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Enhanced Antigen-Specific Antitumor Immunity with Altered Peptide Ligands that Stabilize the MHC-Peptide-TCR Complex

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Summary

T cell responsiveness to an epitope is affected both by its affinity for the presenting MHC molecule and the affinity of the MHC-peptide complex for TCR. One limitation of cancer immunotherapy is that natural tumor antigens elicit relatively weak T cell responses, in part because high-affinity T cells are rendered tolerant to these antigens. We report here that amino acid substitutions in a natural MHC class I–restricted tumor antigen that increase the stability of the MHC-peptide-TCR complex are significantly more potent as tumor vaccines. The improved immunity results from enhanced in vivo expansion of T cells specific for the natural tumor epitope. These results indicate peptides that stabilize the MHC-peptide-TCR complex may provide superior antitumor immunity through enhanced stimulation of specific T cells.

Introduction

A major endeavor in modern medicine is the enhancement of vaccine potency via manipulation of both the antigen and the delivery system. Success in this effort is critical in the development of effective cancer immunotherapy. In particular, enhancement of T cell responses to tumor antigens would lead to more potent treatments (Pardoll, 2000). Endogenous T cell responses to both murine and human cancers can be readily identified (reviewed in De Plaen et al., 1997; Robbins and Kawakami, 1996). Tumor antigens recognized by T cells typically fall into one of three categories (reviewed in Boon et al., 1997; Gilboa, 1999): (1) antigens whose peptide epitopes incorporate the product of a mutation specific to the individual tumor (e.g., mutated oncogenes); (2) nonmutated antigens expressed by a significant proportion of tumors of certain histologic types but silent in most normal tissues in the adult (e.g., MAGE1-3); (3) tissue-specific differentiation antigens specific to the tissue type from which the tumor arose (e.g., gp100 and tyrosinase). The latter two categories of tumor antigen represent attractive candidates for incorporation into antigen-specific cancer vaccines with broad applicability in treatment of many tumors. Despite the growing list of candidate tumor antigens recognized by T cells, antigen-specific vaccination for established cancer has thus far seen relatively limited success. A major limitation to the efficacy of cancer vaccines is that endogenous T cell responses against tumor antigens tend to be relatively weak. In some cases, the weak T cell responses to tumor antigens appear to be related to the low affinity of the peptide antigen for its presenting MHC molecule (Cox et al., 1994). This low affinity results in weak association of peptide with MHC molecules at the surface of antigen-presenting cells resulting in poor presentation of MHC-peptide ligand to T cells. Such antigens are often characterized by an absence of favored residues at critical anchor positions involved in MHC binding. For weak tumor antigens that fall into the low-affinity MHC binding category, replacement or mutation of unfavorable anchor residues with more effective ones may greatly enhance MHC binding properties (Lurquin et al., 1989; Gervois et al., 1996; Parkhurst et al., 1996; Bakker et al., 1997; Dyall et al., 1998; Overwijk et al., 1998; Valmori et al., 1999). These altered peptides may more effectively activate T cell responses against the wild-type peptide antigen by virtue of the increased efficiency of presentation of the MHC-peptide complex to specific T cells. In both mouse and human studies, these anchor-modified peptides can elicit superior T cell responses against the original antigen in vivo (Dyall et al., 1998; Rosenberg et al., 1998). Thus, enhanced stability of the MHC-peptide complex afforded by the favorable anchor residue may result in a more efficacious T cell response due to the presentation of increased numbers of MHC-peptide ligands.

Some tumor antigens bind to their presenting MHC with affinities in a similar range to strong viral antigens yet elicit weak endogenous immune responses (Lee et al., 1999). For tumor antigens with high MHC affinities, the proposed mechanism for weak endogenous immune responses is that high-affinity T cells are actively tolerized via anergy or deletion, thereby leaving a functional repertoire consisting of T cells bearing T cell receptors (TCR) with low affinity for MHC-peptide complexes. This residual T cell repertoire is postulated to have escaped active tolerance induction by virtue of its low affinity for MHC-peptide ligand. This mechanism is particularly relevant for shared tumor antigens, which, because they are self-antigens, have had a long time period to induce tolerance (Morgan et al., 1998).

The current study was designed to investigate the effect of immunization with altered peptides in which amino acids were substituted that affect the affinity of the MHC-peptide complex for TCR rather than the affinity of peptide for MHC. We focused on a nonmutated H-2L^d-

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restricted peptide antigen derived from gp70 amino acids 423–431, referred to as AH1 (SPSYVYHQF), which is the dominant target for the CD8 T cell responses against the CT26 colorectal tumor (Huang et al., 1996). gp70 expression is silent in most normal tissues but is active in many mouse tumors (Huang et al., 1996). According to a computer algorithm developed to rank order the half-time of disassociation of peptide to MHC, AH1 is predicted to bind H-2L^d with higher affinity than other peptides derived from gp70 (Parker et al., 1994). AH1 features a proline in position two and an aliphatic residue in position nine, consistent with the consensus residues for H-2L^d binding peptides (Corr et al., 1992).

Here, we show that AH1 has relatively high affinity for H-2L^d but provides relatively weak immunization against CT26 challenge. An amino acid substitution in AH1 that does not alter MHC binding but rather increases the stability of the MHC-peptide-TCR complex by decreasing the off rate of TCR from MHC-peptide is significantly more potent in activating T cells in vivo specific for the natural tumor epitope, resulting in enhanced systemic antitumor immunity.

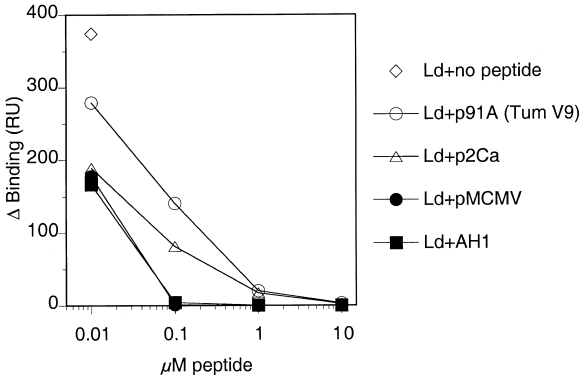

Figure 1. AH1 and pMCMV Peptides Bind H-2L^d with Similar Affinity

Either buffer alone or graded concentrations of the indicated peptide (pMCMV [YPHFMPTNL], p2Ca [LSPFSFDL], p91A-Tum V9 [QNHRALDLV]) were combined with 40 μM H-2L^d produced in mammalian cells and exposed to immobilized pMCMV-C4 peptide on the biosensor surface. The cysteine substitution at position 4 of pMCMV facilitated immobilization of the peptide to the biosensor surface and exhibits similar binding to H-2L^d as the native peptide. The amount of H-2L^d bound to pMCMV-C4 is reflected by the change in resonance units after 4 min.

