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$CD4^+$ T Cell Help Selectively Enhances High-Avidity Tumor Antigen-Specific $CD8^+$ T Cells

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Maintaining antitumor immunity remains a persistent impediment to cancer immunotherapy. We and others have previously reported that high-avidity $CD8^+$ T cells are more susceptible to tolerance induction in the tumor microenvironment. In the present study, we used a novel model where T cells derived from two independent TCR transgenic mouse lines recognize the same melanoma antigenic epitope but differ in their avidity. We tested whether providing $CD4^+$ T cell help would improve T cell responsiveness as a function of effector T cell avidity. Interestingly, delivery of $CD4^+$ T cell help during in vitro priming of $CD8^+$ T cells improved cytokine secretion and lytic capacity of high-avidity T cells, but not low-avidity T cells. Consistent with this observation, copriming with $CD4^+$ T cells improved antitumor immunity mediated by higher avidity, melanoma-specific $CD8^+$ T cells, but not T cells with similar specificity but lower avidity. Enhanced tumor immunity was associated with improved $CD8^+$ T cell expansion and reduced tolerization, and it was dependent on presentation of both $CD4^+$ and $CD8^+$ T cell epitopes by the same dendritic cell population. Our findings demonstrate that $CD4^+$ T cell help preferentially augments high-avidity $CD8^+$ T cells and provide important insight for understanding the requirements to elicit and maintain durable tumor immunity. ***The Journal of Immunology*****, 2015, 195: 3482–3489.**

Adoptive cell therapy (ACT) is a promising immunotherapeutic approach designed to amplify the antitumor immune response. However, several factors have hindered its effectiveness. The T cell repertoire available for ACT is constrained. T cells in the periphery with specificity for self/tumor Ags tend to have relatively low avidity due to the mechanisms of tolerance that delete T cells with high affinity for self-antigen in the thymus; the remaining cells are either of low–intermediate avidity or the high-avidity T cells are maintained as unreactive by mechanisms of peripheral self-tolerance (1). Additionally, T cells that do respond to tumor Ags may be hyporesponsive or tolerant to cells expressing their cognate Ag.

High-avidity CTLs require lower Ag concentration for activation and effector function, and they are thus thought to be more effective than low-avidity cells in both antiviral and antitumor immunity (2–4). However, the complexity and cost of generating these high-avidity T cells for ACT prevent the widespread clinical application of this therapy (5). More recently, there is increasing evidence suggesting that these highly avid T cells are more susceptible to functional impairment in the tumor microenvironment (6, 7). As a result, many studies have focused on preventing suppression of high-avidity T cells (8) or, alternatively, mobilizing the endogenous low-avidity T cell repertoire for cancer immunotherapy (9–11).

$CD4^+$ T cells play a pivotal role in generating effective immune responses by sustaining $CD8^+$ T cell proliferation, preventing exhaustion, and establishing long-lived functional T cell memory (12). This is achieved by providing critical regulatory signals that induce expression of cytokines such as IL-2 and IFN-γ, and through cognate interactions such as CD40 ligation on APCs and CD70 on $CD8^+$ T cells (13). Effector $CD4^+$ T cells also play a critical role by altering the tumor microenvironment (14, 15). We reported that continuous provision of tumor Ag-specific $CD4^+$ T cells can prevent tolerization of $CD8^+$ T cells in a murine model of prostate cancer (8). Several reports have also suggested that $CD4^+$ T cells can enhance $CD8^+$ T cell infiltration into tumors or virus-infected tissues (16, 17). Moreover, in addition to these supporting functions, $CD4^+$ T cells alone were reported to exert antitumor activity in animal models and clinical trials (18, 19). Therefore, it is important to consider including $CD4^+$ T cells when designing cancer immunotherapy protocols.

Using a transgenic (Tg) mouse model in which T cells recognize the same epitope of the melanoma Ag tyrosinase-related protein (TRP)-2, but differ in their avidity, we previously reported that in the ACT setting, lower avidity (TCR^{lo}) $CD8^+$ T cells have minimal effect on B16 melanoma tumor growth, despite possessing and maintaining tumor specificity. In contrast, higher avidity (TCR^{hi}) $CD8^+$ T cells delay tumor growth, but they are more susceptible to tolerization in the tumor microenvironment, which may limit their utility. In the present study, we investigated how $CD4^+$ T cell help modulates antitumor immunity by these tumor Ag-specific T cells. We hypothesized that $CD4^+$ T cell help may prevent tolerization of TCR^{hi} T cells as well as enhance antitumor immunity mediated by TCR^{lo} T cells. Strikingly, this was only partially correct, as our findings demonstrate that $CD4^+$ T cell help only improved tumor immunity by higher avidity T cells.

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Abbreviations used in this article: ACT, adoptive cell therapy; BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; GrB, granzyme B; Tg, transgenic; TRP, tyrosinase-related protein.

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Materials and Methods

Experimental mice

C57BL/6 mice were purchased from the National Cancer Institute Animal Production Area Facility (Charles River Laboratories, Frederick, MD). OT-II TCR Tg mice were a gift from Howard Young (National Cancer Institute, Frederick, MD). The TCR Tg mouse strain 24H9 (TCR^{hi} mice) and 37B7 (TCR^{lo} mice) bear distinct TCR transgenes that recognizes an H-$2K^b$–restricted epitope of TRP-$2_{180–188}$ and were described previously (7, 20). The $CD4^{TRP-1}$ mice were a gift of Dr. Nick Restifo (National Cancer Institute) (18, 19). Mice were housed under specific pathogen-free conditions and were treated in accordance with National Institutes of Health guidelines under protocols approved by the Animal Care and Use Committee of the Frederick National Laboratory for Cancer Research facility.

