CTL Activation Using the Natural Low-Affinity Epitope 222-229 from Tyrosinase-Related Protein 1 Leads to Tumor Rejection

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Abstract

Vaccine strategies for cancer immunotherapy have focused on peptide ligands with high affinity for MHC class I. Largely, these vaccines have not been therapeutic. We have examined the peptide specificity of a strongly protective T-cell response that eradicates established B16 melanoma and find that the recognized epitope is generated by a low-affinity MHC class I ligand from tyrosinase-related protein 1 (TRP1). Cytotoxic T-cell responses are induced against TRP1222-229 by several vaccination schemes using a Toll-like receptor agonist, T regulatory cell depletion, or the immune modulator B7-DCXAb to drive immunity. TRP122 CTL are generated from multiple antigen sources, including antigens expressed by tumors growing in situ, tumor cell lysates, and peptide vaccines. The key finding in this study is that protection from regulatory cell depletion, or the immune modulator B7-DC cross-linking antibody (B7-DCXAb) binds to and cross-links B7-DC on dendritic cells, leading to the generation of strong type I immune responses capable of clearing established tumors. Struck by the robust and rapid response generated against the rather nonimmunogenic B16 tumor, we investigated the target of these CTL. The finding that protective CTL respond to a very weak MHC ligand raises questions about the nature of tumor antigens that can be effectively targeted by immunotherapy and sheds light on an alternative set of natural antigens for use in tumor vaccines.

Introduction

MHC class I restricted self antigens have been selected for tumor vaccines (1–3) using the rationale that high-affinity MHC class I binding peptides will form the best antigens for eliciting tumor-specific cytotoxic lymphocytes (CTLs; ref. 4). One drawback to this approach is that strong binding self-peptides excel in promoting tolerance, leaving only low-affinity T cells as potential responders. Emerging evidence shows that T cells can target low-affinity MHC ligands (3, 5–7). Because these ligands have a low probability of binding MHC, the hypothesis is that only high-avidity T cells can target these peptide antigens. Latent pools of high-avidity T cells that can be primed for antitumor responses have been reported (8). However, approaches to induce responses against these low-affinity ligands are challenging, involving overcoming tolerance using high-affinity heteroclitic peptide mimics or broad-spectrum depletion of regulatory T cells (3, 8).

Although tumors express immunogenic determinants, they often use strategies to escape immune surveillance; these strategies do not necessarily eliminate the T cells targeting the tumor, but instead render them functionally nonresponsive. Several immunotherapeutic strategies target this population of potential effector cells (9, 10), with the intent of reversing tolerance or immunosuppression. We have discovered an immune modulator that robustly activates endogenous CTL capable of targeting tumors (11–13). This immune modulator B7-DC cross-linking antibody (B7-DCXAb) binds to and cross-links B7-DC on dendritic cells, leading to the generation of strong type I immune responses capable of clearing established tumors. Struck by the robust and rapid response generated against the rather nonimmunogenic B16 tumor, we investigated the target of these CTL. The finding that protective CTL respond to a very weak MHC ligand raises questions about the nature of tumor antigens that can be effectively targeted by immunotherapy and sheds light on an alternative set of natural antigens for use in tumor vaccines.

Materials and Methods

Mice. C57BL/6 mice from The Jackson Laboratory were used in compliance with Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Reagents. B16-F1 (B16), C57SV, EL4, and RMAS lines were maintained using standard conditions. Transfected cell lines were maintained in geneticin (Invitrogen) supplemented medium (2.5 mg/ml). Generation of the recombinant human IgM antibody B7-DCXAb and control antibody was described (14). Purified anti-CD25 antibody was prepared from hybridoma PC61 (American Type Culture Collection). Peptides (Supplementary Table S1) were manufactured by Elim Biopharmaceuticals, Inc. ODN-1826 (5′-TCCATGAGCTCTGACGTT-3′; CpG) was synthesized by the Mayo Clinic Advanced Genomics Technology Center. The B16 cell lysate (B16-CL) was prepared as described (15).

B7-DCXAb treatment protocols. Prophylactic B7-DCXAb treatment (30 µg i.v.) was given 1 d before B16 cells (5 × 105), yB16 (12,000 rad) cells, or B16-CL. Therapeutic treatment was administered 4 to 6 d after tumor challenge. Six days after challenge, draining lymph node cells were harvested and pooled for use in cytotoxicity assays. Adoptive transfer of B7-DCXAb-treated dendritic cells has been described (16). Mice were monitored for tumor growth and euthanized when tumor size reached 225 mm3. Typically, 4- to 5-d seeded tumors were not measurable externally but could be observed surgically. By day 7, they could be measured in all mice. Tumor index was determined as the square root of the product of the tumor measured in two dimensions.

