Correction

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The authors note that due to a printer’s error on 4653, left column, second full paragraph, the third sentence is incorrect in part. “Peptides 15 and wavelength of maximum fluorescence (WMF) prevented tumor growth in few or no mice (considered nonprotective); peptides 39 and F1A5 prevented tumor growth in 60 and 90% of the mice, respectively (considered protective)” should read: “Peptides 15 and WMF prevented tumor growth in few or no mice (considered nonprotective); peptides 39 and F1A5 prevented tumor growth in 60 and 90% of the mice, respectively (considered protective).” Additionally, Figs. 1, 3, and 4 appeared incorrectly. The corrected figures and their legends appear below. The online and printed versions have been corrected.

Fig. 1. Peptide variants elicit a range of antitumor immunity. Mice were vaccinated with BV-infected insect cells expressing peptide-MHC molecules 7 and 14 d before challenge with CT26 tumor cells. Mice were killed and scored when the tumor grew to 10 mm. Survival curves of mice vaccinated with the indicated peptides were compared with βgal-vaccinated mice using a log-rank test: ***, P < 0.0001; **, P = 0.0021. The survival curve of the AH1-vaccinated mice (gray dashed line) closely overlaps with the survival curve of the βgal vaccinated (gray solid line). The bold residues indicate where the variant peptides differ from the residues in the AH1 peptide.
Fig. 3. Vaccination with protective peptide variants elicits more endogenous AH1-specific T cells. (A) Splenocytes from mice vaccinated with the indicated peptides were stained with AH1-tet, CD8, and dump antibodies. The number (left axis, black bars) and percentage (right axis, white bars) of AH1-tet CD8+ cells was determined and compared with the number of cells in mice vaccinated with the AH1 peptide using a Student’s t test (n = 3; P ≤ 0.0151 for all peptides). Error bars represent SEM. (B) Splenocytes from A were stained with the corresponding peptide variant-tetramer (variant-tet), CD8, and dump antibodies and analyzed as in A. (C) Splenocytes from A were costained with both AH1-tet (y axis) and the corresponding variant-tet (x axis), CD8, and dump antibodies. The percentage of CD8+ dual tet+ (cross-reactive, Upper Right) or variant-tet+ (Lower Right) cells is indicated. (D) The percentage of variant-tet+ cells that bind to AH1-tet from C.

Fig. 4. Vaccination with protective peptide variants elicits more CTLs that produce IFNγ in response to the AH1 peptide. (A) Splenocytes from Fig. 3 were stimulated with AH1 peptide and stained with IFNγ, CD8, and dump antibodies. The number of CD8+IFNγ+ cells was determined by flow cytometry. (B) The number of CD8+IFNγ+ cells from A was divided by the number of CD8+AH1-tet+ cells from Fig. 3A to determine the percentage of AH1-specific T cells that produced IFNγ. (C) Splenocytes from Fig. 3 were stimulated with the corresponding peptide variant and analyzed as in A. (D) 2.5 x 10⁶ AH1-tet+ cells isolated from mice vaccinated with the F1A5 or WMF peptides were incubated with increasing concentrations of the AH1 peptide or the corresponding peptide variant. The concentration of IFNγ secreted was determined by ELISA 24 h later (performed in duplicate, n = 3; *, P = 0.03; **, P = 0.01).

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Peptide vaccines prevent tumor growth by activating T cells that respond to native tumor antigens

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Peptide vaccines enhance the response of T cells toward tumor antigens and represent a strategy to augment antigen-independent immunotherapies of cancer. However, peptide vaccines that include native tumor antigens rarely prevent tumor growth. We have assembled a set of peptide variants for a mouse-colon tumor model to determine how to improve T-cell responses. These peptides have similar affinity for MHC molecules, but differ in the affinity of the peptide-MHC/T-cell receptor interaction with a tumor-specific T-cell clone. We systematically demonstrated that effective antitumor responses are generated after vaccination with variant peptides that stimulate the largest proportion of endogenous T cells specific for the native tumor antigen. Importantly, we found some variant peptides that strongly stimulated a specific T-cell clone in vitro, but elicited fewer tumor-specific T cells in vivo, and were not protective. The T cells expanded by the effective vaccines responded to the wild-type antigen by making cytokines and killing target cells, whereas most of the T cells expanded by the ineffective vaccines only responded to the peptide variants. We conclude that peptide-variant vaccines are most effective when the peptides react with a large responsive part of the tumor-specific T-cell repertoire.