Cell line and peptides

B16-BL6, hereafter referred to as B16, a TRP-2–expressing murine melanoma cell line, was maintained in culture media as previously described (20). TRP-$2_{180–188}$ (SVYDFFVWL), TRP-$1_{106–130}$ (SGHNCGTCRPGWRGAACNQKILTVR), and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) peptides were purchased from New England Peptide (Gardner, MA).

Coculture of $CD4^+$ and $CD8^+$ T cells in vitro

Lymph node cells and splenocytes from TCR^{hi} or TCR^{lo} Tg mice were cocultured with $CD4^+$ OT-II T cells that recognize an epitope of OVA (hereafter referred to as $CD4^{OVA}$ T cells) in complete DMEM with 1 μM TRP-$2_{180–188}$ and 1 μM $OVA_{323–339}$ peptide. Three to five days later, cells were centrifuged over Ficoll to remove dead cells and were purified with $CD8^+$ T lymphocyte enrichment set (BD Biosciences) according to the manufacturer's instructions and used as effector T cells.

ELISPOT assays

Multiscreen plates (Millipore) were coated with 100 μl IFN-γ Ab (BD Biosciences) or granzyme B (GrB) (R&D Systems) capture Ab overnight at 4°C. Purified $CD8^+$ TCR T cells were added to increasing concentrations of TRP-$2_{180–188}$. After incubation, plates were washed and processed as previously described (8).

CFSE labeling for in vivo cytotoxicity assay and flow cytometric analysis of T cells

Lymph node cells from $TCR^{hi}Thy1.1^+$ and OT-II mice were dispersed into a single-cell suspension and transferred into the recipient mice on day 0. The following day, mice were vaccinated with TRP-$2_{180–188}$ or TRP-$2_{180–188}$ and OVA peptide–pulsed bone marrow–derived dendritic cells (BMDCs). Eleven days after vaccination, splenocytes were labeled with different levels of CFSE (5 or 0.5 μM) for 10 min at room temperature, washed in DMEM supplemented with 2% of FBS, and resuspended in 0.5 ml HBSS. Splenocytes labeled with high doses of CFSE were then pulsed with TRP-2 peptide. Splenocytes were transferred into recipient mice by tail vein injection at a 1:1 (peptide pulsed/unpulsed) ratio. The following day, vaccine-draining lymph nodes were analyzed for the two populations of CFSE-labeled splenocytes. Calculation of specific killing was described previously (20).

Tumor or vaccine-draining lymph node cells (axillary, brachial, and inguinal) or splenocytes were incubated with Abs directed against Thy1.1, CD8, and CD45.1. Intracellular IFN-γ and CD107a (LAMP1) expression from spleen and tumor-infiltrating lymphocytes were analyzed as described previously (20).

Generation of BMDCs and vaccination

On day 0, RBC-depleted bone marrow cells isolated from femurs and tibia were plated in 10-cm tissue culture dishes in complete RPMI 1640 medium supplemented with 15% supernatant from a GM-CSF–secreting EL-4 cell line (20). On day 2, nonadherent cells were washed from dishes and fresh media containing GM-CSF was added. On day 4, cultures were refed with fresh medium supplemented with GM-CSF. Nonadherent cells were harvested from culture dishes on day 7 and pulsed with TRP-$2_{180–188}$ (5 μM) and/or OVA (1 μM) overnight. The following day, the nonadherent cells were harvested, washed twice with HBSS, and resuspended in HBSS. Mice were vaccinated s.c. with control (unpulsed) or peptide-pulsed DCs (2.5×10^5/100 μl HBSS) on each of the left and right dorsal flanks.

Adoptive transfer of Tg T cells to treat s.c. B16 tumor

Four or 11 d after B16 tumor challenge, 2×10^6 Ag-specific $CD8^+$ TCR^{hi} or TCR^{lo} T cells or 4×10^6 $CD4^{OVA}$ T cells were adoptively transferred into tumor-bearing B6 mice. The following day after T cell transfer, mice were vaccinated s.c. with TRP-2 and/or OVA peptide–pulsed BMDCs as previously described (20). Tumor size was estimated by measuring perpendicular diameters using a caliper. Mice were euthanized when tumor area was >exceeded 400 mm^2 and tumor size was recorded as 400 mm^2 thereafter. Mice that died with a smaller tumor were assigned a final measurement of the tumor area at the time of death.

Estimation of DC apoptosis induced during in vitro TCR T cell priming

Lymph node cells from TCR^{hi} or TCR^{lo} Tg mice were cocultured with peptide-pulsed (5 μM TRP-$2_{180–188}$ and 1 μM $OVA_{323–339}$) $CD45.1^+$ BMDCs in the presence or absence of $CD4^{OVA}$ T cells. The cells were cultured in medium supplemented with 2-ME and 100 U/ml mouse IL-2. The adherent and nonadherent cell fractions were collected 48 h after coculture and the frequency of apoptotic cells among the $CD45.1^+$ BMDCs was determined by flow cytometric analysis for annexin V and propidium iodide staining according to the manufacturer's instructions (BD Biosciences).

Statistical analysis

Statistical analyses for differences between group means were performed by an unpaired Student *t* test or one-way ANOVA. A p value <0.05 was considered statistically significant. Prism 5.0 software was used to analyze the data (GraphPad Software). For DC apoptosis studies, a Student *t* test was performed to determine significant differences between the different groups. A randomized-blocks design was used to compare statistical significance across experiments.

