Altered Binding of Tumor Antigenic Peptides to MHC Class I Affects CD8+ T Cell-Effecter Responses

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Abstract

T-cell priming occurs when a naïve T cell recognizes cognate peptide–MHC complexes on an activated antigen-presenting cell. The circumstances of this initial priming have ramifications on the fate of the newly primed T cell. Newly primed CD8+ T cells can embark onto different trajectories, with some becoming short-lived effector cells and others adopting a tissue resident or memory cell fate. To determine whether T-cell priming influences the quality of the effector T-cell response to tumors, we used transnucelar CD8+ T cells that recognize the melanoma antigen TRP1 using TRP1high or TRP1low TCRs that differ in both affinity and fine specificity. From a series of altered peptide ligands, we identified a point mutation (K8) in a nonanchor residue that, when analyzed crystallographically and biophysically, destabilized the peptide interaction with the MHC binding groove. In vitro, the K8 peptide induced robust proliferation of both TRP1high and TRP1low CD8+ T cells but did not induce expression of PD-1. Cytokine production from K8-stimulated TRP1 cells was minimal, whereas cytotoxicity was increased. Upon transfer into B16 tumor-bearing mice, the reference peptide (TRP1-M9)- and K8-stimulated TRP1 cells were equally effective at controlling tumor growth but accomplished this through different mechanisms. TRP1-M9–stimulated cells produced more IFNγ, whereas K8-stimulated cells accumulated to higher numbers and were more cytotoxic. We, therefore, conclude that TCR recognition of weakly binding peptides during priming can skew the effector function of tumor-specific CD8+ T cells.

Introduction

Naïve T-cell priming occurs when a T cell recognizes its cognate peptide complexed with MHC on the surface of an activated antigen-presenting cell (APC), typically a dendritic cell. Costimulatory ligands on the dendritic cell engage CD28 on the T cell to amplify the signal sent via the TCR/CD3 complex, resulting in T-cell activation. Successfully activated T cells proliferate, secrete cytokines such as IL2, and differentiate into effector T cells that may leave the lymph node and traffic to sites of inflammation. Many steps in this initial priming process have ramifications on the ensuing effector cell response. For CD4+ T cells, slight alterations in the cytokine milieu at the time of priming can skew the cells to adopt distinct effector cell fates, with Th1, Th2, and Th17 cells coordinating very different kinds of immune responses (1). CD8+ T cells have discrete effector functions as well, including secretion of IFNγ, TNFs, and IL2, cytolysis, and differentiation into tissue resident or central memory cells. CD8+ T-cell fates are influenced by the circumstances of T-cell priming. This has been best studied for memory cells, where alterations in TCR affinity or duration of contact with the initial APC can affect memory T-cell formation (2–7). CD8+ T-cell–effector functions are also influenced by TCR affinity for peptide–MHC (pMHC) complexes, suggesting that effector functions may be imprinted as early as the priming stage. In a study of influenza-specific T-cell responses, CD8+ T cells with lower TCR affinities produced fewer cytokines, with IFNγ being produced more frequently than IL2, suggesting that CD8+ T cell–effector functions are determined by the quality of TCR engagement. CD8+ T-cell subsets are particularly important in tumors, where T cells may have low affinity or be in various states of exhaustion or dysfunction (8). Higher affinity TCR engagement leads to better antitumor responses, but only to a certain extent. Work by our group and others has shown that adoptive transfer of T cells with the highest affinity TCRs leads to tumor control that is equivalent to or slightly worse than that conferred by more moderate affinity TCRs (9, 10).

We previously reported two lines of mice derived by somatic cell nuclear transfer from nuclei of melanoma-specific CD8+ T cells (9). These two lines of mice contain monoclonal populations of T cells with TCRs that recognize tyrosinase.
related protein 1 (TRP1) with either high or low affinity (TRP1\textsuperscript{high} or TRP1\textsuperscript{low}). Upon adoptive transfer into tumor-bearing mice, both high- and low-affinity TRP1 T cells controlled tumor growth equally well. This is encouraging, because not all patients have robust neoantigen-specific T-cell responses (11). Recruiting self-reactive T cells, which may have a lower affinity, into the antitumor response may be a good strategy for expanding the reach of immunotherapy to a broader patient population.

TRP1 is a self-antigen that can be presented on H-2D\(b\), although the native epitope binds poorly to the MHC groove due to an alanine at the ninth position anchor residue. Substitution of this alanine for methionine dramatically increases the binding to H-2D\(b\), as previously reported for both TRP1 and other H-2D\(b\) binding peptides (12, 13), and T cells generated through vaccination with the altered peptide can mediate rejection of melanomas expressing the native TRP1 peptide (14). The use of altered peptide ligands as cancer vaccines allows for priming and expansion of TCR clonotypes that cross-react with the native peptide but would not ordinarily be primed by the native peptide alone. Typically, the altered peptide contains modified anchor residues to increase stability of the peptide–MHC complex, while presenting a nearly identical TCR contact surface (12, 15). Examples of altered peptide ligands for mouse and human tumor antigens and for MHC class I and class II have been reported (15–18). However, increased stability of the peptide–MHC complex does not always correlate with more efficient T-cell activation. For example, insertion of a proline residue in the third position of a melanoma epitope destabilizes its binding to H-2D\(b\), yet resulted in increased cytokine production from stimulated T cells expressing a transgenic TCR (19).

