

Elevated Frequencies of Self-reactive CD8⁺ T Cells following Immunization with a Xenoantigen Are Due to the Presence of a Heteroclitic CD4⁺ T-Cell Helper Epitope

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Abstract

Immunization of mice with human dopachrome tautomerase (hDCT) provides greater protection against melanoma than immunization with the murine homologue (mDCT). We mapped the CD8⁺ and CD4⁺ T-cell epitopes in both proteins to better understand the mechanisms of the enhanced protection. The dominant CD8⁺ T-cell epitopes were fully conserved between both proteins, yet immunization with hDCT produced frequencies of CD8⁺ T cells that were 5- to 10-fold higher than immunization with mDCT. This difference was not intrinsic to the two proteins because comparable frequencies of CD8⁺ T cells were elicited by both antigens in DCT-deficient mice. Strikingly, only hDCT elicited a significant level of specific CD4⁺ T cells in wild-type (WT) mice. The murine protein was not devoid of CD4⁺ T-cell epitopes because immunization of DCT-deficient mice with mDCT resulted in robust CD4⁺ T-cell immunity directed against two epitopes that were not identified in WT mice. These results suggested that the reduced immunogenicity of mDCT in WT mice may be a function of insufficient CD4⁺ T-cell help. To address this possibility, the dominant CD4⁺ T-cell epitope from hDCT was introduced into mDCT. Immunization with the mutated mDCT evoked CD8⁺ T-cell frequencies and protective immunity comparable with hDCT. These results reveal a novel mechanism by which xenoantigens overcome tolerance. Our data also suggest that immunologic tolerance is more stringent for CD4⁺ T cells than CD8⁺ T cells, providing a mechanism of peripheral tolerance where autoreactive CD8⁺ T cells fail to be activated due to a lack of autoreactive CD4⁺ T cells specific for the same antigen. [Cancer Res 2007;67(13):6459–67]

Introduction

The identification of multiple tumor-associated antigens has greatly spurred interest in developing vaccination strategies for the treatment of cancer. However, most of these antigens are derived from naturally occurring proteins and, as such, immunization strategies must overcome immunologic tolerance, which limits the reactivity of T and B lymphocytes against self-antigen. One strategy

that has been used successfully is “xenoimmunization,” where homologous proteins from different species are used as the basis for the vaccine (1–5). Although the mechanism of action of xenoimmunization remains to be fully understood, it is believed that subtle differences between the native and “xenoprotein” can overcome tolerance by eliciting T-cell and B-cell responses against the xenoantigen that cross-react with the native antigen, providing antitumor immunity.

Previous investigations into the mechanism(s) of action of xenoimmunization showed that heteroclitic CD8⁺ T-cell epitopes present in xenoantigens could elicit CD8⁺ T-cell populations that are reactive to both the xenoantigen and the native antigen (2, 6, 7). Whether heteroclitic CD8⁺ T-cell epitopes underlie the activity of all xenoantigens is not known. Dopachrome tautomerase (DCT; also known as tyrosinase-related protein-2) is a nonmutated antigen that is widely expressed in melanoma. DCT is a promising candidate for the development of melanoma vaccines as it is naturally recognized by melanoma-specific CTL (8–10). Immunization of mice with human DCT (hDCT) resulted in greater antitumor immunity than immunization with murine DCT (mDCT), providing a useful model for studying the mechanisms of xenoimmunization (11–14). Interestingly, the protective effect of hDCT can be recapitulated by immunization with only the dominant CD8⁺ T-cell epitope, SVYDFFVWL, which is 100% conserved within the murine sequence (15), showing that CD8⁺ T-cell immunity can be sufficient in this model. Linkage to strong CD4⁺ T-cell epitopes was required to produce protective immunity with SVYDFFVWL, suggesting that, rather than providing heteroclitic CD8⁺ T-cell epitopes, hDCT may contain CD4⁺ T-cell epitopes that facilitate activation of the SVYDFFVWL-specific CD8⁺ T cells (15–17). However, it is also possible that the increased immunogenicity of hDCT is not due to the SVYDFFVWL epitope. Our previous investigations have shown that both CD4⁺ and CD8⁺ T cells can provide protective immunity following immunization with hDCT (11). Therefore, the increased immunogenicity of hDCT could be due to heteroclitic variants of previously undefined epitopes that give rise to effector CD8⁺ and/or CD4⁺ T cells. Alternatively, immunization with mDCT may give rise to regulatory T cells as shown recently using a series of self-antigens identified by SEREX (18). In that regard, the increased immunogenicity of hDCT may be due to the absence of epitopes that elicit regulatory T cells.

In the current article, we have characterized a series of T-cell epitopes elicited by recombinant adenovirus vaccines expressing DCT. Through combined studies in wild-type (WT) and DCT-deficient mice, we showed that the increased immunogenicity of hDCT relative to mDCT is due to the presence of heteroclitic CD4⁺ T helper epitopes. These studies also revealed a hierarchical

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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regulation of self-reactive T cells where tolerance toward CD4⁺ T-cell epitopes seems to be more stringent than CD8⁺ T cells. The implications of these observations for cancer vaccine design and the current understanding of peripheral tolerance are discussed.

Materials and Methods

Mice. Six- to 8-week-old male and female C57BL/6 mice were obtained from Charles River Laboratories. DCT-deficient mice (19) and CD8-deficient mice (20) were bred at McMaster University. All of our investigations have been approved by the McMaster Animal Research Ethics Board.

Replication-deficient adenoviruses, plasmid vaccines, and immunizations. All replication-deficient adenoviral (rAd) vectors used in this study contain deletions of E1 and E3 regions (21). The expression cassettes were inserted into the E1 region under the control of the murine cytomegalovirus (CMV) promoter and the SV40 polyadenylation sequence. The rAd vectors were propagated and purified as described previously (22). AdhDCT and AdmDCT express hDCT and mDCT, respectively (11). AdhDCT(L/H→Q/N) expresses a mutant hDCT where the H at position 93 was converted to N and the L at position 86 was converted to Q. AdmDCT(Q/N→L/H) expresses a mutant mDCT where the N at position 93 was converted to H and the Q at position 86 was converted to L. AdLCMV expresses the sequence corresponding to residues 33 to 41 and 61 to 80 of the lymphocytic choriomeningitis virus glycoprotein and was included as a negative control. AdRKF-LAMP expresses a synthetic antigen where the dominant CD4⁺ T-cell epitope from hDCT (RKFFHRTCKCTGNFA) is targeted to the lysosomes using the signal peptide and transmembrane domain/sorting signal from LAMP-1 as described by Wu et al. (23). A plasmid vaccine expressing the same synthetic antigen (pRKF-LAMP) was constructed in a modified version of pcDNA3.1 that we have used previously (24). For plasmid immunization, 100 µg of pRKF-LAMP were prepared in 100 µL sterile PBS and injected i.m. in both rear thighs of each mouse (50 µL/thigh) in combination with electroporation as described previously (25). For adenovirus immunizations, 10⁷ or 10⁸ plaque-forming unit (pfu) of adenoviral vector was prepared in 100 µL sterile PBS and injected i.m. in both rear thighs of each mouse (50 µL/thigh).