Results

Tumor Antigen AH1 Binds MHC with High Affinity

To analyze the role of MHC and TCR binding properties on the immunogenicity of a tumor antigen, we chose the well-characterized mouse colorectal cancer, CT26. AH1, an H-2L^d-restricted peptide, was identified as the immunodominant antigen from CT26 recognized by CD8$^+$ T cells in animals immunized with autologous whole cell tumor vaccines (Huang et al., 1996). As with most natural tumor antigens derived from self-proteins, AH1 peptide is a relatively weak immunogen when used independently from the tumor as a vaccine against CT26 even though it is a good target for activated antigen-specific CTL (Huang et al., 1996). To understand why the peptide antigen does not immunize efficiently against tumor formation and to determine if substitutions that alter the stability of the trimeric complex might result in stronger antitumor immunity, we first studied the physical parameters of the interaction between H-2L^d and AH1 as well as the H-2L^d-AH1 complex and a cognate TCR.

To compare binding of the AH1 peptide to the MHC class I molecule H-2L^d relative to other tumor antigens and known H-2L^d binding peptides, we employed a previously described assay using immobilized peptides and surface plasmon resonance (Khilko et al., 1993; reviewed in Khilko et al., 1995). In this assay, the binding of an "empty" soluble H-2L^d to an immobilized peptide is competed for by graded concentrations of a test peptide. The peptide pMCMV-C4 was immobilized, and the indicated competing peptides and H-2L^d were offered in solution phase. AH1 bound H-2L^d as well as pMCMV and better than the other peptides tested as indicated by the decreased change in resonance units, especially at the lower peptide concentrations (Figure 1). pMCMV has previously been shown to be a high-affinity H-2L^d binding peptide (Margulies et al., 1993). p2Ca and p91A-Tum V9, another H-2L^d-restricted tumor antigen, are intermediate affinity MHC binding peptides (Alexander-Miller et al., 1994; Corr et al., 1994). These results indicate that AH1 is a high-affinity binding peptide of H-2L^d. Thus, weak MHC binding does not account for the failure of the AH1 peptide to serve as an effective immunogen.

Alanine Substitution of Residue Five in the AH1 Peptide Enhances Stability of the MHC-Peptide-TCR Complex

To determine the influence of TCR affinity for MHC-peptide complex on immunogenicity, we studied TCR binding to the natural H-2L^d-AH1 complex as well as to a panel of alanine-substituted variant peptides. To overcome the intrinsically low affinity of MHC-peptide for TCR, we used the general approach of making multivalent MHC and TCR complexes (Altman et al., 1996; O'Herrin et al., 1997; Murali-Krishna et al., 1998). We generated dimeric forms of TCR and MHC-peptide using an Ig scaffold to produce fusion proteins (Dal Porto et al., 1993; O'Herrin et al., 1997; Greten et al., 1998). Binding was measured in both directions by flow cytometry by adding peptide-loaded dimeric H-2L^d, Ld-Ig, to AH1-specific T cells and dimeric TCR, CT-Ig, to peptide-loaded antigen-presenting cells. The rank order of binding of either TCR to MHC-peptide on cells or MHC-peptide to TCR expressing cells was essentially the same (Figure 2). Substitution of the consensus MHC anchor residue 2 (proline) resulted in reduced binding, although the interaction was not totally abolished in either the TCR or MHC binding assay. Other amino acids can be tolerated in position 2 in the presence of strong C′ terminal anchor residues as in p2Ca (LSPFSFDL) and Tum (QNHRALDL) (Robinson and Lee, 1996). Peptides with substituted residues 4, 6, 7, 8, and 9 resulted in background-level binding similar to the negative control pMCMV. In contrast, peptides with substituted residues 1, 3, and 5 each mediated more binding than wild-type AH1. Since AH1-A5 mediated the greatest binding of all the peptides tested, we chose to fully characterize the binding of AH1-A5

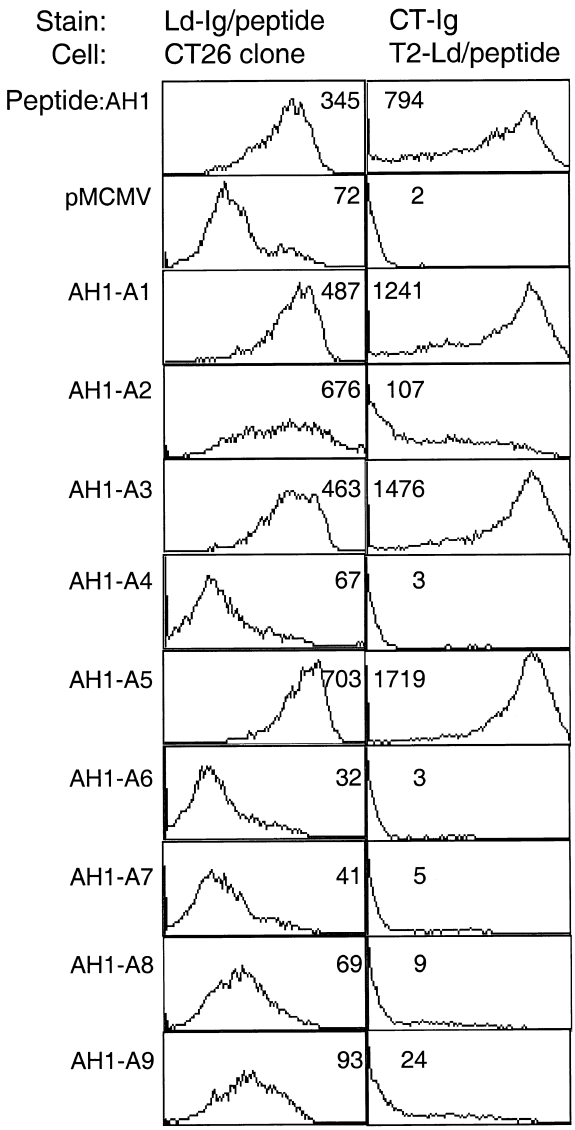

Figure 2. Binding of TCR to Ligand as Mediated by AH1 and AH1 with Alanine Substitutions

In the left panel, the AH1-specific T cell clone was stained with divalent H-2L^d, Ld-Ig, which had been loaded with the indicated peptides, and analyzed by flow cytometry. In the right panel, T2-Ld cells were incubated in 10 μM of the indicated peptide, stained with a divalent single chain form of the TCR from the AH1-specific clone, CT-Ig, and analyzed by flow cytometry. Both Ld-Ig and CT-Ig proteins were added in excess and visualized with IgG1-biotin and Avidin-PE. Histograms of the relative cell number versus fluorescence are shown. The mean channel fluorescence is indicated in each histogram.

for H-2L^d and H-2L^d-AH1-A5 for TCR compared with the wild-type AH1 peptide.