How TCRs recognize pMHC complexes is important (20). The relationships between TCR structure, antigen affinity, signaling strength, and antitumor activity are not fully defined (21). Although a correlation between TCR–pMHC affinity and activity exists, this relationship is not absolute, with both high-affinity/low-activity and low-affinity/high-activity TCR–pMHC pairs identified and characterized (21–23). These relationships are complicated due to factors such as antigen density, mode of structural engagement, and involvement of other molecules in the immune synapse (20). In an immunotherapeutic setting, where antitumor activity must be balanced with the potential for autoimmunity, higher affinity, without consideration for other factors, may not necessarily lead to better outcomes. The affinity of a prospective neoantigen for its cognate MHC and how it compares to the affinity of the original self-antigen may also be important factors in the choice of target antigens (24, 25). Therefore, the efficacy of a candidate target antigen in a tumor may rely upon multivariate considerations, including TCR–pMHC affinity, the mode of engagement between the TCR and pMHC, the peptide’s ability to be processed and presented by MHC, and similarity to self-antigens.

Here, we examined how the initial step of T-cell priming influenced the functions of the ensuing CD8\(^+\) effector T cells. Melanoma-specific CD8\(^+\) T cells were primed \textit{in vitro} using peptides that bound to H-2D\(^b\) with distinct biophysical properties. PD-1 upregulation, cytokine production, and granzyme B expression were differentially regulated by priming with different peptides. These initial differences were propagated long-term and resulted in qualitatively different effector functions \textit{in vitro} and in tumor-bearing mice.

**Materials and Methods**

**Animal care**

Animals were housed at the Dana-Farber Cancer Institute and were maintained according to protocols approved by the DFCI IACUC (#14-019 and #14-037). TRP1\textsuperscript{high} and TRP1\textsuperscript{low} trans-nuclear mouse lines were generated by us as previously reported and maintained in house (9). Both lines are now available through Jackson Labs (stocks #30957 and #30958). TRP1\textsuperscript{high} mice were also crossed to CD45.1\(^+\) mice from Jackson Labs (B6.SJL-Ptprca Pepcb/BoyJ; stock #002014). C57BL/6 mice were purchased from Jackson Labs.

**Cell lines**

For mammalian cell studies, B16-F10 cells were purchased from ATCC. Panc02 cells were obtained from the Division of Cancer Treatment and Diagnosis, National Cancer Institute cell repository. RMAS cells were a gift from Hidde Ploegh (Whitehead Institute for Biomedical Research). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Omega Scientific catalog #FB-11), 2 mmol/L L-glutamine (Gibco), penicillin G sodium (100 U/mL, Gibco), streptomycin sulfate (100 \(\mu\)g/mL, Gibco), 1 mmol/L sodium pyruvate (Gibco), 0.1 mmol/L nonessential amino acids (Gibco), and 0.1 mmol/L \(L\)-mercaptoethanol (Sigma). Cells were passaged 2 to 6 times prior to use and were used for experiments at 80% to 90% confluence. \textit{Mycoplasma} testing was performed by PCR every 4 months and was negative for the entire course of this study. No further authentication was performed.

For in vivo cell production of baculoviruses and protein, S9 and High Five cells were each a gift from K. Christopher Garcia (Stanford University). S9 cells were cultured in SF900-III medium supplemented with 10% FBS (Atlanta Biologicals; S11150) and gentamicin sulfate (Lonza; 17518Z) at 28°C. High Five cells were cultured in Insect Xpress medium (Lonza, BE12-730Q) supplemented with gentamicin sulfate (Lonza; 17518Z) at 28°C. No cell line authentication or \textit{mycoplasma} testing were performed during the course of this study.

**In vivo experiments**

\textit{In vivo} experiments were performed as described (26). Briefly, \(2 \times 10^5\) B16 cells were inoculated by subcutaneous injection into the flank in 100 \(\mu\)L of phosphate-buffered saline (PBS). Tumor size was measured daily using precision calipers. Mice were euthanized when the total tumor volume exceeded 1,500 mm\(^3\). Tumors were excised, minced with scissors, and incubated in RPMI containing digestion enzymes (Milenyi tumor dissociation kit, catalog #130-096-730) at 37°C for 30 minutes. Tumors were filtered through a 40-\(\mu\)m cell strainer, washed with PBS, and centrifuged at 300 \(\times\) g for 5 minutes. The resulting cell pellet containing tumor debris and infiltrating immune cells was resuspended in FACS buffer (PBS with 2% fetal calf serum) and stained with a master mix of antibodies described below. Tumor-draining inguinal lymph nodes were crushed through a 40-\(\mu\)m cell strainer to obtain single-cell suspensions. Spleens were crushed through a 40-\(\mu\)m cell strainer and erythrocytes removed with ACK lysis buffer (150 mmol/L \(NH_4Cl\), 10 mmol/L \textit{KHCO}_3, 0.1 mmol/L \textit{Na}_2EDTA).
Flow cytometry
Cells from 1/10 spleen, total tumor-draining lymph nodes, and approximately 50 mg tumor were incubated with extracellular staining mix including 2% fetal cell serum for 30 minutes at 4°C, washed once in PBS, and either resuspended in 1% formalin in PBS for extracellular analysis only or were fixed, permeabilized, and stained with intracellular antibodies against specific cytokines (intracellular cytokine buffer kit from BioLegend; catalog #421002). Analysis was performed on a BD Fortessa flow cytometer. All tumor infiltrates were first gated on CD45+ cells using SSC low as a proxy for viability. Flow cytometry antibodies used in this study were purchased from BioLegend: CD8 (clone 53-6.7), CD25 (clone 3C7), CD44 (clone IM7), CD45 (clone 30-F11), CD45.1 (clone A20), granzyme B (clone QA16A02), H2-Dk (clone RH95), IFNγ (clone XMG1.2), KLRG1 (clone 2F1), LAG-3 (clone C9B7W), and TIGIT (clone 1C9). Analysis was performed using FlowJo software.