Monoclonal antibodies. The following monoclonal antibodies were purchased from BD Pharmingen: anti-CD3ε-PE, anti-CD8α-PE-Cy5, anti-CD4-PE-Cy7, anti-IFN-γ-APC, anti-TNFα-FITC, anti-CD16/CD32, anti-CD28, anti-CD49d, and biotin-conjugated anti-IFN-γ detection antibody (clone

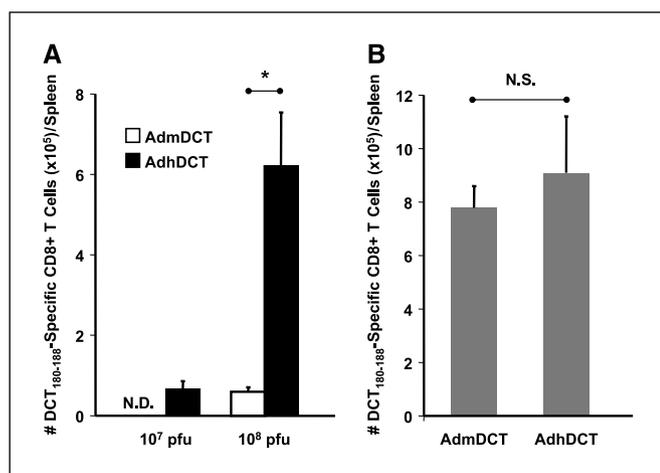


Figure 1. DCT₁₈₀₋₁₈₈-specific CD8⁺ T-cell response elicited by AdhDCT and AdmDCT in WT and DCT-deficient mice. **A**, WT mice were immunized with 10⁷ and 10⁸ pfu of AdmDCT (white columns) or AdhDCT (black columns). Eight days later, the number of DCT₁₈₀₋₁₈₈-specific CD8⁺ T cells in their spleens was quantified by intracellular cytokine staining. **B**, DCT-deficient mice were immunized with 10⁸ pfu of AdmDCT or AdhDCT. Eight days later, the number of DCT₁₈₀₋₁₈₈-specific CD8⁺ T cells in their spleens was quantified by intracellular cytokine staining. *, *P* < 0.01. *N.S.*, not significant; *N.D.*, not detectable.

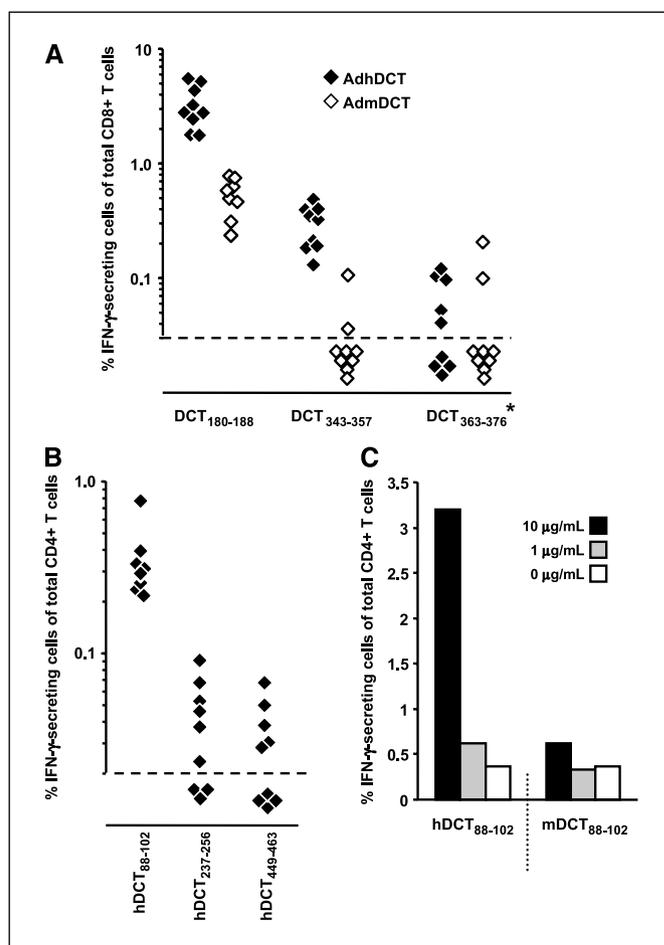


Figure 2. CD8⁺ and CD4⁺ T-cell responses elicited by AdhDCT and AdmDCT in WT mice. WT mice were immunized with 10⁸ pfu of AdhDCT (♦) or AdmDCT (◇). Eight days later, the frequency of epitope-specific T cell in their spleens was quantified by intracellular cytokine staining. **A**, splenocytes were stimulated with CD8⁺ T-cell epitope peptides. Note that AdhDCT-immunized mice were stimulated with hDCT₃₆₃₋₃₇₆ and AdmDCT-immunized mice were stimulated with mDCT₃₆₃₋₃₇₆. **B**, splenocytes were stimulated with CD4⁺ T-cell epitope peptides. **Symbol**, data from an individual mouse; **dashed line**, limit of detection in the assay. **C**, splenocytes from AdhDCT-immunized mice were restimulated with hDCT₈₈₋₁₀₂ for 7 d *in vitro* in the presence of 10 units/mL IL-2. The cells were washed and replated in medium with IL-2 but without hDCT₈₈₋₁₀₂ peptide and allowed to rest for 24 h. Following the rest period, the cells were restimulated with varying doses (1 and 10 µg/mL) of either hDCT₈₈₋₁₀₂ or mDCT₈₈₋₁₀₂ and the frequency of IFN-γ-producing CD4⁺ T cells was quantified by intracellular cytokine staining.

XMG1.2). Purified anti-IFN-γ capture antibody was prepared in the Bramson laboratory.

Peptides. The peptide sequences for the overlapping peptide libraries were generated using PeptGen.³ All peptides were synthesized by Peptscan Systems at 70% purity, resuspended in DMSO, and stored at -20°C.

Intracellular cytokine staining for flow cytometry. Intracellular cytokine staining was done as described previously (22) with the following modifications: (a) purified anti-CD28 and anti-CD49d (BD Pharmingen) were added to each well at 2 µg/mL, (b) the lymphocytes were cultured in the presence of peptide and costimulatory antibodies for 2 h before the addition of brefeldin A, and (c) the restimulated cells were harvested 4 h after the addition of brefeldin A.

³ <http://hiv-web.lanl.gov/content/hiv-db/PEPTGEN/PeptGenSubmitForm.html>

Enzyme-linked immunospot assays. The enzyme-linked immunospot (ELISPOT) assay has been described previously (26). Lymphocytes (5×10^5) were aliquoted into the appropriate wells and stimulated with individual peptides or peptide pools at a final concentration of 2 $\mu\text{g}/\text{mL}$ of each individual peptide. Purified anti-CD28 and anti-CD49d antibodies were included at 2 $\mu\text{g}/\text{mL}$. Spots were enumerated using an ImmunoSpot 3B analyzer (Cellular Technology Ltd.).

