Augmenting Antitumor T-Cell Responses to Mimotope Vaccination by Boosting with Native Tumor Antigens

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Abstract

Vaccination with antigens expressed by tumors is one strategy for stimulating enhanced T-cell responses against tumors. However, these peptide vaccines rarely result in efficient expansion of tumor-specific T cells or responses that protect against tumor growth. Mimotopes, or peptide mimics of tumor antigens, elicit increased numbers of T cells that crossreact with the native tumor antigen, resulting in potent antitumor responses. Unfortunately, mimotopes may also elicit cells that do not crossreact or have low affinity for tumor antigen. We previously showed that one such mimotope of the dominant MHC class I tumor antigen of a mouse colon carcinoma cell line stimulates a tumor-specific T-cell clone and elicits antigen-specific cells in vivo, yet protects poorly against tumor growth. We hypothesized that boosting the mimotope vaccine with the native tumor antigen would focus the T-cell response elicited by the mimotope toward high affinity, tumor-specific T cells. We show that priming T cells with the mimotope, followed by a native tumor-antigen boost, improves tumor immunity compared with T cells elicited by the same prime with a mimotope boost. Our data suggest that the improved tumor immunity results from the expansion of mimotope-elicited tumor-specific T cells that have increased avidity for the tumor antigen. The enhanced T cells are phenotypically distinct and enriched for T-cell receptors previously correlated with improved antitumor immunity. These results suggest that incorporation of native antigen into clinical mimotope vaccine regimens may improve the efficacy of antitumor T-cell responses.

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Introduction

Harnessing the specificity of the adaptive immune system, primarily T cells, to control tumor growth is a major focus of cancer immunotherapy. Tumors provide unique challenges toward inducing effective antitumor T-cell responses, including tumor-induced immune suppression and tolerance toward tumor-associated antigens (TAA; refs. 1, 2). Many TAA are dysregulated, nonmutated self-antigens and, therefore, the majority of T cells with high avidity for TAA are purged from the thymus during negative selection (3). However, negative selection of self-specific T cells is often incomplete and some TAA-specific T cells escape the thymus and enter the periphery (4). Whether expanded by vaccines or other immunologic interventions, these remaining cells, if activated properly, may be instrumental for cancer immunotherapy (5). Although several creative strategies have been attempted, more work is needed to improve antitumor T-cell immunity.

Vaccination with TAA typically elicits minimal expansion of tumor-specific T cells, resulting in a failure to control tumor growth (6). One approach to improve the T-cell response against tumors is vaccination with peptide mimics of tumor antigens, or mimotopes (7). Mimotopes, also referred to as altered peptide ligands, analog peptides, or variant peptides, resemble tumor antigens but have amino acid substitutions that affect peptide-MHC (pMHC) or T-cell receptor (TCR) interactions (7). Initially, mimotopes consisted of amino acid substitutions of MHC-anchor residues, which increased the affinity of the peptide-MHC interaction, resulting in enhanced presentation (8). While these mimotope vaccines increased the frequency of tumor-specific T cells, the T cells often recognized tumor antigens poorly and resulted in minimal tumor protection (9, 10). Current data suggest that even subtle changes in the peptide sequence may dramatically alter T-cell interactions (9, 10). Therefore, mimotopes may be beneficial or inhibitory to the immune response, the results of which may be unknown before vaccination.

We are developing vaccination strategies using mimotopes of the immunodominant CD8+ T-cell epitope, AH1 (SPSYVYHQF), of the mouse colon carcinoma cell line CT26. Using a tumor-specific T-cell clone, we identified mimotopes that afford a range of protection from tumor growth (7, 15–17). We showed that protective mimotopes elicit more high-affinity AH1 crossreactive cells than poorly protective mimotopes (17).
and that the responding T cells from protective vaccines more closely resemble the T-cell repertoire responding to native AH1 (18). Thus, we hypothesized that boosting mimotope-elicited T-cell responses with the native antigen, rather than the mimotope peptide, would select for T cells with increased affinity for the native antigen and improve antitumor immunity. Clinical studies immunizing with an altered peptide (TERT_{G232}) for a cryptic epitope derived from human telomerase (hTERT) followed by boosts with the native peptide (TERT_{P728R}) resulted in higher quality T-cell responses when compared with successive immunization with the altered peptide (19–21). We tested this hypothesis with mimotope-15 (MPKYAYHML), which poorly protects against tumor growth and elicits many AH1-specific T cells that do not function in response to stimulation with AH1 ex vivo (17). Mimotopes are essential to initially expand AH1-crossreactive cells because immunization with native AH1 peptide does not elicit robust expansion of AH1-specific T cells or protect mice from CT26 growth (15, 17).