Results

$CD4^+$ T cell help selectively enhanced TCR^{hi} T cell effector functions

We and others have previously reported that provision of $CD4^+$ T cell help is critical to establish and maintain effective $CD8^+$ T cell–mediated antitumor immunity (8). In this study, we sought to study the differential effects of $CD4^+$ T cell help as a function of CD8 T cell avidity. We therefore cocultured OVA-specific $CD4^+$ T cells ($CD4^{OVA}$) with TRP-2 specific TCR Tg $CD8^+$ T cells with APCs and their respective cognate Ags for 3 d, followed by enrichment of the $CD8^+$ T cells by negative selection. Effector function was tested by determining IFN-γ and GrB production using ELISPOT analysis. Consistent with our previous report (7), the higher avidity TCR^{hi} T cells produced more IFN-γ and GrB than did TCR^{lo} T cells. However, activation of $CD4^{OVA}$ cells in the culture only enhanced the expression of these indicators of effector function by TCR^{hi} T cells (Fig 1A).

We next tested the ability of $CD4^+$ T cell help to improve cytolytic function in vivo. TCR Tg T cells were administered to mice in the presence of $CD4^{OVA}$ T cells and subsequently vaccinated with BMDCs pulsed with TRP-2 and OVA peptides. As target cells, Ag-pulsed splenocytes were labeled with CFSE, and Ag-specific killing was assessed. Our results were again consistent with our previous report (7), demonstrating that TCR^{hi} T cells more efficiently kill TRP-2–pulsed target cells than do TCR^{lo} T cells. Of note, cotransfer with $CD4^{OVA}$ T cells significantly enhanced lysis of targets by TCR^{hi} T cells, but not TCR^{lo} T cells (Fig 1B). Taken together, these data demonstrate that provision of $CD4^+$ T cell help selectively enhances TCR^{hi} T cell effector functions.

$CD4^+$ T cell help differentially enhanced the capacity of TCR T cells to suppress B16 tumor growth

Based on the observed differences in IFN-γ, GrB production, and cytotoxicity between TCR^{hi} and TCR^{lo} T cells, we next sought to determine the effect of $CD4^+$ T cell help on antitumor activity by the TRP-2–specific TCR T cells. Four days after B16 tumor implantation, mice were given TCR Tg T cells and $CD4^{OVA}$ T cells and on the following day, mice were vaccinated with peptide-pulsed BMDCs. As shown in Fig. 2A, and consistent with previously reported results (7), adoptive transfer of TCR^{hi} T cells in

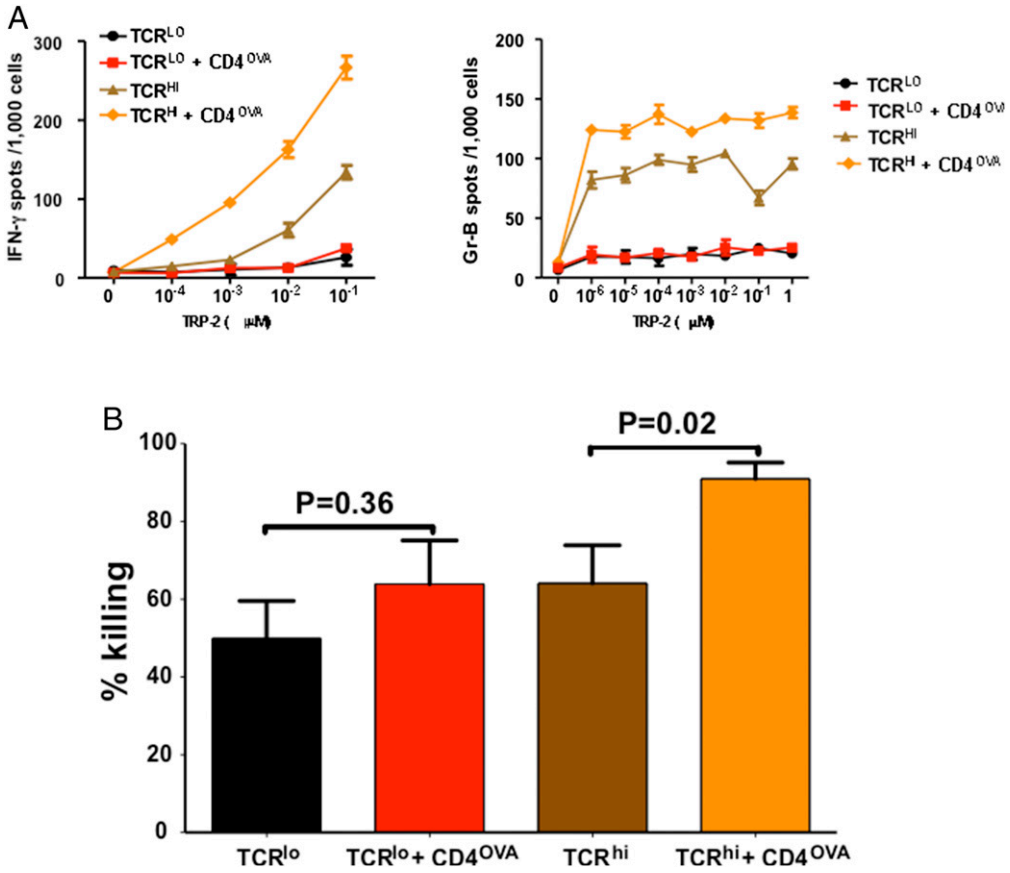

FIGURE 1. CD4$^+$ T cell help enhances TCR^{hi} T cell effector functions. (**A**) TRP-2– specific TCR Tg T cells and CD4^{OVA} T cells were cocultured with APCs and their respective cognate Ags TRP-2 (1 μM) and OVA (1 μM) for 3 d, followed by enrichment of the CD8$^+$ effector cell by negative selection. Production of IFN-γ (*left*) and GrB (*right*) by effector cells was tested by ELISPOT. Data are representatives of three studies with similar results. (**B**) Lysis of TRP-2–pulsed target cells was assessed by injecting mice with CFSE-labeled splenocytes after priming TRP-2–specific TCR T cells in the presence of CD4^{OVA} T cells with BMDCs pulsed with TRP-2 peptide and OVA. Data are presented as pooled results from three independent studies. An unpaired Student *t* test was used to compare groups.

