This information is current as of September 24, 2021.

A Modified Tyrosinase-Related Protein 2 Epitope Generates High-Affinity Tumor-Specific T Cells but Does Not Mediate Therapeutic Efficacy in an Intradermal Tumor Model

Jennifer A. McWilliams, Sean M. McGurran, Steven W. Dow, Jill E. Slansky and Ross M. Kedl

J Immunol 2006; 177:155-161; ;
doi: 10.4049/jimmunol.177.1.155
http://www.jimmunol.org/content/177/1/155

References This article **cites 51 articles**, 28 of which you can access for free at:
http://www.jimmunol.org/content/177/1/155.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2006 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

A Modified Tyrosinase-Related Protein 2 Epitope Generates High-Affinity Tumor-Specific T Cells but Does Not Mediate Therapeutic Efficacy in an Intradermal Tumor Model[1]

Jennifer A. McWilliams,[‡] Sean M. McGurran,* Steven W. Dow,[†] Jill E. Slansky,[‡] and Ross M. Kedl[2‡]

The generation of tumor-specific T cells is hampered by the presentation of poorly immunogenic tumor-specific epitopes by the tumor. Here, we demonstrate that, although CD8$^+$ T cells specific for the self/tumor Ag tyrosinase-related protein 2 (TRP2) are readily detected in tumor-bearing hosts, vaccination of either tumor-bearing or naive mice with an epitope derived from TRP2 fails to generate significant numbers of tetramer-staining TRP2-specific T cells or antitumor immunity. We identified an altered peptide epitope, called deltaV, which elicits T cell responses that are cross-reactive to the wild-type TRP2 epitope. Immunization with deltaV generates T cells with increased affinity for TRP2 compared with immunization with the wild-type TRP2 epitope, although TRP2 immunization often generates a greater number of TRP2-specific T cells based on intracellular IFN-γ analysis. Despite generating higher affinity responses, deltaV immunization alone fails to provide any greater therapeutic efficacy against tumor growth than TRP2 immunization. This lack of tumor protection is most likely a result of both the deletion of high affinity and functional tolerance induction of lower affinity TRP2-specific T cells. Our data contribute to a growing literature demonstrating the ability of variant peptide epitopes to generate higher affinity T cell responses against tumor-specific Ags. However, consistent with most clinical data, simple generation of higher affinity T cells is insufficient to mediate tumor immunity. ***The Journal of Immunology,*** **2006, 177: 155–161.**

It is now abundantly clear that T cell responses play a major role in the immunologic rejection of cancer. In the past, experimental models designed to study tumor responses usually used a "model tumor Ag," a foreign Ag or epitope ectopically expressed in an experimental tumor cell line that contained identified T cell epitopes (1–8). However, there are now many true tumor Ags and their respective class I binding epitopes are known (9–12), which, in most cases, are normal proteins expressed within a peripheral tissue. Examples of these differentiation Ags are the many melanocyte-specific Ags, such as gp100, tyrosinase related protein-1 (TRP1),[3] TRP2 (also known as DCT), tyrosinase and the MAGE Ags (10). Many of these tumor-associated Ags serve as tumor rejection Ags against melanoma despite that fact that they appear to be neither mutated nor aberrantly expressed within the tumor (13–26). T cell responses directed against these Ags are therefore, by definition, self-Ag-specific T cells. As such, the repertoire of T cells specific for these self-Ags is likely to be different than the repertoire of T cells directed against a foreign Ag. For example, T cells specific for a self-Ag may be of too low affinity, in order to escape central and/or peripheral tolerance, to be functionally capable of tumor recognition and destruction (27–30).

In conjunction with the potential effects of central tolerance on the T cell repertoire, tumor-specific T cells may also be difficult to generate because many tumor-specific epitopes are poorly immunogenic. In some cases, this is due to a low affinity of the peptide epitope for binding to the class I MHC (17, 28, 31–34). In these cases, when the MHC binding residues of the epitope are optimized, the resulting modified epitope can restore T cell responses specific for the original wild-type (wt) epitope. In other cases, modification of the wt tumor epitope does not result in enhanced MHC binding but rather in enhanced T cell recognition, presumably by altering the three-dimensional structure of the peptide/MHC to facilitate a more effective interaction with the TCR (35). In either case, immunization using substituted peptide epitopes often generates better antitumor immunity than immunization with the wt epitope. As a result, the identification of new altered peptide epitopes for tumor-specific Ags is a priority in the area of cancer vaccine development.

Using the B16 melanoma cell line and class I MHC tetrameric reagents, we have analyzed the CD8$^+$ T cell response specific for the melanocyte differentiation Ag TRP2. Surprisingly, a population of TRP2-specific T cells was readily detected in tumor-bearing mice. Whereas immunization with the TRP2 peptide only generated low-affinity TRP2-specific T cells, a peptide variant of the TRP2 epitope, called deltaV, induced high-affinity TRP2-specific T cell responses in both naive and tumor-bearing mice. Despite this generation of high-affinity TRP2-specific T cells, deltaV-peptide immunization failed to significantly augment tumor immunity above that observed with TRP2-peptide immunization, the implications of which are discussed below.

*3M Pharmaceuticals, Department of Pharmacology, St. Paul, MN 55144; [†]Departments of Clinical Sciences and Pathology, Colorado State University, Fort Collins, CO 80523; and [‡]Integrated Department of Immunology, University of Colorado, Denver, CO 80206

Received for publication April 7, 2005. Accepted for publication April 17, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by a Seed Grant from the American Cancer Society and the University of Colorado Cancer Center.

[2] Address correspondence and reprint requests to Dr. Ross M. Kedl, Integrated Department of Immunology, University of Colorado Health Science Center, National Jewish Medical and Research Center Goodman K825, 1400 Jackson Street, Denver, CO 80206. E-mail address: ross.kedl@uchsc.edu

[3] Abbreviations used in this paper: TRP1, tyrosinase-related protein 1; i.d., intradermal(ly); wt, wild type.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

Materials and Methods

Tumor cell lines and mouse injections

C57BL/6J (B6) female mice from Charles River or The Jackson Laboratory were anesthetized with Avertin, their rear flanks were shaved, and they were injected with 1×10^5 B16-F10 tumor cells intradermally (i.d.). Mice were monitored for survival over the next 25–100 days.