We show here that boosting the mimotope-15-elicited T cells with native antigen results in fewer AH1-specific cells overall, yet these T cells had increased functional avidity for the tumor antigen. This vaccine strategy elicited T cells that killed AH1-pulsed targets more efficiently, produced more IFN-γ and TNF-α following stimulation with AH1 peptide, and improved antitumor immunity. Ex vivo analysis of these T-cell clonotypes revealed that the dominant TCRβ chains are shared with those from the mimotope-15 vaccine, although at higher frequency. While immunization with the native AH1 antigen in the absence of the mimotope-prime elicited low frequencies of AH1-specific cells, the cells that did respond are high-quality cells and their deficiency in tumor protection may be attributed to their inability to expand sufficiently. These results show that mimotope vaccines expand a broad repertoire of T cells with a range of affinity for the tumor antigen and a subsequent boost with the native antigen selects for T cells with increased functional recognition of the tumor. This simple concept could be readily incorporated into current trials using mimotope vaccines and may potentially improve the quantity and quality of tumor-specific T cells.

Materials and Methods

Mice

Six- to 8-week-old female BALB/cAnNCr mice were purchased from the National Cancer Institute/Charles River Laboratories. Gp70−/− mice were produced by selective breeding as described previously (22). 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 (Invitrogen) and CT26 tumor cells were cultured as described previously (16). CT26 tumor cells are progeny of cells purchased from the American Type Culture Collection in 1996. CT26 expression of the gp70 protein from the endogenous ecotropic murine leukemia virus was verified by flow cytometry as previously described (23).

Peptides

Recombinant baculoviruses (BV) were engineered and produced as described previously (24). Soluble synthetic peptides were of 95% or greater purity (Chi Scientific).

Vaccines

Mice were primed with 10⁷ recombinant-BV infected SF9 insect cells as described previously (16). Mice were boosted 7 days later with 100 μg of the indicated peptide, 50 μg agonistic anti-CD40 antibody (F6k4.5; BioXcel), and 50 μg PolyIC (Amersham) intraperitoneally. 15-15 refers to mice primed with mimotope-15 and boosted with mimotope-15. 15-AH1 refers to mice primed with mimotope-15 and boosted with the AH1 peptide.

H-2Ld tetramer staining

R-PE-conjugated H-2Ld-tetramers were produced in house as described previously (15). Dual tetramer staining was carried out using covalently linked peptide H-2Ld tetramers as described previously (17). Splenocytes were incubated at room temperature for 90 minutes with peptide-loaded tetramer, FcR antibody (2.4G2), viability-discriminating agent 7-aminoactinomycin D (7-AAD; Sigma), and fluorochrome-conjugated Abs (Biologend) against CD6 (53–6.7), CD11a (M17/4), CD4 (RM4-5), B220 (RA3-6B2), and I-A/I-E (M5/114.15.2). CD4/B220/I-A/I-E antibodies and 7-AAD are collectively referred to as the 'Dump' gate. Where indicated, cells were stained with antibody against PD-1 (29F.1A12), KLRG-1 (2F1/KLRG), and IL-7Rα (A7B34). Cells were analyzed on a CyAn flow cytometer (Beckman Coulter) or FACS-Calibur (BD Biosciences) and data were processed using FlowJo software (Tree Star). Tetramer dissociation assays were conducted as described previously (18).

Intracellular cytokine staining

One week following the second vaccination, splenocytes were stimulated with the indicated peptide and GolgiStop in 96-well plates for 5 hours according to the manufacturer’s instructions (BD Cytotox/Cytoperm Plus Fixation/Permeabilization Kit; BD Pharmingen). Following cell surface staining, fixation, and permeabilization, cells were stained with antibody against mouse IFN-γ (APC or PE) for 1 hour at 4°C. In some experiments, antibody for TNFα (AF488) was included.

In vitro killing assay and IFN-γ ELISA

Splenocytes from multiple mice were combined and CD8+ T cells were enriched using the Dynal CD8+ Negative Selection Kit (Invitrogen). AH1-tet+, AH1-tet−, and AH1-tet−/− CD11a− cells were sorted to approximately 70% to 95% purity on the iCy Synergy cell sorter. GP70−/− splenocytes were isolated and labeled as target cells. Splenocytes were pulsed for 3 hours in the absence of FBS with 10 μg/mL AH1, 15, or μg-μg peptides. Cells were washed and labeled with either 5 μmol/L carboxyfluorescein succinimidyl ester (CFSE) (AH1, 15) or 0.5 μmol/L CFSE (μg). Equal numbers of AH1 or 15-targets were incubated with 5 × 10⁵ sorted AH1-tet+ effectors. Twelve hours following incubation, the cells were washed and stained with 7-AAD to exclude dead cells and the number of CFSE+7-AAD-target cells was determined. Specific killing was determined as

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described previously (17). Supernatant from the killing assay was collected and IFN-γ production was measured using the BD OptEIA IFNγ ELISA kit.