combination with a peptide-pulsed BMDC vaccine delayed B16 tumor progression. Cotransfer of CD4^{OVA} cells significantly improved suppression of tumor growth by TCR^{hi} cells (Fig. 2A, *left*). Tumor incidence was ~10% among mice treated with CD4^{OVA} help and TCR^{hi} T cells; this is in contrast to a frequency of 100% in mice treated with TCR^{hi} T cells alone. Surprisingly, no significant change in tumor growth or tumor incidence was observed when TCR^{lo} T cells were delivered with CD4^{OVA} help. Similar results were observed when tumor Ag-specific CD4 T cells (CD4$^{TRP\text{-}1}$) were cotransferred and primed with their cognate Ag, TRP-1, in combination with the TCR Tg T cells (Fig. 2A, *bottom*). Consistent with published results (19), the CD4$^{TRP\text{-}1}$ T cells display inherent antitumor activity and, therefore, subsequent studies were performed using the CD4^{OVA} T cells.

We next tested the effectiveness of CD4 T cell help to enhance antitumor activity by TCR^{hi} T cells in a more established tumor model. TCR^{hi} T cell and CD4^{OVA} T cells were transferred 11 d after tumor implantation, when the tumor is palpable, and then sensitized with a DC vaccine the following day. We found that addition of CD4^{OVA} cells was again able to improve TCR^{hi} T cell control of tumor growth (Fig 2A, *right*), which alone only slightly delayed tumor growth.

To study the effect of CD4$^+$ T cell help on the expansion of TCR Tg T cells after transfer into tumor-bearing hosts, we evaluated T cell numbers in the lymph node, spleen, and tumor based on expression of the Thy1.1 congenic marker by the transferred T cells, which distinguishes them from the Thy1.2$^+$ host T cells. Surprisingly, compared with TCR^{hi} T cells, we observed significantly higher numbers of TCR^{lo} T cells in the lymph node, spleen, and tumor. Although cotransfer of CD4^{OVA} T cells significantly increased both TCR^{hi} and TCR^{lo} T cell number in the lymph node and spleen, provision of T cell help only enhanced TCR^{hi} T cell infiltration of B16 tumors (Fig. 2B).

The increased infiltration of TCR^{hi} T cells raises the possibility that improved tumor immunity may be due to increased effector numbers in the tumor microenvironment following priming in the presence of CD4$^+$ T cell help. To address this possibility, and in an attempt to mimic the frequency of cells that infiltrate tumor following CD4^{OVA} transfer and priming, we increased the number of TCR^{hi} T cells transferred into tumor-bearing hosts. As shown in Fig. 2C, doubling the number of TCR^{hi} T cells did not reduce tumor growth to levels observed following cotransfer of CD4$^+$ T cell help, despite increasing the number of TCR^{hi} cells that infiltrate the B16 tumors (Fig 2D). Collectively, our data suggest that rather than simply improving T cell expansion, CD4$^+$ T cell help selectively enhanced high-avidity T cells by conditioning them to provide more potent antitumor immunity.

CD4$^+$ T cells and TCR^{hi} T cells must be primed by the same DC population to provide optimal responses

We next sought to determine whether both effector and Th cells need to be primed by the same APC. Tumor-bearing mice were transferred with TCR^{hi} T cells and CD4^{OVA} T cells. The following day, they were vaccinated with either DCs that were pulsed with