Peptide immunizations

Peptides for the wt TRP2 epitope (SVYDFFVWL), deltaD (SVYIFFVWL), deltaY (SVIDFFVWL), deltaV (SIYDFFVWL), and OVA (SIINFEKL) were synthesized by the Molecular Biology Core facility at National Jewish Medical Research Center (Denver, CO). Naive or tumor-bearing mice were immunized with 50 μg of anti-CD40 Ab (FGK4.5 or 1C10), 100 μg of the TLR3 agonist poly(I:C), or 150 μg of the TLR7 agonist S-27609 (36, 37), and 100 μg of peptide as previously described (37). In some cases, mice were boosted 14 days after primary immunization with the TRP2 peptide, anti-CD40 and poly(I:C).

Cell preparation, tetramers, and cell staining

Five to 7 days after immunization, tissues were removed and processed for tetramer staining and/or intracellular IFN-γ production as previously described (37). Alternatively, peripheral blood was taken by tail vein bleed at various times after immunization and/or tumor challenge. Cells isolated from draining nodes (periaortic, inguinal, axillary, and brachial), spleen, tumor, or peripheral blood were homogenized into single-cell suspensions. In the case of spleen, tumor, and blood, the RBC were lysed by brief treatment with ammonium chloride buffer followed by washing with balanced salt solution buffer. All cells were finally suspended in complete MEM, and total cell numbers were counted using a Coulter Counter.

Anti-CD8-allophycocyanin, CD44-FITC, and B220-PerCP were all purchased from BD Pharmingen. K^b tetramer production was performed as previously described (38). A 5–10 M excess of SIINFEKL peptide (OVA residues 257–264, negative control peptide) or TRP2 peptide SVYDFFVWL (residues 180–188) were added directly to newly constructed K^b-SA-PE tetramer for at least 30 min at 4°C. Tetramer staining was performed as previously described (37, 38).

For intracellular IFN-γ staining, cells were treated with Golgiplug (BD Pharmingen) (brefeldin A) in the presence or absence of peptide Ag for 4–6 h in complete medium at 37°C. For peptide dose responses (see Fig. 3), spleen cells were isolated from mice immunized with either TRP2 or deltaV as described above and were incubated for 4 h in vitro in the presence of Golgiplug and decreasing amounts of TRP2 peptide. The cells were then stained for CD8, washed, fixed, permeabilized, and stained for the presence of intracellular and IFN-γ according to the BD Biosciences protocol. Four-color FACS data were collected on a BD Biosciences FACSCalibur flow cytometer and analyzed using CellQuest software.

Class I MHC stabilization assay

Class I H-2K^b stabilization assays were performed as previously described (17) using the TAP-deficient RMAS cell line. Briefly, RMAS cells were incubated at room temperature with the indicated concentrations of peptide for 4–6 h (39) and then returned to 37°C for 4–6 h. The cells were then stained with anti-K^b-PE Ab (BD Pharmingen) and analyzed by FACS. For analysis of the half-life of peptide MHC binding, RMAS cells were incubated with 50 μg/ml peptide at room temperature for 4 h. The cells were then washed extensively, resuspended in complete medium, and incubated at 37°C. At various time points, representative cells were removed, stained for K^b as described above, and fixed in paraformaldehyde. Cells from all time points were then analyzed for cell surface expression of K^b.

Results

TRP2-specific T cells respond to B16-F10 but are tolerant

The TRP2 protein is an enzyme in the melanin synthesis pathway and is expressed in the melanoma cell line B16-F10 (B16) (9, 15, 40). Mice were challenged with B16 i.d., and at various times thereafter, cells were taken from lymphoid and tumor tissue and stained with an MHC tetrameric reagent to detect K^b/TRP2-specific T cells. Interestingly, TRP2-specific T cells were detectable within the tumor 15–18 days after tumor challenge (Fig. 1). Consistent with the growth of the tumor, these TRP2-specific T cells did not demonstrate any lytic activity or ability to produce IFN-γ in response to antigenic stimulation (data not shown). Thus, in the absence of any other intervention, the growth of the B16 melanoma appears to stimulate the expansion of a small population of short lived, hyporesponsive TRP2-specific T cells.

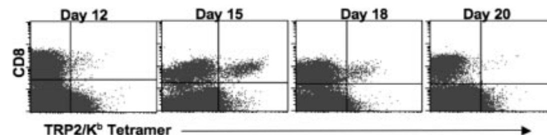

FIGURE 1. TRP2-specific T cells are detectable within the tumor of B16-F10-bearing mice. B6 mice were shaved and challenged in the flank with 1×10^5 B16-F10 cells i.d. At the time points indicated after tumor challenge, the mice were sacrificed, and the tumors were removed and assessed for the presence of tetramer-staining cells as described in *Materials and Methods*. The dot plots shown were gated on all live, $B220^-$ events. The quadrants were set by staining the sample with control tetramer containing an irrelevant peptide (data not shown). These data are representative of four separate experiments performed.

The altered TRP2 epitope, deltaV, elicits TRP2-specific T cells

Because TRP2-specific T cells spontaneously expanded in tumor-bearing hosts, we reasoned that immunization of the tumor-bearing host with the TRP2 peptide Ag might result in greater expansion, function, and/or survival of these T cells. Using an immunization strategy that we recently described (37), we immunized mice with the TRP2 peptide Ag and determined the degree of TRP2-specific T cell expansion by TRP2 tetramer and intracellular IFN-γ staining. Whereas TRP2 tetramer-staining cells were rarely observed (Fig. 2*A*), a significant population of TRP2-specific T cells (~10–20% of total $CD8^+$ T cells) was identifiable by intracellular IFN-γ staining (Fig. 2*B*). These data suggested that the T cell response following TRP2 peptide immunization may be of too low affinity to be identified by tetramer staining (28, 41–43).