**TCR sequencing**

Between 8 and 10 mice were immunized as described and splenocytes combined on the basis of the vaccine. AH1-tet+ T cells were sorted (2–3 × 10⁶), mRNA was isolated using TRIzol Reagent, and first-strand cDNA was generated using random hexamers and SuperScript III Reverse Transcriptase (Invitrogen). High-throughput sequencing PCR reactions were carried out as described for all Vβ8 family members (with barcode identifiers for each vaccine type; ref. 18). Amplicons presenting the expected size of 300 to 400 bp were quantified by fluorescent measurement using the Qubit ds DNA HS assay (Invitrogen). Equimolar pools of barcoded amplicons were produced and used in an emulsion PCR. The emPCR, corresponding to clonal amplification of the purified amplicon pool, was carried out using the 454 GS FLX Titanium (Roche Diagnostics) according to the manufacturer’s recommendations. Sequencing data were analyzed as described previously (18).

**Tumor infiltrating CD8+ T cell isolation**

Mice were vaccinated as described earlier. After 1 day of the boost, mice were challenged with 5 × 10⁶ CT26 tumor cells subcutaneously and monitored until 8th-gal-vaccinated control tumors reached approximately 25 mm². Tumors were collected and cut into small pieces, incubated with 0.1 mg/mL Liberase (Roche) for 30 minutes at 37°C, and large clumps were broken up by trituration with an 18 G needle. Single-cell suspensions were either stained directly or stimulated with 10 μg/mL AH1-peptide for 5 hours for intracellular cytokine staining.

**Tumor challenge**

One week following the second vaccination (day 0), mice were challenged with 5 × 10⁶ CT26 tumor cells subcutaneously in the left hind flank (22). Tumor-free survival was assessed by palpation of the injection site and mice were sacrificed when tumors reached 100 mm².

**Statistical analyses**

Tumor-free survival was analyzed on Kaplan–Meier survival plots and statistical significance was analyzed with Prism version 4.0. GraphPad Software, using the log-rank test. Other analyses were conducted using an unpaired 2-tailed Student t test. A P value of less than 0.05 was considered statistically significant and error bars represent the standard error of the mean unless otherwise noted.

**Results**

**An increased proportion of T cells elicited by vaccination with mimotope-15 followed by native AH1 boost crossreact with AH1**

Vaccination with the AH1-peptide tumor antigen elicits few AH1-specific T cells and does not provide protection against subsequent CT26 challenge (17). Conversely, vaccination with mimotope-15 expands significantly more AH1-specific T cells yet only 20% of mice survive following CT26 tumor challenge (17). We previously reported that a majority of mimotope-15 elicited T cells do not recognize AH1, and therefore, we hypothesized that we could improve antitumor immunity elicited by poorly protective mimotopes, such as mimotope-15, by boosting with the native AH1 peptide. Using a prime-boost protocol, we immunized initially with baculovirus-infected Sf9 insect cells expressing either mimotope-15 or the AH1 peptide and, 7 days later, boosted this response with either of the peptides in conjunction with Poly-IC and cCD40 (16, 25). This regimen elicited more antigen-specific T cells than either insect cells or PolyIC/cCD40 alone in the prime and boost (data not shown). Using H-2Ld tetramers loaded with AH1-peptide (AH1-tet), we determined the total number of AH1-specific cells, and the frequency, in the spleen 7 days following the boost (Fig. 1A and B). Surprisingly, the number and frequency of AH1-tet+ cells in the spleen was decreased in mice boosted with the AH1 peptide. As expected, the AH1-AH1 vaccine generated very few AH1-specific cells. However, 1 mouse of 18 tested had a substantial response to the AH1-AH1 vaccine. If these data are analyzed using the Mann–Whitney test, or if the outlier is excluded, significant differences (P < 0.0001) between the AH1-AH1 group and either 15-15 or 15-AH1 are observed.

We next determined the frequency of T cells that cross-react with the AH1 peptide. We vaccinated mice as described earlier and stained splenocytes with either AH1- or 15-loaded H-2Ld tetramer. The frequency of 15-specific cells that cross-reacted with AH1 was significantly greater following the 15-AH1 vaccination (Fig. 1C and D). Similar results were observed in experiments where cells were co-cultured with covalently linked AH1 or 15-tetramers (17) conjugated to different fluorophores (Fig. 1E). Experiments using an irrelevant peptide (8-gal) to prime the immune response, followed by a boost with the AH1 peptide, resulted in the detection of background levels of AH1-specific cells (data not shown), suggesting that the AH1-boost is not generating detectable AH1-specific cells in the absence of the mimotope-15 prime and that crossreactive T cells are necessary for the response. Thus, the 15-AH1 vaccine did not increase overall cell numbers, but focused the response toward the AH1-tumor antigen.

