Microenvironment and Immunology

High-Avidity T Cells Are Preferentially Tolerized in the Tumor Microenvironment

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Abstract

One obstacle in eliciting potent antitumor immune responses is the induction of tolerance to tumor antigens. TCR^{lo} mice bearing a TCR transgene specific for the melanoma antigen tyrosinase-related protein-2 (TRP-2, *Dct*) harbor T cells that maintain tumor antigen responsiveness but lack the ability to control melanoma outgrowth. We used this model to determine whether higher avidity T cells could control tumor growth without becoming tolerized. As a part of the current study, we developed a second TRP-2–specific TCR transgenic mouse line (TCR^{hi}) that bears higher avidity T cells and spontaneously developed autoimmune depigmentation. In contrast to TCR^{lo} T cells, which were ignorant of tumor-derived antigen, TCR^{hi} T cells initially delayed subcutaneous B16 melanoma tumor growth. However, persistence in the tumor microenvironment resulted in reduced IFN-γ production and CD107a (*Lamp1*) mobilization, hallmarks of T-cell tolerization. IFN-γ expression by TCR^{hi} T cells was critical for upregulation of MHC-I on tumor cells and control of tumor growth. Blockade of PD-1 signals prevented T-cell tolerization and restored tumor immunity. Depletion of tumor-associated dendritic cells (TADC) reduced tolerization of TCR^{hi} T cells and enhanced their antitumor activity. In addition, TADCs tolerized TCR^{hi} T cells but not TCR^{lo} T cells *in vitro*. Our findings show that T-cell avidity is a critical determinant of not only tumor control but also susceptibility to tolerization in the tumor microenvironment. For this reason, care should be exercised when considering T-cell avidity in designing cancer immunotherapeutics. *Cancer Res; 73(2); 595–604. ©2012 AACR.*

Introduction

Many tumor antigens are derived from self-antigens that are tissue-specific differentiation antigens. These include melanoma antigens such as Melan-A, Gp-100, tyrosinase, tyrosinase-related protein-1, and tyrosinase-related protein-2 (TRP-2; refs. 1–3). These melanocyte differentiation antigens (MDA) are expressed by both melanoma cells and normal melanocytes. Immune responses against MDAs are regulated by central and peripheral tolerance mechanisms. Most high-avidity, self-reactive CD8^{+} T cells are deleted during thymic selection (4, 5). Some lower avidity, self-reactive T cells escape thymic deletion and persist in the periphery under the control of various peripheral tolerance mechanisms, including anergy, suppression, ignorance, and deletion (6–8). We and others have reported that tolerance to TRP-2 could be broken with various vaccination protocols, and provision of granulocyte macrophage colony-stimulating factor (GM-CSF) was able to improve the weaker autoimmune response to TRP-2 into a more potent antitumor response (9, 10).

While a longstanding goal of cancer immunotherapy is the generation of an adequate number of tumor-specific CD8^{+} T cells that are capable of effectively clearing the tumor, not only the quantity but also the "quality" of the cytotoxic T lymphocytes (CTL) is critical. Accordingly, CTLs can be classified on the basis of their avidity for antigen-bearing targets. High-avidity CTLs require lower antigen concentration for activation and effector function and are thought to be more effective than low-avidity cells in both antiviral and antitumor immunity (11–13). In fact, a significant effort in adoptive T cell therapy has focused on generating tumor-specific T cells with high avidity for tumor antigens either by isolating them based on MHC/antigen tetramer staining, or more recently, through transduction of genes encoding high-affinity, tumor-reactive T cell receptors (TCR) into patients' peripheral blood T cells (14, 15).

Adoptive immunotherapy with *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) has achieved objective clinical responses in a significant fraction of patients with metastatic

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melanoma (16, 17). However, the failure of many patients to develop long-term tumor control may be, in part, due to tolerization of transferred T cells (18). Our laboratory has reported that in an experimental model of prostate cancer, tumor antigen–specific CD8^{+} T cells become tolerized after infiltrating tumor tissue (19–21). One previous study suggested that recognition of a xenogeneic, tumor-associated antigen by higher avidity T cells leads to increased susceptibility to tolerization (22). Given the recent excitement about genetic transfer of higher affinity TCRs to confer higher avidity to T cells for adoptive immunotherapy (23, 24), a better understanding of the role of T-cell avidity in T-cell tolerance to self/tumor antigen would be beneficial to generating more durable antitumor immune responses.