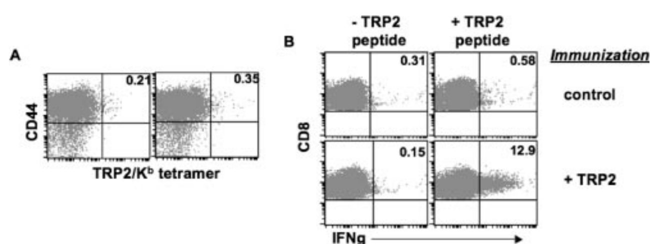

FIGURE 2. Immunization of tumor-bearing mice with TRP2 peptide generates TRP2-specific T cells detectable by intracellular cytokine staining, but not tetramer staining. B6 mice were challenged with B16-F10 and 7 days later were immunized i.p. with TRP2 peptide, the TLR7 agonist S-27609, and anti-CD40 Ab (FGK45) as described in *Materials and Methods*. Seven days after immunization (14 days after tumor challenge), lymph nodes, spleen, tumor, and peripheral blood were removed, and the cells were stained with TRP2 tetramer or intracellular cytokine staining for IFN-γ. The plots in *A* and *B* are from spleen cells and are representative of cells from blood and tumor. *A*, Spleen cells from two separate animals were stained with TRP2 tetramer as described in Fig. 1 with the addition of CD44 staining as an activation marker (activated/memory T cells express high levels of CD44). The dot plots are gated on live, $B220^-$ events, and the quadrants set based on staining with control tetramer (data not shown). *B*, Spleen cells were incubated with brefeldin A only (−TRP2 peptide) or with brefeldin A and 5 μg/ml TRP2 peptide (+TRP2 peptide) for 4 h at 37°C. The cells were then stained for surface expression of CD8 and washed, fixed, permeabilized, and stained for intracellular IFN-γ as described in *Materials and Methods*. The dot plots shown are gated on $CD8^+$ events. The data are representative of three separate experiments performed.

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

We speculated that the inability of TRP2 immunization to generate T cells detectable by tetramer staining might be because the TRP2 epitope was suboptimal with respect to its ability to bind MHC, its ability to facilitate TCR interaction with the peptide/MHC complex, or both. Previous data from Chen et al. (44) demonstrated the importance of the no. 2 position of the epitope, in the context of K^b, in facilitating T cell recognition of the complex. We therefore created a number of peptide variants of the TRP2 epitope at or around the no. 2 position (Table I). Using these peptides, we immunized naive B6 mice and stained the resulting spleen cells with the TRP2 tetramer to determine which peptide generated T cells that were cross-reactive with the original wt peptide. The deltaD and deltaY peptides were found to be poor at generating TRP2-specific T cells (Fig. 3*A*). However, the deltaV peptide elicited a T cell response cross-reactive with the wt TRP2 epitope as demonstrated by binding to the respective tetramers (Fig. 3*A*). These cells produced IFN-γ and TNF-α upon Ag stimulation with the TRP2 epitope (Fig. 3*B*), demonstrating that they were functionally responsive to the TRP2 epitope as well. Interestingly, based on the intracellular IFN-γ staining, deltaV immunization actually generated fewer TRP2-specific T cells (Fig. 3*B*).

deltaV immunization elicits high-affinity TRP2-specific T cells

Using either K^b- or D^b-expressing 3T3 cell lines, we observed that the $CD8^+$ T cells from TRP2-immunized mice did not recognize TRP2 peptide in the context of H-$2D^b$ (data not shown), ruling out the possibility that the lack of TRP2/K^b tetramer staining was due to their specificity for TRP2/D^b. Therefore, our observations were consistent with the prediction that deltaV immunization generated T cells with higher affinity for TRP2/K^b than did immunization with TRP2. We therefore tested the relative affinity of the TRP2-specific T cells responding to TRP2 or deltaV immunization. Cells from mice immunized with either peptide were incubated with decreasing amounts of TRP2 peptide in the presence of brefeldin A and stained for their production of intracellular IFN-γ. Although the majority of the dose-response curve was essentially the same between cells derived from mice immunized with either peptide (Fig. 4*A*), the response of cells derived from the TRP2-immunized mice fell to essentially zero at the lower end of the peptide titration, whereas at least 20% of the cells derived from deltaV-immunized mice continued to produce IFN-γ (Fig. 4*B*). Our interpretation from this representative experiment is that the polyclonal response to deltaV immunization contains a small subset of high-affinity TRP2-specific T cells compared with the polyclonal response generated by TRP2 immunization. We have repeated these experiments with bulk T cell cultures, derived from immunization with either TRP2 or deltaV, and obtained similar results (Fig. 4*C*). This further suggests that the difference in response at the low end of the dose-response curve (between cells derived from TRP2 and deltaV immunization) is real, because it can be propagated in vitro. Interestingly, the response of the cells from the deltaV-immunized mice was slightly lower throughout most of the curve, suggesting that TRP2 immunization generates a greater number of intermediate-affinity TRP2-specific T cells than deltaV immunization. Thus, the data demonstrate that TRP2 immunization generates a high number of lower affinity T cells, whereas deltaV immunization generates a smaller number of higher-affinity TRP2-specific cells.

Table I. *Modified peptide epitope key*

	Epitope
wt TRP2	SVYDFFVWL
deltaD	SVYIFFVWL
deltaV	SIYDFFVWL
deltaY	SVIDFFVWL

deltaV and TRP2 have similar affinities for class I K^b

We concluded from these studies that the deltaV epitope generated higher affinity TRP2 T cells from the host either because the deltaV peptide bound H-$2K^b$ better or because it facilitated a stronger TCR/peptide/MHC interaction than did the wt TRP2 peptide. We therefore performed assays to determine the relative affinity of either peptide for binding to H-$2K^b$ (Fig. 5*A*) and the relative half-life of peptide MHC binding (Fig. 5*B*). The deltaV epitope had, at best, a 2- to 3-fold higher affinity for H-$2K^b$ (Fig. 5*A*), but still had at least a log lower affinity for class I than the dominant H-$2K^b$ binding epitope from OVA, SIINFEKL. Surprisingly, the TRP2 peptide, once bound to K^b, demonstrated a similar half-life of binding than the deltaV or ova peptide (Fig. 5*B*). It is possible to rule out the possibility that the small increase in MHC binding favorably contributes to the response to deltaV. However, it is unlikely that differences in MHC binding, especially when one considers the results on the half-life of peptide/MHC interaction, contributes significantly to the ability of the deltaV peptide to generate a higher affinity TRP2-specific T cell response than the TRP2 peptide. This observation actually fits well with the prediction of Chen et al. (44) that changes at the no. 2 position influence T cell recognition without influencing MHC binding, and is consistent with observations made of altered peptide tumor epitopes in other model systems (35).