**15-AH1 vaccination elicits CD8+ T cells with greater affinity for AH1-tet than the 15-15 vaccine**

While examining the AH1-specific T cells in Fig. 1, we observed differences in AH1-tet+ staining intensity that warranted further analysis. The mean fluorescence intensity (MFI) of tetramer staining has been correlated with increased T-cell affinity for antigen (26, 27). We observed that AH1-specific cells expanded by the AH1-boost stained more intensely with AH1-tet as determined by geometric MFI (Fig. 2A and B). Interestingly, the small frequency of AH1-specific cells elicited by the AH1-AH1 vaccine stained with similar intensity as the 15-AH1 cells, suggesting a similar avidity for AH1-tet. Importantly, costaining with a TCR-specific antibody suggested that TCR expression was slightly lower on the AH1-boosted cells, indicating that their increased staining intensity for AH1-tet was not because of increased TCR expression (data not shown). We extended this analysis by examining the kinetics...
of the pMHC:TCR interaction with a tetramer dissociation assay. Tetramer dissociation correlates with the off-rates of monomeric pMHC:TCR affinity as measured by surface-plasmon resonance (28). AH1-specific cells from mice boosted with the AH1 peptide had a slower rate of tetramer decay following the addition of an MHC Fab-specific competitor and significantly longer half-lives of tetramer association than those that received the 15-15 vaccine (Fig. 2C and D). Combined, these results suggest that the T cells elicited by 15-AH1 vaccination have increased avidity for the native AH1-peptide.

**AH1-specific cells following 15-AH1 vaccination produce more pro-inflammatory cytokines following ex vivo stimulation**

Increased pMHC:TCR interaction detected by tetramer binding does not always correlate with increased function (29). Therefore, we determined if the T cells elicited by each vaccine strategy produced cytokines upon antigen recognition. We stimulated splenocytes from vaccinated mice with increasing concentrations of AH1-peptide and measured intracellular IFN-γ production. We observed an increase in the total number of IFN-γ-producing cells elicited by the 15-15 vaccine at high peptide concentrations (Fig. 3A). This result was not surprising because the 15-15 vaccine elicited more total AH1-tet+ T cells (Fig. 1). The total number of IFN-γ+ cells from AH1-AH1 vaccination was low, again attributed to the low number of AH1-tet+ cells that respond to this vaccine (Fig. 1). However, when IFNγ production is plotted as a frequency of AH1-tet+ cells, a greater frequency of the AH1-tet+ cells from the 15-AH1 and AH1-AH1 vaccines produced IFN-γ (Fig. 3B). Thus, a greater proportion of the AH1-tet+ cells elicited by these regimens functionally recognize AH1. Consistent with the increased avidity as detected by tetramer staining (Fig. 2), the 15-AH1 vaccine elicited IFN-γ-producing T cells with increased sensitivity for the AH1 peptide (Fig. 3C and D). AH1-AH1 vaccination, albeit low in frequency, elicited T cells with enhanced functional avidity toward AH1. Furthermore, analyses of the geometric MFI revealed that stimulation of AH1-boosted T cells resulted in significant increases in the amount of IFN-γ produced by each T cell, suggestive of a higher quality response (Fig. 3E).

Increased T-cell function and quality has been correlated not only to the production of IFN-γ, but also to the production of other cytokines, such as TNF-α and IL-2 (30). More of the IFN-γ+CD8+ T cells in the AH1-boosted mice produced TNF-α (Fig. 3F). However, we observed no difference in IL-2 production (data not shown). Importantly, when splenocytes were stimulated with mimotope-15 ex vivo, we saw no differences in cytokine production, suggesting these cells were not defective, but indeed, had lower sensitivity for the AH1-peptide (data not shown). Overall, these results support the hypothesis that the 15-AH1 vaccine strategy elicits a higher quality AH1-specific T-cell population than
the 15-15 vaccine and the AH1-AH1 vaccination elicits very low frequencies of high-quality T cells.

15-AH1 vaccination elicits an increased frequency of "short-lived effector" AH1-specific T cells

We next determined if the 15-AH1 vaccine altered the effector phenotype of AH1-specific T cells. KLRG-1 and IL-7Rα markers have been commonly used to identify short-lived effector cells (SLEC; KLRG-1+IL-7Rα-) or memory-precursor effector cells (MPEC; KLRG-1-IL-7Rα+; ref. 31). SLEC are terminally differentiated T cells with immediate cytolytic effector function and increased expression of the transcription factor T-bet (32, 33). Interestingly, a greater frequency of the AH1-tet+ T cells from the 15-AH1 mice were identified as SLEC compared with cells from the 15-15 group (Fig. 4A and B). T cells from the 15-15 group also had significant increases in the frequency of MPEC and early-effector cells (EEC; KLRG-1-IL-7Rα/C0). The phenotype of the AH1-tet+ cells from AH1-AH1 vaccination had equal frequencies of both MPEC and SLEC, and increased IL7Rα+KLRG-1+ cells (Fig. 4A and B). Lastly, we examined PD-1 expression on CD8+ T cells, which increases on activation and remains high on exhausted or anergic cells (34). AH1-tet+ cells from the 15-AH1 and AH1-AH1 groups had significantly lower levels of PD-1 expression compared with homologous 15-15 vaccination (Fig. 4C and D). In combination, these results suggest that the AH1-specific cells from each vaccine may be receiving different stimuli during vaccination and, therefore, have different activation profiles.