cancer | immunotherapy | mimotopes

Of the many factors that affect tumor growth, detection of tumor-infiltrating CD8\(^+\) T cells correlates with improved patient survival (reviewed in ref. 1). In many cases, tumor-infiltrating lymphocytes (TIL) recognize tumor/self-tumor antigens or tumor-associated antigens (TAA), whose expression is up-regulated in tumors (2). These are usually low-affinity T cells that escaped negative selection in the thymus and are suboptimally activated by the native TAA expressed by the tumor (3, 4). Various vaccine strategies have been developed to improve the response to tumors, including the use of strong adjuvants (5) or stimulatory cytokines (6). Another approach to improve this response is the use of peptide variants of TAA (7, 8), also referred to as mimotopes, heteroclitic peptides, altered-peptide ligands, and superagonists.

Although the rules of designing effective peptide variants are as yet undetermined, a number of approaches have been used for their identification, including MHC-anchor residue modifications (9, 10), systematic residue substitutions (11), chemically synthesized combinatorial peptide libraries (12, 13), peptide libraries with synthetic amino acids (14), and genetically encoded peptide libraries (15–17). Some of these peptides more completely activate the low-affinity TAA-specific T cells that have escaped central tolerance by driving stronger T-cell receptor (TCR) signaling cascades than the native antigens (18, 19). A number of these peptide vaccines have been tested in clinical trials, although the results have not met expectations. Amino acid substitutions that improve binding of the peptide to MHC molecules (5, 20) and change interactions with the cognate TCR have been tested (21, 22). In both cases, changes in the peptide-MHC-binding surface lead to changes in the repertoire of responding T cells (20, 23–25). Importantly, for effective antitumor responses, some of the T cells activated by the variant peptides must cross-react with the native TAA.

To determine how particular peptide variants generate effective T-cell responses, we have used the CT26 transplantable colon tumor model (11, 13, 26). The immunodominant antigen of CT26, gp70\(_{223-431}\) (AH1), is derived from a self-antigen encoded by the genome of BALB/c mice (27). Though often highly expressed in tumors, the gp70 antigen is poorly expressed in normal, young tissues and, therefore, some AH1-specific T cells escape negative selection (3). These T cells are suboptimally activated by vaccination with the AH1 peptide and require stimulation with higher-affinity peptides to overcome this defect (3, 11). We have identified peptide variants that activate an AH1-specific T-cell clone better than AH1 itself, both in vitro and in vivo (11, 13, 26).

Our previous studies concluded that nonprotective peptides elicit T cells from the endogenous tumor-specific repertoire that do not respond to ex vivo peptide stimulation (13). To determine whether the lack of response by these TIL was related to the tumor environment or to qualities of the responding T cells, we analyzed the AH1-specific T cells in the spleen following vaccination. We showed that vaccination with protective peptides results in the expansion of AH1-specific T cells that produced the cytokine IFN\(\gamma\) and killed AH1-loaded target cells. The lack of functional responses by some peptide-variant elicited T cells is likely the result of poor recognition of the wild-type tumor antigen, rather than an intrinsic defect of these cells. Thus, some peptides expand a fully functional and cross-reactive portion of the AH1-specific repertoire, while others expand a poorly cross-reactive portion. These results highlight the need not only to identify peptide variants that bind to tumor-specific T-cell clones with higher affinity, but also elicit functional T cells that respond to both the wild-type and variant peptides. Finally, we determined that the ineffective peptides did not reduce the tumor protection of the effective peptides after coadministration, indicating that vaccination with multiple peptide variants may be a practical therapeutic approach to peptide vaccines.