AH1 and AH1-A5 Peptides Bind MHC with Similar Affinities

One possible mechanism to explain the increased binding of TCR to MHC-peptide complexes containing AH1-A5 relative to the wild-type AH1 peptide is enhanced MHC binding. We therefore compared the ability of the peptides to bind H-2L^d as described in Figure 1. Again, the AH1 peptide competed for soluble H-2L^d as well as pMCMV and nominally better than the other peptides tested including AH1-A5 (Figure 3). Thus, the enhanced binding mediated by the AH1-A5 peptide relative to the AH1 peptide shown in Figure 2 is not a result of an increased affinity for MHC.

H-2L^d-AH1-A5 Binds TCR with Higher Affinity than H-2L^d-AH1

Surface plasmon resonance was used to directly measure the binding properties between TCR and the different peptides complexed with H-2L^d. The CT26 TCR-Ig was immobilized to the biosensor surface to measure the binding characteristics of graded concentrations H-2L^d complexed with AH1, AH1-A5, AH1-A7, and pMCMV. Binding of MHC-peptide complexes to the immobilized TCR was visualized as the time-dependent increase in resonance units (Figure 4A). Based on steady-state binding levels, the equilibrium constants for dissociation, K_D for the interaction of the TCR-Ig protein to the soluble H-2L^d complexes were determined (Figure 4B; Table 1). H-2L^d-AH1-A5 resulted in more binding than H-2L^d-AH1 reflecting the stronger affinity (K_D). Significant binding of pMCMV-H-2L^d to the TCR was not detected as expected demonstrating specificity. Independently, the kinetic dissociation rate constants (k_{off}) for each interaction were determined using a direct fit algorithm (Table 1) with the assumption of 1:1 Langmuir binding. Based on the measured K_D and k_{off}, the kinetic association rate constants, k_{on}, were calculated. As shown in Table 1, the on rates of the AH1 and AH1-A5 peptide complexes were similar. However, the off rate of the AH1-A5 complex was about 3-fold slower than the AH1 complex accounting for the difference in affinity. The on rate of the H-2L^d-AH1-A7 complex was about 4-fold slower than the H-2L^d-AH1 complex, although the off rates were similar. Table 1 also summarizes the half-time of binding of the MHC-peptide binding to the TCR based on the k_{off}.

AH1-A5 Peptide Improved Cytolytic Activity Mediated by an AH1-Specific T Cell Clone

As an initial step toward correlating TCR binding properties with immunogenicity, we analyzed the reactivity of the AH1-specific T cell clone to the AH1 peptide as well as the panel of alanine-substituted variant peptides in chromium release assays (Figure 5). 10 ng/ml (8.3 nM) AH1 peptide resulted in maximal specific lysis by the AH1-specific clone. When the anchor residues were changed to an alanine (AH1-A2 and AH1-A9), peptide-dependent sensitization for lysis was abolished. Substitution at positions 4, 6, and 7 also resulted in background-level lysis similar to the negative control pMCMV. Substitution in positions 1, 3, and 8 mediated approximately the same level of lysis at AH1. However, the alanine substitution at position 5 sensitized target cells for maximal lysis at ~10-fold lower concentration of peptide (1 ng/ml) and shifted the entire dose response curve. With the exceptions of AH1-A2 and AH1-A8, the cytolysis results correlated with those obtained in the binding experiments (Figure 2). Thus, the increased affinity of the AH1-A5-mediated MHC-TCR complex correlated with increased sensitization of targets for cytolysis by the AH1-specific T cell clone.

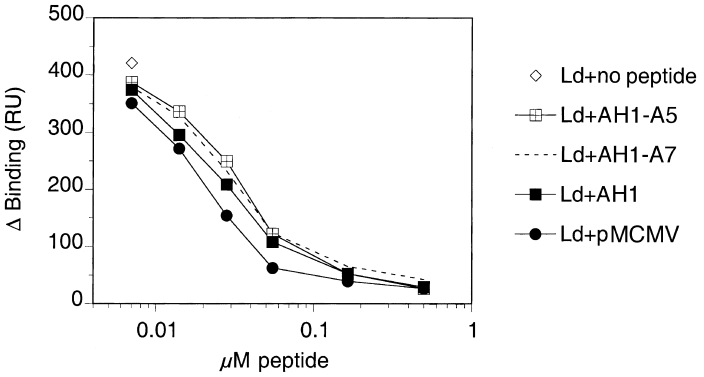

Figure 3. AH1 and AH1-A5 Bind H-2L^d with Similar Affinity

Either buffer alone or graded concentrations of indicated peptide were combined with 40 μM H-2L^d and exposed to immobilized pMCMV-C4 peptide on the biosensor surface, as in Figure 1. The amount of H-2L^d bound to pMCMV-C4 is reflected by the change in resonance units on the Y axis after 1 min.

Vaccination Using AH1-A5 Peptide Improved In Vivo T Cell Immunity to AH1 Peptide and CT26 Tumor

The enhanced apparent TCR affinity and stimulatory capacity of AH1-A5 for an individual T cell clone prompted us to evaluate in vivo immunogenicity of AH1-A5 relative to the wild-type AH1 peptide. At issue is whether a significant proportion of the AH1-specific T cell repertoire in vivo mimics that of the isolated CD8$^+$ T cell clone. Of relevance to cancer immunotherapy is whether the increased binding between the cloned T cell receptor and the H-2L^d-AH1-A5 complex as compared to the H-2L^d-AH1 complex can result in a more robust immune response in vivo against the AH1 epitope and ultimately the tumor, CT26. To determine if AH1-A5