H-2Db tetramers containing a photocleavable ligand as described (27) were obtained from the NIH Tetramer Core Facility and photo-crosslinked in the presence of an altered peptide library (A1, A3, A4, K4, E8, F8, K8, TRP1-M9, and S9) and the native TRP1 peptide (TAPDNLGYA). An irrelevant peptide was included as a negative control. Tetramer (1 μg) and peptide solutions (1 mmol/L final concentration) were placed in a 96-well plate (U-bottom, 100 μL per well). The plate was placed on ice and irradiated for 15 minutes in a Stratalinker 2400 UV cross-linker equipped with 365-nm UV-lamps at an ~10-20 cm distance. Peptides were synthesized by the MIT Koch Institute Biopolymers Facility.

Cell culturing, proliferation assay, and cytokine analysis
Primary cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Omega Scientific; catalog #FB-11), 2 mmol/L L-glutamine (Gibco), penicillin G sodium (100 U/mL, Gibco), streptomycin sulfate (100 μg/mL, Gibco), 1 mmol/L sodium pyruvate (Gibco), 0.1 mmol/L nonessential amino acids (Gibco), and 0.1 mmol/L β-mercaptoethanol (Sigma).

B cells were isolated from C57BL/6 mouse spleen and lymph nodes using magnetic bead enrichment (Thermo Fisher Dynabeads Mouse CD43; catalog #11422D) and stimulated with anti-CD40 (clone HM40-3, 2 μg/mL Dynabeads Mouse CD43; catalog #11422D) and stimulated with lymph nodes using magnetic bead enrichment (Thermo Fisher catalog #553721) for 48 hours. CD8+ T cells were isolated from spleen and lymph nodes of TRP1high mice and plated at a 1:1 ratio with activated B cells, TRP1-M9 or K8 peptide (1 μg/mL), and human IL2 (10 U/mL; day 0). After coculture for 48 hours, B cells are removed using the same CD8+ T-cell–negative selection kit as above (day 2). T cells were rested in culture for 1 week, with fresh media containing IL2 (10 U/mL) supplied every 48 hours. On day 8, B16 and Panco2 (negative control tumor cell line) were plated with IFNγ (100 ng/mL, PeproTech; catalog #315-05) for 24 hours to enhance MHC class I expression. On day 9, T cells were plated in media as a negative control (no restimulation), with B16, or with Panco2 at various ratios as indicated in the figure legends. Wells with no T cells, including media-only, B16-only, and Panco2-only wells, were used as negative controls. After coculture for 24 hours (day 10), GolgiStop was added according to the manufacturer's protocol, and the cells were further incubated for 3.5 hours. T cells were removed by vigorous pipetting and analyzed by flow cytometry using the antibodies indicated above. After T cells were removed, the remaining tumor cells were lysed with CellTiter-Glo (Promega) as previously reported and analyzed using a PerkinElmer Envision plate reader (28). Luminiscence values were compared with values from wells containing tumor cells only (no T cells). Media-only values were subtracted from experimental values to eliminate background signal.

Restimulation and cytotoxicity assay
On day 2, B cells were isolated by CD43 magnetic bead selection and stimulated with anti-CD40 as described for the proliferation assay. CD8+ T cells were isolated from spleen and lymph nodes of TRP1high mice and plated at a 1:1 ratio with activated B cells, TRP1-M9 or K8 peptide (1 μg/mL), and human IL2 (10 U/mL; day 0). After coculture for 48 hours, B cells are removed using the same CD8+ T-cell–negative selection kit as above (day 2). T cells were rested in culture for 1 week, with fresh media containing IL2 (10 U/mL) supplied every 48 hours. On day 8, B16 and Panco2 (negative control tumor cell line) were plated with IFNγ (100 ng/mL, PeproTech; catalog #315-05) for 24 hours to enhance MHC class I expression. On day 9, T cells were plated in media as a negative control (no restimulation), with B16, or with Panco2 at various ratios as indicated in the figure legends. Wells with no T cells, including media-only, B16-only, and Panco2-only wells, were used as negative controls. After coculture for 24 hours (day 10), GolgiStop was added according to the manufacturer's protocol, and the cells were further incubated for 3.5 hours. T cells were removed by vigorous pipetting and analyzed by flow cytometry using the antibodies indicated above. After T cells were removed, the remaining tumor cells were lysed with CellTiter-Glo (Promega) as previously reported and analyzed using a PerkinElmer Envision plate reader (28). Luminiscence values were compared with values from wells containing tumor cells only (no T cells). Media-only values were subtracted from experimental values to eliminate background signal.

Adoptive transfer
CD8+ T cells were isolated from TRP1high CD45.1+ mice and cocultured with CD40-activated B cells in the presence of TRP1-M9 or K8 peptide and IL2 as already described. After 48 hours, 2 × 106 CD45.1+ CD8+ T cells were transferred by intravenous injection (150 μL volume, diluted in sterile PBS) into B16 tumor-bearing mice at either 2 days or 5 days after tumor inoculation as indicated in the figure legend. For some experiments, tumor size was monitored daily by precision calipers. For other experiments, tumor size was not measured and mice were euthanized 6 days after adoptive transfer, and spleens, tumor-draining lymph nodes, and tumors were dissociated and analyzed by flow cytometry as above. Transferred TRP1 T cells were identified as CD45+ CD8+ CD45.1+ cells.

