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. TRP1222 CTL are generated from regulatory cell depletion, or the immune modulator B7-vaccination schemes using a Toll-like receptor agonist, T immunotherapeutic CTL. We propose that when administered appropriately, peptide antigens for cancer vaccines are high-affinity MHC mediated by TRP1222-specific CTL and not by CTL specific for freshly implanted or established B16 tumors is primarily in situ multiple antigen sources, including antigens expressed by tumor measured in two dimensions.

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 (CTL; ref. 4). One drawback to this approach is that strong binding self-peptides excite promoting tolerance, leaving only low-affinity T cells as potential responders. Emerging evidence shows that T cells can target lower-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 the 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′-TCCATGACGTTCCTGACGTT-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 iv.) was given 1 d before B16 cells (5 × 106), γB16 (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 mm2. 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 size 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 iv. 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 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-2Kb (B8-24-3) and H-2Dd (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 tyrosine226 to alanine. C57SV cells were stably transfected with linearized cDNA using the Fugene 6 Transfection reagent (Roche Diagnostics). TRP1 expression was verified by real time reverse transcription-PCR.

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

Peptide inhibition. We used a peptide inhibition strategy to block peptide-specific T-cell response (18). Mice were injected thrice i.v. with 0.1 mg of TRP1222, TRP2180, or VSV52 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 Bonf-Sidak method. Significance was determined by P values <0.05.

Results

CTL generated with live B16 or B16 tumor cell lysate recognize the peptide TRP1222-229. 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 assess 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 TRP1222 (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 any of the traditionally defined B16 epitopes (Fig. 1B; Supplementary Fig. S1), but instead effectively lysed cells pulsed with TRP1222 (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 determined by direct sequence analysis.

Struck by the unexpected focus on a cryptic self-antigen, we sought to rule out the possibility that B7-DCXAb treatment leads to the generation of CTL that are distinctive to this immune modulator. Accordingly, we elicited CTL using a vaccine strategy based on the TLR9 agonist CpG. This vaccine was administered with or without prior depletion of CD25+ T-reg. Immunization led to a detectable CTL response against B16 following CpG treatment alone (Fig. 1B). By combining CD25 depletion with CpG, a more robust response against B16 and TRP1222 (Fig. 1B) was elicited.

We next tested whether immunization using more traditional techniques could lead to the generation of these CTL by challenging mice with B16-CL emulsified in Titermax adjuvant and restimulated in vitro with either TRP1222 or TRP2180 pulsed stimulators. Splenocytes from B16-CL immunized mice that were restimulated with TRP1222 or TRP2180 generated effectors that could target their cognate peptide, demonstrating that both of 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 TRP1222 epitope. However, only the TRP1222-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-2K^b 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 TRP1222, 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.

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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. A, EL4 target cells were pulsed with H-2K^b-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. B, CTL generated from the spleens of TRP1222-immunized mice were used to assess presentation of the TRP1222 epitope. Targets were TRP1222-pulsed EL4 or B16 tumor cells (left) or C57SV fibroblasts transduced with a full-length TRP1, a TRP1 H-2K^b anchor residue mutant, or nontransfected C57SV (right). C, peptide binding to H-2K^b was assessed by the change in mean fluorescence intensity (MFI) from peptide- versus nonpeptide-pulsed RMSAs. 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).

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-2K^b 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 EC_{50} values using regression analysis. We chose VSV52 as a reference (22), which stabilized H-2K^b in the
n nanomolar range (Fig. 2C). The previously identified melanoma peptide TRP2180 stabilized H-2Kb in the 100 nmol/L range, whereas 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 10^3 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 vitro 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 TRP1222- and gp100+ 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. 3A). 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 tumor growth. Accordingly, we used the peptide to block the induction of TRP1222-specific T cells before challenge with B16 tumors. 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 gp100 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 gp100+ (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

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**Figure 4.** Gamma irradiation and tumor growth affects B16 immunogenicity and antigen presentation. A, CTL induced with irradiated B16 cells (γIR-B16) were assessed for specificity using B16 or peptide-pulsed C57SV targets. B, TRP1222- and TRP1222-depleted CTL were used to assess antigen presentation by unmanipulated B16, γIR-B16, and control C57SV targets. C, CTL as in 8 were used to determine antigen presentation in unmanipulated B16, IFNγ-treated B16, and control C57SV targets. D, B16 tumors grew in situ for 5 d before B7-DCXAb treatment. Six days later, CTL were tested for specificity using B16 and EL4 (left) and TRP1222-, TRP2180-, or gp100+ pulsed EL4 (right).
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, IFN-γ is often used to up-regulate MHC class I before use as in vitro targets. To determine whether IFN-γ was important for targeting TRP1222 or TRP2180 on the B16 line, we pretreated our cells with IFN-γ before use as targets. TRP1222 CTL killed both the unmanipulated B16 cells as well as the IFN-γ-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 IFN-γ resulted in TRP2180-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 tumors (Fig. 1A). Mice were challenged with B16 tumors 5 days prior to transfer 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 induce with B7-DCXAb eradicate established B16 tumors. A, animals were depleted with TRP1222, TRP2180, or VSV52 1 d before challenge with B16. On day 5, the depleted animals were treated with B7-DCXAb; CTL activity was assayed 6 d later for specificity using B16 and TRP1222, TRP2180-pulsed, or VSV52-pulsed 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 (DCvAb). Peptide-pulsed DCvAb vaccine was administered 4 d after B16 tumor challenge. D, tumor-free survival in groups treated with DCvAb or control IgM-treated dendritic cells (DCab) pulsed with TRP1222, TRP2180, gp10025, or VSV52 before transfer into mice, challenged 4 d prior to challenge with B16 (P < 0.001 DCvAb TRP1222 versus DCvAb VSV52).](image)