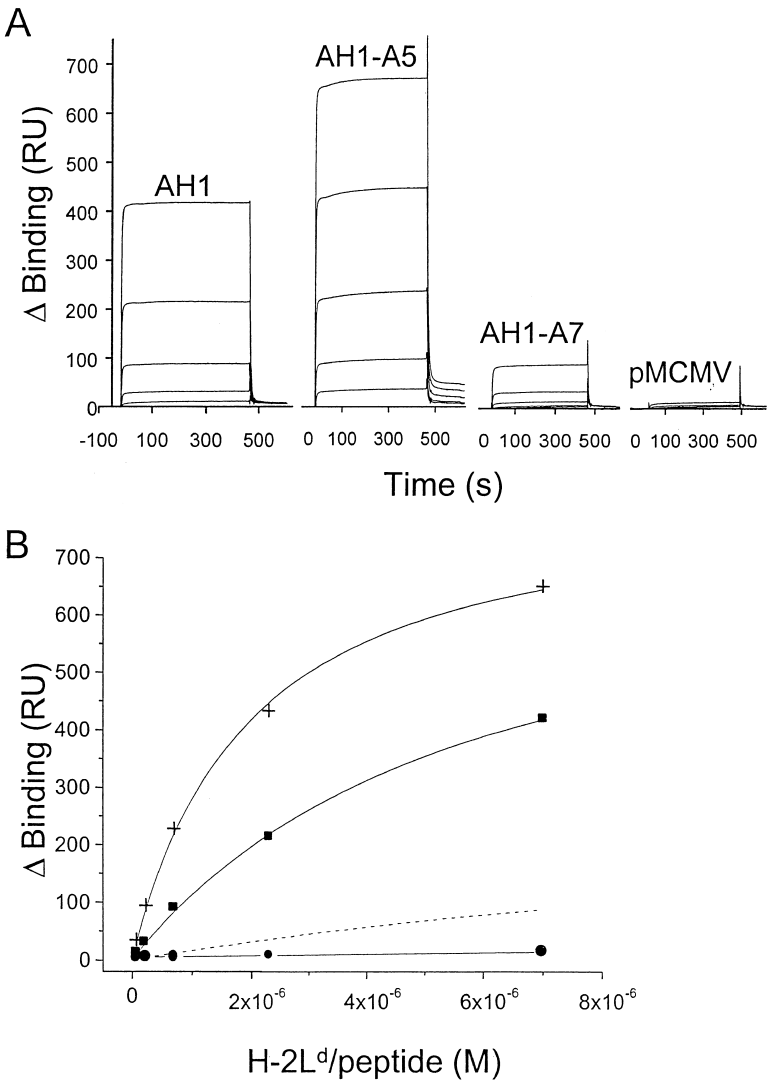

Figure 4. AH1-A5 Has a Stronger Affinity and a Longer Dissociation Time Than AH1

(A) Profiles from the biosensor of H-2L^d-peptide binding to the TCR derived from the AH1-specific clone are shown. Graded concentrations (7.00, 2.33, 0.70, 0.233, and 0.07 mM) of H-2L^d produced in bacteria complexed with the indicated peptide were injected over 5300 resonance units of immobilized CT-Ig at a flow rate of 5 μl/min. The change in resonance units (RU) reflects binding of H-2L^d-peptide to the surface over time. Data extrapolated from these curves were used to determine the dissociation rates (k_{off}) using Biaevaluation 3.0 software and to the graph in Figure 4B. Results of these analyses are shown in Table 1.

(B) Equilibrium binding constants (K_D) were derived from these curves using a nonlinear 1:1 Langmuir binding curve-fit on the Origin program from Microcal 1.4 software (+, AH1-A5; square, AH1; dotted line, AH1-A7; and circle, pMCMV).

Table 1. Affinity and Kinetic Constants for Binding of H-2L^d/Peptide[a] to T Cell Receptor[b]

Ld/Peptide	k_{off} (s^{-1})[c]	k_{on} ($s^{-1}M^{-1}$)[d]	K_D (μM)[e]	$t_{1/2}$ (s)[f]
AH1	0.35 ± 0.025	$6.1 \times 10^4 \pm 6.5 \times 10^3$	5.7 ± 0.45	2.0 ± 0.14
AH1-A5	0.11 ± 0.017	$5.8 \times 10^4 \pm 1.0 \times 10^4$	1.9 ± 0.18	6.3 ± 0.97
AH1-A7	0.28 ± 0.023	$1.6 \times 10^4 \pm 6.4 \times 10^3$	18 ± 7.3	2.4 ± 0.20
pMCMV[g]			<<60 ± 50	

[a] H-2L^d protein was produced in *E. coli* and folded separately around each peptide.
[b] The single-chain CT26 T cell receptor was produced in plasmacytoma cells and immobilized to a CM5 chip with amine coupling.
[c] The k_{off} values were determined using the BIAevaluation 3.0 program for 1:1 Langmuir dissociation kinetics.
[d] The k_{on} values were calculated from k_{off} and K_D.
[e] The K_D values were obtained from equilibrium binding data.
[f] $T_{1/2}$ equals ln 2 divided by k_{off}.
[g] H-2L^d/pMCMV interaction with CT26 T cell receptor was too weak to permit accurate assessment of binding parameters.

is a stronger tumor antigen than endogenous AH1, we compared these two peptides in a vaccination study.

For this study, we used dendritic cells as antigen-presenting cells (Mayordomo et al., 1995) derived from the spleen and loaded overnight with peptide in serum-free medium. The phenotype of these cells is similar to that of dendritic cells described by others (reviewed in Banchereau and Steinman, 1998); they express high levels of MHC class II, costimulatory molecules (B7.1 and B7.2), and CD11c (Figure 6A). By staining the cells with the dimeric TCR, CT-Ig, as described in Figure 2, we determined the most efficient conditions for peptide loading. Then, using the same method, we determined that the dendritic cells were loaded with similar amounts of AH1 and AH1-A5 peptides.

To determine the relative effect of these peptide-loaded dendritic cells on the tumor-specific T cell response, we primed and boosted mice with dendritic cells before removing the spleen. After a single in vitro stimulation, we analyzed the cells for both CT26-specific lysis and binding to the Ld-Ig loaded with AH1. Vaccination with AH1-A5 peptide resulted in significantly greater lysis of CT26 targets (Figure 6B). This increased activity was associated with an increase in the percentage of CD8$^+$ H-2L^d-AH1-specific T cells as assessed by staining with Ld-Ig loaded with AH1 (Figure 6C). Vaccination with AH1-A5 loaded dendritic cells resulted in a 5- to 10-fold greater expansion of AH1-specific T cells relative to the AH1-loaded dendritic cell vaccine. Although qualitative differences of the T cell populations after vaccination with different dendritic cells are not measured here, these results show a significant quantitative difference in the T cell populations.