deltaV immunization alone does not enhance tumor immunity

We next tested whether deltaV immunization augmented the therapeutic efficacy relative to TRP2 immunization in tumor-bearing mice. Mice were immunized with either TRP2 or deltaV 7 days after tumor challenge and boosted with the TRP2 epitope at 21 days after tumor challenge (14 days after primary immunization). The magnitude of the TRP2-specific T cell response, determined by tetramer or IFN-γ staining of cells isolated from peripheral blood at various time points, and the survival of the mice were monitored. Consistent with previous results, TRP2-specific T cells were detectable by tetramer staining only in mice immunized with deltaV but not in mice immunized with TRP2 (Fig. 6*A*). Surprisingly, despite this difference in TRP2 tetramer-staining T cells, deltaV-immunized mice did not demonstrate any increase in survival over TRP2-immunized mice (Fig. 6*B*). Further assessment of the TRP2-specific T cells from the immunized mice revealed that the T cells from deltaV-immunized mice were able to directly recognize the tumor, whereas T cells from TRP2-immunized mice did not (Fig. 6*C*) (45–47). This further supports the conclusion that deltaV immunization promotes higher affinity TRP2-specific T cells, but does not explain why immunization with deltaV peptide does not promote greater antitumor immunity.

We next investigated whether the TRP2-specific T cells retained their ability to make IFN-γ over time. Consistent with previous results, TRP2-specific IFN-γ production was observable in a significant population of $CD8^+$ T cells 7 days after primary immunization with either the TRP2 or deltaV epitopes, although, again, more so with TRP2 immunization (Fig. 6*D*, primary). However, after boosting the mice with the TRP2 epitope, little TRP2-specific tetramer staining or IFN-γ production was observed in mice previously immunized with either epitope (Fig. 6, *A* and *D*, secondary). Therefore, both peripheral deletion of the high-affinity cells (based on the loss of tetramer staining; Fig. 6*A*) as well as peripheral tolerance of the low-affinity cells (based on loss of detectable intracellular IFN-γ production; Fig. 6*C*) are actively subduing the

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

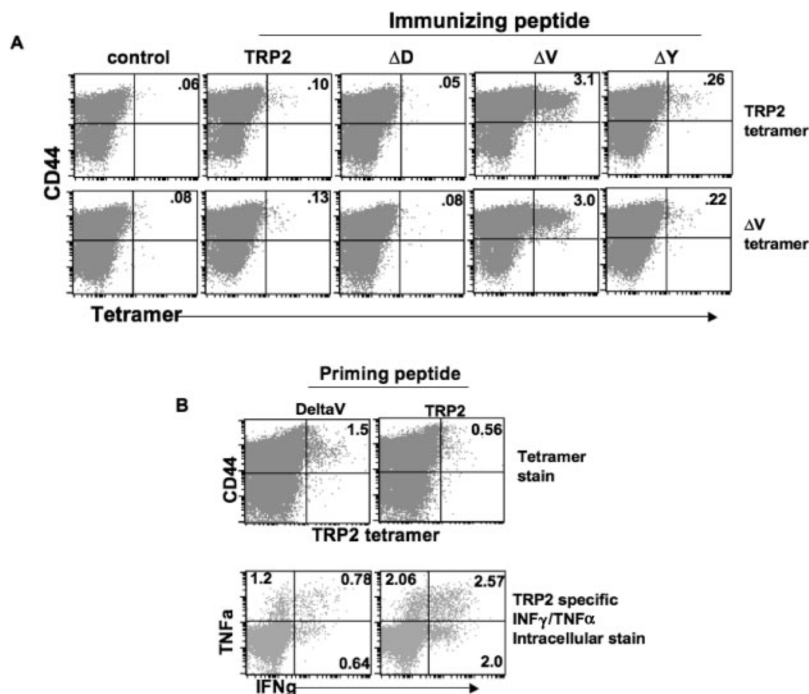

FIGURE 3. Immunization with the deltaV variant peptide elicits TRP2-specific T cells. A, Mice were immunized with the TRP2, deltaY, deltaD, or deltaV peptide in the context of anti-CD40 and poly(I:C) as described in *Materials and Methods*. Seven days later, spleen cells were removed and analyzed for either TRP2 (*top row*) or deltaV (*bottom row*) tetramer staining. The cells were stained and analyzed as in Fig. 2. *B*, Mice were immunized as in *A* with either TRP2 or deltaV peptide. Seven days later, spleen cells were removed and analyzed for either TRP2 tetramer (*top row*) or deltaV-loaded tetramer staining (*bottom row*).

TRP2-specific T cell response elicited from either deltaV or TRP2 immunization. It is currently unclear what is responsible for this peripheral deletion/tolerance. We have also observed the same phenomenon in non-tumor-bearing mice (data not shown), suggesting that self-tissue (skin or eye) expression of the Ag likely plays a significant role in enforcing peripheral tolerance on the TRP2-specific T cell response.

Discussion

In the studies presented here, we have described the identification of a novel modified peptide epitope for the TRP2 tumor Ag that is capable of inducing high-affinity TRP2-specific T cell responses. Analysis of peptides containing amino acid substitutions in the TRP2 epitope identified a single variant, termed deltaV, which is capable of eliciting high-affinity T cell responses in both naive and