Tumor challenge. Mice were immunized with 10^8 pfu of recombinant adenovirus vaccine and challenged i.d. with either 10^5 or 10^6 B16F10 cells 7 days later. Tumor growth was monitored daily and measured twice weekly using calipers.

Statistical analysis. Student's *t* tests were conducted using Microsoft Excel, and differences were considered significant at $P < 0.05$. Data are presented as mean \pm SE.

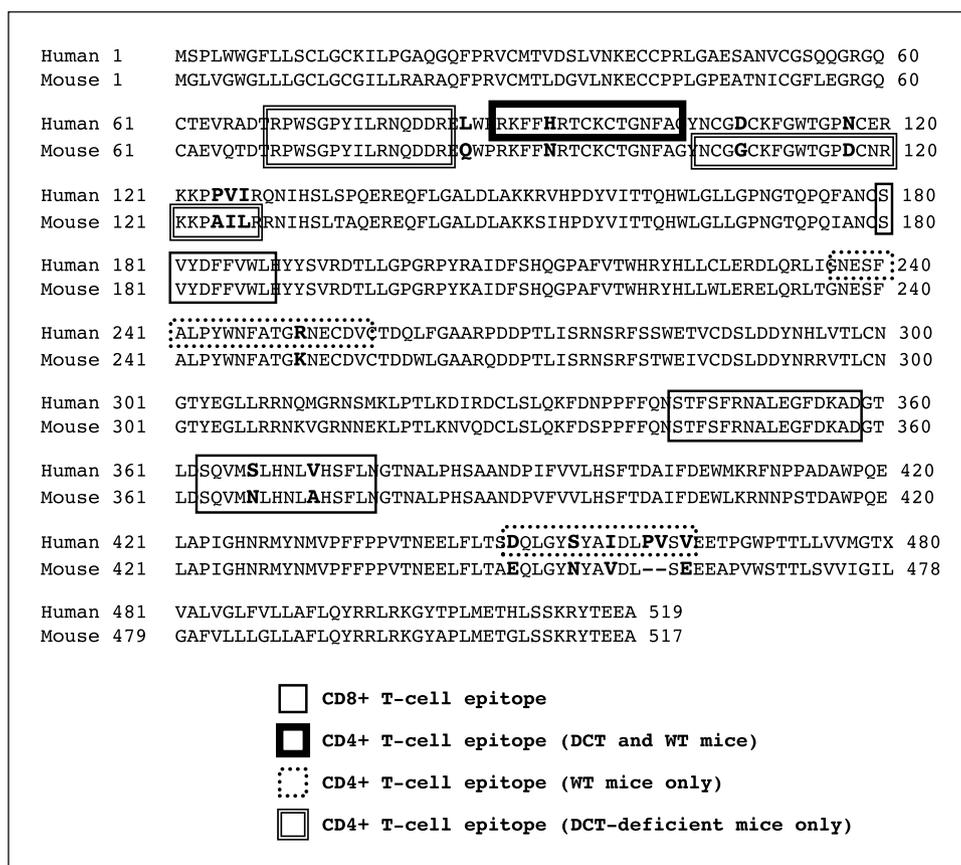
Results and Discussion

Measurement of CD8⁺ T cells specific for DCT₁₈₀₋₁₈₈ following xenoinmunization in WT mice and DCT-deficient mice. Our previous report showed that immunization with an adenoviral vector expressing hDCT elicited greater protective immunity than a vector expressing mDCT (11). To understand the enhanced protection by hDCT, we examined the CD8⁺ T-cell response elicited by AdhDCT and AdmDCT. Splenocytes from mice immunized with either 10^7 or 10^8 pfu of the recombinant adenovirus vaccines were restimulated with the dominant CD8⁺ T-cell epitope from DCT (DCT₁₈₀₋₁₈₈). The DCT₁₈₀₋₁₈₈-specific response was dose dependent for both vectors (Fig. 1). AdhDCT clearly evoked a more robust CD8⁺ T-cell response, although the sequence of DCT₁₈₀₋₁₈₈ is fully conserved in both proteins (Fig. 1A). Thus, the difference in the magnitude of the CD8⁺ T-cell response

following AdmDCT immunization was not due to insufficient availability of CD8⁺ T-cell precursors but rather to reduced immunogenicity of mDCT. Both vectors expressed the same level of DCT *in vitro* (data not shown), so the lower frequency of DCT₁₈₀₋₁₈₈-specific CD8⁺ T cells was not due to insufficient protein production. These results suggested that the weaker response of the AdmDCT may be due to some form of tolerance toward the mDCT antigen. To address this possibility, we immunized DCT-deficient mice with both vectors. Strikingly, both vectors elicited the same frequency of DCT₁₈₀₋₁₈₈-specific T cells in DCT-deficient mice (Fig. 1B). These data indicate that the reduced immunogenicity was not due to an intrinsic property of the mDCT protein because both AdhDCT and AdmDCT produced similar CD8⁺ T-cell responses in DCT-deficient mice. Furthermore, the reduced CD8⁺ T-cell response following immunization of WT mice with mDCT was not due to central deletion of DCT₁₈₀₋₁₈₈-specific T cells, as these cells were available for the AdhDCT immunization. Therefore, it seems that some form of peripheral tolerance is limiting the development of the CD8⁺ T-cell response to mDCT in WT mice.

Mapping of CD8⁺ and CD4⁺ T-cell epitopes in hDCT and mDCT following immunization of WT mice. Steitz et al. (16) have suggested that a robust CD4⁺ T-cell epitope may exist within hDCT that is able to enhance the CD8⁺ T-cell response relative to mDCT. However, no such CD4⁺ T-cell epitope has been identified to date. Another possible explanation for the increased activity of hDCT could be the presence of heteroclitic CD8⁺ T-cell epitopes independent of DCT₁₈₀₋₁₈₈. Alternatively, there may be suppressor epitopes in mDCT that trigger regulatory T cells provoking greater suppression of the CD8⁺ T-cell response. To address these

Figure 3. Location of CD8⁺ and CD4⁺ T-cell epitopes in hDCT and mDCT. The complete sequences for hDCT and mDCT are shown aligned. *Bold*, nonidentical residues. *Thin-line boxes*, CD8⁺ T-cell epitopes; *thick-line boxes*, CD4⁺ T-cell epitopes observed in WT and DCT-deficient mice; *dashed boxes*, CD4⁺ T-cell epitopes that are observed only in WT mice; *double-lined boxes*, CD4⁺ T-cell epitopes that are detected only in DCT^{-/-} mice.



possibilities, we used a strategy using a library of overlapping peptides spanning the DCT proteins to map CD8⁺ and CD4⁺ T-cell epitopes as described (27–29). We used two libraries of 15-mer peptides overlapping by 9 amino acids composed of 97 peptides spanning hDCT and 95 peptides spanning mDCT. The peptides were distributed into pools using a two-dimensional format as described (29) such that each peptide was present in only two pools. Splenocytes were harvested from mice 8 days after immunization with AdhDCT or AdmDCT, which is the peak of the CD8⁺ T-cell response (data not shown), and restimulated with the peptide pools. Peptide-specific reactivity was measured using an IFN- γ ELISPOT. Candidate epitopes were identified based on coincident stimulation by two pools carrying the same peptide. In repeat experiments, the splenocytes were stimulated with single candidate epitope peptides to identify the specific peptides carrying T-cell epitopes. To characterize the phenotype of the responding T-cell (i.e., CD4⁺ or CD8⁺), we repeated the stimulations with individual peptides and visualized IFN- γ and tumor necrosis factor- α production by CD8⁺ and CD4⁺ T cell using flow cytometry.