Using the same vaccination protocol, we evaluated whether the increased in vivo stimulation of AH1-specific T cells by the AH1-A5 peptide resulted in enhanced antitumor immunity (Figure 6D). Development of tumors in mice treated with unpulsed dendritic cells, dendritic cells pulsed with pMCMV, or AH1-A7 occurred at the same rate as in untreated mice. Vaccination with AH1-loaded dendritic cells induced a significant delay in tumor growth relative to untreated mice ($p = 0.001$). Tumor-free survival was also significantly increased with the treatment of AH1-A5-pulsed dendritic cells relative to AH1-pulsed dendritic cells ($p = 0.025$). Thus, selection of a peptide variant that stabilized the trimolecular complex by decreasing TCR off rate resulted in enhanced in vivo activation and expansion of antigen-specific T cells and superior antitumor immunity.

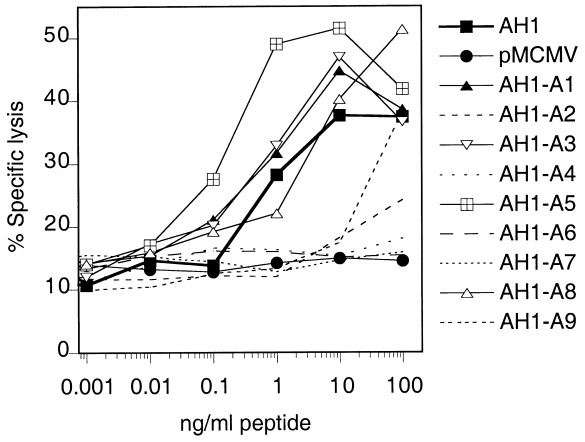

Figure 5. AH1-A5 Mediates More Cytolytic Activity Than the Wild-Type Tumor Antigen, AH1

^{51}Cr-labeled MC57G-Ld cells (3×10^3) were combined with the AH1-specific T cell clone (1.5×10^4) and graded concentrations of peptide as indicated on the X axis. Percent lysis was calculated after 4 hr at 37°.

Discussion

A structural understanding of the formation of a ternary MHC-peptide-TCR complex suggests that particular amino acid residues of the bound peptide contribute predominantly either to MHC interactions (anchor or agretopic residues) or to TCR interactions (epitopic or antigenic residues). This compartmentalized view of peptide interactions with MHC and TCR is most likely incorrect in detail but nevertheless offers a simplified framework of conceptualizing the effects of single residue amino acid substitutions of antigenic peptides. With the initial demonstrations that amino acid alterations in T cell epitopes could enhance stimulation of T cell populations specific for the original epitope, epitope modification has become an attractive approach to augment antigen-specific immunotherapies (Solinger et al., 1979; Boehncke et al., 1993; Chen, 1999). Indeed, amino acid substitutions that enhance binding affinity of peptide for the presenting MHC molecule have been demonstrated to improve the immunogenicity of a number of MHC class I–restricted

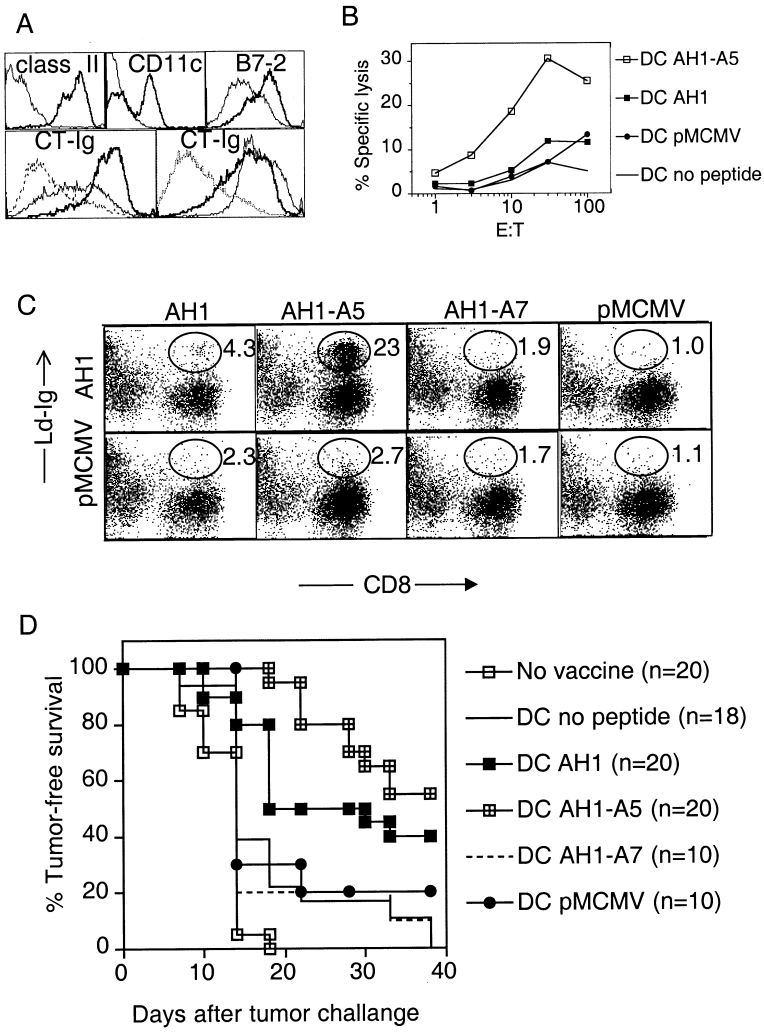

Figure 6. Immunization with AH1-A5-Loaded Dendritic Cells Results in Enhanced Lytic Activity, Frequency of $CD8^+$ CT26-Specific T Cells, and Tumor-Free Survival Relative to Immunization with AH1-Loaded Dendritic Cells

(A) Characteristics of cell surface markers and peptide loading of dendritic cells derived from the spleen are shown. Dendritic cell populations from the spleen were enriched to 60%–80% with a BSA gradient and adherence to plastic. After a 12 hr incubation with 100 or 300 μg/ml peptide, the cells were analyzed for cell surface markers. Expression of MHC class II, CD11c, and B7-2 were examined. In the first row, the thick line represents staining with the indicated antibody, and the thin line represents background fluorescence from an isotype-matched control antibody. In the second row, dendritic cells are stained with CT-Ig. In the left panel, the thick line represents cells incubated with 300 μg/ml AH1 peptide, the thin line represents cells incubated with 100 μg/ml AH1 peptide, and the dotted line represents cells incubated without peptide. In the right panel, the thick line represents cells incubated with 300 μg/ml AH1 peptide, the thin line represents cells incubated with 300 μg/ml AH1-A5 peptide, and the dotted line represents cells incubated without peptide.