CpG immunization and CD25 depletion. Mice were injected with 100 µg of CpG on days −2, −1, 0, +1, and +2 in the right flank and with 200 µg of B16-CL emulsified in IFA on day 0. For T regulatory cell depletion, we injected 0.5 mg of anti-CD25 antibody i.v. on days −3 and −2. Day 7 draining lymph nodes were harvested for cytotoxicity assays. On day 7, CD4+CD25+ cells continued to be reduced by at least 40%. TRP1222- and TRP2180-specific CTL were generated as described previously (3). Mice were injected in the footpad with 5 µg of peptide or 20 µg of B16-CL emulsified in Titermax classic adjuvant (Sigma). Splenocytes were harvested 7 d later and mixed with irradiated (30 Gy) splenocytes pulsed with 1 µg/mL of peptide for 1 h at 37°C. TRP1222 and TRP2180 effector cells were used in cytotoxicity assays 5 d later. Lineage specificity was performed using magnetic bead separation (Miltenyi Biotec) and...
MHC I restriction using blocking antibodies to H-2K<sup>b</sup> (B8-24-3) and H-2D<sup>b</sup> (B22-249.R1 and 28-14-8).

**Cytotoxicity assays.** CTL specificity was assessed using 4-h chromium release assays. An array of peptides (Supplementary Table S1) pulsed onto EL4 or C57SV (10 μg/mL) were used as targets. Where indicated, B16 tumor cells were pretreated for 18 h with 200 ng/mL of IFNγ (Invitrogen). Data represent triplicate measurements ± SD.

**Cloning, mutagenesis, and transfection.** TRP1 cDNA corresponding to bases 121-1631 (NM 031202) was cloned from B16 and ligated into the pcDNA 3.1 TOPO vector (Invitrogen). We used site-directed mutagenesis to change tyrosine<sub>226</sub> to alanine. C57SV cells were stably transduced with linearized cDNA using the Fugene 6 Transfection reagent (Roche Diagnostics). TRP1 expression was verified by real time reverse transcription-PCR.

**RMS stability assay.** MHC class I stability assays were performed as described (17). Briefly, H-2K<sup>b</sup> was stabilized by overnight incubation at 30°C and diluted peptides were co-incubated at 0°C for 1 h before transfer to 37°C for 4 h. H-2K<sup>b</sup> stabilization was measured by flow cytometry using anti-H-2K<sup>b</sup> antibody (B8-3-24).

**Peptide inhibition.** We used a peptide inhibition strategy to block peptide-specific T-cell responses (18). Mice were injected i.v. with 0.1 mg of TRP1<sub>222</sub>, TRP2<sub>180</sub>, or VSV<sub>52</sub> peptide. One day following depletion, mice were injected i.v. with 30 μg of B7-DCXAb. On the following day, mice were challenged with live B16 tumor cells or 200 mg of B16-CL.

**Statistical analysis.** Kaplan-Meier survival analysis was performed using SigmaStat3.1 software (Systat Software). Multiple comparisons were performed using the Holm-Sidak method. Significance was determined by P values <0.05.

**Results**

CTL generated with live B16 or B16 tumor cell lysate recognize the peptide TRP1<sub>222-229</sub>. B7-DCXAb given 1 day before B16 challenge or with B16-CL as a vaccine generates CTL that target and eliminate B16 melanoma (15). To learn which antigens are targeted by these vaccines, we used peptide pulsed targets to screen MHC class I epitopes identified in B16 models (1, 2, 19, 20). B7-DCXAb was administered 1 day before s.c. B16 or B16-CL and 6 days later CTL harvested from lymph nodes were tested for antigen specificity. CTL targeting B16 were observed exclusively in the draining lymph nodes (not shown), demonstrating vaccine specificity. These lymphocytes did not recognize targets presenting any of the traditionally defined B16 epitopes (Fig. 1A; Supplementary Fig. S1), but instead effectively lysed cells pulsed with TRP1<sub>222</sub> (Fig. 1A), a peptide previously identified as a weak MHC class I binding ligand targeted using a heteroclitic mimic vaccine (3). Our studies use a B16 tumor line expressing wild-type TRP1, as studies use a B16 tumor line expressing wild-type TRP1, as opposed to a B16 cell line expressing the TRP1<sub>222</sub> mutant used in the original studies (2).