Using this epitope mapping strategy with splenocytes from immunized WT mice, we identified two previously defined CD8⁺ T-cell epitopes [DCT₁₈₀₋₁₈₈ and DCT₃₆₃₋₃₇₁ (30, 31)] and a novel epitope within the peptide DCT₃₄₃₋₃₅₇. As with DCT₁₈₀₋₁₈₈, the CD8⁺ T-cell reactivity to the other epitopes was markedly reduced following immunization with AdmDCT relative to AdhDCT (Fig. 2A). Following immunization with AdhDCT, we observed a clear hierarchy of dominance for the CD8⁺ T-cell epitopes (Fig. 2A). DCT₁₈₀₋₁₈₈ was confirmed as the dominant epitope. DCT₃₄₃₋₃₅₇ was subdominant and responses to DCT₃₆₃₋₃₇₆ were only observed in a fraction of the mice. Interestingly, DCT₃₄₃₋₃₅₇ (STSFNRLAEGFDKAI) was fully conserved between mDCT and hDCT (Fig. 3). Only DCT₃₆₃₋₃₇₅ exhibited some sequence differences [SQVM(S/N) LHN(L/V/A)HSFL], which were suggestive of a heteroclitic epitope (Fig. 3). However, mice immunized with mDCT were reactive to mDCT₃₆₃₋₃₇₅ and mice immunized with hDCT were reactive with hDCT₃₆₃₋₃₇₅; therefore, it is unlikely that the hDCT₃₆₃₋₃₇₅ is heteroclitic. Thus, unlike the previous reports (2, 6, 7), we did not identify any heteroclitic CD8⁺ T-cell epitopes in hDCT to explain the increased immunogenicity of this antigen in murine models. Because AdhDCT was able to elicit robust CD8⁺ T-cell immunity against two epitopes conserved between hDCT and mDCT, it seems that the reduced CD8⁺ T-cell response following AdmDCT immunization was not due to insufficient CD8⁺ T-cell precursors but rather to some form of peripheral tolerance that prevented activation of the circulating CD8⁺ T cells.

The results for CD4⁺ T-cell epitopes were quite different. Only AdhDCT immunization gave rise to specific CD4⁺ T cells in WT mice (Figs. 2B and 3). We were not able to measure any CD4⁺ T-cell immunity toward mDCT following immunization of WT mice with AdmDCT. The dominant CD4⁺ T-cell response following AdhDCT immunization was targeted to hDCT₈₈₋₁₀₂ (Fig. 2B). CD4⁺ T cells responsive to the other epitopes, hDCT₂₃₇₋₂₅₆ and hDCT₄₄₉₋₄₆₃, were only measurable in a fraction of the immunized mice (Fig. 2B). Unlike the dominant CD8⁺ T-cell epitopes, all the CD4⁺ T-cell epitopes in hDCT identified using WT mice exhibited some degree of sequence divergence from the sequences in mDCT (Fig. 3), suggesting that they all may be heteroclitic. As direct evidence that the dominant CD4⁺ T-cell peptide (hDCT₈₈₋₁₀₂) is heteroclitic relative to the murine peptide, we stimulated short-term T-cell cultures of hDCT₈₈₋₁₀₂-specific CD4⁺ T cells with both hDCT₈₈₋₁₀₂

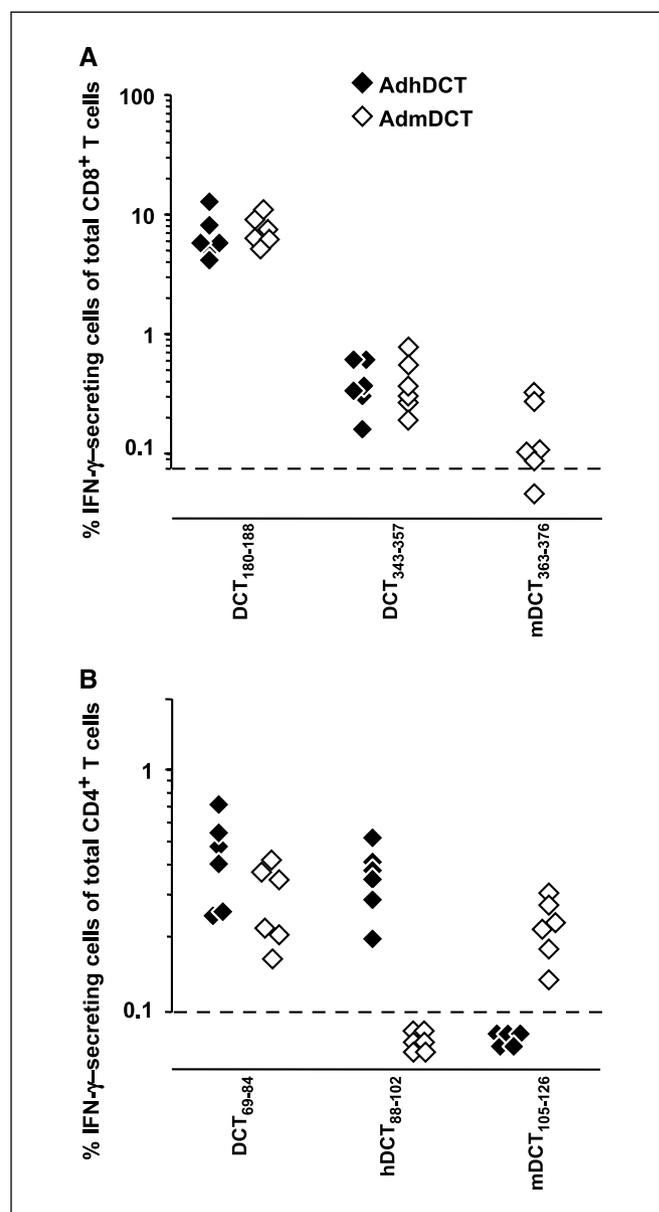


Figure 4. CD8⁺ and CD4⁺ T-cell responses elicited by AdhDCT and AdmDCT in DCT-deficient mice. DCT-deficient mice were immunized with 10^8 pfu of AdhDCT (\blacklozenge) or AdmDCT (\circ). Eight days later, the frequency of epitope-specific T cell in their spleens was quantified by intracellular cytokine staining. *A*, *X* axis, splenocytes were stimulated with CD8⁺ T-cell epitope peptides. *B*, *X* axis, splenocytes were stimulated with CD4⁺ T-cell epitope peptides. *Symbol*, data from an individual mouse; *dashed line*, limit of detection in the assay.

and mDCT₈₈₋₁₀₂. As shown in Fig. 2C, the hDCT₈₈₋₁₀₂ peptide displayed ~10-fold greater stimulatory activity than the mDCT₈₈₋₁₀₂ peptide. Likewise, although we did not observe any IFN- γ production by splenocytes from AdmDCT-immunized mice stimulated with mDCT₈₈₋₁₀₂, we did observe some weak responses following stimulation with hDCT₈₈₋₁₀₂ (data not shown). Therefore, it seems that the increased immunogenicity of hDCT may be due to the presence of heteroclitic CD4⁺ T-cell epitopes.