Results

Vaccination with Peptide Variants Elicits Variable Antitumor Immunity.

We used several different techniques to identify variants of the AH1 peptide bound to the MHC class I molecule, H-2L\(^d\) (L\(^d\)). Alanine substitutions of the AH1 peptide (11), a combinatorial library consisting of mixtures of synthetic peptides (13), and a genetically encoded mixture of peptides expressed by BV-infected insect cells (26, 28) were screened for stimulation of an AH1-specific CT T-cell clone. This clone was used for the initial identification and evaluation of our peptides because all of the T-cell clones previously isolated from CT26-GM-vaccinated BALB/c mice expressed this TCR (11, 29). Peptides that activated the CT T-cell clone better than the AH1 peptide itself were selected as tumor-vaccine candidates. Like the AH1 peptide itself, most of

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the peptides in this panel had consensus L\textsuperscript{d} anchor residues at positions 2 (pro) and 9 (phe or leu) (27, 30). They also all shared the tyr residues at positions 4 and 6, which are critical for T cell recognition (Fig. 1) (11).

We tested the panel of peptides for antitumor immunity using a BV-infected insect-cell vaccine (26). The BV-infected insect-cell vaccine provides both the antigenic epitope and the adjuvant, resulting in large peptide-specific T-cell responses. The peptides are likely processed through a conventional cross-priming pathway that involves presentation of the mimotopes on the L\textsuperscript{d} molecules of host antigen-presenting cells.

In agreement with previous studies, the peptides elicited variable antitumor immunity (Fig. 1) (11, 13, 26). As we previously showed, vaccination with the AH1 peptide, even when used with this powerful insect-cell adjuvant, did not prevent tumor growth, similar to no vaccination or vaccination with the irrelevant βgal peptide. Peptides 15 and WMF prevented tumor growth in few or no mice (considered nonprotective); peptides 39 and F1A5 prevented tumor growth in 60 and 90% of the mice, respectively (considered protective). Remarkably, peptide A5, which differs from the AH1 peptide in a single amino acid (val to ala in position 5), protected all of the mice from tumor growth. Thus, we studied the properties of these peptides to determine the characteristics that correlate with protection.

**Tumor Protection Does Not Correlate with the Relative Affinity of the Peptide for the L\textsuperscript{d} Molecule or the Peptide-L\textsuperscript{d} Complex for the CT-TCR.** The peptide variants were designed to bind well to L\textsuperscript{d} and were selected because of improved interactions with the CT-TCR relative to the AH1 peptide. However, to determine if the differences in protection were related to variations in these interactions, we first measured the relative L\textsuperscript{d}-binding affinity of each peptide using a competition assay (31). The amount of competing peptide required to reduce binding of L\textsuperscript{d} molecules to the immobilized reference peptide (MCMV-C4) was determined (Fig. 2A). All of the peptide variants competed with the reference peptide for binding to L\textsuperscript{d} molecules at similar peptide concentrations, indicating that they bind to L\textsuperscript{d} molecules with similar high affinity.

We next determined the relative affinities of the peptide-L\textsuperscript{d} molecules for the CT-TCR. We previously showed that the relative binding of multimeric TCR to a peptide-MHC complex expressed on BV-infected insect cells correlates with the affinity of monomeric TCR for the peptide-MHC complex as determined by surface plasmon resonance (32). Therefore, we stained insect cells infected with BV, encoding each of the mimotope-L\textsuperscript{d} complexes, with increasing concentrations of soluble fluorescent multimeric CT-TCR and determined the mean fluorescence intensities at each concentration (Fig. 2B). The CT-TCR bound to all of the peptide-L\textsuperscript{d} complexes on the surface of the insect cells better than to the AH1-L\textsuperscript{d} complex. The binding at the highest CT-TCR concentration correlated with the CT-TCR affinities measured by surface plasmon resonance (AH1, A5, 39, and 15) (11, 26) and the proliferative response of CD8\textsuperscript{+} T cells from CT-TCR transgenic mice stimulated in vitro (Fig. 2C). However, these affinity measurements did not correlate with tumor protection.