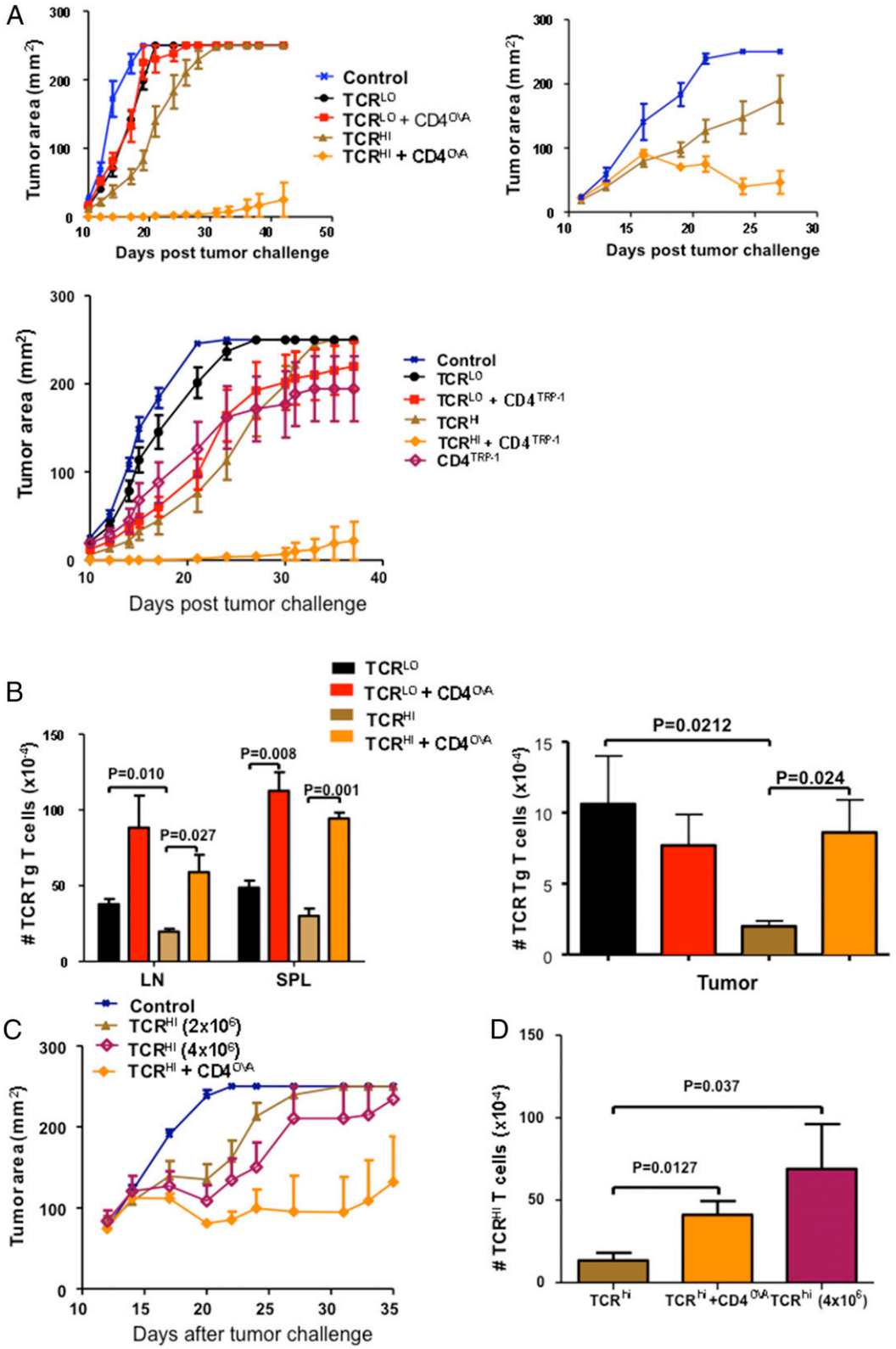

FIGURE 2. CD4$^+$ T cell help enhances antitumor activity of TCR^{hi} T cells. (**A**, *top*) Mice were injected s.c. with B16 tumor cells on day 0, and four (*left*) or 11 (*right*) days later, mice were transferred with TCR Tg T cells and CD4^{OVA} T cells. The following day, mice were vaccinated with peptide-pulsed BMDCs. Tumor size was monitored. Data are representative of at least four similar studies. (A, *bottom*) Mice were injected s.c. with B16 tumor cells on day 0. Four days later, mice were transferred with 2×10^6 TCR Tg T cells and 1×10^5 purified CD4^{TRP-1} T cells. On the following day, mice were vaccinated with TRP-2 and TRP-1 peptide–pulsed BMDCs. Tumor size was monitored. Data are representative of at least three similar studies. (**B**) Six days after DC vaccine, TCR Tg T cells numbers were calculated in lymph node and spleen (*left*) and tumor (*right*). Data are representative of at least four separate studies. (**C** and **D**) Mice were injected s.c. with B16 tumor cells on day 0. On day 11, the indicated doses of TCR^{hi} T cells were delivered i.v. The following day, mice were vaccinated with peptide-pulsed BMDCs. Tumor size was monitored (C) or tumor-infiltrating TCR^{hi} T cell numbers were calculated on day 25 (D). The experiment was performed three times with similar results. (C) presents data from one representative study and (D) presents pooled data for all three studies.

both TRP-2 and OVA peptides (as above) or a mix of DCs pulsed with either Ag, alone. As shown in Fig. 3A, antitumor activity was significantly reduced when TRP-2 and OVA were not presented by the same DC. We also observed a significantly greater number of TCR^{hi} T cells in the lymph node and spleen when the mice were vaccinated with DC loaded with both Ags (Fig. 3B). Thus, optimal activation of $CD8^+$ T cells and induction of antitumor activity depend on priming of both effector $CD8^+$ T cells and helper $CD4^+$ T cells by the same APC.

Some studies suggest that DCs can become resistant to apoptosis following licensing by CD40 ligation or interactions with activated T cells (21, 22). If priming of TCR^{hi} T cells in the presence of T cell help leads to greater DC survival, this might explain their selective enhancement by provision of T cell help. Therefore, we tested whether providing $CD4^+$ T cells altered susceptibility of DCs to apoptosis following priming of TcR Tg T cells. Our initial studies attempted to track DCs following in vivo priming; however, we were unable to reliably detect vaccine DCs by flow cytometry. Therefore, we performed in vitro studies using mixed cultures of T cells and DCs. As shown in Supplemental Fig. 1, levels of DC apoptosis were similar in cultures with TCR^{hi} or TCR^{lo} T cells. Surprisingly, the addition of $CD4^{OVA}$ T cells and their cognate Ag resulted in great apoptosis of DCs in mixed cultures with TCR^{hi} T cells compared with those with TCR^{lo} T cells. These findings suggest that DC survival at the time of priming was not a critical factor in the enhancement of tumor control by TcR^{hi} T cells by CD4 T cells.

$CD4^+$ T cell help reduces tolerization of adoptively transferred TCR^{hi} T cells

We previously demonstrated that TCR^{hi} T cells are more susceptible to tolerization in the tumor microenvironment, which limited tumor immunity (7). Given our observation that $CD4^+$ T cell help improved tumor immunity, we next tested the possibility that provision of $CD4^+$ T cell help could prevent tolerization of TCR^{hi} T cells. We were unable to accurately assess the function of tumor-infiltrating TCR^{hi} T cells ex vivo, so we studied splenic TCR^{hi} T cells, which we reported also display progressive loss of function (7). As indicated in Fig. 4A, a significant increase in the frequency of IFN-γ–expressing TCR^{hi} T cells in the spleen was observed following priming in the presence of $CD4^{OVA}$ T cells. However, no change in the already high level of CD107a mobilization was noted. Additionally, delivery of a 2-fold dose of TCR^{hi} T cells, which did not reduce tumor growth but mimicked the level of infiltration of B16 by TCR^{hi} T cells following priming with $CD4^+$ T cell help (Fig. 2C), did not alter the frequency of splenic IFN-γ–producing TCR^{hi} T cells. Taken together, these data suggest that priming TCR^{hi} T cells in the presence of $CD4^+$ T cell help may program the TCR^{hi} T cells to resist or delay tolerization.

