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

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
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. Kedl2‡

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.

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., intrader- mal(ly); wt, wild type.
Materials and Methods

Tumor cell lines and mouse injections

C57BL/6d (B6) female mice from Charles River or The Jackson Laboratory were anesthetized with Avertin, their rear flanks were shaved, and they were injected with \(1 \times 10^6\) 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 (SVYIKFFVWL), deltaA (SVIFDFVWL), deltaV (SYDFDFVVLW), and OVA (SHINEKFL) 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 \(\mu\)g of anti-CD40 Ab (FGK4.5 or IC10), 100 \(\mu\)g of the TLR3 agonist poly(I:C), or 150 \(\mu\)g of the TLR7 agonist S-27609 (36, 37), and 100 \(\mu\)g 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-\(\gamma\) production as previously described (37). Alternatively, peripheral blood was taken by tail vein bleed at 37°C. At various time points, representative cells were removed, stained with anti-Kb-PE Ab (BD Pharmingen) and analyzed by FACS. For peptide dose responses (see Fig. 2A), spleen cells were isolated from mice immunized with either TRP2 or deltaA 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-\(\gamma\) staining (Fig. 2A). 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).

Class I MHC stabilization assay

Class I H-2K\(^{\beta}\) 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\(^{\beta}\)-PE Ab (BD Pharmingen) and analyzed by FACS. For analysis of the half-life of peptide MHC binding, RMAS cells were incubated with 50 \(\mu\)g/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\(^{\beta}\) as described above, and fixed in paraformaldehyde. Cells from all time points were then analyzed for cell surface expression of K\(^{\beta}\).

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\(^{\beta}\)/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-\(\gamma\) 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.

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-\(\gamma\)-staining. Whereas TRP2 tetramer-staining cells were rarely observed (Fig. 2A), a significant population of TRP2-specific T cells (~10–20% of total CD8\(^{+}\) T cells) was identifiable by intracellular IFN-\(\gamma\)-staining (Fig. 2B). 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).
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 based on the intracellular IFN-\(\gamma\), as well as the relative half-life of peptide MHC binding (Fig. 5B). The deltaV epitope had, at best, a 2- to 3-fold higher affinity for H-2K\(^b\) (Fig. 5A) and the relative half-life of peptide MHC binding (Fig. 5B). The deltaV epitope, once bound to \(K\(^b\), demonstrated a similar half-life of binding than the deltaV or ova peptide (Fig. 5B). 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-\(\gamma\)-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. 6A). 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. 6B). 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. 6C) (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-\(\gamma\) over time. Consistent with previous results, TRP2-specific IFN-\(\gamma\) 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. 6D, primary). However, after boosting the mice with the TRP2 epitope, little TRP2-specific tetramer staining or IFN-\(\gamma\)-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. 6A) as well as peripheral tolerance of the low-affinity cells (based on loss of detectable intracellular IFN-\(\gamma\) production; Fig. 6C) are actively subduing the high number of lower affinity T cells, whereas deltaV immunization generates a smaller number of higher-affinity TRP2-specific cells.

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. 5A) and the relative half-life of peptide MHC binding (Fig. 5B). The deltaV epitope had, at best, a 2- to 3-fold higher affinity for H-2K\(^b\) (Fig. 5A), 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. 5B). 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 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-\(\gamma\). Although the majority of the dose-response curve was essentially the same between cells derived from mice immunized with either peptide (Fig. 4A), 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-\(\gamma\) (Fig. 4B). 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. 4C). 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

<table>
<thead>
<tr>
<th>Epitope</th>
<th>SVYYDDFFVWL</th>
<th>SVYYDDFFVWL</th>
<th>SIYDFVWL</th>
<th>SVIYDFVWL</th>
</tr>
</thead>
</table>

Table I. Modified peptide epitope key
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...
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 affect TCR recognition but have little effect on overall binding to MHC. In a tumor model system using H-2L\(^d\) rather than H-2K\(^b\), Slansky et al. (35) similarly demonstrated that tumor epitopes can be modified to induce T cell responses without affecting peptide binding. 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.

**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.

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
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