We next tested whether immunization using more traditional techniques could lead to the generation of these CTL. By combining CD25 depletion with CpG, a more robust response against B16 and TRP1<sub>222</sub> (Fig. 1B) was elicited. We considered the possibility that these T cells were primed with the B16-CL (Fig. 1C). Thus, several vaccine strategies can give rise to T-cell responses against the native TRP1<sub>222</sub> epitope. However, only the TRP1<sub>222</sub>-specific CTL, generated in this manner, targeted B16.

**Live tumor cells elicit responses against a low-affinity MHC ligand processed from TRP1.** We considered the possibility that...
other TRP1 peptides might be targeted after challenge with B16. We used an algorithm-based strategy (21) to choose five additional H-2Kb peptides from TRP1 and tested whether challenge with B16 tumor or B16-CL elicited a response against these epitopes. Our live cell therapy elicited a focused response primarily directed against the epitope TRP1222 (Fig. 2A). Consistent with previous findings (15), immunization with B16-CL and immune-modulating B7-DCXAb generated a broader response, targeting TRP17, TRP1175, TRP1222, and TRP1396.

Immunization can lead to the generation of CTL that may not necessarily target antigens presented by MHC class I on tumors. Therefore, we generated TRP1222-specific CTL using peptide immunization to determine whether these CTL can target the TRP1222 epitope processed from B16. The TRP1222 CTL were able to kill B16 (Fig. 2B), demonstrating that this antigen is indeed presented by B16 and is potentially a relevant tumor target.

Homologous sequences derived from other melanoma antigens could potentially mimic TRP1222 surface presentation. Therefore, we transfected the syngeneic fibroblast line C57SV with a full-length TRP1 clone to confirm that CTL can target the epitope derived from TRP1. TRP1222 CTL killed the TRP1-transfected cells but not the untransfected control line nor a line transfected with a mutant TRP1 containing a noncanonical H-2Kb anchor residue at position 226 of the TRP1222 epitope (Fig. 2B), demonstrating that the low-affinity TRP1222 epitope is processed and presented from the TRP1 molecule.

One factor governing the presentation of a particular peptide is its ability to stabilize MHC class I before presentation on the cell surface. We performed stabilization assays using the transporter associated with the antigen processing-deficient line RMAS to evaluate the MHC binding affinity of potential TRP1-derived epitopes (17) and determined EC50 values using regression analysis. We chose VSV52 as a reference (22), which stabilized H-2Kb in the

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**Figure 2.** B16 challenge induces CTL that recognize a low-affinity MHC class I ligand presented by the B16 tumor line. Cytotoxicity assays were performed as in Fig. 1. EL4 target cells were pulsed with H-2Kb-binding peptides from TRP1 and CTL were from draining lymph nodes of mice treated with B7-DCXAb using live B16 (black columns) or B16-CL (gray columns) as antigen. A, CTL generated from the spleens of TRP1-immunized mice were used to assess presentation of the TRP1222 epitope. Targets were TRP1222- and VSV52-pulsed EL4 or B16 tumor cells (left) or C57SV fibroblasts transfected with a full-length TRP1, a TRP1 H-2Kb anchor residue mutant, or nontransfected C57SV (right). B, peptide binding to H-2Kb was assessed by the change in mean fluorescence intensity (MFI) from peptide- versus nonpeptide-pulsed RMAS cells. Data are representative of three independent trials. Symbols represent the change in MFI over a range of peptide concentrations for the indicated peptides.

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**Figure 3.** TRP1222-specific depletion inhibits killing of B16 melanoma in vitro and in vivo. A, lysis of peptide-pulsed EL-4 targets from mice pretreated with TRP1222 or VSV52 before vaccination with B7-DCXAb and live B16 tumor cells (left) or B16-CL (right). B, percent of B16-challenged animals surviving after TRP1222 depletion and B7-DCXAb treatment compared with treatment with control peptide and B7-DCXAb or control antibody (P < 0.001 TRP1 versus VSV-treated animals). C, percent of surviving animals immunized with B16-CL and depleted before immunization as in B are shown (P < 0.001 TRP1 versus VSV depletion).

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the TRP1222 peptide was effective in the 7,000 nmol/L range, confirming the reported low affinity of this ligand for the H-2Kb allele. We also analyzed the array of TRP1 peptides for their ability to stabilize H-2Kb (Fig. 2A). All of the epitopes targeted by B16-CL–induced CTL stabilized H-2Kb with affinities in the 500 to 7,000 nmol/L range as determined by nonlinear regression analysis (Fig. 2C). Those not targeted by CTL have affinities lower than 10^4 nmol/L. Therefore, whereas the ability to bind MHC class I molecules may be an important feature of immunogenic peptides, the range of required binding affinity seems to be quite large.