We previously reported that the continuous provision of tumor Ag-specific $CD4^+$ T cell help enhanced $CD8^+$ T cell–mediated antitumor immunity in a murine model of prostate cancer (8). Because we also observed that a small fraction of $CD4^{OVA}$ T cells infiltrate B16 tumors after in vivo priming with DC vaccination (Fig. 4B), we tested whether multiple transfers of $CD4^{OVA}$ T cells would maintain TCR^{hi} control of B16 tumor growth. To our surprise, no additional antitumor effects were observed when sustained $CD4^+$ T cell help was delivered (Fig. 4C). These results suggest that $CD4^{OVA}$ T cell help may principally influence the priming of TCR^{hi} T cells and at the given numbers were unable to prevent the eventual tolerization in the tumor microenvironment.

Discussion

In this study, we tested whether $CD4^+$ T cell help would promote more robust T cell responses directed against a defined melanoma Ag. We observed that provision of $CD4^+$ T cell help enhanced cytokine secretion and cytolytic capacity of high-avidity T cells in vitro and antitumor activity in vivo, but it had only minimal effects on low-avidity T cells with identical specificity. The lower

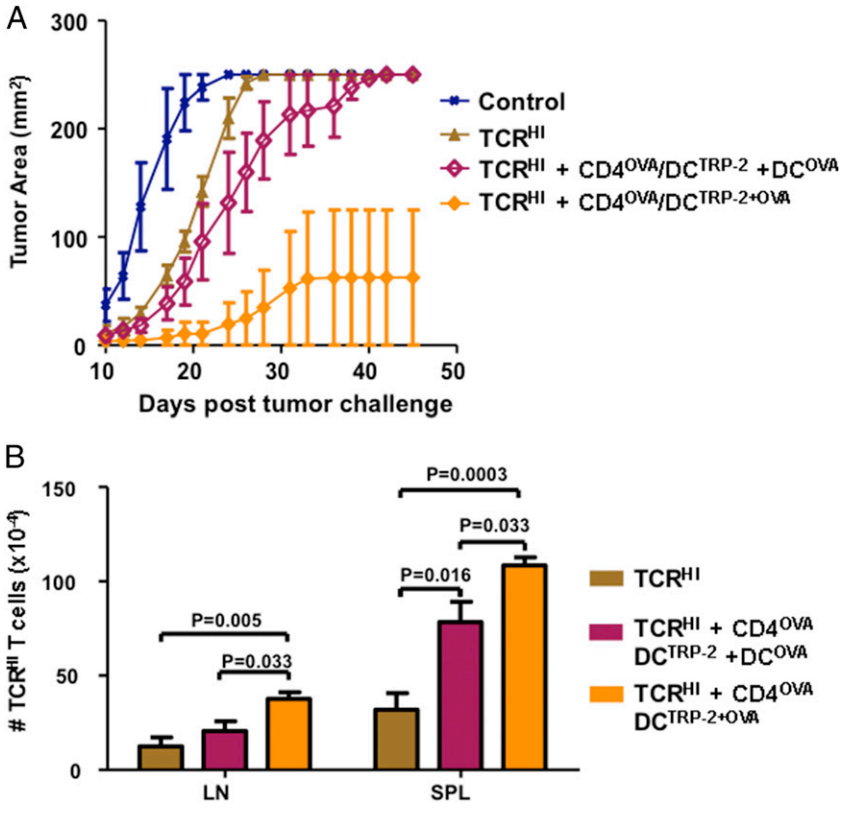

FIGURE 3. Optimal activation of TCR^{hi} T cells depends on priming of both $CD8^+$ and $CD4^+$ T cells by the same DC. (**A**) Mice were injected s.c. with B16 tumor cells on day 0. Four days later, mice were transferred with either TCR^{hi} T cells in the presence or absence of $CD4^{OVA}$ T cells. The following day, mice were vaccinated with BMDCs that were pulsed with both TRP-2 and OVA peptides or vaccinated with a mix of BMDCs pulsed with either Ag, alone. Tumor size was monitored. The experiment was performed three times with similar results and one representative study is presented. (**B**) The number of TCR^{hi} T cells in the lymph node and spleen was calculated for mice treated as described in (A). An unpaired Student *t* test was used to compare the groups. The experiment was performed three times with similar results and one representative study is presented.