The 15-AH1 vaccine stimulates T cells that more efficiently lyse AH1+ target cells

We next determined if AH1-specific cells from each group killed AH1-expressing targets cells with similar efficiency. We sorted AH1-tet+ cells from 15-15 and 15-AH1 mice (Fig. 5A) and showed that AH1-tet+ cells from the 15-AH1 mice killed more effectively than AH1-tet+ cells from 15-15 mice (Fig. 5B). Closer examination of the AH1-tet+ cells elicited from 15-15 vaccination revealed 2 populations of cells with different staining intensities for AH1-tet (AH1-tethi and AH1-tetlo; Fig. 5A). Only 1 population of AH1-tet+ cells was detected after the AH1-boost (AH1-tethi). To determine if the AH1-tetlo cells were inhibiting the function of AH1-tethi cells in the 15-15 group, we sorted the AH1-tethi cells from both groups with and without the AH1-tetlo cells in killing assays. In the absence of AH1-tetlo cells, the AH1-tethi cells from the 15-15 mice were less effective than AH1-tethi from the 15-AH1 (Fig. 5B). The dysfunction in killing from 15-15 vaccine-elicited T cells was specific for AH1-loaded targets as they effectively killed mimotope-15-loaded targets (Fig. 5B). To confirm that these groups of T cells

Figure 2. AH1-specific T cells following AH1-boost have increased affinity for AH1-tetramer. A, mice were immunized as in Fig. 1 and AH1-tet+ T cells were analyzed by flow cytometry. B, AH1-tet+ T cells were analyzed as in A and the geometric MFI was determined. Symbols represent individual mice and data reflect 1 of 5 independent experiments (*, P = 0.0068) with similar results. C, splenocytes from 15-15 (●) or 15-AH1 (▲) vaccinated mice were stained with AH1-tet and used in a tetramer decay assay. D, half-life of tetramer decay from C was determined from a 1-phase exponential decay curve. Each symbol represents an individual mouse and different colors represent 3 independent experiments (**, P = 0.0054).
produced and secreted IFN-γ as assumed in Fig. 3D, we collected supernatant from the killing assays and measured IFN-γ by ELISA. As expected, the T cells from the 15-AH1 group generated more IFN-γ than the other groups (Fig. 5C). Overall, these results suggest that the AH1-tet+ cells elicited by each vaccine have different phenotypes and functional reactivity toward AH1.

**Heterologous AH1-boost preferentially expands a subset of T cells elicited by priming with the mimotope-15 vaccine**

Previous analyses of AH1-specific cells responding to mimotope vaccines revealed a positive correlation between tumor protection and Vβ8.3-expressing TCRs paired with Jβ2.6 gene fragments (18). Increased representation of Vβ8.3-Jβ2.6+ cells was also identified in the responses to the native AH1 vaccine. Data presented earlier suggest that the heterologous 15-AH1 vaccine elicits high-quality T cells, similar to the cells elicited by AH1. Thus, we determined if the AH1 boost selected specific T cells elicited by the mimotope-15 prime and whether these cells were similar to those described. We pooled sorted AH1-tet+ cells from several mice, generated cDNA, and carried out high-throughput sequencing using Vβ8.3-family specific primers. More than 22,000 sequences per group were analyzed as described previously (18). The heterologous AH1-boost significantly enhanced the frequency of Vβ8.3+ clones expressing Jβ2.6 compared with AH1-tet+ T cells from the 15-AH1 group (Fig. 5D). Not surprisingly, the frequency of Vβ8.3+ sequences expressing Jβ2.6 was similar to those of the AH1-tet+ cells from AH1-AH1 vaccination. These results suggest that the robust stimulation provided by the mimotope-15 vaccine may elicit a broad repertoire of T cells and the native AH1 boost focuses the response toward T cells expressing Jβ2.6.

