.
To address whether E75 and S5K stimulation affected expansion, TCR expression, and Bcl-2 expression in E75/H11001 TCR cells, S5K-CTL were stimulated with T2 cells pulsed with either E75 or S5K or not pulsed with peptide (T2-NP). The number of E75/H11001 TCR cells was determined. One week later, to determine whether the affinity of the TCR for E75 was affected by the stimulation, we assessed expression of E75/H11001 TCR cells both immediately after staining and after an additional 50-min incubation of dE75-stained cells in PBS (Fig. 7, C and D, and Fig. 8A). For further refinement, E75+ TCR expression and Bcl-2 expression were analyzed separately in two gated populations of smaller size (FW scatter: 380–600) and of larger size (FW scatter: 640–1000). In the small lymphocytes (Fig. 7C), the percentages of E75+ TCR cells were similar in all three stimulation groups and the E75 and S5K-stimulated S5K-CTL appeared to have similar affinities for dE75, which were stable 50 min. In contrast, in the larger lymphocytes, the percentage of E75+ TCR cells was higher in the E75-stimulated than in S5K-stimulated S5K cells (Fig. 7D). The affinity for E75 also seemed to be higher in the E75-stimulated group than in the S5K-stimulated group (Fig. 8A). Because E75-stimulated cells proliferated better than S5K-stimulated cells, we calculated the number of E75+ -TCR cells in each stimulated culture. The number of E75+ -TCR cells in both small and large lymphocytes stimulated by E75 was higher than in the S5K-stimulated S5K-CTL (Fig. 8B). The percentage increase was similar to the increase observed in CD8+ cells (Fig. 6, A and B). This finding confirmed that S5K-induced CTL expanded better when restimulated with E75 than when restimulated with S5K. The levels of E75+ TCR and Bcl-2 in the E75-stimulated S5K-CTL in the large lymphocytes were also higher than in the S5K-stimulated S5K-CTL (Fig. 8C and D). This suggested that stimulation of S5K-CTL with E75 resulted in changes in receptor distribution or conformation that increased the binding of dE75 as suggested by Braciale and Spencer (32). These effects were not observed in the small E75+ TCR lymphocytes. Bcl-2 levels were higher in the small lymphocytes after stimulation with

**FIGURE 5.** S5K-CTL recognized endogenous E75 presented by ovarian tumor cells. A and B, Cold target inhibition of cytolysis of OVA-16 (HLA-A2, HER-2+). Cold targets were T2 pulsed with E75, using as specificity control T2 which were not pulsed with peptide (T2-NP). Numbers in the parentheses indicate the percentage of inhibition of lysis of S5K-CTL by T2-E75 compared with lysis of tumor in the presence of T2-NP. *, p < 0.05. E:T ratio was 10:1; the ratio of cold to hot targets was 1:1. C, IFN-γ induction. IL-12 was used at 3 IU (300 pg/ml); the responders to SKOV3. A2 stimulator ratio was 40:1.

**FIGURE 6.** Expansion of CD8+ cells from S5K-CTL after stimulation with E75 (A) or S5K (B) in the absence (○) or presence (●) of CH11 mAb. Equal numbers of S5K-CTL were stimulated with DCs pulsed with 0, 25, and 50 µg/ml of each peptide. The number of CD8+ cells was determined by flow-cytometry using anti-CD8 mAb-FITC conjugated. C, Ag-induced resistance to CD95-mediated apoptosis. S5K-CTL were stimulated with autologous DCs pulsed with E75 or S5K at 5 and 25 µg/ml or control no peptide (0). CH11 mAb was added 1 h later. The number of apoptotic cells was determined 1 and 4 days later. D, Restimulation with E75 and S5K-induced resistance to CD95-mediated apoptosis in S5K-CTL stimulated 1 wk before with S5K. Apoptotic cells are shown in the panel subG1. Results are from one experiment representative of three independently performed experiments. Bars indicate unstimulated (∥), E75 stimulated (●), E75 + anti-Fas mAb stimulated (□), S5K-stimulated (●), and S5K+ anti-Fas mAb stimulated (□).
S5K compared with E75. E75-stimulated S5K-CTL recognized E75 both as peptide and when endogenously presented by tumor (data not shown). Together these results indicate that priming CD8 cells with agonists for induction of cytolysis that are weaker than the nominal wild-type Ag followed by restimulation with the wild-type Ag can bypass induction of apoptosis either by the wild-type Ag (at priming) or by the weak agonist (at restimulation). This effect leads to increased survival and expansion of antitumor effectors.