RMS stabilization assay
The TAP-deficient cell line RMS was incubated at 30°C overnight. TRP1-altered peptides were used at the concentrations indicated in Fig. 1F and incubated with 100,000 RMS cells at 37°C for an additional 3 hours as previously described (29). Peptide binding was assessed by surface expression of H-2Db, as measured by flow cytometry of cultured RMS cells and analyzed using FlowJo.

RNA-seq
TRP1high or TRP1low CD8+ T cells (106) were stimulated with 106 peptide-pulsed B-cell blasts. CD8+ T cells were sorted by FACS from cocultures after 48 hours, and RNA was prepared (Qiagen RNeasy Plus Mini Kit; catalog #74134). Library preparation and Illumina sequencing was performed by the Dana-Farber Molecular Biology Core Facility Kapa stranded mRNA Hyper Prep kit (KAPA Biosystems) was used for library
construction, followed by multiplex sequencing on a single lane of HiSeq (Illumina). Paired sequencing (75 bp) was performed. Data were analyzed using Viper software and deposited in GEO (accession number GSE120892).

**Protein expression of recombinant H-2D^b**

Recombinant H-2D^b is expressed both as a peptide β2M-H-2D^b single-chain trimer and as exogenously loaded MHC molecules. The single-chain trimers were constructed as previously described (30), with the C-terminus of the TRP1-M9 or K8 peptide linked to the N-terminus of mouse β2M via a 15-amino acid (GGGGS)_4 linker, the C-terminus of β2M linked to the N-terminus of H-2D^b with a 20 amino acid (GGGSG)₄ linker, and with C-terminal BAP and His₈ tags for site-specific biotinylation and purification, respectively. The construct was cloned into the pET28a vector (Novagen 69864-3), then linked to the N-terminus of mouse β2M-H-2D^b via a 15-amino acid (GGGGS)_4 linker, the C-terminus of β2M linked to the N-terminus of H-2D^b with a 20 amino acid (GGGSG)₄ linker, and with C-terminal BAP and His₈ tags for site-specific biotinylation and purification, respectively. The construct was cloned into the pET28a vector (Novagen 69864-3), then

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**Figure 1.**

A spectrum of TRP1-altered peptide ligands identifies agonists, selective agonists, and null peptides. A–C, CD8^+ T cells were isolated from spleens and lymph nodes of TRP1^{High} or TRP1^{Low} mice and cocultured at a 1:1 ratio with CD40-activated B-cell blasts and 1 ng/mL of the indicated peptides. A, Cells were stained with antibodies to CD8 and CD25 and analyzed by flow cytometry 48 hours after activation. B, IL2 and (C) IFNγ in culture supernatants were quantified by ELISA after 48 hours. Error bars are ± SD. All data are representative of at least 3 independent replicates. D, TRP1^{High} and TRP1^{Low} CD8^+ T cells were labeled with Celltrace Violet and cocultured at a 1:1 ratio with CD40-activated B-cell blasts and 1 nmol/L of the indicated peptides for 72 hours. Cells were stained with antibodies to CD8 and analyzed by flow cytometry. Proliferation index was quantified as the number of mitotic events divided by the number of progenitor cells. All data are representative of at least 5 independent replicates. E, 10^6 RMAS cells were incubated at 30°C overnight. TRP1-altered peptides were added at the indicated concentrations, and cells were incubated at 37°C for an additional 3 hours. Peptide binding was assessed by surface stabilization of H-2D^b measured by flow cytometry. F, H-2D^b tetramers containing a photocleavable ligand were photo-exchanged in the presence of the indicated peptides. An irrelevant peptide was included as a negative control. Total spleen cells from TRP1^{High} and TRP1^{Low} mice were stained with antibodies to CD8 and the indicated tetramers and analyzed by flow cytometry. Plots are gated on CD8^+ cells and are representative of 5 mice across 2 independent experiments. Percentages of tetramer^+ cells are shown in bold. Red numbers: positive tetramer staining, black indicates lack of tetramer staining. Yellow numbers: conditions where the peptide activated TRP1 T cells and no tetramer staining was observed.

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expressed in BL21 DE3 E. coli (New England Biolabs, C25271) at 37°C for 5 hours. Bacteria were pelleted via centrifugation (20 minutes at 6,000 × g), resuspended in Heps Buffer saline (10 mM HEPES pH 7.2, 150 mM NaCl), and lysed by adding 2× volume of lysis buffer (50 mM/L Tris-HCl pH 8.0, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholic acid, 100 mM/L NaCl) and 10 μL Benzonase. Cells were then sonicated and centrifuged for 15 minutes at 10,000 × g, leaving protein inclusion bodies. The inclusion bodies were then purified via resuspension followed by centrifugation three times in a detergent-containing wash buffer (50 mM/L Tris-HCl pH 8.0, 100 mM/L NaCl, 0.5% (v/v) Triton X-100, 1 mM/L sodium EDTA, 1 mM/L DTT, 0.2 mM/L/L PMSF), followed by a final wash in a detergent-free buffer (50 mM/L Tris-HCl pH 8.0, 1 mM/L sodium EDTA, 1 mM/L DTT). The β2M and H-2Dβ inclusion bodies were then then co-refolded in the presence of TRP1-M9 or K8 peptides via the dilution method in refolding buffer (100 mM/L Trit pH 8.0, 400 mM/L Arginine hydrochloride, 0.5 mM/L oxidized glutathione, 5 mM/L reduced glutathione, 0.2 mM/L/L PMSF) as previously described (32). All proteins were then exchanged into HBS (100 mM/L HEPES pH 7.2, 150 mM/L NaCl) and lysed by adding 10 to 15 mg/mL. Initial screening was conducted using a Bio-Rad CFX384 Real-Time PCR system. The proteins were exposed to increasing temperature from 20°C to 95°C at 1 degree/minute increments, and fluorescence was monitored at 525 nm wavelength. The protein melting temperature (Tm) was determined by calculating the minimum of the −dF/dt measurement at 525 nm as previously described (33).