Another common feature identified within AH1-specific T cells identified from protective vaccines was a relatively short CDR3b length of 10 amino acids (18). More than 70% of all Vβ8.3+ sequences following the heterologous AH1 boost had a CDR3b length of 10 amino acids, although that frequency was lower in all mimotope-15 vaccine groups (Fig. 5E). Surprisingly, the majority of Vβ8.3+ sequences from the AH1-AH1 vaccine were 9 amino acids. A single dominant clone that was identified in a total out of 8,188 Vβ8.3+ sequences largely influenced these results. Our previous observations also identified a motif within the CDR3b, consisting of a small hydrophobic residue, followed by a large polar residue, and the tyrosine residue unique to Jβ2.6 (18). On analysis of the Vβ8.3+ sequences identified within each population, the AH1-boost significantly enriched for T cells expressing this CDR3b motif (Fig. 5F). In contrast to our previous data indicating that AH1 elicits the same motif-expressing cells (18), the AH1-specific cells elicited in this experiment did not meet the strict definition of the motif (Fig. 5F). Several differences in experimental design, including pooled T cells from multiple mice
and tetramer design, may account for these differences. In addition, the dominant clone identified in AH1-AH1 cells was 1 amino acid short of the motif, dramatically impacting the results.

The majority of the CDR3β sequences identified from the heterologous AH1-boost (88.5%) were also identified in the homologous 15-15 cells, albeit at lower frequencies (Table 1). Only 1 sequence (light grey) from the dominant 15-AH1 clones was identified in the AH1-AH1 vaccine but not 15-15 (0.76%), suggesting that most of the AH1-specific T cells from the 15-AH1 vaccine originated from the 15-prime. Thirty percent of the dominant 15-AH1 sequences were identified in both AH1-AH1 and 15-15 groups, suggesting the possibility that either vaccine could elicit the same cell, although the frequencies are more consistent with the mimotope priming these cells.

**Increased frequency of functional AH1-tet + T cells infiltrate the tumor, express less PD-1, and enhance tumor-free survival following 15-AH1 vaccination**

Next, we determined the difference between the vaccine groups in T-cell infiltration and function within the tumor. To ensure tumor growth, we challenged mice with CT26 tumors immediately following the boost. When control tumors (βgal vaccinated or unvaccinated) were approximately 25 mm², the infiltrating CD8+ T cells, or TIL, were examined. Significant differences in the number of AH1-tet + TIL per milligram of tumor were not observed between the 2 groups, although there was a trend toward more cells following 15-15 vaccination (Fig. 6A). However, similar to the periphery, we observed a greater percentage of AH1-specific cells that produced IFN-γ and TNF-α when stimulated with AH1 peptide following 15-AH1 vaccination (Fig. 6B). Examination of the phenotype of TIL from mice boosted with the AH1-peptide showed significantly less PD-1 than T cells boosted with mimotope-15 (Fig. 6C and D). Both vaccine strategies elicited cells with lower PD-1 expression than unvaccinated mice, consistent with previous reports (34). These data suggest that AH1-specific cells elicited by 15-15 vaccination can infiltrate the tumor; however, the majority does not functionally recognize AH1. In addition, increased expression of PD-1 on T cells within the tumor of 15-15 vaccinated and unvaccinated mice may further contribute to functional defects observed within the tumor.

Next, we determined if the 15-AH1 peptide vaccine enhanced tumor protection. Indeed, the AH1 boost significantly improved tumor-free survival (Fig. 6E). Importantly, AH1-AH1 vaccination does not result in expansion of AH1-specific cells (Fig. 1) and does not protect mice from tumor challenge (Fig. 6E; ref. 17). Consistent with these results, T cells from the 15-AH1 vaccine kill CT26 tumor cells efficiently in vitro (data not shown) Therefore, in systems wherein the native antigen elicits weak T-cell expansion, use of mimotopes to expand crossreactive cells followed by a native-antigen boost may better select the highest avidity and most effective tumor-specific T cells.

**Discussion**

For CT26, as well as in other mouse (35, 36) and human tumors (37), vaccination with native tumor antigen results in weak expansion of tumor-specific T cells. Mimotopes are 1
example of several vaccine strategies aimed at overcoming this problem, as mimotopes either enhance peptide-MHC binding and antigen presentation (8, 38), or alter TCR interactions (39). While mimotopes have been used effectively to expand tumor-specific T cells, it is not well understood how alterations in peptide sequence may affect the function or repertoire of the responding T-cell populations (9, 10, 14). We hypothesized that boosting mimotope-elicited T cells with the native tumor-expressing antigen would select for high-affinity tumor-specific T cells initially expanded by the mimotope. This hypothesis is not without precedent, as immunization with the cryptic epitope mimotope (TERT572R) from human telomerase followed by the native peptide (TERT572Y) from human telomeraseesis is not without precedence, as immunization with the same antigen (41, 42). Therefore, a heterologous boost with the native tumor antigen may remove the competition and expand tumor-specific T cells, boosting with native antigen selects for T cells with greater affinity for tumor antigen and improves antitumor immunity.