To eliminate the possibility that the lack of correlation between in vitro responses of the T-cell clone and tumor protection was the result of differential processing and presentation of the peptides in vivo, we transferred CT-TCR Tg T cells into BALB/c Thy1.1 congenic mice and vaccinated them with BV-infected insect cells expressing the variant peptide-L\textsuperscript{d} complexes. All of the peptide vaccines induced proliferation of the transferred CT-TCR Tg T cells, while vaccination with insect cells expressing the negative control peptide, βgal, did not (Fig. 2D). In agreement with the in vitro response of the CT-TCR Tg T cells, the extent of proliferation induced by the peptide variant vaccines was greater than that of the AH1 vaccine. Therefore, the differences in tumor protection were not a result of differential presentation of the nonprotective peptides.

**Protective Peptide Variants Elicit More T Cells that Cross-React with the AH1 Peptide.** We previously reported that vaccination with protective peptides elicits functional AH1-specific TIL, and vaccination with nonprotective peptides elicits TIL with functional defects (13). Here, we characterize splenic T cells responding to the vaccines directly ex vivo to determine whether the functional defects of the T cells elicited by nonprotective peptides were related to the tumor environment, or to intrinsic properties of the T cells recruited to each response.

First, using fluorescent AH1-L\textsuperscript{d} tetramers, we determined the number and frequency of AH1-specific CD8\textsuperscript{+} T cells in the spleens of the vaccinated mice (Fig. 3A). In every case, vaccination with the peptide variant elicited significantly more AH1-specific T cells than vaccination with the wild-type AH1 peptide. Overall, the protective peptides elicited more AH1-specific T cells than the nonprotective peptides. In particular, the two most protective peptides, A5 and F1A5, produced the most AH1-specific T cells, remarkably accounting for about 20% of the CD8\textsuperscript{+} T cells in the spleens of immunized mice. However, the nonprotective peptides 15 and WMF elicited statistically similar numbers of AH1-specific T cells as the protective peptide 39, indicating that frequency alone does not account for all of the differences in tumor protection.

Using fluorescent L\textsuperscript{d} tetramers containing the peptide variant rather than the AH1 peptide, we next determined the number and frequency of variant-specific CD8\textsuperscript{+} T cells in the spleens of the vaccinated mice (Fig. 3B). All of the vaccines produced strong responses with statistically similar numbers of variant-specific T cells. Therefore, we hypothesized that the variation in the frequency of AH1-specific T cells was primarily caused by the number of variant-elicited T cells that cross-reacted with the AH1 antigen, rather than differences in the magnitude of the total T-cell response to each peptide.

To determine directly the proportion of peptide variant-elicited T cells that cross-reacted with the AH1 TAA, we costained splenocytes with tetramers linked to the AH1 peptide and to the respective variant peptide (Fig. 3C). This costaining resulted in a slight decrease in the number and percentage of cells that...
stained with the tetramers, likely because of competition between tetramers for the TCR binding. However, the ratio of AH1-tetramer-positive to peptide variant-tetramer-positive cells was the same as in samples that were individually stained with each tetramer. The percentage of cells that bound to both tetramers was higher in mice vaccinated with the most protective peptides, A5 and F1A5, than with nonprotective peptides (Fig. 3D). Again, the percentage of cross-reactive T cells in mice vaccinated with the protective 39 and the nonprotective 15 and WMF peptides was statistically similar, confirming that the frequency of AH1-specific T cells alone does not account for all of the differences in protection among the peptide vaccines.