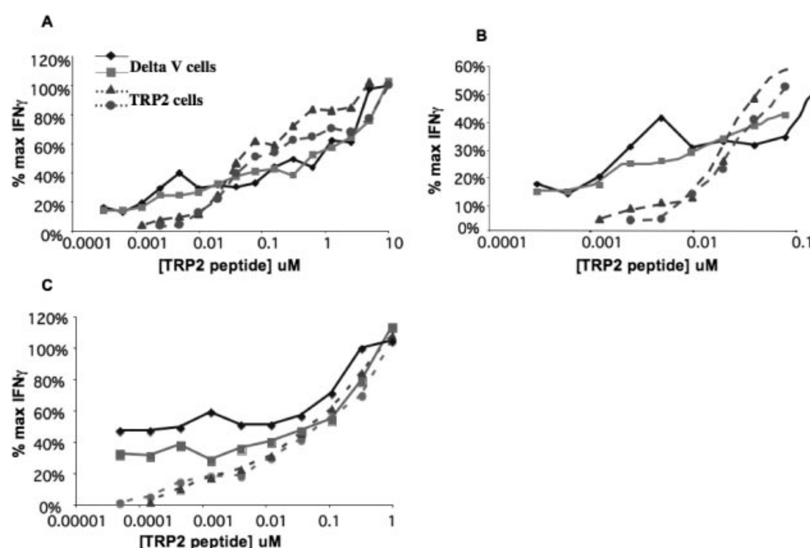

FIGURE 4. TRP2-specific T cells from deltaV-immunized mice have higher affinity than cells from TRP2-immunized mice. Mice were immunized with either TRP2 or deltaV peptide as described in Fig. 3. Seven days later, spleen cells were removed and incubated with the indicated concentrations of TRP2 peptide in the presence of brefeldin A for 4 h at 37°C. The cells were then stained for CD8 and intracellular IFN-γ as described in Fig. 2 and *Materials and Methods*. The percentage of $CD8^+$ T cells expressing IFN-γ was calculated by FACS analysis. These percentages were normalized against the percentage expressing IFN-γ at the highest peptide dose from each immunized mouse; the data are presented as the percent maximal IFN-γ production at a given peptide dose. Two mice per peptide immunization are shown (♦ and ■ for deltaV, ● and ▲ for TRP2-immunized mice) and are representative of three experiments performed. The data shown in *B* are the same as in *A* but focused in on the lower end of the curve to more easily observe the differences between the cells derived from the two immunizations. In *C*, the cells were first expanded in vitro with TRP2 peptide for 1 wk followed by a TRP2 peptide dose response, as described above for *A* and *B*, on the bulk TRP2-specific T cell culture.

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

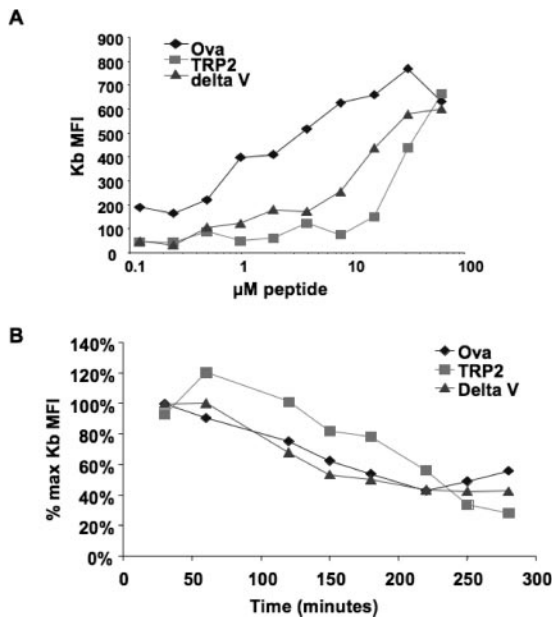

FIGURE 5. deltaV and TRP2 have relatively similar affinities, and half-lives, for binding to class I K^b. *A*, RMAS cells (TAP deficient) were incubated with the indicated concentrations of TRP2, deltaV, or OVA peptide (SIINFEKL) at room temperature for 4 h. The cells were then placed at 37°C for 2 h and stained for the surface expression of H-2K^b. The presence of K^b on the cell surface at the given peptide concentration is an indication of the degree to which the peptide is able to bind to the MHC and therefore maintain its retention on the cell surface. The data shown are representative of eight separate experiments performed. *B*, RMAS cells were incubated with the indicated peptides (50 μg/ml) at room temperature for 4 h. The cells were then washed and incubated in complete medium at 37°C. At the times indicated in the *x*-axis, cells were removed, stained for K^b as in *A*, and analyzed by FACS for the degree of K^b on the cell surface. The data are expressed as the percentage of maximum K^b mean fluorescence intensity (as determined at the 0 time point) for the cells at the given time point.

tumor-bearing hosts. Importantly, deltaV-initiated responses are cross-reactive with the wt TRP2 epitope and are even of comparable affinity to T cells generated against the completely foreign OVA epitope SIINFEKL (data not shown).

Our data suggest that immunization with the deltaV peptide elicits a T cell response independent of its properties of MHC binding. Peptides with poor affinity for MHC are poor at stimulating T cell responses (17, 28, 31–33, 48, 49). In these cases, the ability of the epitope to elicit a T cell response, and therefore mediate antitumor effects, can be elevated by optimizing the MHC binding residues of the peptide (17, 28, 33). However, work by Chen et al. (44) suggested that certain epitope modifications could result in enhanced TCR recognition without affecting peptide/MHC binding. They noted that, for H-2K^b binding epitopes, the residue in the P2 position could effect TCR recognition but have little effect on overall binding to MHC. In a tumor model system using H-2L^d rather than -K^b, Slansky et al. (35) similarly demonstrated that tumor epitopes can be modified to induce T cell responses without affecting the peptide's overall affinity for the class I. They showed that the peptide/MHC complex containing a variant epitope had an increased affinity for binding to the TCR compared with the wt peptide/MHC complex, but that both wt and variant epitopes had equivalent MHC binding affinities. The data we have presented for the deltaV peptide suggest that it is another example of a peptide modification that facilitates TCR/MHC/peptide interactions rather than significantly affecting the peptide's binding to the MHC. We have begun investigating the possibility that modifications of residues at the P2 position could be a general way of enhancing tumor epitope immunization, at least for H-2K^b binding epitopes, but as yet the results are inconclusive.

T cells responding to a self-Ag can have lower affinity for the Ag than those responding to foreign Ags (28, 29, 31, 32). This is at least partly due to the deletion of high-affinity, self-reactive cells by central tolerance. Our data indicate that high-affinity TRP2-specific T cells must escape central tolerance. However, their activation and expansion in the periphery seem to require stimulation with the deltaV epitope. In contrast, stimulation with the wt TRP2 epitope appears to only induce the expansion of lower affinity TRP2-specific T cells. This observation is somewhat paradoxical given that challenge with the B16 tumor readily generates TRP2 tetramer-staining cells during the natural process of tumor growth with no intervention whatsoever (see Fig. 1). Indeed, immunization of a tumor-bearing host with the TRP2 peptide usually results in the loss of TRP2 tetramer-staining T cells from within the tumor, even at the time points that nonimmunized, tumor-bearing mice demonstrate TRP2 tetramer staining T cells (data not shown). This suggests that TRP2 immunization of tumor-bearing mice may lead to the deletion or inhibition of the high-affinity TRP2 cells that are normally in the tumor. The reason immunization with the deltaV epitope is able to generate tetramer staining cells is unclear but suggests that this modified epitope either delivers a qualitatively different signal to TRP2-specific T cells, thereby avoiding their deletion, or raises a different population of tetramer-staining T cells from that seen responding to tumor challenge alone.