Using 2 CD8^{+} T-cell clones that recognized the same self-antigen (TRP-$2_{180-188}$) but differed in their functional avidity, we generated 2 lines of TRP-2–specific TCR transgenic (Tg) mice—TCR[lo] and TCR[hi]. Our previous studies showed that despite infiltrating B16 melanoma tumors and remaining reactive against TRP-2, the lower avidity TCR^{lo} T cells did not reduce subcutaneous B16 tumor growth (25). In the current study, we compared the difference between T cells derived from the 2 lines of TCR Tg mice. We report that the higher avidity TCR^{hi} T cells generated superior antitumor activity and caused autoimmune depigmentation. However, they were more susceptible to tolerization, both in the tumor microenvironment (TME) as well as ex vivo following stimulation by tumor-associated dendritic cells (TADC). Given the trend for exploiting higher avidity T cells for cancer therapy, these findings suggest that selection of T cells based solely on elevated avidity may not be optimal for maintaining immunity to tumor antigens. Rather, strategies that target the tolerization of T cells and sustain antigen responsiveness may yield more durable antitumor responses.

Materials and Methods

Experimental mice

C57BL/6 female mice were purchased from National Cancer Institute Animal Production Area Facility (Frederick, MD). The TCR Tg mouse strain 37B7 (TCR[lo] mice) bears a TCR transgene that recognizes an H-2K[b]–restricted epitope of TRP-$2_{180-188}$ and was described previously (25). Mice were housed under specific pathogen-free conditions and were treated in accordance with NIH Guidelines under protocols approved by the Animal Care and Use Committee of the NCI-Frederick facility.

Generation of high-avidity TRP-$2_{180-188}$ TCR Tg mice (TCR^{hi} mice)

Vα5 and Vβ7 TCR chain usage by a TRP-$2_{180-188}$ peptide-specific CD8^{+} T cell clone (clone 24; ref. 26) was identified by spectratype analysis (27) and were subsequently cloned and sequenced. TCR^{hi} mice were generated by similar method as described for TCR[lo] mice (25). In some experiments, TCR[hi] mice were crossed to IFN-γ–deficient background (gift, Dr. Robert Wiltrout, NCI, Frederick, MD) to generate IFN-γ^{−/−}TCR[hi] mice.

Cell line and peptide

B16-BL6, hereafter called B16, a TRP-2^{+} murine melanoma cell line, was maintained in culture media as previously described (25). TRP-$2_{180-188}$ (SVYDFFVWL) peptides were purchased from New England Peptide.

Tetramer decay assay

The tetramer decay assay and subsequent analysis were conducted as described previously (28, 29). Briefly, splenocytes from TCR Tg mice were incubated with anti-CD8 and clonotype-specific anti-Vβ antibodies for 30 minutes at 4°C and then washed 3 times with fluorescence-activated cell-sorting (FACS) buffer [PBS + 0.5% bovine serum albumin + 0.1% sodium azide]. Cell suspensions were then incubated with TRP-2 tetramer (courtesy, NIAID Tetramer facility, Emory University, Atlanta, GA) for 2 hours at 4°C. A competing unlabeled anti-H2K[b] Ab (clone Y3) was added to the cell suspension and aliquots were taken at 0, 10, 20, 40, 60 minutes up to 24 hours afterward. Cells were fixed in 1% paraformaldehyde/FACS buffer for analysis on BD LSR II.

IFN-γ ELISPOT Assay

Multiscreen plates (Millipore) were coated with 100 μL of IFN-γ capture antibody (BD Biosciences) overnight at 4°C. T cells were added to increasing concentrations of TRP-$2_{180-188}$. After incubation, plates were washed and processed as previously described (19).

^{51}Cr Release assay

The ^{51}Cr release assay was conducted as described previously with some modification (25). Briefly, B16 cells were treated with IFN-γ (20 ng/mL) overnight and labeled with ^{51}Cr and used as targets. Effector cells were generated by culturing TCR Tg T cells with TRP-2 Ag (1 μmol/L) and 20 IU interleukin (IL)-2 for 6 days and purified using CD8^{+} T Lymphocyte Enrichment Set (BD Biosciences) according to the manufacturer's instructions. Graded numbers of effectors were added to target cells in a 96-well plate to achieve the indicated effector: target (E:T) ratio. Four hours later, supernatants were harvested and radioactivity assessed using a WALLAC 1470 Gamma counter.