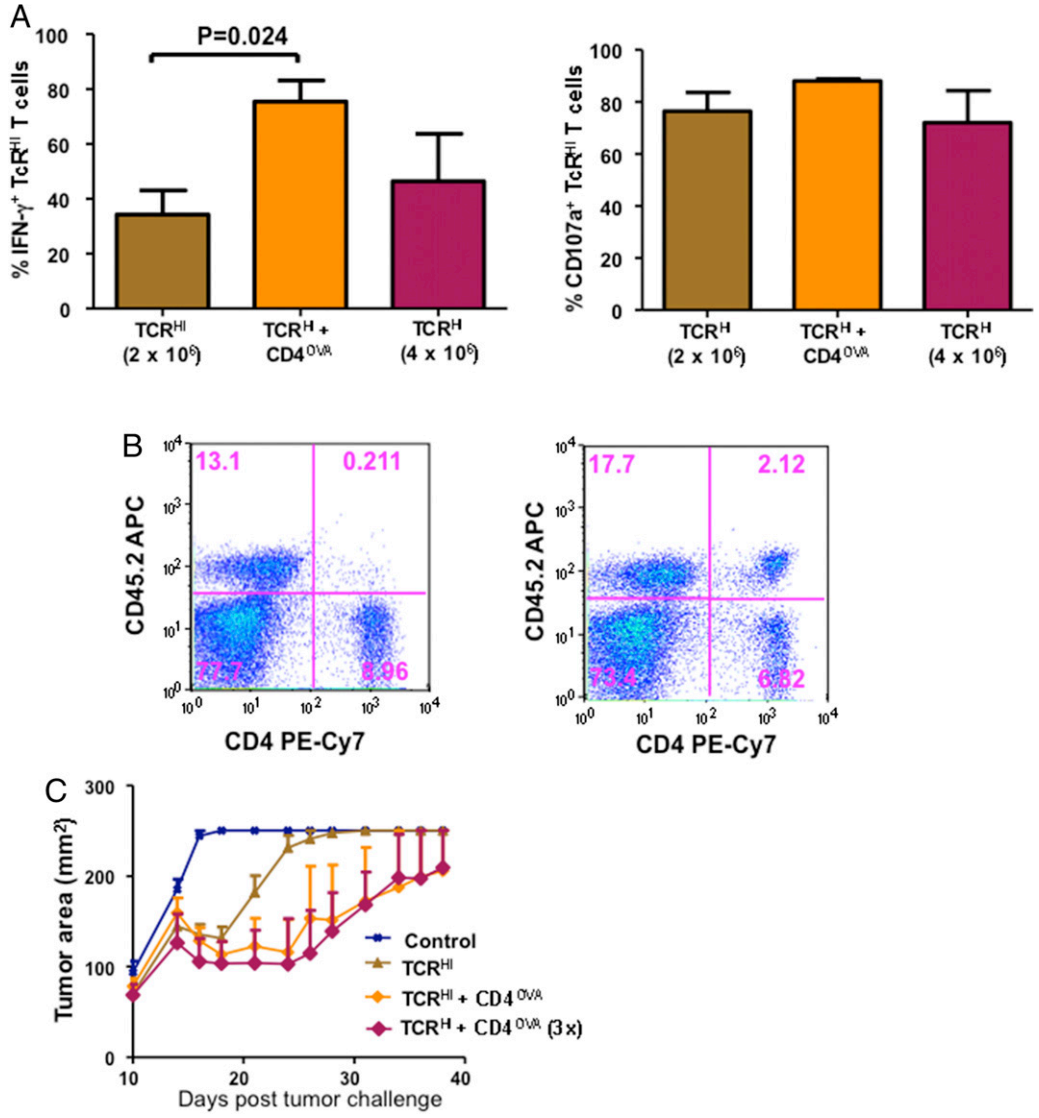

FIGURE 4. CD4 T cell help reduces TCR^{hi} T cell tolerization. (**A**) Wild-type mice were injected s.c. with 1×10^5 B16 tumor cells on day 0. On day 11, mice were transferred with TCR^{hi} T cells alone or with $CD4^{OVA}$ T cells. The following day, mice were vaccinated with peptide-pulsed BMDCs. Twelve days after DC vaccination, mice were euthanized and splenocytes were analyzed for IFN-γ expression and CD107a mobilization. Data are pooled from three experiments using three mice per group. (**B**) $CD45.1^+$ mice were injected as in (A), with the exception that $CD45.2^+$ $CD4^{OVA}$ T cells were used. Five days after BMDC vaccination, mice were euthanized and tumors were tested for infiltration by $CD4^{OVA}$ T cells ($CD4^+CD45.2^+$). The *left panel* shows control mice that did not receive transfer of $CD4^{OVA}$ T cells; the *right panel* shows mice that received $CD4^{OVA}$ T cells. The experiment was performed twice with similar findings. (**C**) Mice were challenged with tumor and treated as in (A). In one group, two additional doses of $CD4^{OVA}$ cells were adoptively transferred on days 16 and 24, followed by vaccination with OVA-pulsed BMDCs the following day. Tumor size was monitored. Data are representative of two similar studies.

avidity TCR^{lo} T cells exhibit strong proliferative expansion in vitro (data not shown) and in vivo, but they exert weaker effector functions than the higher avidity TCR^{hi} T cells. The mechanism by which this functional uncoupling of expansion and effector function remains unknown. Interestingly, to achieve greatest enhancement of TCR^{hi} T cell function, both $CD4^+$ T cells and effector $CD8^+$ T cells required priming by DCs pulsed with both epitopes.

A dominant role for $CD4^+$ T cells in promoting tumor immunity has been studied extensively (12). We and others have reported that provision of exogenous $CD4^+$ T cell help enhances antitumor immunity (8, 17, 23, 24), in part by enhancing $CD8^+$ T cell responses. However, many of these studies have focused on tumor Ag-specific $CD4^+$ T cells, where it is well known that similar to viral infections, tumor Ag-specific $CD4^+$ T cells may be tolerized or exhausted (25–27) or converted into suppressive regulatory T cells, leading to reduced $CD8^+$ T cell responses, as well. In our present study, we demonstrated that provision of $CD4^{OVA}$ T cells, which principally provide help during $CD8^+$ T cell priming, selectively enhanced high-avidity $CD8^+$ T cell antitumor immunity. These findings are consistent with a previous report demonstrating that heterospecific $CD4^+$ T cells could sustain reactivity of memory tumor-specific $CD8^+$ T cells (28). Interestingly, the effector $CD8^+$ T cells in that study were endogenous T cells that responded to Lewis lung carcinoma Ags, suggesting they may be a mix of both low- and high-avidity T cells. As suggested by the authors, those findings cannot rule out the possibility that the $CD4^+$ T cells may also support endogenous tumor-specific $CD4^+$ T cells, as well. However, taken together with our present studies, these findings support the possibility that provision of bystander $CD4^+$ T cell help may provide a more effective way of maintaining tumor-specific $CD8^+$ T cells.