![Figure 6. TRP1222-specific CTL are sufficient for clearing established B16 tumors. A, TRP1222 and TRP2180-specific effector CTL were depleted of CD4 or CD8 T cells before assessing CTL specificity to TRP1222 or TRP2180-pulsed EL4. B, blocking antibodies to H-2Kb (B8-24-3) and H-2Db (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).](image)
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 VSV25-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 VSV25 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
(DC×Ab) or control IgM (DC×M) ex vivo before pulsing with peptide
and adoptive transfer into tumor-bearing mice. Animals vaccinated
with DC×Ab pulsed with TRP1222 remained free of measurable
tumor, whereas tumors grew progressively in mice treated with
DC×M pulsed with the melanoma peptides TRP2180 and gp10025 or
the control peptide VSV25 (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-avidity MHC class I ligand
TRP1222 tumor clearing response infused with native tumor antigens
was remarkable, particularly when 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 the live antigens bind efficiently to
MHC-encoded antigen-presenting molecules has been used to
identify several immunomodulating viral antigens (24). Therefore,
high-avidity 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 remain-
ning low- to medium-avidity T cells using high-affinity MHC pep-
tide 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-avidity MHC peptide
antigens. Low-avidity MHC ligands do not naturally deplete the
high-avidity T cells, making appropriate T cells available for
generating an effective antitumor response. One strategy to elic-
tive 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 (27–32) have identified TRP1 as a target for
tumor immunotherapies. 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 find 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
allowing 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
as suggesting that negatively charged moieties, consistent with
surface 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 (31). 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
been biased toward the generation of low-avidity T cells (31), this
study shows that at equimolar concentrations of the peptide,
TRP2180 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, B16-0VA, 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 immunogenicity, including the biochemical processing and subcellular characteristics of the antigens that liberate these MHC ligands. For example, we have found an increased sensitivity of TRP1222 generation to protein synthesis inhibitors compared with other melanoma antigens, demonstrating that rapid protein turnover may be important for presentation of this epitope.

The finding that a weak-binding self-peptide can be the target of effective antitumor immunity could have far-reaching implications. Genetic changes that lead to cancer, particularly those that escape immune surveillance, will not likely generate new high-affinity MHC ligands. In the rare cases when high-affinity ligands are generated, they would be encountered first by the immune system in the absence of costimulation, leading to tolerance. The likelihood of generating new peptides with low affinity for MHC antigen-presenting molecules is higher, and the expectation is that high-affinity T cells present in the peripheral repertoire would be available for activation provided optimal activation signals are delivered. Here, we show that a protective and therapeutic CTL response against the native form of a low-affinity MHC ligand can be elicited using a variety of immunization schemes, including the immune modulator B7-DCXAb. These studies emphasize the selection of effective tumor antigens goes beyond the identification of epitopes that can bind MHC with high affinity. Additional factors, including the structure of the processed antigen, availability of the antigen to the immune system, and the ability to recruit high-avidity T cells, can be crucial to the development vaccines that are not only effective immune stimulants but are also curative.

**Disclosure of Potential Conflicts of Interest**

Mayo Clinic holds intellectual property rights to B7-DCXAb, and L.R. Pease could receive monetary compensation at some future time. The other authors disclosed no potential conflict of interest.

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References

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

The authors wish to retract the article titled “CTL Activation Using the Natural Low-Affinity Epitope 222-229 from Tyrosinase-Related Protein 1 Leads to Tumor Rejection,” which was published in the April 1, 2009 issue of Cancer Research (1).

In the course of investigating suspicious patterns of experimental results in the laboratory, a systematic and in-depth study of key findings in this article was carried out using blind protocols. In these repeat studies, no evidence was found to support our original conclusions that B7-DCXAb modulates dendritic cell functions. We do not believe our failure to reproduce our earlier findings is the result of a technical problem. A member of the B7-DCXAb investigative team, Dr. Suresh Radhakrishnan, who was involved in or had access to all work on this subject, was found in a formal institutional investigation to have engaged in scientific misconduct in unpublished experiments by manipulating another investigator’s experiment involving the B7-DCXAb reagent. This finding of misconduct together with our inability to reproduce key findings using blinded protocols has undermined our confidence in our published reports. We seek therefore to retract this body of work.

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