CFSE labeling and flow cytometric analysis

Lymph node (LN) cells from TCR^{hi}-Thy1.1^{+} mice were dispersed into a single-cell suspension. CD8^{+}CD44^{lo} were enriched using biotin-conjugated anti-CD44 antibody (clone: IM7) and biotinylated CD8 T cell enrichment cocktail, followed by streptavidin magnetic beads (BD Pharmingen). The resulting cell population was labeled with 5,6-carboxyfluorescein-diacetate succinimidyl ester (CFSE) as previously described (25), and 2.0×10^6 antigen-specific CD8^{+} T cells were transferred into recipient mice by tail vein injection. Mice were euthanized on indicated days after adoptive transfer. Tumor or vaccine-draining lymph nodes (axillary, brachial, and inguinal) or spleens were incubated with antibodies directed against Thy1.1, CD8, and CD44. Intracellular IFN-γ, Granzyme B, and CD107a expression from TILs were analyzed as described previously (25). MHC-I (H-2K[b]) expression on tumor cells was

analyzed by immunostaining of enzymatically digested tumors, gating on CD44+CD45− tumor cells.

Adoptive transfer of transgenic T cells to treat subcutaneous B16 tumor

A total of 2.5×10^6 antigen-specific CD8+ lymph node T cells from TCRhi or TCRlo Rag−/− mice were adoptively transferred into B6 mice. The day after T cell transfer, mice were vaccinated s.c. with TRP-2 peptide–pulsed, bone marrow–derived dendritic cells (BMDC) as previously described (25). Alternatively, mice were injected with similar number of transgenic T cells 1 day after tumor challenge. Similar outcomes were noted irrespective of T cell transfer. In some studies, to study T cell responses in the context of treating established tumors, T cells were transferred into mice 9 days after tumor implantation.

Some mice were treated with *in vitro* activated TCR Tg T cells. In those studies, mice were injected s.c. with B16 tumor cells (1×10^5). Three, 7, and 11 days after tumor challenge, mice received an intravenous injection of *in vitro* generated TRP-2–specific effector cells (1×10^7). Effector cells were generated as described above for the ^{51}Cr release assay. In all studies, tumor size was estimated by measuring perpendicular diameters using a caliper. Mice were euthanized when tumor area exceeded 250 mm^2 and tumor size was recorded as 250 mm^2 thereafter.

Blocking and depletion antibodies

A blocking antibody directed against IFN-γ (XMG6, kindly provided by Dr. Giorgio Trinchieri, NCI, Frederick, MD) was injected intraperitoneally (i.p.; 0.5 mg) on days 9 and 13 after B16 tumor challenge. Anti-CD317 (PDCA-1) antibody (0.5 mg/injection; kindly provided by Drs. Trinchieri and Marco Colonna, Washington University, St. Louis, MO) was injected i.p. on day 7, 8, and 15, with respect to B16 tumor challenge (30). Anti-PD-1 (clone RMP1-14) antibody was generously provided by Dr. Hideo Yagita (Juntendo University, Tokyo, Japan). Mice received 250 μg of anti-PD-1 starting 10 days after tumor challenge and every 3 days thereafter.

TADC isolation and *in vitro* tolerance assay

Subcutaneous B16 tumors were digested in 5 mL dissociation buffer (100 U/mL collagenase IV and 100 μg/mL DNase in RPMI/10% FBS) for 30 minutes at 37°C. DCs were isolated from single-cell suspensions of the tumor using phycoerythrin (PE)-coupled anti-PDCA-1 antibody and anti-PE magnetic beads with the Miltenyi MACS cell separation system (31). Splenic plasma DCs were isolated in the same way and used as control. Cell separations were conducted according to the manufacturer's instructions and consistently yielded purity of more than 95% CD11c+/CD317+.

For the *in vitro* tolerance assay, naïve TCRhi or TCRlo Thy1.1+ cells were cocultured for 72 hours with TRP-2 antigen–pulsed B16 TADCs (CD11c+/B220+/BST2+) isolated from subcutaneous B16 tumors. The T cells were re-isolated via negative selection against the DCs using magnetic beads (31). TCR Tg T cells were delivered secondary stimulation using splenocytes pulsed with TRP-2 peptide. After 48 hours, wells were pulsed with 1 μCi [^{3}H] thymidine (Amersham) and harvested 16 hours later. Alternatively, intracellular staining for IFN-γ expression was tested as described above.

Statistical analysis

Statistical analyses for differences between group means were conducted by unpaired Student t test or ANOVA. Tumor growth was compared using a 2-way ANOVA. $P < 0.05$ was considered statistically significant. PRISM 5.0 software was used to analyze the data (GraphPad Software, Inc.).