Single-chain trimer sequences

Please see the Supplemental Methods for the protein and DNA single-chain trimer sequences for M9 and K8.

Differential scanning fluorimetry

All H-2Dβ proteins were diluted to 1 μmol/L in HBS (10 mM/L HEPES pH 7.2, 150 mM/L NaCl) in the presence of Sypro Orange dye (Thermo Fisher, S6650) diluted 1,000-fold from its 5,000× stock to 5×. Twenty microliters of each protein/dye mixture were then placed in triplicate in a Bio-Rad CFX384 Real-Time PCR system. The proteins were exposed to increasing temperature from 20°C to 95°C at 1 degree/minute increments, and fluorescence was monitored at 525 nm wavelength. The protein melting temperature (Tm) was determined by calculating the minimum of the −dF/dt measurement at 525 nm as previously described (33).

Crystallization, structural determination, and refinement

TRP1-M9- and K8-single-chain trimer proteins were concentrated to 10 to 15 mg/mL. Initial screening was conducted using the PACT premier and JCSG crystallization screens (Hampton Research). One hundred nanoliters of each condition in the crystallization screens were individually combined with 100 nL of protein in sitting drops using a Phenix crystallization robot (Art Robbins). Crystals were then further optimized in 1 μL + 1 μL protein:crystallant mixture sitting drops. For the TRP1-M9 single-chain trimer, crystals formed in 16% PEG-6000, 200 mM/L LiCl, and 100 mM/L Tris-HCl (pH 8.0). For the K8 single-chain trimer, crystals formed in 30% PEG-1500 and 100 mM/L MiB buffer (Hampton Research), pH 7.0. Crystals were flash frozen in liquid nitrogen in crystallant supplemented with 20% to 25% ethylene glycol.

For TRP1-M9, X-ray diffraction data were collected at the MIT Structural Biology Service Center using a Rigaku MicroMax-007HF X-ray source and collected via a Rigaku Saturn CCD. For K8, data were collected at Advanced Photon Source beamline 24-ID-E via an Eiger X 16M detector. Data were then indexed, integrated, and scaled using XDS and XSCEAL (34).

Structures were solved via molecular replacement using the program Phaser (35). The molecular replacement search models used were a previously solved H-2Dβ structure [Protein Data Bank (PDB) ID 4HUU; ref. 36], with the α1α2, α3, and β2M domains each placed separately. The peptide was not included when solving the structure to avoid model bias. Manual model building of the peptide was performed in COOT followed by rounds of refinement using Phenix (37, 38). Simulated annealing composite omit maps were generated using Phenix (38). Structures were visualized and figures made using PYMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Accession numbers

TRP1-M9-H-2Dβ and TRP1-K8-H-2Dβ coordinates and structure factors are deposited in the PDB at https://www.rcsb.org/structure/6MPO under accession numbers 6MPO and 6MP1, respectively.

Statistical analysis

Unpaired Student t test with Welch correction was used throughout. Error bars are SD; P values <0.05 after Bonferroni correction were considered significant. Data were analyzed using GraphPad Prism software.

Results

TRP1-altered peptide ligands K8 is a weak agonist that binds poorly to MHC class I

Using the heterocytic TRP1 peptide as our reference (TRP1-M9), we generated a panel of altered peptide ligands with single amino acid substitutions in the nonanchor residues (39, 40). We used this peptide library to stimulate CD8+ T cells derived from TRP1high or TRP1low transnuclear mice (9). As predicted, several of these had abolished recognition by both TRP1high and TRP1low TCRs, and selective agonists of each TCR were also identified (Table 1 and Fig. 1A–D). One peptide, K8, was weakly agonistic for both TRP1high and TRP1low TCRs, resulting in robust proliferation but diminished CD25 expression and diminished IL2 and IFNγ production from both TCR
clonotypes (Fig. 1A–D). Given the complete absence of sequence similarity between TRP1\textsuperscript{high} and TRP1\textsuperscript{low} TCRs and TCR\textbeta CDR3 regions (9), we had not expected to find peptides that had diminished recognition to a similar extent between the two different TCRs. Weak agonists that bind strongly to TCRs but weakly to the MHC have been reported (41). To determine whether K8 bound to H-2D\textsuperscript{b}, we performed an RMAS stabilization assay (Fig. 1E). RMAS cells are deficient in the peptide transport complex TAP and, therefore, cannot properly stabilize MHC class I peptide complexes on their cell surface (29). Exogenously added peptides can compensate for this defect, leading to surface display of H-2D\textsuperscript{b} that is directly proportional to the binding capabilities of the added peptide. Addition of TRP1-altered peptides resulted in stabilization of surface H-2D\textsuperscript{b}, consistent with the peptides being H-2D\textsuperscript{b} binders. As expected, native TRP1 peptide with its ninth position alanine performed poorly in the RMAS stabilization assay, whereas TRP1-M9 peptide and most of the other altered peptide ligands stabilized H2-D\textsuperscript{b} expression on RMAS cells, consistent with the alterations affecting nonanchor residues. K8 and A3 peptides, however, bound poorly to H-2D\textsuperscript{b}, despite the mutations being leading to surface display of H-2D\textsuperscript{b} (30). With these constructs, we crystallized the structures of K8 and TRP1-M9 single-chain trimer constructs (Fig. 2; Supplementary Table S1). When the structures of K8 and TRP1-M9 were compared, it was observed that both peptides were presented in the MHC–peptide binding groove in a canonical orientation, with the P2, P5, and P9 positions serving as MHC-contacting “anchor” residues. Essentially no global alterations to MHC structure (the RMSD between H-2D\textsuperscript{b} in each structure was 1.38 Å), the peptide’s backbone, or any side chain conformation aside from the defining mutation at P8 was observed (Fig. 2A; Supplementary Fig. S1A–S1B). These structures, therefore, suggest that no gross alteration in peptide presentation exists and that the alteration at P8 is likely the cause of the observed difference in peptide stability.