Vaccination with Protective Peptides Elicits More T Cells that Respond to AH1 Stimulation. Numerous studies have shown that more effective cytotoxic T lymphocytes (CTLs) have increased sensitivity to antigen, as measured by effector molecule production (1, 25, 33). The protective peptide vaccines elicited more T cells that produced the effector molecule IFN-γ after stimulation with the AH1 peptide (Fig. 4A). Because of internalization of the TCR

Fig. 2. Peptide variants bind to Ld similarly and stimulate T cells expressing the CT-TCR more effectively than the native AH1 peptide. (A) The amount of competing peptide required to reduce the Ld binding by 50%, the EC50 value, was determined by fitting the relative RU values to a sigmoidal dose-response curve using a one site-competition equation (Prism Graphpad). (B) Insect cells were infected with recombinant baculovirus encoding membrane-bound peptide-MHC complexes and stained with an Ld antibody and increasing concentrations of soluble fluorescent multimerized CT-TCR. To control for infection differences, the CT-TCR mean fluorescence intensity (MFI) was determined at a constant level of MHC-expression. The CT-TCR MFIs at 500 μg/mL are listed. (C) CFSE-labeled splenocytes from CT-TCR Tg mice were incubated with increasing concentrations of peptide in vitro. Proliferation was determined by CFSE dilution of CD8+ T cells. (D) CFSE-labeled Thy1.2+ splenocytes from CT-TCR Tg mice were transferred into naive Thy1.1+ BALB/c mice and vaccinated 1 day later with infected insect cells expressing the indicated peptide-MHC molecules. Proliferation was determined by CFSE dilution of Thy1.2+ CD8+ T cells.

Fig. 3. Vaccination with protective peptide variants elicits more endogenous AH1-specific T cells. (A) Splenocytes from mice vaccinated with the indicated peptides were stained with AH1-tet, CD8, and dump antibodies. The number (left axis, black bars) and percentage (right axis, white bars) of AH1-tet+CD8+ cells was determined and compared with the number of cells in mice vaccinated with the AH1 peptide using a Student’s t test (n = 3; P < 0.0151 for all peptides). Error bars represent SEM. (B) Splenocytes from A were stained with the corresponding peptide variant-tetramer (variant-tet), CD8, and dump antibodies and analyzed as in A. (C) Splenocytes from A were costained with both AH1-tet (y axis) and the corresponding variant-tet (x axis), CD8, and dump antibodies. The percentage of dumpCD8+ dual tet* (cross-reactive, Upper Right) or variant-tet* (Lower Right) cells is indicated. (D) The percentage of variant-tet* cells that bind to AH1-tet from C.
after antigen stimulation, IFNγ-producing T cells could not be analyzed directly by AH1-tetramer staining; however, the ratio of the number of T cells producing IFNγ in response to the AH1 peptide versus the number binding the AH1-L3 tetramer was higher in mice immunized with the protective peptides (Fig. 4B). Thus, the protective peptides elicited more AH1 cross-reactive T cells and a higher proportion of these cells produced IFNγ in response to the AH1 peptide.

It was possible that AH1 cross-reactive T cells elicited by the nonprotective peptides made a poor IFNγ response to the AH1 peptide, either because of their previous exposure to AH1 in vivo or because they had incompletely differentiated after immunization with the variant peptide (34–36). To test these possibilities, we examined IFNγ production by the T cells in response to the immunizing variant peptide itself (Fig. 4C). Most of the vaccine-elicited T cells produced IFNγ after stimulation with the vaccinating peptides. Therefore, most of the T cells elicited by the peptide variants differentiated into effector T cells capable of IFNγ production, but those elicited by the nonprotective peptides did not respond to stimulation with the AH1 peptide.

It was still possible that the IFNγ-producing cells in Fig. 4C may have been confined to variant peptide-specific cells that did not cross-react with the AH1 peptide. To determine if the AH1-specific T cells produced IFNγ after stimulation with variant peptides, we sorted AH1-tetramer+ cells from mice vaccinated with protective (F1A5) or nonprotective (WMF) peptides. An equal number of AH1-specific CD8+ T cells were cultured with irradiated BALB/c splenocytes and either the AH1 peptide or the corresponding peptide variant and IFNγ production was measured by ELISA. As with the intracellular IFNγ staining results, WMF-elicited T cells produced less IFNγ after stimulation with the AH1 peptide than F1A5-elicited T cells (Fig. 4D). However, these cells produced similar amounts of IFNγ after stimulation with the respective peptide variant. Thus, although the AH1-specific T cells elicited by the WMF mimotope bound to the AH1-tetramer, most of them were not responsive to stimulation with the AH1 peptide. However, they produced IFNγ after stimulation with the WMF peptide, indicating that these cells had differentiated into CTLs.