(B–D) BALB/c mice were injected on day –14 and day –7 subcutaneously in both hind flanks with 1×10^5 dendritic cells pulsed with 300 μg/ml of the indicated peptides. (B) Lytic activity specific for CT26 targets from mice immunized with peptide-pulsed dendritic cells. CTL derived from mice were examined 5 days after in vitro stimulation with CT26 expressing B7. Background lytic activity from spleen cells of a naive mouse treated in parallel was subtracted. A representative experiment of two mice per group is shown. (C) Frequency of AH1-specific T cells was measured 7 days after in vitro stimulation. The peptide used to pulse the dendritic cells is indicated on top. The numbers in the right-hand corner indicate the percent antigen-specific $CD8^+$ cells measured. (D) On day 0, 5×10^4 CT26 tumor cells were injected subcutaneously in the left hind flank and tumor-free survival was monitored for 38 days. N represents the number of mice in each group pooled from two independent experiments performed under identical conditions. The survival differences analyzed on a Kaplan-Meier curve with Mantel-Cox statistics indicates that the difference between treatment with DC pulsed with no peptide verses DC pulsed with AH1 ($p = 0.001$) and DC pulsed with AH1 verses DC pulsed with A5 ($p = 0.025$) are significant.

tumor antigens (Gervois et al., 1996; Parkhurst et al., 1996; Bakker et al., 1997; Dyall et al., 1998; Overwijk et al., 1998; Valmori et al., 1999). Here, we have demonstrated that an amino acid substitution that enhances the affinity of the MHC-peptide complex for the TCR also enhances expansion and activation of polyclonal T cell responses specific for the wild-type antigen. In the case of the immunodominant AH1 tumor antigen studied here, this enhanced in vivo immunization results in superior immunity against challenge with the CT26 tumor.

Substitution of alanine for valine at position five (P5) of the AH1 peptide does not significantly change peptide binding to MHC but increases the affinity of MHC-peptide for TCR, suggesting that it is epitopic. Consistent with our interpretations, analysis of H-$2L^d$ binding to the tum peptide suggests that arginine at P5 also binds the TCR (Robinson and Lee, 1996). Amino acids at this position are predicted to have high solvent accessibility that correlates with increased TCR interactions (Zhang et al., 1992; Balendiran et al., 1997). However, using an H-$2L^d$ crystal structure to predict the orientation of the fifth residue of the QL9 peptide (phenylalanine) suggests that it is buried in pocket C of H-$2L^d$ (Speir et al., 1998). The crystal structure of p29 peptide complexed with H-$2L^d$ shows the asparagine at P5 is pointing toward the α1 helix of the MHC (Balendiran et al., 1997). Pocket C of the mid-cleft region of H-$2L^d$ is shallow, hydrophobic, and does not require binding of a consensus residue (Balendiran et al., 1997). Given these disparities in orientation of P5 for different H-$2L^d$ binding peptides, one cannot accurately predict the orientation of P5 in the AH1 peptide. It is thus unclear whether the altered TCR affinity afforded by the valine to alanine substitution of AH1 is due to direct contact of P5 with the TCR or rather through affects on the relative orientation of the adjacent tyrosines.

Since the AH1-A5 peptide was originally identified

using only a single AH1-specific T cell clone in vitro, the heteroclitic properties were somewhat unexpected. Differences in in vitro reactivity using a single T cell clone might not necessarily predict the relative reactivities between wild-type AH1 and AH1-A5 among the diverse in vivo polyclonal T cell response. The finding that AH1-A5 was so much more efficient at expanding and activating T cells specific for wild-type AH1 suggests that a large T cell repertoire exists in vivo whose TCR displays common structural features and reactivity patterns to the original T cell clone.

As with many other antigens, certain Vβ segments dominate the AH1-specific T cell response. We found a predominance of Vβ8.3, the Vβ segment expressed by the T cell clone, within polyclonal AH1-specific T cell lines. Colombo and colleagues have identified T cell populations specific to AH1 expressing other Vβ regions (personal communication). We are currently determining if there are qualitative differences in the repertoire of AH1-specific T cells activated by in vivo immunization with AH1-A5 versus wild-type AH1. It is possible that the AH1-reactive T cell repertoire specifically expanded by AH1-A5 represents a subset of the total AH1-reactive repertoire. Whether or not this is the case, the enhanced TCR binding properties of AH1-A5 relative to wild-type AH1 clearly provides enough of an advantage at the population level in vivo to result in a net enhancement in activation and expansion of AH1-specific T cells.

The overall antitumor immune response afforded by various immunotherapies is a combination of multiple types of responses to multiple tumor antigens not limited to, for example, a single antigen. The finding that modification of a single epitope can significantly affect antitumor immunity raises the possibility that additive effects of multiple antigenic modifications in a polyvalent vaccine may provide more potent protection. While substitution of MHC binding anchor residues is a reasonable approach to modify epitopes displaying low MHC affinities, it is likely that the poor immunogenicity of most tumor antigens is due instead to tolerization of high-affinity T cells, thereby leaving an available repertoire of low-affinity T cells. The feasibility of activating these remaining low-affinity populations of T cells was demonstrated in studies using HA as a model self- and tumor antigen (Morgan et al., 1998).

The gp70 antigen from which the AH1 epitope is derived represents a prototype example of a shared tumor antigen whose expression has been selectively upregulated in CT26 and in many other mouse tumors (Jaffee and Pardoll, 1996). Although gp70 expression was not detected in the thymus in our previous study (Huang et al., 1996), it is quite possible that natural expression of gp70 tolerizes high-affinity T cells. Evidence for such a mechanism has indeed been observed with other endogenous retroviral gene products such as the MMTV-derived superantigens (Marrack et al., 1993). Consistent with this idea, AH1 peptide is a weak immunogen despite the fact that it is the dominant target for CT26-specific CTL responses. For example, immunization with recombinant vaccinia expressing either full-length gp70 or an AH1 minigene downstream of an endoplasmic reticulum signal sequence fails to protect animals against CT26 challenge (K. Gorski, E. M. J., and D. M. P., unpublished data). In contrast, immunization with vaccinia virus expressing β-galactosidase efficiently protects animals against challenge with CT26-expressing β-galactosidase (Bronte et al., 1995).