Biophysical and structural analyses of K8 reveals destabilized binding to H-2D\textsuperscript{b}

Although P8 is predicted to be a solvent-exposed, TCR contact residue, class I MHCs can accommodate a distribution of peptide lengths (42), and mutations within a peptide can cause unpredicted changes in overall peptide geometry in the MHC groove, including changing the TCR contact epitope (22). Therefore, to verify the results from the RMAS stabilization assay (Fig. 1F) and to better understand how a point mutation might destabilize binding to H-2D\textsuperscript{b}, we conducted a series of structural and biophysical studies comparing recombinantly expressed H-2D\textsuperscript{b} bound to either the TRP1-M9 or K8 peptides. We first set out to structurally characterize the TRP1-M9 and K8 peptides bound to H-2D\textsuperscript{b} via X-ray crystallography. Because MHCs refolded from bacterial inclusion bodies and exogenously loaded with peptide did not readily crystallize, we expressed insect cell peptide–β2M–H-2D\textsuperscript{b} “single-chain trimers” that contain MHC molecules physically tethered to the peptide via a glycine-serine linker to ensure that even weakly binding peptides could be stably presented (30). With these constructs, we crystallized the peptide's backbone, or any side chain conformation aside from the defining mutation at P8 was observed (Fig. 2A; Supplementary Fig. S1A–S1B). These structures, therefore, suggest that no gross alteration in peptide presentation exists and that the alteration at P8 is likely the cause of the observed difference in peptide stability.

We further compared the peptides using a simulated annealing composite omit map and observed the electron density of the K8 peptide was substantially worse at the P4, P6, and P8
side chains, as well as the P8 peptide backbone (Fig. 2B). However, because K8 and TRP1-M9 crystallized in different crystal forms (Table 1), it was difficult to establish a causal relationship. Our structural and biophysical analyses indicated that it was the difference in contact between H-2Db and the solvent-exposed “TCR contact” residue P8 that drove the differences in peptide presentation. Although the P8 position was exposed to solvent, it still made contact with amino acids on the MHC helices (Supplementary Fig. S1C), potentially providing a mechanism for the observed differences.

Because we did not observe any gross alterations to peptide conformation and to ensure linking the peptide and MHC did not inadvertently affect the noted peptide stability changes (Fig. 1), we analyzed each pMHC by determining their Tm via ThermoFluor thermal stability shift assay shows a large difference in protein stability. H2-Db/K8 single-chain trimer (orange, solid line) demonstrated a Tm 6 degrees lower than that of H2-Db/TRP1-M9 (teal, solid line) (39.5°C vs. 45.5°C). When unlinked peptide (dashed line) was compared with peptides linked as a single-chain trimer (solid line), a similar Tm was observed for each peptide studied.

Figure 3.

Stimulation with TRP1-M9 peptide generates stronger cytokine output and differential gene expression from TRP1high and TRP1low cells compared with K8 peptide. A, TRP1high or TRP1low CD8+ T cells were cultured at a 1:1 ratio with CD40-activated B-cell blasts and 1 nmol/L of either TRP1-M9 or K8 peptides for 48 hours. Culture supernatants were collected and analyzed by cytokine bead array. n = 3 biological replicates. Heat map shows log2 expression values (pg/mL). B, Total RNA was collected from TRP1 CD8+ T cells stimulated as in A and subjected to RNA-seq analysis. Genes found to be significantly differentially expressed between TRP1-M9- and K8-activated T cells are presented as a heat map. Genes of particular relevance to T-cell effector function are shown in bold. C, Top 25 enriched gene sets identified by preranked GSEA in K8-activated T cells (blue) versus TRP1-M9-activated T cells (red).
differential scanning fluorimetry (DSF), a method that enables accurate determination of protein folding and stability (43) and has been shown to correlate with other measurements of pMHC stability, such as peptide half-max inhibitory concentration (IC50) (33). DSF measurements found the H-2Db-pMHC stability, such as peptide half-max inhibitory concentration, to be higher for TRP1-M9 than for TRP1-K8 (Fig. 2), demonstrating the constructs used for crystallography imparted a stability difference despite their superimposable structures (Fig. 2). Refolded MHC loaded with exogenously loaded peptide showed similar measurements to the peptide-linked single-chain trimers (Fig. 2), additionally suggesting that the stability difference between the peptides is not altered by peptide linkage. These differences confirmed the results of the RMAS stabilization assay (Fig. 1F) and further demonstrated an alteration of the peptide’s ability to bind to MHC caused by the P8 Tyr-Lys mutation, despite no gross structural changes (Fig. 2).