In addition to production of effector cytokines, killing of antigen-expressing target cells is another important function of CTLs (reviewed in ref. 1). More T cells from mice vaccinated with the protective peptides expressed CD107a (LAMP-1), a marker of degranulation (37), on the cell surface after stimulation with the AH1 peptide than T cells from mice vaccinated with the nonprotective peptides (Fig. 5A). Furthermore, purified AH1-specific T cells from mice vaccinated with the protective F1A5 peptide were more effective at killing AH1-loaded target cells than T cells from mice vaccinated with the nonprotective WMF peptide (Fig. 5B). This trend was the same at multiple effector to target ratios (Fig. 5C). These data demonstrate that the protective peptide vaccines generate CTLs that recognize and kill TAA-loaded target cells.

The inability to induce functional AH1-specific T cells raised the possibility that the nonprotective peptides might actively or competitively suppress the expansion and differentiation of T cells elicited by the protective peptides. Because this could be a major stumbling block to the development of peptide variant vaccines, we tested this possibility directly. We monitored tumor growth in mice vaccinated with both the nonprotective WMF peptide and the protective F1A5 mimotope (Fig. S1A). As shown in Fig. 1, F1A5 vaccination protected nearly all mice from tumor challenge and WMF immunization was ineffective. However, covaccination with the WMF vaccine did not suppress the antitumor immunity afforded by the F1A5 vaccine. Furthermore, the percentage of AH1-specific T cells in peripheral blood of the F1A5 peptide immunized mice at the time of tumor challenge was unaffected by covaccination with the WMF peptide (Fig. S1B). This result has important implications for therapeutic peptide vaccines because it demonstrates that vaccination with mixtures of peptides (some protective and some nonprotective) may not reduce the therapeutic effect.
Discussion

Peptide vaccines are a promising strategy to mobilize the immune system against tumor-associated antigens. However, in clinical trials to date, wild-type and variant peptide vaccines rarely eliminated tumors (3, 4, 38, 39). Here, we use a panel of peptides for the immunodominant epitope of the CT26 tumor—the AH1 peptide—that have substitutions predicted to either directly or indirectly change the peptide-MHC surface recognized by T cells. We maintained optimal MHC-anchor amino acids at positions 2 and 9 and the two main TCR-interacting tyr residues at positions 4 and 6. Our rationale was that changes in the remaining five amino acids might introduce subtle changes that create a stronger ligand for the AH1-specific T-cell repertoire.

We selected peptides for this study that bound to and activated the AH1-specific CT T-cell clone (11) better than the AH1 peptide itself (Fig. 2). The perceived risk in using a single T-cell clone to select peptide variants is that, in tailoring the peptides to interact optimally with this clone’s TCR, the interactions with the rest of the native repertoire to the tumor antigen may be curtailed, resulting in a net loss of immunogenicity. In fact, the T-cell clone selected both protective and nonprotective peptides. It is clear from this study that the most effective peptide variants elicited more T cells that cross-reacted with the tumor antigen. We would predict from these results that screening peptide variants with a polyclonal population of T cells would yield peptides that have a broad reactivity to many tumor-specific T cells, increasing the likelihood that they would elicit a large cross-reactive response in vivo.