Results

TCRhi T cells display higher functional avidity than TCRlo T cells

Our previous studies showed that TRP-2-specific TCRlo T cells were unable to control B16 tumor growth despite infiltration of the tumor and retention of cytolytic ability (25). We subsequently prepared a second TCR transgenic line based on a T cell clone described to have high functional avidity (26); these T cells required lower doses of antigen for stimulation than the T cell clone from which the TCRlo mice were derived and displayed greater IFN-γ expression at indicated antigen doses (Supplementary Fig. S1). We observed a striking phenotypic difference in TCRhi mice compared with TCRlo mice (Fig. 1A): TCRhi mice spontaneously developed autoimmune depigmentation as initially revealed by diminished pigmentation of the ear at weaning. Over time, progressive vitiligo-like

Figure 1. TCRhi T cells display higher functional avidity than TCRlo T cells. A, TCRhi Tg mice spontaneously developed autoimmune depigmentation. B, tetramer decay assay revealed T cells from TCRhi mice bound tetramer with higher avidity. Data are representative of 4 experiments with similar results.

TCRhi (3 mo)   TCRhi (13 mo)   TCRlo (13 mo)
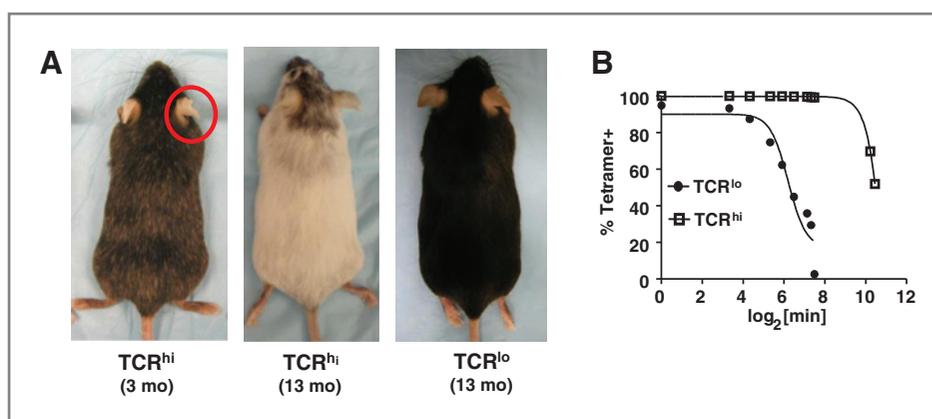
depigmentation was observed in the coat hair of TCR^{hi} mice, which was rarely observed in TCR^{lo} mice (Fig. 1A). Consistent with these observations, we detected T cells in the skin of TCR^{hi} mice, but not in the skin of TCR^{lo} mice (Supplementary Fig. S2).

We next characterized the phenotype of CD8$^+$ T cells from TCR^{hi} mice. We observed a distinct population of cells that were CD25$^-$, CD44^{hi}, and Ly6c$^+$ that was not observed in TCR^{lo} mice, which instead displayed a uniformly naïve phenotype: CD62L^{hi} (data not shown), CD25$^-$, CD44^{lo}, and Ly6c^{neg} while displaying comparable levels of TCR (Supplementary Fig. S3A). This phenotype suggested that these were antigen-experienced TCR^{hi} T cells, presumably responding to endogenous TRP-2 antigen.

To confirm that TCR^{hi} T cells respond to endogenous antigen, we enriched the CD44^{lo} TCR Tg T cells using magnetic beads by negative selection. TCR Tg T cells were labeled with CFSE to monitor proliferation and transferred to immuno-competent naïve C57BL/6 mice that express TRP-2. Consistent with our previous findings from the TCR^{lo} transgenic mice (25), only T cells from TCR^{hi} mice transferred to WT mice showed detectable CFSE dilution. Among those cells that diluted CSFE, most were CD44^{hi} (Supplementary Fig. S3B). These findings confirm that only TCR^{hi} T cells responded to endogenous TRP-2 antigen in the absence of exogenous prim-ing, presumably due to their elevated avidity.

We next conducted tetramer decay analysis to confirm that TCR^{hi} T cells truly possess higher avidity than TCR^{lo} T cells. This assay measures the binding strength of a soluble form of the TCR ligand, a TRP-2$_{180-188}$/H-2K^b tetramer. The decay time to 50% maximal tetramer binding for the TCR^{lo} T cells was 65 minutes but was 2,610 minutes for the TCR^{hi} T cells (Fig. 1B). Thus, as predicted, T cells from TCR^{hi} mice bound tetramer ligand with higher avidity than TCR^{lo} T cells.