Replacing the mimotope-15 boost with the AH1-peptide boost resulted in fewer AH1-specific T cells overall, but a greater frequency of 15-specific T cells that crossreacted with the AH1 peptide. How mimotope-specific T cells that do not crossreact with the native antigen may impact AH1-specific T-cell expansion or function is not yet known. One possibility is that a subsequent mimotope boost preferentially expands T cells with high affinity for the mimotope but low affinity for the tumor antigen. This possibility implies that mimotope-specific cells that do not crossreact with native peptide have increased affinity for mimotope over their crossreactive counterparts. Competition between T cells of differing affinities is well documented, with high-affinity T cells out-competing low-affinity T cells specific for the same antigen (41, 42). Therefore, a heterologous boost with the native tumor antigen may remove the competition.
between tumor- and mimotope-specific T cells and preferentially expand mimotope-elicited cells that have high affinity for the tumor antigen.

One explanation for the decrease in number of AH1-tet+ cells observed in the 15-AH1 vaccine may be that the AH1-boost is expanding only a small population of high-affinity cells. We have observed distinct differences in AH1-tet+ staining profiles with 2 populations of AH1-tet+ cells in mice boosted with mimotope-15, AH1-tethi, and AH1-teto. We observed lower frequencies of AH1-tethi cells in mice boosted with AH1-peptide, possibly because of AH1-teto cells outcompeting the AH1-tethi cells following vaccination. In addition, AH1-tethi cells lost tetramer staining faster during the tetramer decay assay, indicating that these cells have lower affinity for AH1 and may not proliferate sufficiently to the AH1 boost. Therefore, the difference in cell numbers and overall relative affinity may be attributed to the absence of AH1-tethi in the 15-AH1 mice. Our sequencing data support that the majority of 15-15 AH1-tethi and 15-AH1 AH1-tethi sequences are shared; however, their overall representation of the total sequences is different with the AH1-boost (Fig 5D–F, Table 1). This suggests that the AH1 boost expands cells from the AH1-tethi population, while AH1-teto cells are not expanded.

Although the number and staining intensity of AH1-tethi cells is similar between the 2 vaccines, AH1-teto cells from the

Table 1. Most frequent Vβ8.3 TCR sequences identified following the AH1 boost are elicited by the mimotope-15 vaccine at lower frequency

<table>
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<tr>
<th>Jβ-region</th>
<th>Codonsb</th>
<th>CDR3β regionc</th>
<th>% of 15-AH1 tet</th>
<th>% of 15-15 Hi Lo tet</th>
<th>% of 15-15 Hi</th>
<th>% of 15-15 Lo</th>
<th>% of AH1-AH1</th>
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<td>cASSDGQDYEQYf</td>
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<td>6.76</td>
<td>10.29</td>
<td>1.40</td>
<td>1.18</td>
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<td>+ 10.28</td>
<td>0.90</td>
<td>1.68</td>
<td>0.13</td>
<td>-</td>
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<tr>
<td>Jβ 2.6</td>
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<td>+ 5.90</td>
<td>1.04</td>
<td>0.91</td>
<td>0.36</td>
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<td>cASSPSGQYEQYf</td>
<td>+ 3.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>cASSDGQDYEQYf</td>
<td>+ 2.88</td>
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<td>0.39</td>
<td>0.01</td>
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<td>cASSDGQDYEQYf</td>
<td>+ 2.16</td>
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<tr>
<td>Jβ 2.6</td>
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<td>cASSDGQDYEQYf</td>
<td>+ 1.85</td>
<td>0.35</td>
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<td>Jβ 2.6</td>
<td>2</td>
<td>cASSDGQDYEQYf</td>
<td>+ 1.75</td>
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<td>Jβ 2.6</td>
<td>6</td>
<td>cASSDGQDYEQYf</td>
<td>+ 1.71</td>
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<td>1.00</td>
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<td>Jβ 2.6</td>
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<td>0.79</td>
<td>1.37</td>
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<tr>
<td>Jβ 2.6</td>
<td>7</td>
<td>cASSDGQDYEQYf</td>
<td>+ 1.29</td>
<td>0.03</td>
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<td>cASSDGQDYEQYf</td>
<td>+ 1.23</td>
<td>0.01</td>
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<tr>
<td>Jβ 2.4</td>
<td>3</td>
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<td>+ 0.77</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>cASSDGQDYEQYf</td>
<td>+ 0.65</td>
<td>0.07</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>0.05</td>
<td>0.02</td>
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<tr>
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<td>cASSDGQDYEQYf</td>
<td>+ 0.41</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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<td>cASSDGQDYEQYf</td>
<td>+ 0.31</td>
<td>0.07</td>
<td>0.22</td>
<td>-</td>
<td>0.10</td>
</tr>
</tbody>
</table>

NOTE: Dark gray rows indicate sequences from 15-AH1 that were not identified in either 15-15 or AH1-AH1 vaccines. Light gray rows indicate sequences from 15-AH1 unique to the AH1-AH1 vaccine. Bold numbers indicate the highest observed representation of total sequences for each CDR3β.

aTotal number of codons identified refers to the number of unique DNA sequences identified for each CDR3β.

bAmino acids between V and J regions are in bold.