The same strategy for epitope mapping was also applied to splenocytes from immunized DCT-deficient mice to determine whether the epitope patterns were influenced by tolerance to mDCT in WT mice (Fig. 4). Surprisingly, we did not uncover any

additional CD8⁺ T-cell epitopes. We only measured reactivity to the same three CD8⁺ T-cell epitopes previously identified in WT mice. Moreover, the hierarchy of epitope dominance for the CD8⁺ T-cell response in the DCT-deficient mice was identical to the hierarchy in WT mice, indicating that epitope dominance was not influenced by self-tolerance (Fig. 4A). Importantly, the frequencies of CD8⁺ T cells reactive to DCT₁₈₀₋₁₈₈ and DCT₃₄₇₋₃₅₇ were identical in DCT-deficient mice immunized with either AdhDCT or AdmDCT, providing additional evidence that the lack of CD8⁺ T-cell response in WT mice following AdmDCT immunization was not due to intrinsic differences between mDCT and hDCT but rather to some aspect of peripheral tolerance. Surprisingly, we did not observe CD8⁺ T-cell immunity to hDCT₃₆₃₋₃₇₆ following immunization with AdhDCT, although we did observe a response to the corresponding epitope in mDCT following immunization with AdmDCT (Fig. 4A). These data present an interesting picture of the mechanisms of CD8⁺ T-cell tolerance with regard to DCT. It seems that central tolerance is not a major factor in limiting the CD8⁺ T-cell response against DCT because we did not observe T-cell clones with novel specificity following immunization of DCT-deficient mice. Rather, our data argue that limiting CD4⁺ T-cell help is the major mechanism controlling CD8⁺ T-cell immunity to this antigen as suggested previously (15, 16).

Unlike the CD8⁺ T-cell response, a novel CD4⁺ T-cell epitope was uncovered in DCT-deficient mice that was 100% conserved between hDCT and mDCT (hDCT₆₉₋₈₃ and mDCT₇₀₋₈₃; Fig. 3). CD4⁺ T cells reactive to this epitope were present at a frequency comparable with those reactive to the previously defined CD4⁺ T-cell epitope, hDCT₈₈₋₁₀₂ (Fig. 4B, *closed diamonds*). An additional CD4⁺ T-cell epitope was identified in mDCT (mDCT₁₀₅₋₁₂₆), which seemed to be unique to mDCT. Splenocytes from mice immunized with AdhDCT did not react with hDCT₁₀₅₋₁₂₆ (data not shown) nor did they cross-react with mDCT₁₀₅₋₁₂₆ (Fig. 4B). It is interesting to note that, unlike the CD8⁺ T-cell responses, there was no obvious hierarchy among the CD4⁺ T-cell responses in DCT-deficient mice. Thus, the lack of CD4⁺ T-cell response following immunization with AdmDCT in WT mice was not due to a lack of CD4⁺ T-cell epitopes in mDCT. Further, these results show that tolerance toward CD4⁺ T-cell epitopes in mDCT is considerably more stringent than tolerance toward CD8⁺ T-cell epitopes.

CD4⁺ T-cell immunity plays a key role in the CD8⁺ T-cell response and protective immunity produced by AdhDCT immunization. In addition to the lower magnitude of CD8⁺ T-cell immunity produced by AdmDCT relative to AdhDCT, we were struck by the observation that we could essentially measure no CD4⁺ T-cell immunity following AdmDCT. To assess the role of CD4⁺ T-cell help in the CD8⁺ T-cell response to AdhDCT, we compared the CD8⁺ T-cell response elicited by AdhDCT in WT and MHC class II-deficient mice that lack CD4⁺ T cells. Although robust CD8⁺ T-cell immunity was generated in WT mice, we could not measure any CD8⁺ T-cell response above background in the MHC class II-deficient mice (Supplementary Fig. S1A). Furthermore, immunization of CD4-deficient mice or MHC class II-deficient mice with 10⁸ pfu AdhDCT failed to provide protective immunity against B16F10 challenge (Supplementary Fig. S1B). These results are similar to those of Steitz et al. (16) who used a variant of mDCT that was fused to enhanced green fluorescent protein to augment the CD8⁺ T-cell response to mDCT. However, it should be noted that we have recently shown that CD4⁺ T cells are required for optimal expansion of CD8⁺ T cells following immunization with rAd vectors (32). Therefore, we cannot

conclude whether the defective CD8⁺ T-cell response and lack of protective immunity is due to lack of CD4⁺ T-cell help for the self-reactive CD8⁺ T cells or lack of CD4⁺ T-cell help for the rAd vaccine.

Assessing the role of hDCT₈₈₋₁₀₂ in the CD8⁺ T-cell response evoked by AdhDCT. To this point, our results have shown a critical role for CD4⁺ T cells in the immunogenicity of AdhDCT. Given the observation that AdmDCT immunization failed to evoke substantial CD4⁺ T-cell immunity in WT mice, we hypothesized that the increased CD8⁺ T-cell response elicited by AdhDCT may be due to the dominant heteroclitic CD4⁺ T-cell epitope between residues 88 and 102 (Fig. 3). The human sequence across this area only differs from the corresponding sequence in mDCT by a single amino acid at position 92. We also identified a single amino acid that was different immediately upstream at position 86, which could be involved in epitope processing. Therefore, to assess the role of this CD4⁺ T-cell epitope in the CD8⁺ T-cell response, we prepared mutants of DCT with reciprocal mutations at positions 86 and 92, which we have named hDCT(L/H→Q/N) and mDCT(Q/N→L/H). These mutations had no effect on protein production *in vitro* (data not shown). We used our epitope mapping strategy to examine the specificity of the T-cell response elicited by immunization of WT and DCT^{-/-} mice with these mutant DCT antigens. We did not identify any novel epitopes (data not shown). Therefore, we will only present the data for the aforementioned epitopes.