The epitope TRP1222 is an immunodominant antigen mediating B16 tumor protection. To determine whether CTL specific for this low-affinity peptide are important for tumor clearance, we depleted TRP1222–specific T-cell responses in vivo by pretreating animals with soluble peptide before immunization. In mice pretreated with an irrelevant peptide, B16 or B16-CL challenge elicited CTL that targeted B16 as well as TRP1222 (Fig. 3A). Pretreatment with TRP1222 blocked the development of TRP1222–specific CTL. This blockade completely inhibited in vitro killing of B16 when mice were challenged with B16 and B7-DCXAb (Fig. 3A). Mice challenged with B16-CL and antibody generated TRP2180 and gp10025 CTL when a response to the immunodominant peptide was suppressed, indicating that TRP1222 CTL outcompete these responses as no reactivity against these epitopes was observed with B16-CL immunization (Fig. 1A). In addition, B16 killing was inhibited by >50% when B16-CL was used as the vaccine antigen, demonstrating that TRP1222 CTL represent a substantial component of this response.

Because TRP1222 CTL are important for killing B16 in vitro, we tested whether these T cells were important for modulating antitumor protection. Accordingly, we used the peptide to block the induction of TRP1222–specific T cells before challenge with B16 tumor. Tumors failed to grow in animals pretreated with control peptide before B7-DCXAb immune modulation (Fig. 3B). In contrast, mice depleted with TRP1222 grew tumors despite B7-DCXAb treatment, with tumor growth kinetics similar to the growth in mice treated with control antibody. Because TRP1222 depletion did not completely suppress B16 killing by CTL (Fig. 3A), we tested whether the response against TRP1222 was critical for the protection in B16-CL–induced vaccines. After peptide depletion, animals were treated with B16-CL and B7-DCXAb then challenged with live B16. Animals pretreated with the control peptide were protected from tumor outgrowth by the B16-CL vaccine. However, the TRP1222–depleted group developed tumors, similar to control mice, demonstrating a critical role of TRP1222 CTL in B16-CL induced vaccines (Fig. 3C).

The antigenicity of B16 melanoma tumor changes in response to irradiation, IFNγ and in vivo growth. In contrast to our findings, previous reports have identified the melanoma differentiation proteins TRP2180 and gp10025 as important antigens in B16 melanoma (1, 2) and had designated unmodified TRP1222 as a very weak antigen (3). We hypothesized that a vaccine using irradiated cells may target different antigens than vaccines elicited with nonirradiated cells. To test this, we used irradiated B16 cells (γIR-B16) in conjunction with B7-DCXAb as a vaccine. The elicited CTL targeted B16 as well as TRP1222, TRP2180 and gp10025 (Fig. 4A). Because γIR-B16 can induce a T-cell response against TRP2180 we wondered whether this treatment would also lead to the presentation of TRP2180 on the surface of B16. TRP1222–specific CTL targeted the B16 cell line and γIR-B16 (Fig. 4B). In contrast, TRP2180 CTL did not kill the unmanipulated B16 tumor cells or the control line C57SV but killed γIR-B16. Therefore, irradiation of B16 tumor cells changes the spectrum of expressed antigens.

The induction of epitope-specific CTL after challenge with B16 melanoma tumors using various vaccine strategies have been
reported (3, 23). However, few studies have shown a strong antigen-specific correlate in vitro using unmanipulated tumor cells. Because B16 tumors are very low MHC class I–expressing cells, IFNγ is often used to up-regulate MHC class I before use as in vitro targets. To determine whether IFNγ was important for targeting TRP1222 or TRP2180 on the B16 line, we pretreated our cells with IFNγ before use as targets. TRP1 222 CTL killed both the unmanipulated B16 cells as well as the IFNγ-treated cells, but not the syngeneic fibroblast line (Fig. 4C). Consistent with previous results, TRP2180 CTL did not target the unmanipulated B16 cells; however, pretreatment with IFNγ resulted in TRP2 180-specific killing of the melanoma line.