Of greatest interest in these studies is the observation that immunization with the deltaV epitope, despite generating higher affinity TRP2-specific T cells, fails to significantly augment tumor immunity in this i.d. tumor model relative to immunization with the TRP2 epitope. As mentioned above, productive tumor immunity suffers from a paucity of high-affinity tumor-specific T cells able to recognize weakly presented Ags by the tumor (11, 50). The corollary to this prediction is that the generation of higher and higher affinity T cells should have an increasingly positive effect on promoting tumor immunity; i.e., the efficacy of a tumor vaccine to enhance tumor immunity increases in direct proportion to the affinity of T cells it generates. Our data demonstrate that this corollary may not always hold true and predicts that increasing affinity may become a liability for the tumor-specific T cells, possibly due to the T cells' increased recognition of Ag within normal peripheral tissues. If true, then some tumor vaccines may fail because they generate T cells of either too low or too high affinity. A successful vaccine may follow a "Goldilocks principle"; much like the porridge, the affinity of tumor-specific T cells must be just right. The accuracy of this prediction for clinical application is necessary to confirm or deny before we assume that the success of a tumor vaccine depends on the production of the T cells with the highest affinity possible.

The identification of modified tumor-associated antigenic epitopes is an important, if not crucial, aspect of generating therapeutic antitumor T cell responses. Our data contribute to a growing list of modified tumor Ag epitopes able to augment the production of tumor-specific T cells. However, our results demonstrate that the initiation of a high-affinity tumor-specific T cell response is alone ineffective at mediating therapeutic efficacy against tumor growth. Identifying the mechanisms of peripheral tolerance and deletion that limit tumor-specific responses becomes more important as our capability of generating higher affinity T cell responses increases. It is interesting to note that the TRP2 epitope we have used in these studies is the same for HLA-A2 binding as it is for K^b binding (51). The possibility that the deltaV

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

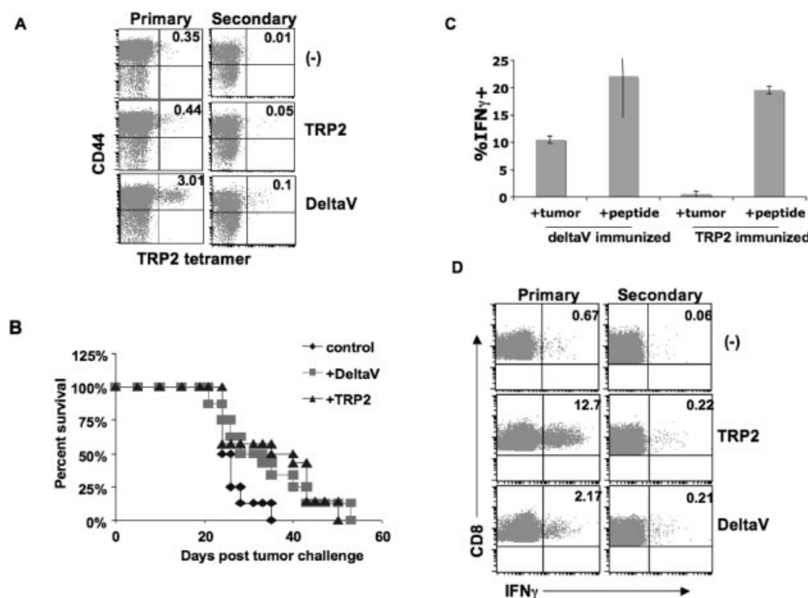

FIGURE 6. Despite the initial generation of high-affinity TRP2-specific T cells, deltaV immunization does not promote increased survival, of either the host or the T cells, against tumor challenge compared with TRP2 immunization. B6 mice were challenged i.d. with 1×10^5 B16-F10 cells in the flank. Seven days later, the mice were immunized s.c. with poly(I:C) and anti-CD40 alone or in combination with either TRP2 or deltaV peptide. Fourteen days later, the mice were boosted with poly(I:C), anti-CD40, and TRP2 peptide. Peripheral blood was taken by tail vein 7 days after the primary immunization and 5 days after the secondary immunization, and the cells were analyzed for TRP2 tetramer staining (*A*), tumor-free survival (*B*), and intracellular IFN-γ staining (*D*) as described in *Materials and Methods*. The data shown are representative of five experiments performed. To examine direct tumor recognition (*C*), spleen cells were removed 7 days after immunization and the cells were expanded in vitro for 7 days by restimulation with TRP2 peptide. The cells were then washed and incubated in the presence of brefeldin A and B16 tumor cells without (+tumor) and with (+peptide) TRP2 peptide. Because B16-F10 expresses little to no class I in vitro (45–47), the tumor was incubated overnight with 10 U/ml rIFN-γ (PeproTech) to induce detectable levels of class I expression (45–47). Intracellular IFN-γ expression in the CD8$^+$ T cells was determined by FACS analysis as described in *Materials and Methods* and is expressed as percentage of IFN-γ^+ cells of the total cells in culture. The data shown are representative of two independent experiments performed.

modification may be also beneficial as a modified tumor epitope for human T cell responses is an intriguing possibility and is currently under investigation.

Disclosures

The authors have no financial conflict of interest.