Discussion
In this paper, we investigated the possibility of using molecular models of peptide:HLA-A2 complexes to select side chains that can induce CTL responses in T cells recognizing the HER-2 CTL epitope E75. The E75-HLA-A2 model identified several residues, Lys\(^1\), Ser\(^5\), Phe\(^8\), that point upwards and may contact the TCR. We found that Ser\(^5\) variants affected activation of T cell effector functions and produced differential levels of effector activity in CTL against the wild-type peptide E75. Modifications that removed the OH group led to variants that induced higher levels of IFN-\(\gamma\) at priming. In addition, CTL primed by the variant S5A recognized E75 better in lytic assays than did CTL induced by E75. In contrast, modification of E75 by extending its side chain with an aminopropyl group lead to the S5K variant, which induced IFN-\(\gamma\) in two strong responders to E75 but was not a better inducer of E75-CTL-specific activity. In a third donor, weak responder to E75, the potency of E75 and S5K to induce IFN-\(\gamma\) was similar at priming and restimulation. S5K-CTL recognized E75 with lower affinity than E75-CTL. Only at high concentrations of E75, its recognition by E75-CTL and S5K-CTL was similar. Sequential stimulations S5A\(\Rightarrow\)S5A, S5G\(\Rightarrow\)S5G, and E75\(\Rightarrow\)E75 led to death rather than to CTL expansion.

A possible explanation for these effects may be provided if the effects of water (H\(_2\)O) molecules are considered. The OH group of the Ser\(^5\) can form H-bonds with residues in TCR. Intercalation of water molecules and formation of H-bonds with the OH group of Ser may decrease the affinity of binding to the TCR, while elimination of OH group may increase the number of hydrophobic interactions. Because the sequence of the TCR and crystal structures of TCR-E75-HLA-A2 complexes are not yet available, we could not define the role of water molecules in the stimulation. Thus, deletion of the OH and CH\(_2\)-OH (hydroxymethyl) groups induced death by overstimulation. Repeated stimulations with S5K minimized S5K-CTL losses due to apoptosis compared with stimulations with S5A, S5G, and E75. E75 and S5K were similar in their ability to induce IFN-\(\gamma\). The signal from S5K was weaker than the signal from E75 in that S5K induced significantly lower levels of IL-2 in the S5K-CTL than did E75 (A. Castilleja and C. G. Ioannides, unpublished observations).

Once S5K-CTL were established and were protected from apoptosis by restimulation with S5K, signals from the wild-type
FIGURE 8. Stimulation of SSK-CTL with E75 significantly increased the number of E75^+ TCR cells. A, Percentage of E75^+ TCR cells in the large (□) and small (●) TCR-dE75 complexes. Most small lymphocytes recognized E75 with $t_{1/2}$ of >50 min, while ~50% of large lymphocytes had a $t_{1/2}$ of 50 min for E75. B, Increase in the numbers of E75^+ TCR cells of SSK-CTL after stimulation with E75 and SSK large (□) and small (●) lymphocytes. The numbers of live cells recovered after stimulation with T2-NP, T2-E75, and T2-SSK, and expansion in IL-2 were 2.7, 3.2, and 2.9 x 10^6 cells, respectively. C, Increased levels of expression of E75^+ TCR in large lymphocytes stimulated with E75 compared with SSK. The differences in MCF in small lymphocytes were minimal: 202 for E75, 180 for SSK. D, Increased levels of expression of Bcl-2 in E75^+ TCR large lymphocytes but not in small lymphocytes at stimulation with E75 or SSK. All determinations were performed in the same experiment. Results are from one determination representative of two with similar results.

E75, or variants with Ala^1 side chain extended with 3CH2 groups, or Phe^8 with side chain shortened with 1CH2 group, induced even higher Bcl-xL:Bad ratios. In SSK-CTL, E75 also increased the levels of TCR expression and Bcl-2 expression more than SSK. Considering that SSK was recognized with lower affinity than E75 by E75-induced CTL, it is possible that SSK is a weak CTL activator similar to homeostatic inducers (33, 34). A possible explanation for the low affinity of SSK-CTL for E75 is that the stimulus is not sufficiently strong to bring TCR together in the appropriate conformation for wild-type Ag recognition. This may have the advantage of extending the life of such CTL. Further studies with distinct agonists should address this question.