The CD4⁺ T-cell response to hDCT₈₈₋₁₀₂ following immunization with AdhDCT(L/H→Q/N) was reduced to a level comparable with the response produced by AdmDCT immunization (compare Fig. 5A, *open diamonds* with Fig. 5B, *closed circles*), although this mutation did not affect immunity to other CD4⁺ T-cell epitopes in hDCT (Fig. 5A). Consistent with the potential role for hDCT₈₈₋₁₀₂ as a helper epitope, the CD8⁺ T-cell response elicited in WT mice following immunization with AdhDCT(L/H→Q/N) was significantly reduced compared with AdhDCT (Fig. 5C) but still higher than the response elicited by AdmDCT (Fig. 5D) possibly due to the presence of other CD4⁺ T-cell epitopes in the mutant hDCT. As expected, AdmDCT(Q/N→L/H) elicited a CD4⁺ T-cell response to hDCT₈₈₋₁₀₂ that was comparable with AdhDCT (compare Fig. 5B, *open circles* with Fig. 5A, *closed diamonds*), confirming that this epitope was appropriately processed and presented by the virus expressing the mutant protein. Strikingly, AdmDCT(Q/N→L/H) elicited a CD8⁺ T-cell response to all three epitopes that was significantly elevated relative to AdmDCT (Fig. 5D) and indistinguishable from the response elicited by AdhDCT (compare Fig. 5D, *open circles* with Fig. 5C, *closed diamonds*), confirming a crucial role for hDCT₈₈₋₁₀₂ in the immunogenicity of hDCT.

Consistent with the role of the DCT₈₈₋₁₀₂ epitope in overcoming tolerance against mDCT, we observed little difference between immunization with the WT and mutant proteins in DCT-deficient mice (Supplementary Fig. S2). We observed a slight decrease in the frequency of CD4⁺ T cells specific for DCT₆₉₋₈₄ in mice immunized with AdhDCT(L/H→Q/N) that we cannot explain at this time. Likewise, we observed a small but significant increase in frequencies of CD8⁺ T cells reactive to DCT₃₄₃₋₃₅₇ following immunization with AdmDCT(Q/N→L/H) possibly due to the additional CD4⁺ T-cell help.

Although these data strongly support the role for hDCT₈₈₋₁₀₂ as a helper epitope, it is also possible that mDCT₈₈₋₁₀₂ is an epitope for regulatory T cells and that the reciprocal mutations ablate the suppressive activity of the regulatory T cells, permitting a more robust CD8⁺ T-cell response by the AdmDCT(Q/N→L/H) vector.

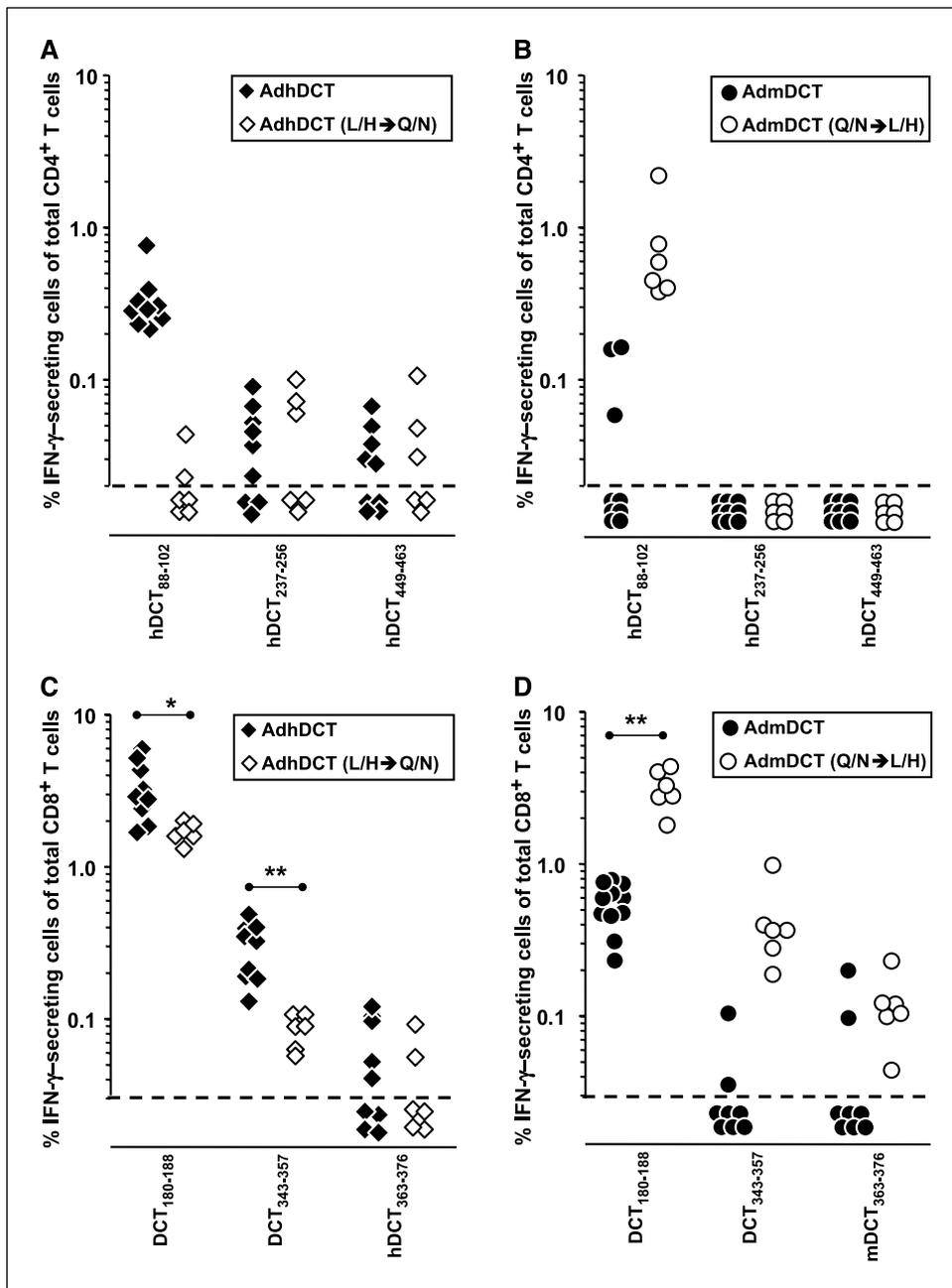


Figure 5. CD8⁺ and CD4⁺ T-cell responses elicited by AdhDCT, AdhDCT(L/H → Q/N), AdmDCT, and AdmDCT(Q/N → L/H) in WT mice. WT mice were immunized with 10⁸ pfu of AdhDCT (◆), AdhDCT(L/H → Q/N) (◇), AdmDCT (●), or AdmDCT(Q/N → L/H) (○). Eight days later, the frequency of epitope-specific T cells in their spleens was quantified by intracellular cytokine staining. *A and B*, X axis, splenocytes were stimulated with CD4⁺ T-cell epitope peptides. *C and D*, X axis, splenocytes were stimulated with CD8⁺ T-cell epitope peptides. *Symbol*, data from an individual mouse; *dashed line*, limit of detection in the assay. *, *P* < 0.05; **, *P* < 0.001.