K8 stimulation of TRP1high or TRP1low CD8+ T cells leads to an altered activation profile

To determine whether the stability of peptide binding to MHC affects the functional properties of stimulated CD8+ T cells, we activated TRP1high or TRP1low CD8+ T cells in vitro with either TRP1-M9 or K8 peptides. Cytokine production was increased in TRP1-M9 activated cultures, regardless of which TCR was present (Fig. 4A). Transcriptional analysis likewise showed a signature of K8 activation that was distinct from activation with TRP1-M9 peptide. Figure 4B shows a heat map of differentially expressed genes between TRP1-M9- and K8-stimulated conditions, with OX40 (Tnfrsf4), Nur77 (Nrl1), IFNγ, and PD-1 (Pdcd1) increased in TRP1-M9 conditions and cell-trafficking molecules and granzyme A (Gzma) having higher expression in K8-stimulated conditions. Overall, gene signature enrichment analysis confirmed higher IL2/STAT5 signaling in TRP1-M9-stimulated cells, but higher interferon signaling and chemokine signaling in K8-stimulated cells (Fig. 4C).

In addition to its role in T-cell exhaustion, PD-1 is an acute activation marker that is upregulated on activated T cells. However, PD-1 expression remained low on K8-stimulated TRP1high and TRP1low cells, even at high peptide concentrations (Fig. 5A and B). Although TRP1-M9-stimulated cells may seem more activated, K8-stimulated cells displayed a skewed activation profile that could not be easily classified as simply less activated. We considered whether TRP1-M9–pulsed APCs simply contained more stable pMHC complexes than APCs pulsed with K8 at the same concentration. To evaluate this, we titrated TRP1-M9 and K8 peptides and found that lower concentrations with K8 at the indicated times. Proliferation was assessed by flow cytometry. D and E, CD8+ T cells from C were stained with the indicated antibodies and analyzed by flow cytometry. N = 3/group. Error bars are SD. P values were calculated by ratio paired t test across all three time points.

Figure 5.
Weak agonist peptide K8 induces robust proliferation from TRP1high and TRP1low cells without acute induction of PD-1. A, TRP1high – CD8+ T cells were labeled with Celltrace Violet and cocultured at a 1:1 ratio with CD40-activated B-cell blasts and the indicated peptides at 1 μg/mL for 72 hours. Cells were stained with antibodies to CD8, PD-1, and CD25 and analyzed by flow cytometry. B, TRP1high or TRP1low CD8+ T cells were cocultured at a 1:1 ratio with CD40-activated B-cell blasts pulsed with either TRP1-M9 or K8 peptides at the indicated concentrations. PD-1 expression was determined by flow cytometry. N = 4/group. C, TRP1high – CD8+ T cells were labeled with CFSE and cocultured at a 1:1 ratio with CD40-activated B-cell blasts pulsed with 1 μg/mL TRP1-M9 or K8 for the indicated times. Proliferation was assessed by flow cytometry. D and E, CD8+ T cells from C were stained with the indicated antibodies and analyzed by flow cytometry. N = 3/group. Error bars are SD. P values were calculated by ratio paired t test across all three time points.
We next investigated the effect of priming TRP1<sup>high</sup> cells with either TRP1-M9 or K8 peptide on overall effector function. For these experiments, CD8<sup>+</sup> T cells were cocultured with APCs and peptide for 48 hours, and then reisolated with magnetic beads, washed, and plated in fresh media containing IL2 and no further peptide stimulation. TRP1-M9– or K8-primed CD8<sup>+</sup> T cells were rested for 1 week to generate effector cells, and then restimulated with B16 melanoma cells expressing endogenous levels of TRP1 protein (Fig. 6). At every effector-to-target ratio, TRP1-M9–primed CD8<sup>+</sup> T cells expressed more IFNγ upon restimulation with B16 (Fig. 6A and B). However, K8-primed cells were more cytolytic, expressed higher levels of intracellular granzyme, and killed B16 melanoma cells more effectively than TRP1-M9–primed cells (Fig. 6A and B). In addition, K8-primed cells showed equivalent levels of PD-1, LAG3, and TIGIT expression between TRP1-M9– or K8-stimulated cells alone, suggesting that the two kinds of stimulated cells acted additively, rather than synergistically (Fig. 7A). Analysis of tumor-infiltrating TRP1<sup>high</sup> cells showed equivalent levels of PD-1, LAG3, and TIGIT expression between TRP1-M9– and K8-primed conditions (Fig. 7B). Thus, although K8 priming led to consistently low PD-1 expression in vitro, PD-1 was restored in vivo, possibly due to interactions with native TRP1 peptide displayed on H-2D<sup>B</sup> in the tumor microenvironment.

Cell-trafficking molecules, including sphingosine-1-phosphate receptor (S1pr1), were increased in K8-stimulated cells (Fig. 4B), which suggested altered localization of these cells in vivo. Mice with B16 tumors received an adoptive transfer of TRP1-M9– or K8-activated TRP1<sup>high</sup>CD45.1<sup>+</sup> cells 1 week after tumor inoculation, at a time point when tumors were approximately 50 mm<sup>3</sup>. Six days later, tumor-draining lymph nodes were isolated, and transferred cells were quantified based on their expression of the CD45.1 congenic marker. Three-fold more K8-stimulated cells
were recovered from the tumor-draining lymph nodes as compared with TRP1-M9–stimulated cells (Fig. 7C). Consistent with in vitro experiments, TRP1-M9–stimulated cells produced more IFNγ in tumor-draining lymph nodes (Fig. 7D and E). No difference in IFNγ production was observed when gating on endogenous CD8+ T cells (Fig. 7E), suggesting that the increased IFNγ was a function of prior activation with the TRP1-M9 peptide rather than alterations in the microenvironment.