Both the number of cross-reactive T cells and the sensitivity to the native tumor antigen correlated with tumor protection. Although the magnitude of the TAA-specific CD8\(^+\) T cell response (40) and the precursor frequency of TAA-specific T cells (41) correlates with prolonged tumor-free survival in some studies, even very large numbers of TAA-specific T cells do not alter tumor progression in others (42). Therefore, variant peptide vaccines also need to expand an effective portion of the TAA-specific T-cell repertoire, which is dependent on the structure of the variant peptide and the cross-reactivity of the responding T cells (23, 24). We hypothesize that protective peptide variants prevent tumor growth because they elicit endogenous tumor-specific T cells that have an increased affinity for the native tumor antigen relative to the T cells elicited by the nonprotective peptides. We are currently characterizing the repertoire of T cells elicited by the peptide variants to determine if they express different TCRs, and whether the affinity of the responding T cells predicts the overall anti-tumor response.

Materials and Methods

Mice. Six- to 8-week-old female BALB/cAnNCr mice were purchased from the National Cancer Institute/Charles River Laboratories. Thy1.1\(^+\) BALB/c mice were a generous gift from Charles Suri (Scripps Research Institute). CT26 Rag2-deficient transgenic mice have been described (26). All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at National Jewish Health.

Cells. Sf9 and High Five insect cells (15) (Invitrogen), CT26 tumor cells (43), and CT-T-cell clone cells (11) were cultured as described.

Antibodies and Staining Reagents. To increase the resolution between the tetramer-positive and -negative T cells, L\(^2\) tetramers with covalently linked peptides were constructed. Cysteine residues were inserted in the sequence encoding the L\(^2\) molecule and the linker of the peptide to form a disulfide trap as shown by Truscott et al. (44). Fluorescent L\(^4\) tetramers (13) and soluble CT-TCR (15, 26) were prepared as described. Tetramers loaded with variant peptides were assembled with streptavidin-labeled AlexaFlour-647 molecules (Invitrogen). Two million splenocytes were stained with 20 \(\mu\)L of conjugated tetramer (25 \(\mu\)g/mL) at 4 °C for 2 h. For costaining experiments, 2 million splenocytes were stained with AH1-tet for 1 h at 37 °C and the respective variant-tet was added and incubated for 1 h at 4 °C.

Antibodies specific for the L\(^1\) molecule (28.14.8b; ATCC, CDB (53-6.7; Biolegend), IFNy (XMG11.2; eBioscience), CD107a (1D4B; BD Pharmingen), B220 (RA3-6B2; Biolegend), CD4 (RM4-5; Biolegend), MHC-II (MSI14.15.2; Biolegend), and the compounds 7-amino-actinomycin D (7-AAA; Sigma) and carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Molecular Probes) were used for flow cytometric analyses. The B220, CD4, and MHC-II antibodies were analyzed in the “dump gate.”

Peptides. MCMV-C4 (31), βgal (27), AH1 (27), A5 and A9 (11), 39 and 15 (13), and WMF (26) peptides were identified as described. The F1A5 peptide variant was identified from a BV-expressed peptide-MHC library (28). CT-TCR\(^\#\) peptide-MHC complexes were identified from the library using soluble fluorescent multimeric CT-TCR proteins. BV-s that encoded peptides that bound to the CT-TCR were cloned by limiting dilution and DNA from infected cells was sequenced. Soluble synthetic peptides were ≥ 95% pure (Chispecific).

Proliferation Assays. In vitro and in vivo proliferation assays were performed as described (26). Briefly, 5 × 10\(^5\) CFSE-labeled splenocytes from CT-TCR Tg mice (Thy1.2\(^+\)) were incubated in 96-well plates at 37 °C with increasing concentrations of soluble peptides, or 1 × 10\(^5\) cells were transferred into Thy1.1\(^+\) BALB/c mice and vaccinated one day later. Cells were harvested 3 d later and CFSE dilution of CD8\(^+\) Thy1.2\(^+\) cells was analyzed by flow cytometry.