We have shown that treatment with B7-DCXAb alone can lead to the clearance of established B16 tumors (12). Because antigen expression patterns by growing tumors can be dynamic, we compared the antigen specificity of CTL induced by established and freshly challenged tumor (Fig. 1A). Mice were challenged with B16 tumors. A, animals were depleted with TRP1222- or TRP2180-specific effector CTL were depleted of CD4 or CD8 T cells before assessing CTL specificity using CD4 or CD8 T cells as targets. B, blocking antibodies to H-2Kb (B8-24-3) and H-2Dd (B22-249.R1 and 28-14-8) were added to peptide pulsed targets before TRP1222 and TRP2180 effector CTL. C, in vitro generated TRP1222 or TRP2180 CTL were infused into mice challenged with B16 5 days prior. Mice receiving no vaccine were used as controls (P < 0.001 TRP1222 versus TRP2180 or none).

Figure 5. TRP1222-specific CTL induced with B7-DCXAb eradicate established B16 tumors. A, animals were depleted with TRP1222- or TRP2180-pulsed, or unpulsed EL4 targets. B, tumor-challenged animals were monitored for survival as in Fig. 2 (P < 0.001 TRP1222 versus VSV52 or TRP2180 depletion). C, tumor growth in individual mice treated with TRP1222-, TRP2180-, gp10025-, or VSV52-pulsed dendritic cells treated with B7-DCXAb (DCXAb). Peptide-pulsed DCXAb vaccine was administered 4 d after B16 tumor challenge. D, tumor-free survival in groups treated with DCXAb or control IgM-treated dendritic cells (DCXAb) pulsed with TRP1222-, TRP2180-, gp10025-, or VSV52-pulsed dendritic cells treated with B7-DCXAb (DCXAb).
B16 for 6 days before treatment with B7-DCXAb. On day 12, CTL effectively killed the B16 tumor, as well as cells pulsed with TRP1222, TRP2180, and gp10025 (Fig. 4D), demonstrating that melanoma growing in situ can prime T cells to several antigens.

**Clearance of established B16 tumors using B7-DCXAb therapy is dependent on TRP1222 CTL.** Our hypothesis that TRP1222 is the major tumor antigen in this model is challenged by the finding that the TRP2180 and gp10025 antigens can serve as potential targets in established tumors. Because TRP1-specific responses are elicited by established tumor, we asked whether depletion of TRP1222 CTL would inhibit tumor rejection after B7-DCXAb therapy. To address this, we depleted TRP1222, TRP2180, or VSV52-specific T cells with peptide 1 day before B16 challenge and treated the mice with B7-DCXAb 4 days later. Sentinel mice were assessed on day 10 verifying peptide-specific depletion of CTL. TRP1222-depleted mice did not target TRP1222 or B16, but targeted TRP2180. Similarly, TRP2180-specific responses were inhibited by TRP2180 peptide, whereas the TRP1222 and B16 responses remained. Pretreatment with irrelevant VSV52 resulted in CTL that continued to target TRP1222 and TRP2180-pulsed EL4 cells, as well as the B16 tumor, but not EL4 cells (Fig. 5A). TRP1222-depleted animals evaluated for tumor growth succumbed by day 20. In contrast, mice depleted with TRP2180 survived for the extent of the observation period (Fig. 5B), similar to the B7-DCXAb treatment group.

We then asked whether the generation of a monospecific TRP1222 response is sufficient to clear established tumors. We treated bone marrow–derived dendritic cells with B7-DCXAb (DCXAb) or control IgM (DCCtrl) in vitro before pulsing with peptide and adoptive transfer into tumor-bearing mice. Animals vaccinated with DCXAb pulsed with TRP1222 remained free of measurable tumor, whereas tumors grew progressively in mice treated with DCXAb pulsed with the melanoma peptides TRP2180 and gp10025 on the control peptide VSV52 (Fig. 5C and D). This shows the importance of antigen specificity in this treatment regimen.

As a complementary approach, we generated TRP1222 and TRP2180 monospecific T cells and assessed their ability to lyse melanoma peptide–pulsed cells in a CD8-dependent and H-2Kb–dependent manner (Fig. 6A and B), demonstrating their classic CTL functions. When these cells were transferred into mice 5 days after challenge with B16 tumor, animals receiving either no T cells or cells specific for TRP2180 succumbed to tumor by day 20, whereas those receiving TRP1222 T cells remained tumor-free (Fig. 6C).