Identical amino acid sequences identified in 15-15 using a different Jβ-region.
I5-AH1 vaccine killed AH1-pulsed target cells more efficiently, suggesting that the 2 AH1-tet cells populations have inherent functional differences. Furthermore, the presence of AH1-tet cells did not inhibit AH1-tet cells from killing AH1-pulsed targets. Further ex vivo clonotype analyses of I5-AH1 cells revealed preferred Vβ3.3 usage with an enrichment in Jβ2.6 gene fragments. Previously, we identified a CDR3 motif in T cells responding to the more protective mimotopes in our model (18). This motif was found less frequently after vaccination with poorly protective mimotopes, such as mimotope-I5, and was heavily selected for within AH1-tet+ cells following the heterologous AH1-boost (Fig. 5F, Table 1).

In addition, we observed different phenotypic profiles between the AH1-specific T cells elicited by each vaccine. Most AH1-specific cells following the AH1-boost expressed KLRG-1 and downregulated IL-7Rα, defined as short-lived effector T cells. Both SLEC and MPEC are abundant producers of cytokines and have immediate cytolytic function (31), as do the AH1-specific T cells following the AH1 boost. However, SLEC differentiation is thought to be driven by a longer duration of antigen stimulation and increased proliferation (43). Given that AH1-tet+ cells following I5-AH1 vaccination are predominantly SLEC, the AH1 boost may be driving increased proliferation of the high-avidity AH1-specific cells elicited by mimotope-I5 prime. Activated effector cells have a lower ligand affinity threshold for activation than naive T cells (44), which might explain why activated AH1-specific T cells proliferate in response to AH1 while naive T cells do not.

Increased expression of PD-1 on activated CD8+ T cells is often an indication of functional exhaustion or anergy (45). In addition, T cells infiltrating tumors often express high levels of PD-1, while surrounding tumor stroma expresses the PD-1 ligand, PDL-1 (34, 46). AH1-specific T cells from the spleens of the I5-AH1 vaccinated mice expressed higher levels of PD-1 on the day of tumor challenge compared with their AH1-boosted counterparts (Fig. 4C and D). Similarly, the TIL from mimotope-I5-boosted mice expressed higher levels of PD-1, which may be associated with poor antitumor T-cell responses observed by these lower affinity cells. It would be interesting to determine if treatment with antibodies that block PD-1/PD-L1 interactions restore the function in AH1-specific T cells following I5-AH1 vaccination. It is not surprising that AH1-tet cells...
cells following 15-AH1 vaccination, being predominantly SLEC, express lower levels of PD-1. SLEC have increased levels of T-bet expression, which directly represses PD-1 expression (47). Interestingly, it has also been proposed that, as competition for antigen during T-cell activation increases, the level of PD-1 expression decreases, suggesting that a weaker overall activation signal may result in less PD-1 (48). If true, the AH1-boost may be delivering a weaker signal to AH1-tet+ cells compared with the boost with mimotope-15, which may still drive sufficient proliferation of the activated AH1-specific cells (44). Considering the boost vaccination occurs at the peak of the expansion phase, the AH1 boost may provide not only a sufficient signal, but also the optimal signal for these high-affinity AH1-specific cells (15, 49).

Ideally, well-designed mimotopes will preferentially expand only tumor-specific cells with high avidity for the native antigen. It is important to note, however, that there have been reports of detectable, high-avidity T-cell responses to native peptide vaccines in combination with improved adjuvants. Using the unmodified self-antigen Melan-A/MART-I and a combination of CpG oligodeoxynucleotides in Montanide, Speiser and colleagues showed enhanced T-cell function despite lower T-cell frequencies when compared with vaccination with a common mimotope peptide (10). In these experiments, the “altered peptide” vaccine elicited T cells that were less functionally reactive against the tumor. The results here support that immunization with the native antigen elicits high-quality cells, although the total number or frequency may be too low to afford tumor protection. Conversely, vaccination with native-antigen in combination with mimotope vaccines may be beneficial, when immunization with the native antigen alone is insufficient.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J.D. Buhrman, K.R. Jordan, J.E. Slansky
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.D. Buhrman
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.D. Buhrman, L. U’Ren, J. Sprague, J.E. Slansky
Writing, review, and/or revision of the manuscript: J.D. Buhrman, K.R. Jordan, L. U’Ren, C.B. Kemmler, J.E. Slansky
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Sprague, J.E. Slansky, J.D. Buhrman
Study supervision: J.E. Slansky

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References
Native Antigen Boost Improves Mimotope-Elicited T Cell Response


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