References

1. Diehl, L., A. T. den Boer, S. P. Schoenberger, E. I. van der Voort, T. N. Schumacher, C. J. Melief, R. Offringa, and R. E. Toes. 1999. CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat. Med.* 5: 774–779.
2. Marzo, A. L., R. A. Lake, B. W. Robinson, and B. Scott. 1999. T-cell receptor transgenic analysis of tumor-specific CD8 and CD4 responses in the eradication of solid tumors. *Cancer Res.* 59: 1071–1079.
3. Morgan, D. J., H. T. Kreuwel, S. Fleck, H. I. Levitsky, D. M. Pardoll, and L. A. Sherman. 1998. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol.* 160: 643–651.
4. Prevost-Blondel, A., C. Zimmermann, C. Stemmer, P. Kulmburg, F. M. Rosenthal, and H. Pircher. 1998. Tumor-infiltrating lymphocytes exhibiting high ex vivo cytolytic activity fail to prevent murine melanoma tumor growth in vivo. *J. Immunol.* 161: 2187–2194.
5. Shrikant, P., and M. F. Mescher. 1999. Control of syngeneic tumor growth by activation of CD8$^+$ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. *J. Immunol.* 162: 2858–2866.
6. Sotomayor, E. M., I. Borrello, E. Tubb, F. M. Rattis, H. Bien, Z. Lu, S. Fein, S. Schoenberger, and H. I. Levitsky. 1999. Conversion of tumor-specific CD4$^+$ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat. Med.* 5: 780–787.
7. Bachmann, M. F., T. M. Kundig, G. Freer, Y. Li, C. Y. Kang, D. H. Bishop, H. Hengartner, and R. M. Zinkernagel. 1994. Induction of protective cytotoxic T cells with viral proteins. *Eur. J. Immunol.* 24: 2228–2236.
8. Kedl, R. M., M. Jordan, T. Potter, J. Kappler, P. Marrack, and S. Dow. 2001. CD40 stimulation accelerates deletion of tumor-specific CD8$^+$ T cells in the absence of tumor-antigen vaccination. *Proc. Natl. Acad. Sci. USA* 98: 10811–10816.
9. Wang, R. F., E. Appella, Y. Kawakami, X. Kang, and S. A. Rosenberg. 1996. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *J. Exp. Med.* 184: 2207–2216.
10. Rosenberg, S. A. 2000. Identification of cancer antigens: impact on development of cancer immunotherapies. *Cancer J.* 6(Suppl. 3): S200–S207.
11. Restifo, N. P., and S. A. Rosenberg. 1999. Developing recombinant and synthetic vaccines for the treatment of melanoma. *Curr. Opin. Oncol.* 11: 50–57.
12. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.* 74: 181–273.
13. Gattoni-Celli, S., and D. J. Cole. 1996. Melanoma-associated tumor antigens and their clinical relevance to immunotherapy. *Semin. Oncol.* 23: 754–758.
14. Mazzocchi, A., W. J. Storkus, C. Traversari, P. Tarsini, M. J. Maeurer, L. Rivoltini, C. Vegetti, F. Belli, A. Anichini, G. Parmiani, and C. Castelli. 1996. Multiple melanoma-associated epitopes recognized by HLA-A3-restricted CTLs and shared by melanomas but not melanocytes. *J. Immunol.* 157: 3030–3038.
15. Bloom, M. B., D. Perry-Lalley, P. F. Robbins, Y. Li, M. el-Gamil, S. A. Rosenberg, and J. C. Yang. 1997. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. *J. Exp. Med.* 185: 453–459.
16. Overwijk, W. W., D. S. Lee, D. R. Surman, K. R. Irvine, C. E. Touloukian, C. C. Chan, M. W. Carroll, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1999. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4$^+$ T lymphocytes. *Proc. Natl. Acad. Sci. USA* 96: 2982–2987.
17. Overwijk, W. W., A. Tsung, K. R. Irvine, M. R. Parkhurst, T. J. Goletz, K. Tsung, M. W. Carroll, C. Liu, B. Moss, S. A. Rosenberg, and N. P. Restifo. 1998. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med.* 188: 277–286.
18. van Elsas, A., R. P. Sutmuller, A. A. Hurwitz, J. Ziskin, J. Villasenor, J. P. Medema, W. W. Overwijk, N. P. Restifo, C. J. Melief, R. Offringa, and J. P. Allison. 2001. Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. *J. Exp. Med.* 194: 481–489.
19. Xiang, R., H. N. Lode, T. H. Chao, J. M. Ruehlmann, C. S. Dolman, F. Rodriguez, J. L. Whitton, W. W. Overwijk, N. P. Restifo, and R. A. Reisfeld. 2000. An autologous oral DNA vaccine protects against murine melanoma. *Proc. Natl. Acad. Sci. USA* 97: 5492–5497.
20. Colella, T. A., T. N. Bullock, L. B. Russell, D. W. Mullins, W. W. Overwijk, C. J. Luckey, R. A. Pierce, N. P. Restifo, and V. H. Engelhard. 2000. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: implications for tumor immunotherapy. *J. Exp. Med.* 191: 1221–1232.