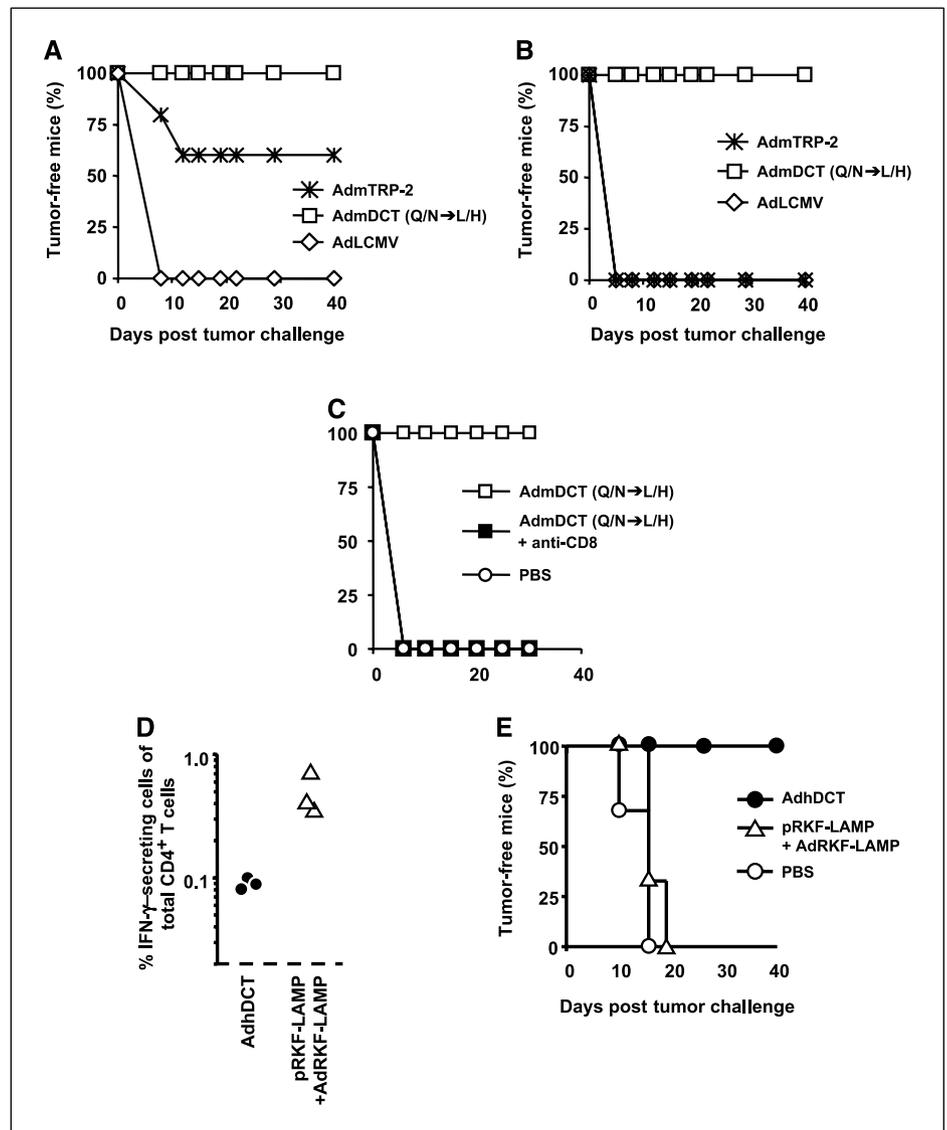
The latter hypothesis is unlikely because mice immunized with adenoviral vectors carrying the mDCT₈₈₋₁₀₂ epitope mount a low-level CD4⁺ T-cell response against DCT₈₈₋₁₀₂ that can be measured as IFN- γ secretion, a marker of conventional CD4⁺ T cells, following stimulation with the heteroclitic hDCT₈₈₋₁₀₂ epitope but not the native mDCT₈₈₋₁₀₂ epitope (Fig. 5A, open diamonds and Fig. 5B, closed circles). Furthermore, if this alternate hypothesis were correct, we would expect to measure immunosuppressive cytokines, such as interleukin (IL)-10 and transforming growth factor- β (TGF- β), following restimulation of splenocytes from AdmDCT-immunized mice with mDCT₈₈₋₁₀₂. We have restimulated splenocytes from AdmDCT with the entire library of mDCT peptides for a period of 24, 72, and 120 h *in vitro* and we were not able to measure production of IL-2, IL-4, IL-10, or TGF- β but we were able to measure low-level IFN- γ production from stimulations with

DCT₁₈₀₋₁₈₈.⁴ Thus, we believe that our data strongly support hDCT₈₈₋₁₀₂ as a helper epitope rather than mDCT₈₈₋₁₀₂ as a suppressor epitope.

hDCT₈₈₋₁₀₂ elicits CD4⁺ T helper cells that augment protective immunity but do not provide direct antitumor effector function. Although the previous data clearly show a helper role for hDCT₈₈₋₁₀₂, it is also possible that hDCT₈₈₋₁₀₂ may contribute to the CD4⁺ T-cell-mediated protection that we have observed previously (11). To characterize the role of the heteroclitic hDCT₈₈₋₁₀₂ epitope in antitumor immunity, mice were immunized with either AdmDCT or AdmDCT(Q/N → L/H) and challenged with

⁴ K. Kianizad, unpublished data.

Figure 6. Protective immunity provided by AdhDCT, AdmDCT, and AdmDCT(Q/N→L/H) in WT mice. *A* and *B*, WT mice were immunized with 10^6 pfu of AdhDCT (circles), AdmDCT (asterisks), AdmDCT(Q/N→L/H) (squares), or AdLCMV (diamonds). Eight days later, mice were challenged with either a low dose (10^5 cells; *A*) or a high dose (10^6 ; *B*) of B16F10 cells. *C*, mice were immunized as in (*B*). ■, one group of mice immunized with AdmDCT(Q/N→L/H) was treated with anti-CD8 antibody (2.43) as described previously (11) on days -2, 0, and +2 relative to challenge with 10^6 B16F10 cells. *D* and *E*, CD8-deficient mice were immunized with 10^6 pfu AdhDCT (●) or a prime-boost regimen consisting of 100 μ g pRKF-LAMP followed 28 d later by 10^6 pfu AdRKF-LAMP (Δ). ○, mice injected with PBS served as a positive control for tumor growth. Seven days later, the frequency of hDCT₈₈₋₁₀₂-specific CD4⁺ T cells in the peripheral blood was assessed (*C*) and mice were challenged with 2×10^4 B16F10 tumor cells (*D*).



a low dose (10^5) and a high dose (10^6) of B16F10 melanoma cells. With regard to low-dose challenge, AdmDCT(Q/N→L/H) provided protection against tumor growth in 100% of immunized mice, whereas AdmDCT only provided protection in 60% of the mice (Fig. 6A). At the higher dose of tumor challenge, none of the AdmDCT-immunized mice were protected from tumor growth, whereas all of the mice immunized with AdmDCT(Q/N→L/H) remained tumor-free (Fig. 6B). Thus, the inclusion of hDCT₈₈₋₁₀₂ in mDCT increases the potency of the immunogen by at least 10-fold relative to mDCT. Consistent with the observation that AdmDCT(Q/N→L/H) evokes a more robust CD8⁺ T-cell response than AdmDCT, we found that protection against the high-dose tumor challenge following AdmDCT(Q/N→L/H) immunization was dependent on CD8⁺ T cells (Fig. 6C). Thus, it seems that the improved protective immunity achieved by AdmDCT(Q/N→L/H) is due to the augmented CD8⁺ T-cell response.