The measured affinity of the H-2L^d-AH1 complex for the cognate TCR was at the low end of the range of reported affinities for other agonist MHC class I–restricted antigen TCR pairs (reviewed in Davis et al., 1998). However, given the variabilities in construction of soluble TCRs and MHCs used in the different studies as well as variations affecting surface plasmon resonance measurements, it is difficult to make reliable comparisons among different studies. The roughly 3-fold enhanced TCR affinity of H-2L^d-AH1-A5 relative to H-2L^d-AH1 is reflected in a 3-fold decreased k_d; the k_a for H-2L^d-AH1 and H-2L^d-AH1-A5 is essentially identical. This 3-fold difference in affinity and off rate results in a roughly 10-fold enhancement in activation of the AH1 T cell clone. The elements of MHC-peptide binding to TCR that are most relevant to T cell stimulation are still not yet completely resolved. While some groups have presented data favoring a strong correlation between off rate and stimulatory capacity (Chen et al., 1994; Savage et al., 1999), others have argued for an important contribution of on rate and/or overall affinity in determining functional T cell reactivity (Alam et al., 1996). For other cases, a clear cut correlation is lacking and other characteristics may be involved (Al-Ramadi et al., 1995; reviewed in Manning and Kranz, 1999). Of note, the measured off rates of H-2L^d-AH1-A7 and H-2L^d-AH1 for TCR are similar though AH1-A7 fails to stimulate the AH1-specific T cell clone. This result further suggests that off rate of MHC-peptide from TCR is not the sole determinant of biological activity.

While the TCR affinity of H-2L^d-AH1 and its biological activity in stimulating the AH1 clone are consistent with AH1 being a weak agonist, it is possible that AH1 has partial antagonist properties as well. Indeed, it has been suggested that low TCR affinities for tumor antigens could lead to tolerance in tumor-specific T cells. A recent study in melanoma patients identified an unresponsive or "anergic" phenotype among populations of tyrosinase-specific T cells from draining lymph nodes containing melanoma metastases (Lee et al., 1999). If tolerance is being induced, amino acid substitutions in tumor antigens that result in increased TCR affinity may enhance immunogenicity by converting peptides from partial antagonists to full agonists for populations of specific T cells in vivo. Further functional analysis of the populations of AH1-specific T cells activated in vivo by wild-type AH1 versus AH1-A5 may shed light on how peptide substitutions effecting the stability of the MHC-peptide-TCR complex affect the balance between activation and tolerance of tumor-specific T cells.

Experimental Procedures

Peptides

All peptides were purified to greater than 95% purity and purchased from Macromolecular Resources (CO), Chiron (CA), or Princeton BioMolecules (OH). The sequences of the peptides are SPSYVYHQF (AH1), **A**PSYVYHQF (AH1-A1), S**A**SYVYHQF (AH1-A2), SP**A**YVYHQF (AH1-A3), SPS**A**VYHQF (AH1-A4), SPSY**A**YHQF (AH1-A5), SPSY**V**AHQF (AH1-A6), SPSYVY**A**QF (AH1-A7), SPSYVYH**A**F (AH1-A8), SPSYVYHQ**A** (AH1-A9), YPHFMPTNL (pMCMV), and YPH**C**MPTNL (pMCMV-C4), LSPFSFDL (p2Ca), and QNHRALDLV (p91A-Tum V9).

Biosensor Analysis

The real-time surface plasmon resonance experiments utilized the Biacore 2000. All immobilization and binding experiments were performed at 25°C at a flow rate of 5 μl/minute. The binding buffer, HBST (10 mM Hepes [pH 7.5], 3.4 mM EDTA, 15 mM sodium chloride, and 0.005% Tween 20), was used to dilute the solution phase ligand. The cell culture–produced H-2L^d used in the peptide binding experiments have been described (Boyd et al., 1992; Goldstein et al., 1997). 40 mM H-2L^d was used in each reaction. The change in binding (resonance units) was determined 1 min after addition to the peptide surface. Sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (Pierce) was used to couple between 100 and 200 resonance units of peptides through a cysteine to the dextran-coated CM5 sensor chip as previously reviewed and described (Margulies et al., 1996). After binding the MHC to the peptide surface, the surface was regenerated with 50 mM phosphoric acid. Approximately 5000 resonance units of the single chain TCR in 25 mM sodium acetate (pH 5.1) was coupled to the CM5 chip with amine chemistry (NHS/EDC) as determined by the BIAevaluation 3.0 Application Wizard. A blank surface was prepared using the same method without protein. The TCR surface was cleared of MHC-peptide binding with a 5 min wash with HBST. The fit of curves with a standard error of less than 5% of the calculated k_{off} and residuals less than 2 were considered.

To obtain equilibrium constants, the data were nonlinearly fit to the following equation using the Origin program from Microcal software: $R = (R_{max}c)-(K_D+c)$ where R = resonance units, R_{max} = maximum resonance units as determined by the nonlinear fit, c = concentration of analyte, and K_D = the affinity constant.

Cells

CT26, CT26-B7 that express human B7.1, and MC57G-Ld were cultured in complete medium (RPMI-1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 1× MEM nonessential amino acids [Sigma], 1 mM sodium pyruvate, 10 mM Hepes [pH 7.5], 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol) as described (Griswold and Corbett, 1975; Huang et al., 1996). T2-Ld cells were cultured in the same medium (O'Herrin et al., 1997).

The AH1-specific T cell clone was generated from limiting dilution of a CT26 CTL line that was isolated from a spleen of a BALB/c mouse vaccinated with CT26 transduced with GM-CSF (Huang et al., 1996). The T cell clones were stimulated in vitro every 7 days as described (Huang et al., 1996). Splenocytes were stimulated in vitro by the same method. In brief, the spleens were crushed and red blood cells were lysed (Kruisbeek, 1993). After two washes in complete medium, the 4×10^6 cells were combined with 1×10^5 mitomycin C-treated CT26-B7 and 10 units/ml IL-2 in 2 ml complete medium in 24-well plates.

Dendritic cells were generated from the spleen as described (Inaba et al., 1998). The dendritic cells were loaded overnight in 100 or 300 μg/ml peptide in serum-free AIMV medium supplemented with 0.1 mM β-mercaptoethanol, 100 units/ml penicillin, and 100 units/ml streptomycin. Excess peptide was removed in three washes of Hanks buffer (Gibco).

Construction, Expression, and Purification of Ld-Ig and CT-Ig

Ld-Ig, the divalent H-2L^d protein was constructed, expressed, and purified using described methods (Schneck et al., 1999). In brief, the BALB/c H-2L^d cDNA from pLd.444 (Huang et al., 1996) was amplified using primers 5′LdMluI ATACGCGTCGCAGATGGGGGC GATGGCTCC and 3′LdXhoI ACCTCGAGTGCGGCCGCCCATCTC AGGGTGAGGGG. The fragment was digested with MluI and XhoI and inserted into the same sites of pX-Ig (Dal Porto et al., 1993). The sequence was verified. The resulting plasmid was cotransfected by electroporation with a human β2-microglobulin expression plasmid into J558L plasmacytoma cells. A clone that secreted relatively high levels of protein, as determined by ELISAs specific for H-2L^d or the IgG1 portion of the molecule, was grown in Hybridoma-SFM (Gibco), and protein was purified from supernatants by affinity chromatography to the variable region of the Ig portion. Purified Ld-Ig was loaded with peptide by incubating it with ~200-fold excess peptide for 1–5 days. The resulting protein was used to stain antigen-specific T cells.