Discussion

The nuances of T-cell priming matter. Here, we showed that priming with peptide ligands differing by a single amino acid can imprint disparate fates on the resulting effector CD8+ T cells. Although priming with TRP1-M9 peptide led to predominantly cytokine-producing cells, priming with K8 peptide led to effector CD8+ T cells specializing in cytolytic function. CD8+ T-cell effector subsets are less clearly defined than the well-known CD4+ T-cell subsets, yet we demonstrated not only that functionally distinct lineages can form, but also that the fate decisions made during priming can propagate over multiple cell divisions. To that point, phenotypic differences imparted during in vitro activation with TRP1-M9– versus K8-primed cells were maintained 6 days after adoptive transfer into B16 tumor–bearing mice.

How T cells mediate tumor rejection is still unclear. Although the role of CD8+ T cells in tumor control is well documented, CD8+ T cells may act through their production of IFNγ, their direct cytolysis of tumor cells, or both. Previous reports have shown that growth inhibition via IFNγ can be as important as direct cytolysis (44). Acquired resistance to checkpoint blockade in both mice and humans is frequently mediated by the loss of signaling through the IFNγ receptor in tumor cells (45, 46). In our model, we showed preferential production of IFNγ by TRP1-M9–primed cells. We hypothesized that this IFNγ contributed to the overall control of tumor growth in vivo, although we did not exclude the possibility that TRP1-M9–primed cells were also engaging in direct cytolysis. Tumor-specific CD8+ T cells, particularly those of more moderate affinity, do not always produce multiple cytokines and have high expression of granzymes (47). Loss of this polyfunctional state has been assumed to be part of T-cell exhaustion, and in some cases, previously polyfunctional CD8+ T cells lose effector capacity as a result of signaling through coinhibitory ligands, such as PD-1 or exposure to nutrient starvation or suppressive myeloid cells. A complementary hypothesis may

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be that CD8+ effector T cells become polarized to execute only one distinct effector function during priming. Here, we demonstrated, using T cells specific for a melanoma self-antigen, that fates are determined at the level of T-cell priming. TRP1<sup>high</sup> and TRP1<sup>low</sup>-T cells, by virtue of having been selected at random from a mouse during primary vaccination with a melanoma vaccine, represent physiologically achievable efficiencies of self-antigen reactive TCRs. Adoptive transfer of activated TRP1 cells alone does not cure mice with preestablished B16 melanoma (9), and attempts to use higher affinity T cells only improve responses to a certain point (10). Higher affinity priming leads to higher expression of PD-1 on activated cells, possibly explaining the paradox that vaccination with high-affinity peptides stimulates robust CD8+ T-cell responses but impairs tumor control compared with vaccination with lower affinity peptides (48). PD-1 may also play a role in restraining cytolytic capacity of newly primed effector cells. In mice acutely infected with lymphocytic choriomeningitis virus Armstrong, early blockade of PD-1 led to increased granzyme B expression in virus-specific CD8+ T cells and increased viral clearance (49). Here, we found that K8-primed T cells, which do not acutely upregulate PD-1, similarly showed increased granzyme B expression and increased cytolytic capacity in the effector phase.

T-cell priming is influenced by multiple factors, including TCR affinity, but also contact time, the number and density of cognate MHC–peptide complexes, costimulation, and the local cytokine milieu. We used in vitro priming with equivalently activated APCs to investigate the role of the particular pMHC-TCR dynamics on T-cell priming. This three-molecule interaction was controlled on the TCR side by showing that two structurally distinct TCRs recognizing K8-H-2Db<sup>+</sup> complexes led to the same lower PD-1 expression and skewed toward cytotoxicity. We, therefore, focused our attention on the pMHC interaction and discovered that K8 forms unusually unstable complexes with H-2Db.<sup>6</sup>

Our results further showed the requirement of the entire peptide, rather than only the MHC anchor positions, in determining T-cell recognition and function. Although it has been established that peptide binding to the MHC is an important factor for antigenicity (12, 50) and that the interplay between affinities of neoantigens and unmutated self-antigens can affect antitumor responses (24, 25), our data suggested that even relatively subtle peptide alterations that do not obviously affect MHC anchor positions may affect immune response and effector phenotypes. These effects have been previously observed for both class I and class II MHCs (51, 52). Although individual point mutants did have large effects in isolation, the “TCR contact epitope” as a whole made significant contact to the MHC and could, in aggregate, affect peptide presentation and the resulting T-cell responses. A previous report of influenza peptide binding to H-2D<sup>b</sup> examined MHC binding affinities upon single amino acid substitutions at all nonanchor positions and found that mutations to the TCR contact positions, such as position 8, had relatively modest, yet potentially impactful influences, on peptide stability (53, 54). The mutation we study here (Tyr to Lys in position 8) caused a 10-fold increase in IC<sub>50</sub>. Factors beyond changes to pMHC stability, including the overall rigidity of the pMHC complex, have also been observed and may potentially have similar effects, as we observed between TRP1-M9 and K8 (19, 55, 56). Collectively, these data showed the need to better understand and design peptide targets for immunotherapies because simply maximizing either peptide affinity for MHC or TCR effector response may not lead to a maximally effective antitumor response.

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

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1534 Cancer Immunol Res; 6(12) December 2018

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Altered Binding of Tumor Antigenic Peptides to MHC Class I Affects CD8+ T Cell–Effector Responses

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