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021

21. Bronte, V., E. Apolloni, R. Ronca, P. Zamboni, W. W. Overwijk, D. R. Surman, N. P. Restifo, and P. Zanovello. 2000. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res.* 60: 253–258.
22. Overwijk, W. W., and N. P. Restifo. 2001. Creating therapeutic cancer vaccines: notes from the battlefield. *Trends Immunol.* 22: 5–7.
23. Chaux, P., B. Lethe, J. Van Snick, J. Corthals, E. S. Schultz, C. L. Cambiaso, T. Boon, and P. van der Bruggen. 2001. A MAGE-1 peptide recognized on HLA-DR15 by $CD4^+$ T cells. *Eur. J. Immunol.* 31: 1910–1916.
24. Chaux, P., R. Luiten, N. Demotte, V. Vantomme, V. Stroobant, C. Traversari, V. Russo, E. Schultz, G. R. Cornelis, T. Boon, and P. van der Bruggen. 1999. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by in vitro stimulation with dendritic cells transduced with MAGE-A1. *J. Immunol.* 163: 2928–2936.
25. Chaux, P., V. Vantomme, V. Stroobant, K. Thielemans, J. Corthals, R. Luiten, A. M. Eggermont, T. Boon, and P. van der Bruggen. 1999. Identification of MAGE-3 epitopes presented by HLA-DR molecules to $CD4^+$ T lymphocytes. *J. Exp. Med*. 189: 767–778.
26. Schultz, E. S., B. Lethe, C. L. Cambiaso, J. Van Snick, P. Chaux, J. Corthals, C. Heirman, K. Thielemans, T. Boon, and P. van der Bruggen. 2000. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by $CD4^+$ cytolytic T lymphocytes. *Cancer Res.* 60: 6272–6275.
27. Lee, P. P., C. Yee, P. A. Savage, L. Fong, D. Brockstedt, J. S. Weber, D. Johnson, S. Swetter, J. Thompson, P. D. Greenberg, et al. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med.* 5: 677–685.
28. Yee, C., P. A. Savage, P. P. Lee, M. M. Davis, and P. D. Greenberg. 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J. Immunol.* 162: 2227–2234.
29. de Visser, K. E., T. A. Cordaro, H. W. Kessels, F. H. Tirion, T. N. Schumacher, and A. M. Kruisbeek. 2001. Low-avidity self-specific T cells display a pronounced expansion defect that can be overcome by altered peptide ligands. *J. Immunol.* 167: 3818–3828.
30. de Visser, K. E., T. A. Cordaro, D. Kioussis, J. B. Haanen, T. N. Schumacher, and A. M. Kruisbeek. 2000. Tracing and characterization of the low-avidity self-specific T cell repertoire. *Eur. J. Immunol.* 30: 1458–1468.
31. Clay, T. M., M. C. Custer, M. D. McKee, M. Parkhurst, P. F. Robbins, K. Kerstann, J. Wunderlich, S. A. Rosenberg, and M. I. Nishimura. 1999. Changes in the fine specificity of $gp100_{209-217}$-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. *J. Immunol.* 162: 1749–1755.
32. Dionne, S. O., M. H. Smith, F. M. Marincola, and D. F. Lake. 2003. Functional characterization of CTL against gp100 altered peptide ligands. *Cancer Immunol. Immunother.* 52: 199–206.
33. Denkberg, G., E. Klechevsky, and Y. Reiter. 2002. Modification of a tumor-derived peptide at an HLA-A2 anchor residue can alter the conformation of the MHC-peptide complex: probing with TCR-like recombinant antibodies. *J. Immunol.* 169: 4399–4407.
34. Thompson, L. W., C. F. Garbee, S. Hibbitts, L. H. Brinckerhoff, R. A. Pierce, K. A. Chianese-Bullock, D. H. Deacon, V. H. Engelhard, and C. L. Slingluff, Jr. 2004. Competition among peptides in melanoma vaccines for binding to MHC molecules. *J. Immunother.* 27: 425–431.
35. Slansky, J. E., F. M. Rattis, L. F. Boyd, T. Fahmy, E. M. Jaffee, J. P. Schneck, D. H. Margulies, and D. M. Pardoll. 2000. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC-peptide-TCR complex. *Immunity* 13: 529–538.
36. Doxsee, C. L., T. R. Riter, M. J. Reiter, S. J. Gibson, J. P. Vasilakos, and R. M. Kedl. 2003. The immune response modifier and TLR7 agonist S-27609 selectively induces IL-12 and TNF-α production in $CD11c^+CD11b^+CD8^-$ dendritic cells. *J. Immunol.* 171: 1156–1163.
37. Ahonen, C. L., C. L. Doxsee, S. M. McGurran, T. R. Riter, W. F. Wade, R. J. Barth, J. P. Vasilakos, R. J. Noelle, and R. M. Kedl. 2004. Combined TLR and CD40 triggering induces potent $CD8^+$ T cell expansion with variable dependence on type I IFN. *J. Exp. Med.* 199: 775–784.
38. Kedl, R. M., W. A. Rees, D. A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *J. Exp. Med.* 192: 1105–1113.
39. Ljunggren, H. G., N. J. Stam, C. Ohlen, J. J. Neefjes, P. Hoglund, M. T. Heemels, J. Bastin, T. N. Schumacher, A. Townsend, K. Karre, et al. 1990. Empty MHC class I molecules come out in the cold. *Nature* 346: 476–480.
40. del Marmol, V., and F. Beermann. 1996. Tyrosinase and related proteins in mammalian pigmentation. *FEBS Lett.* 381: 165–168.
41. Rees, W., J. Bender, T. K. Teague, R. M. Kedl, F. Crawford, P. Marrack, and J. Kappler. 1999. An inverse relationship between T cell receptor affinity and antigen dose during $CD4^+$ T cell responses in vivo and in vitro. *Proc. Natl. Acad. Sci. USA* 96: 9781–9786.
42. Crawford, F., E. Huseby, J. White, P. Marrack, and J. W. Kappler. 2004. Mimotopes for alloreactive and conventional T cells in a peptide-MHC display library. *PLoS Biol.* 2: E90.
43. Kedl, R. M., B. C. Schaefer, J. W. Kappler, and P. Marrack. 2002. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat. Immunol.* 3: 27–32.
44. Chen, W., J. McCluskey, S. Rodda, and F. Carbone. 1993. Changes at peptide residues buried in the major histocompatibility complex (MHC) class I binding cleft influence T cell recognition: a possible role for indirect conformational alterations in the MHC class I or bound peptide in determining T cell recognition. *J. Exp. Med.* 177: 869–873.
45. McEarchern, J. A., D. G. Besselsen, and E. T. Akporiaye. 1999. Interferon γ and antisense transforming growth factor β transgenes synergize to enhance the immunogenicity of a murine mammary carcinoma. *Cancer Immunol. Immunother.* 48: 63–70.
46. He, X., P. Luo, T. C. Tsang, T. Zhang, and D. T. Harris. 2005. Immuno-gene therapy of melanoma by tumor antigen epitope modified IFN-γ. *Cancer Immunol. Immunother.* 54: 741–749.
47. Seliger, B., U. Wollscheid, F. Momburg, T. Blankenstein, and C. Huber. 2001. Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. *Cancer Res.* 61: 1095–1099.
48. Kedl, R. M., J. W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr. Opin. Immunol.* 15: 120–127.
49. Yewdell, J. W., and J. R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17: 51–88.
50. Restifo, N. P. 2001. Hierarchy, tolerance, and dominance in the antitumor T-cell response. *J. Immunother.* 24: 193–194.
51. Parkhurst, M. R., E. B. Fitzgerald, S. Southwood, A. Sette, S. A. Rosenberg, and Y. Kawakami. 1998. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). *Cancer Res.* 58: 4895–4901.

Downloaded from http://www.jimmunol.org/ by guest on September 24, 2021