CT-Ig was generated similarly to Ld-Ig and to other divalent TCRs constructed (Plaksin et al., 1997; Lebowitz et al., 1998). In place of the MHC fragment in Ld-Ig, a fragment encoding a single chain TCR from the AH1-specific T cell clone was inserted. The variable domain of the β chain of the T cell clone was identified by recognition of the vβ8.3 antibody (1B3.3). The variable region family of the α chain of the T cell clone was identified by PCR (as determined by David Woodland). The family member was identified as vα4.11 by sequencing. The α and β chains were amplified from cDNA from the AH1-specific clone using these primers: 5′vα4.11.AseI GGAATTCATT AATGACTCAGTAACCCAGATGCAA, 3′vαHindIII ATAAGAAAGCTT TCCTGGTTTTACTGATAATTT, 5′β8.3NdeI ATAAGAATGCGGCCG CCGAGGTCGCAGTCACCCAAAGCCCTA, and 3′cβXhoI CCGCCTC GAGTCAGTCTGCTCGGCCCCAGGCCTCTGC. The fragments were inserted into pCR2.1 (Invitrogen) to verify the sequence. The NotI-XhoI fragment harboring the coding region of v-d-j-c of the β chain was inserted into the same sites of pET-scTCR to replace the existing β chain (Plaksin et al., 1997). The AseI-HindIII fragment encoding the v-j region of the α chain was then inserted NdeI-HindIII of pET-scTCR leaving the linker between the α and the β chain (Chung et al., 1994) and the rest of the vector, pET21+ (Novagen) intact. The single chain TCR was amplified with primers 5′MluITCR GATCACG CGTCGATGGACTCAGTAACCCAGA and 3′SalITCR GATCGTCGA CGTCTGCTCGGCCCCAGGCCTC. The resulting fragment was inserted into pCR2.1 for sequencing and then the MluI-SalI fragment was inserted into the MluI and XhoI sites of pX-Ig. As with Ld-Ig, this plasmid was electroporated into J558L cells, and a clone that reacted with antibodies to the cβ region (H57-597, PharMingen), vβ8 (F23.1, PharMingen), and IgG1 (Caltag) (data not shown), was expanded to produce protein for staining antigen presenting cells and binding experiments.

Antibodies, Cell Staining, and Flow Cytometric Analysis

To examine peptide-mediated binding, 2×10^5 CT26 T cell clone was incubated on ice with ~1 μg Ld-Ig loaded with different peptides for 1–2 hr in flow cytometry wash buffer (1×HBSS (Gibco), 2% fetal calf serum, 10 mM Hepes [pH 7.5], and 0.1% sodium azide). The Ld-Ig-peptide was visualized with IgG1-biotin (PharMingen) and avidin-PE (Caltag) on a Becton-Dickinson FACScan flow cytometer. When visualizing antigen-specific T cells after an in vitro stimulation, CD4-FITC (RM2501-3, Caltag), B220-FITC (PharMingen), and CD8-cychrome (PharMingen) were also included with the avidin-PE to facilitate detection of the CD8$^+$ cells.

For visualization of peptide-loaded H-2L^d complexes, T2-Ld cells were incubated overnight at 25°C. T2-Ld cells were then incubated with 10 μM peptide for 2 hr at 37°C. Approximately 1 μg CT-Ig was added to either 3×10^5 T2-Ld cells or peptide-pulsed dendritic cells, the cells were incubated on ice for 1–2 hr, and, then visualized by flow cytometry as described with Ld-Ig. The antibody 14-4-4, which recognizes MHC class II (American Type Cell Collection), anti-CD80, which recognizes B7.1 (PharMingen), and CD11c (PharMingen) were used in analyzing the cell surface marker of the dendritic cells.

Chromium Release Assays

One million target cells were labeled in 100 μl complete medium and 200 μCi ^{51}Cr at 37°C for 1–1.5 hr. Target cells were washed three times to remove excess chromium. To determine which peptides mediate lysis of the AH1-specific clone, ^{51}Cr-labeled MC57G-Ld cells (3×10^3) and AH1-specific T cell clone (1.5×10^4) were added to 10-fold serial dilutions of the indicated peptides in a total of 200 μl in a round-bottom 96-well plate for 4 hr at 37°C. Half of the supernatant was removed and the gamma irradiation was counted. To determine the tumor specific lysis from splenocytes after vaccination, 3×10^3 ^{51}Cr-labeled CT26 cells were added to varying concentrations of splenocytes that had been stimulated in vitro for 5 days at a 40:1 ratio with mitomycin C-treated CT26-B7. Each data point was performed in triplicate and then averaged. The shown experiments are a representative of an example of at least two experiments performed with each peptide. Data are expressed as % specific lysis = (measured release − spontaneous release) − (maximum release − spontaneous release) × 100. Activity of splenocytes from naive mice was subtracted from activity of splenocytes from vaccinated mice.

Protein Expression and Purification

H-2L^d (Balendiran et al., 1997) and β2-microglobulin (Shields et al., 1998) were produced in bacteria similarly to a described procedure (Kurucz et al., 1993; Li et al., 1998). The H-2L^d preparations were made in parallel; each of the indicated peptides were folded with H-2L^d and β2-microglobulin separately. The H-2L^d-β2-microglobulin-peptide complexes were purified from aggregates on a Sephadex 75 gel filtration column, dialyzed against HBST, and quantitated using the UV-1601 Shimadzu spectrophotometer before using in binding experiments.

Mice and Immunizations

Six- to eight-week-old female BALB-CanNCr mice were purchased from the National Cancer Institute. Mice were injected with 1×10^5 dendritic cells in the left- and right-hind flank subcutanously on days –14 and –7. On day 0, mice were injected subcutanously in the left flank with 5×10^4 CT26 cells. Tumor-free survival was assessed by detection of a palpable solid tumor at the site of injection. At 6 days, we began to monitor for palpable tumors. Tumors developed in most of the untreated mice within 2 weeks. The statistics were analyzed using the software StatView 5.0.1 (SAS Institute).

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