Although the previous data support a role for hDCT₈₈₋₁₀₂ as a helper epitope, we wanted to determine whether hDCT₈₈₋₁₀₂-specific CD4⁺ T cells can provide some degree of protection against B16F10 tumors. To this end, we prepared vaccination vectors

expressing only the hDCT₈₈₋₁₀₂ epitope. A synthetic antigen was constructed (RKF-LAMP) where hDCT₈₈₋₁₀₂ was targeted to the lysosomes using the signal peptide and sorting signal from LAMP-1 as described previously (24). Studying CD4⁺ T-cell-dependent immunity following AdhDCT immunization in WT mice is challenging because CD8⁺ T cells represent the dominant anti-tumor mechanism. We have determined that AdhDCT-mediated protection in CD8-deficient mice is fully CD4⁺ T cell dependent (11),⁵ providing a more suitable model for studies of CD4⁺ T-cell-mediated protection. We have mapped the T-cell epitopes in CD8-deficient mice following AdhDCT immunization and observed that only the three CD4⁺ T-cell epitopes described in this article appear and CD4⁺ T cells reactive against hDCT₈₈₋₁₀₂ dominated the response.⁶ We did not observe any new CD4⁺ T-cell epitopes nor did we measure reactivity to any of the described CD8⁺ T-cell epitopes.⁵ Therefore, we believe that the CD8-deficient mice

⁵ S. Cheng and D. Bernard, in preparation.

⁶ D. Bernard, unpublished data.

accurately reflected the CD4⁺ T-cell component of the AdhDCT immunization. Although a single immunization with 10⁸ pfu AdhDCT completely protected CD8-deficient mice from B16F10 challenge, the same dose of AdRKF-LAMP did not provide any protection against B16F10, although the frequency of hDCT₈₈₋₁₀₂-specific CD4⁺ T cells elicited by AdRKF-LAMP was ~2-fold higher than AdhDCT (data not shown). We subsequently used a prime-boost approach, where CD8-deficient mice were primed with pRKF-LAMP followed 28 days later by a boost with AdRKF-LAMP, resulting in frequencies of hDCT₈₈₋₁₀₂-specific CD4⁺ T cells that were five times greater than the frequencies elicited by AdhDCT (Fig. 6D). In spite of the high frequencies of hDCT₈₈₋₁₀₂-specific CD4⁺ T cells, the mice immunized with RKF-LAMP showed no protection against challenge with a very low dose of B16F10 cells (2 × 10⁴), whereas mice immunized with AdhDCT showed 100% protection (Fig. 6E). Thus, our combined data show that hDCT₈₈₋₁₀₂ serves only as a helper to increase the immunogenicity of DCT but does not contribute directly to the antitumor effector response.

Final comments. Our data have revealed a novel mechanism underlying the increased immunogenicity of xenoantigens where heteroclitic CD4⁺ T-cell epitopes augment T-cell help for the development of an antitumor response. Unlike previous reports where the increased immunogenicity of xenoantigens was due to heteroclitic CD8⁺ T-cell epitopes, our study has shown that xenoantigens can also provide heteroclitic CD4⁺ helper T-cell epitopes that augment CD8⁺ T-cell immunity against conserved CD8⁺ T-cell epitopes. Similarly, a recent publication determined that CD4⁺ T-cell epitopes created by random mutagenesis of mDCT could enhance protective immunity, although the T cells reactive to these epitopes did not cross-react with sequences within the native protein (31). Likewise, insertion of a foreign CD4⁺ T-cell epitope could break tolerance to chicken egg ovalbumin in ovalbumin-transgenic mice and increase immunity against ovalbumin-expressing tumors (33). Additionally, immunization with a vaccine that included a tumor antigen-specific CD4⁺ T-cell epitope was necessary to evoke antitumor CD8⁺ T cells against a virally induced tumor (34); however, in this case, the antigen was a foreign viral antigen, not a self-antigen. We were surprised that the CD4⁺ T-cell epitopes provided by the incoming adenovirus capsid proteins were not sufficient to augment the response to mDCT. However, it is possible that the virion antigens were short lived, whereas the CD4⁺ T-cell epitopes provided by hDCT following AdhDCT immunization should persist for 7 to 14 days based on our previous studies of gene expression following adenoviral infection (22).

These studies have also advanced our understanding of the mechanisms of peripheral tolerance for CD8⁺ T cells. Although negative selection in thymus represents a major mechanism for the elimination of autoreactive CD8⁺ T cells (35), central tolerance is not complete and circulating autoreactive T cells must be controlled through peripheral tolerance (36). An emerging model of peripheral tolerance suggests an active pathway, consisting of tolerogenic dendritic cells and regulatory T cells, is responsible for preventing activation of T cells with reactivity to self (37, 38). Our data provide evidence of a passive method by which the absence of conventional CD4⁺ T cells can regulate autoreactive CD8⁺ T cells in the periphery. As a result of insufficient CD4⁺ T-cell precursors specific for mDCT in WT mice, the available repertoire of DCT-specific CD8⁺ T cells could not be activated by the AdmDCT vaccine. This was not due to an absence of CD4⁺ T-cell epitopes in mDCT. We interpret these results to suggest that the tolerogenic mechanisms that limit the availability of autoreactive CD4⁺ T cells are considerably more stringent than the mechanisms that control the presence of autoreactive CD8⁺ T cells. A heightened stringency for autoreactive CD4⁺ T cells is an effective method for controlling autoreactivity in the periphery, as lack of cognate CD4⁺ T-cell help would also serve to suppress spurious engagement of autoreactive CD8⁺ T cells and B cells, which both require CD4⁺ T-cell help for proper activation. Although we suspect that the frequencies of mDCT-specific CD4⁺ T cells were limited by central tolerance in WT mice, we cannot discount the possibility that the mDCT-reactive CD4⁺ T cells are kept in check through peripheral mechanisms.

In summary, our results have provided novel insight into the mechanism of action of xenoimmunization and reinforce the concept that optimal cancer vaccine design should include robust CD4⁺ T-cell epitopes.

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Elevated Frequencies of Self-reactive CD8⁺ T Cells following Immunization with a Xenoantigen Are Due to the Presence of a Heteroclitic CD4⁺ T-Cell Helper Epitope

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