One important consideration now emerging from lymphocyte activation studies is that the CTL response to an Ag first expands and then contracts to bring the number ofactivated effectors (35, 36). Reduction in the number of activated CTL is initiated by Ag and manifests by induction of apoptosis at restimulation a phenomenon that is amplified by IL-2 (37). The development of agonistic variants that more strongly activate antitumor effector CTL is a necessary requirement for immunotherapy. Such CTL may be useful if they can mediate intermediate effects, i.e., tumor eradication upon activation. Repeated stimulations/vaccinations with strong agonistic variants may lead to depletion of highly activated effectors (38, 39). This raises concerns regarding the use of agonistic variants that are stronger than the nominal Ag in cancer vaccination for induction of central and peripheral memory CTL, because the life span of T cells activated by agonistic variants may be limited. An additional consideration emerging from activation studies is that agonist-induced effectors should survive and maintain their lytic function at encounter with the wild-type tumor Ag. We noted that CTL induced by wild-type E75 showed poor viability after two to three rounds of stimulation. This pattern of response is in agreement with the general pattern of responses to activation by self-specific T cells to avoid induction of autoimmunity (40).

Activation of antitumor effector CTL by weak agonists followed by wild-type Ag is a novel approach to promote their expansion and functional competence that has not been described before in human tumor systems. Similarly, protection from apoptosis and expansion of these cells by the self-peptide tumor Ag is also a novel finding for tumor systems. Such effectors may be useful for controlling the growth of tumors that express high levels of tumor Ag (e.g., HER-2). In addition, low-level activation of effector functions by weak agonists that can also induce homeostatic proliferation may be useful for immunotherapy after chemotherapy or radiation treatments, both of which are known to reduce leukocyte counts. This possibility is supported by studies with experimental models showing that activated low-avidity CTL that are specific for a self Ag can induce tumor rejection (40), and that stimulation of low-avidity clones can break tolerance to T cell epitopes (41, 42). Our previous studies demonstrated that differential TCR signaling can regulate functional activation and apoptosis in T cells (43). High-strength TCR-Ag interactions lead to activation-induced cell death, while low-strength TCR-Ag interactions can promote death by neglect. However, depending on the nature of TCR-Ag interactions, a range of cellular responses can be induced to avoid cell death (44). Recent manipulations of such responses have involved the use of “null ligands” to attenuate the signaling by strong agonists for high-avidity CTL activation (45), the use of stronger agonists to improve the proliferative capacity of low-avidity CTL (46), and the use of molecular modeling to direct repairs in weak/partial agonists (10).

Priming a CTL response to an immunodominant epitope simultaneously results in priming to variants of the peptide sequence that the individual has not encountered (47, 48). Our previous studies demonstrated that ovarian and breast tumor-associated lymphocyte, which recognized E75, also recognized better variants S5A and S5G (49). This suggested that CTLs that recognized these variants were present in patients. The possible contribution of such clones to the immune response against tumor is still unknown (50–52). Our results show that CTL survival and effector function can be enhanced by sequential stimulation with Ag variants followed by wild-type Ag. This strategy allows the response to be followed or shifted to clones that may be endowed with better survival capacity and can differentiate to peripheral memory cells, or clones with better effector function as needed. Because S5A and S5G are stronger agonists than S5K, while F8Y and F8K appear to be weaker agonists than S5K, it will be important to determine how exposure to these variants can maintain the focus of the CTL response to the wild-type tumor Ag, and which vaccination strategy is more effective in maintaining a response against tumors in patients with persistent disease. Ongoing studies in our laboratory aim to address the effects of sequential stimulation with homeostasis inducers, strong agonists, and weak agonists in developing an antitumor response.

Acknowledgments

We thank Dr. Martin Campbell for HLPC analysis of peptides, and Dr. Christine Wogan for editing this paper. We thank the volunteers who enthusiastically donated blood for these studies.
References

5. Kuhns, J. J., M. A. Batalia, Y. Shuqin, and E. J. Collins. 1999. Poor binding of a HER-2/neu epitope (GP2) to HLA-A2.1 is due to a lack of interaction with the center of the peptide. J. Biol. Chem. 274:36422.