Fig. 5. Vaccination with protective peptide variants elicits more CTLs that kill AH1-loaded target cells. (A) Splenocytes from Fig. 3 were stimulated with AH1 peptide in the presence of CD107a (LAMP-1) antibody and stained with CD8 and dump antibodies. The number of CD107a CD8\(^+\) cells was determined by flow cytometry. (B) Sorted AH1-tet cells (as in Fig. 4D) were incubated with CFSE-labeled peptide-loaded target cells. Three days later, the number of live (7AAD\(^–\)) target cells was determined by flow cytometry. The number of live CFSE-labeled (CFSE\(^++\)) or AH1 peptide-loaded (CFSE\(^–\)) target cells are indicated. (C) AH1-tet CD8\(^+\) cells were incubated at different effector to target ratios (E:T) as in B. Groups were compared using a Student’s t-test (n = 3; **, \(P = 0.006\); *, \(P = 0.02\)).
Vaccination. SF9 insect cells were infected as described with recombinant BV encoding the indicated peptides (26). SF9 insect cells were infected for 3 d with recombinant BV, washed three times, resuspended in HBSS (Mediatech), and 5 × 10^6 cells were injected intraperitoneally twice, 1-week apart. Splenocytes were harvested 1 week after the second injection for flow cytometric and functional analyses. Statistical analyses were performed with Prism version 4.0, GraphPad Software, using an unpaired two-tailed Student's t test. A P value of < 0.05 was considered statistically significant.

Tumor Challenge. One week after the second vaccination, on day 0, mice were injected subcutaneously in the left hind flank with 5 × 10^6 CT26 tumor cells (27). Tumor-free survival was assessed by palpation of the injection site and mice were killed upon development of 10-mm tumors. Tumor-free survival was analyzed on Kaplan-Meier survival plots and statistical significance was determined with Prism version 4.0, GraphPad Software, using the log-rank test.

IFNγ Production. One million splenocytes were stimulated with the indicated peptide in 96-well plates for 5 h and stained for intracellular cytokine as per the manufacturer's instructions (GolgiStop, BD CytoFix/Cytoperm Plus Fixation/Permeabilization Kit; BD Pharmingen). The percentage of IFNγ+ CD8+ T cells in vaccinated mice was determined by subtracting the background IFNγ+ and AH1-tet staining cells in i.g.-vaccinated mice and dividing the number of IFNγ+ CD8+ cells by the number of AH1-tet+ CD8+ cells.

For the ELISA, CD8+ T cells from mice vaccinated with F1A5 or WMF peptides for 3 h at room temperature, washed in HBSS, and labeled with 10 μM (AH1 target cells) or 1 μM (i.gal target cells) CFSE for 10 min at room temperature. After washing, 5 × 10^6 AH1-loaded targets, (i.gal-loaded targets, and AH1-tet+ CD8+ T cells were combined (1:1 effector to target ratio) and incubated for 3 d at 37°C. 7AAD target cells were excluded and the number of live CFSE+ cells in each target peak was determined by flow cytometry. The percentage of specific killing was determined using the following equation: % specific killing = 100 × (1 - # of 7AAD+ AH1 targets)/(# of 7AAD+ i.gal targets).

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Fig. S1. Vaccination with the nonprotective WMF (SPSYAYVMF) peptide does not suppress protective F1A5 (FPSYAYHQF)-elicited antitumor immunity. (A) Mice were vaccinated with F1A5, WMF, or both peptides. One group was injected with vaccine produced with both peptides (coinf) and another with vaccine produced separately (mixed). A similar amount of vaccine was administered to the F1A5, WMF, and βgal controls. Mice were challenged with a subcutaneous injection of $5 \times 10^4$ CT26 tumor cells 1 week after the second vaccination. Tumor growth was monitored for 60 days and mice were killed and scored when the tumors reached 10 mm. Groups were compared to βgal-vaccinated mice using a log-rank test; ***, $P < 0.0001$. (B) One week after the second injection, peripheral blood mononuclear cells from the mice in A were stained with AH1-tet, and CD8 and dump antibodies. The percentage of CD8$^+$ T cells that bound to the AH1-tet was determined. The background tetramer staining, determined in the mice vaccinated with βgal peptide, was subtracted.