We further showed that cotransfer of the $CD4^+$ T cells significantly increased the number of both TCR^{hi} and TCR^{lo} T cells in the lymph node and spleen, but only enhanced the frequency of TCR^{hi} T cells infiltrating B16 tumors. These findings highlight the importance of $CD4^+$ T cells in the accumulation of $CD8^+$ T cells in a variety of different tissues. This may involve multiple mechanisms, including enhanced proliferation, trafficking, and infiltration, as well as reduced apoptosis of CTLs. In vitro studies suggested that provision of T cell help did not reduce DC apoptosis during effector T cell priming. Bos and Sherman (17) showed that $CD4^+$ T cells improved survival of $CD8^+$ T cells in the tumor microenvironment. This is consistent with the studies of Schoenberger and colleagues (29), who demonstrated that helpless $CD8^+$ T cell express elevated levels of TRAIL, which contributes to their apoptotic death. However, we did not observe any effect of $CD4^{OVA}$ T cells on apoptosis of TCR^{hi} T cells (data not shown). Interestingly, when we transferred increased numbers of TCR^{hi} T cells, in an effort to mimic the increased infiltration of TCR^{hi} T cells in the tumors following cotransfer of the $CD4^{OVA}$ T cells, we did not detect any improvement of antitumor immunity. These findings suggest that beyond enhanced expansion, $CD4^+$ T cell help programs the TCR^{hi} T cells for improved antitumor activity, which may also include resistance to immune suppression within the tumor microenvironment. This loss of T cell functionality may occur through a variety of mechanisms, including tolerization, suppression, or exhaustion through engagement of inhibitory checkpoint receptors.

We previously reported that compared with TCR^{lo} T cells, TCR^{hi} T cells were more susceptible to tolerization in the tumor microenvironment, as marked by reduced mobilization of CD107a and expression of IFN-γ (7). In the present study, we demonstrated that provision of $CD4^+$ T cell help delayed TCR^{hi} T cell tolerization, similar to our previous studies using the TRAMP model of prostate cancer (8). However, repeated delivery of $CD4^{OVA}$ T cells was unable to sustain immunity to B16 melanoma, unlike our observations in the TRAMP model. This may be due, in part, to the lack of OVA expression by the tumor, as well as the relatively weak infiltration of $CD4^{OVA}$ cells into the B16 tumors. Consistent with this, at least one previous report suggested that tumor infiltration by $CD4^+$ T cell help is required for augmenting antitumor activity (17). Tumor-specific $CD4^{TRP\text{-}1}$ T cells infiltrate tumors in appreciable numbers, but as mentioned above, they also display inherent antitumor activity.

In the present study, we also demonstrated that to generate the most efficient antitumor response, both $CD4^+$ and $CD8^+$ T cells must be primed by the same DC. Two possible mechanisms may explain this observation. The first is the classical three cell interaction, where $CD4^+$ T cells are brought into close proximity to the $CD8^+$ T cells because the same DC presents both epitopes. In this scenario, the $CD4^+$ T cells may secrete cytokines that help prime (or condition) the $CD8^+$ T cells. Alternatively, the $CD4^+$ T cells may activate, or license, the DCs, which then subsequently prime the $CD8^+$ T cells. This mechanism might involve the CD40 axis, which is known to render DCs more potent for priming T cells (30, 31). Our data do not rule out either possibility, or the combination of both mechanisms, and ongoing studies are testing these possibilities.

Although our study demonstrates that provision of CD4 help preferentially augmented TCR^{hi} T cells both in vitro and in vivo, Sherman and colleagues (14, 32) reported that tumor Ag-specific $CD4^+$ T help increased expansion as well as effector functions of both high- and low-avidity tumor Ag-specific $CD8^+$ T cells, which resulted in tumor eradication. In those studies, both $CD4^+$ and $CD8^+$ T cells recognized a surrogate tumor Ag that was expressed as a transgene by an autochthonous tumor. The discrepancies between these studies may be related to the relative avidity of the T cells, the expression level of the tumor Ag, or the overall complexity of the tumor microenvironment. In a recent study, using melanoma Ag-specific $CD4^+$ T cells, Church et al. (24) reported that $CD4^+$ T cells improved $CD8^+$ T cell–mediated tumor immunity by reducing PD-1 expression by the $CD8^+$ T cells. Interestingly, our previous study demonstrated that PD-1 limits the reactivity of higher avidity T cells (7). If the TCR^{lo} T cells are not limited by PD-1 expression, then this may explain why $CD4^+$ T cell help cannot further enhance their ability to control tumor growth, which may principally be limited by their lower avidity.

Some limitations of our model system exist. Although easy to use to track T cell responses, the use of monoclonal populations of TCR Tg T cells restricts the magnitude and diversity of the response. Moreover, we have only tested one clone of each population of T cell. Expansion of the studies to multiple clones for each avidity class would further validate the findings, but they are technically challenging. However, we are exploring the possibility of using display approaches to diversify the TCR^{lo} T cells and increase their avidity to match that of TCR^{hi} T cells.

Taken together, our findings demonstrate that provision of $CD4^+$ T cell help preferentially augmented higher avidity $CD8^+$ T cell–mediated antitumor immunity. Because high-avidity T cells are commonly used in clinical trials for adoptive T cell therapy, these findings may have important implications for cancer immunotherapy. A greater understanding of the mechanisms by which $CD4^+$ T cells help boosts tumor immunity by higher avidity T cells will lead to approaches that confer more durable tumor immunity.

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Disclosures

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