**Discussion**

The immunodominance of the low-affinity MHC class I ligand TRP1222 tumor clearing response induced with native tumor antigens was remarkable, particularly as it occurred in the relative absence of responses against important targets of cellular immunity previously defined using the B16 melanoma tumor model. The paradigm that effective antigens bind efficiently to MHC-encoded antigen-presenting molecules has been used to identify several immunodominant viral antigens (24). Therefore, high-affinity peptide ligands of tumor origin have been targeted in vaccine development. However, these antigens are naturally presented to the immune system in the absence of frank danger signals, before vaccination, resulting in the induction of tolerance. Consequently, high-avidity T cells specific for these tumor antigens may have been depleted from the immune repertoire. Tumor immunotherapy strategies have focused on recruiting the remaining low- to medium-avidity T cells using high-affinity MHC peptide ligands, with the hope that these T cells might be effective. Unfortunately, therapies based on this approach have failed.

Our findings support an alternative approach, eliciting high-avidity, protective T-cell responses using low-affinity MHC peptide antigens. Low-affinity MHC ligands do not naturally deplete the high-avidity T cells, making appropriate T cells available for generating an effective antitumor response. One strategy eliciting strong T-cell responses to weak MHC ligands has been to use self-peptide heteroclitic mimics as antigens. However, recent studies show that immunization strategies using native or heteroclitic antigens elicit distinct CTL responses, and immunization with native antigen preferentially induces high-avidity T-cell responses (25) with increased tumor recognition (26) in comparison with immunization with altered peptides.

Previous reports (3, 6, 27, 28) have identified TRP1 as a target for tumor immunotherapy. Melanoma patients develop IgG antibodies to TRP1, implying recognition of this melanoma differentiation antigen by CD4+ T cells. CD8+ T cells capable of recognizing TRP1 peptides are present in the immune repertoire and have been activated using xenogenic human and heteroclitic variants of mouse peptides. We found that native antigen derived from live, irradiated, or lysed tumor cells can activate strong CTL responses against TRP1. The precise nature of the TRP1 antigen that reaches the lymph node is not known. TRP1 can be secreted (29), possibly gaining access to the lymph node faster than cell-associated antigens. We previously identified biochemical differences in recognized antigens derived from live tumors versus tumor lysates (15), suggesting that negatively charged moieties, consistent with membrane sialylated TRP1 molecules, may be targeted more efficiently after challenge with live tumor cells. Importantly, native TRP1 is processed and cross-presented by dendritic cells in a manner promoting the activation of CD8+ T cells specific for this low-affinity MHC class I ligand.

Another important finding in this report is that other well-known melanoma antigens were not targeted using B7-DCXAb to incite immunity against B16 tumor cells. Earlier studies used manipulated B16 cells to identify tumor antigens (1). Gamma irradiation can modulate the repertoire of MHC class I peptides presented by tumor cells (30). We also find that irradiated B16 tumor cells or tumors grown in situ for 6 days elicited responses against several antigens. Nevertheless, depletion of the TRP2180-specific T-cell response did not affect B7-DCXAb therapy in the established tumor model, whereas T cells targeting TRP1222 were effective at eliminating the tumor. Although our protocol for generating adoptively transferred TRP2180-specific T cells may have biased toward the generation of low-avidity T cells (31), this study shows that at equimolar concentrations of the peptide, TRP1222 peptide elicits T cells that are very effective at targeting and clearing B16. The importance of TRP1 in providing a survival advantage to melanomas has been suggested in studies of TRP1 mutations (32) and should be considered as a factor in the therapeutic effectiveness of this particular antigen. In our survey of B16 melanomas, TRP1-specific CTL kill B16, B16-OVA, B16-F10, as well as B16 cells that have been irradiated or established for 6 days in vivo. In contrast, TRP2180-specific CTL do not kill freshly cultured B16 but recognize tumor cells that have been irradiated, treated with IFNγ, or established in vivo. We surmise that TRP1222-specific CTL responses can clear all tumor cells, whereas TRP2180-specific CTL may only be able to kill stressed cells, leaving behind seeds of healthy cells that sustain tumor growth.
This study supports challenges to the paradigm that high-affinity peptide ligands make effective vaccines for tumor therapy. It may be useful to develop strategies that can identify lower-affinity peptides that elicit curative T-cell responses. This task is difficult because the generation of MHC/tetramers or pentamers needed to track T cells specific for low-affinity MHC ligands is problematic. Identification of relevant peptides by acid elution and mass spectrometry would be expected to generate a bias toward peptides that bind to MHC class I with higher affinity, therefore missing peptides with the binding affinity characteristic of TRP1222. Further investigation must focus on alternative aspects of peptide immunity, including the biochemical processing and subcellular investigation must focus on alternative aspects of peptide immuno-


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