Given this difference in avidity for TCR ligand, we next assessed the functional differences between TCR^{lo} and TCR^{hi} T cells. We generated CD8$^+$ effector cells by antigen stimulation *in vitro* and tested antigen-specific cytotoxicity. Consistent with higher functional avidity of the original T-cell clones (Supplementary Fig. S1), TCR^{hi} effector T cells displayed greater cytotoxicity than TCR^{lo} effector T cells (Supplementary Fig. S3C). Taken together, these data show that TCR^{hi} T cells represented a higher avidity population than TCR^{lo} T cells and this increased avidity resulted in increased responsiveness to TRP-2 Ag, including increased IFN-γ production and Ag-specific lytic potential.

T cell avidity determines the magnitude of response to B16-derived antigen

On the basis of their different responses to endogenous antigen as well as *in vitro* function, we next sought to determine the response of TCR^{hi} and TCR^{lo} T cells to B16 tumor–derived TRP-2 antigen. CFSE-labeled TCR Tg T cells were transferred into WT B6 mice, and the following day, mice were challenged with B16 tumor cells. This enabled us to study T cell responses to tumor-derived TRP-2 antigen. Twenty days later, we ana-lyzed CFSE dilution and expression of CD44 and IFN-γ by tumor-infiltrating TCR T cells. As shown in Fig. 2A, only TCR^{hi} T cells recognized B16 tumor–derived Ag, as shown by a

prominent fraction of tumor-infiltrating TCR^{hi} T cells display-ing diminished CFSE levels and elevated CD44 expression. Approximately 15% of TCR^{hi} T cells that infiltrate the B16 tumors expressed IFN-γ. When comparing CFSE dilution, TCR^{hi} T cells in the draining lymph node displayed more robust proliferative responses to B16 tumor–derived antigen than to endogenous TRP-2 antigen (Fig. 2A, top right, naïve vs. tumor); IFN-γ production was similarly elevated in LN TCR^{hi} cells from tumor-bearing mice (22.6% vs. 5.7%, $P < 0.0001$). In contrast, in the absence of any exogenous stimulation, TCR^{lo} T cells did not infiltrate B16 tumors (Fig. 2A), dilute CFSE, or generate effector function as measured by IFN-γ production, as we previously reported (25). Not surprisingly, vaccination with TRP-2 peptide–pulsed DCs 15 days after T-cell transfer induced a marked increase in infiltration of TCR^{hi} T cells within the tumor and an elevated frequency of TCR^{hi} T cells that expressed IFN-γ (Fig. 2B). This was again consistent with our previous report showing infiltration of TCR^{lo} T cell into B16 tumors following DC vaccination (25).

Given our observation that TCR^{hi} T cells generated more robust responses than TCR^{lo} T cells to both endogenous and tumor-derived antigen, we next tested whether transfer of TCR^{hi} T cells could control B16 tumor. Despite modest generation of effector function (Fig. 2A), no reduction of tumor growth was noted in mice that were transferred with TCR^{hi} T cells alone (Fig. 2C). However, transfer of TCR^{hi} T cells in combination with a TRP-2–pulsed DC vaccine 9 days after tumor challenge significantly delayed tumor progres-sion. This effect was not observed for TCR^{lo} T cells (Fig. 2C; ref. 25). Similar results were obtained when treating s.c. B16 tumor cells with *in vitro* activated effector cells (Supple-mentary Fig. S4A). In addition, we also observed that s.c. B16 tumor growth was delayed in TCR^{hi} Tg mice, but not in TCR^{lo} Tg mice (Supplementary Fig. S4B). Taken together, these results indicate that TCR^{hi} T cells generate superior antitumor activity compared with the lower avidity TCR^{lo} T cells.

TCR^{hi} T cells infiltrating subcutaneous B16 tumors are tolerized

Given that both *in vivo* and *in vitro* primed TCR^{hi} T cells initially slowed subcutaneous B16 tumor growth, but all mice eventually developed progressive tumor growth, we hypothe-sized that TCR^{hi} T cells may be progressively tolerized within the developing B16 TME. Therefore, we sequentially analyzed TCR^{hi} T cell reactivity after tumor infiltration. As indicated in Fig. 3 and Supplementary Fig. S5, TCR^{hi} TILs were highly responsive to TRP-2 antigen 5 days after vaccination. This included expression of IFN-γ and Granzyme B as well as CD107a mobilization, an indicator of CTL granule exocyto-sis. These observations were consistent with our previous studies examining TCR^{lo} TILs (25). However, at later time points (e.g., 15 days after vaccination), a significant reduc-tion in the frequency of TCR^{hi} TILs expressing IFN-γ and mobilizing CD107a was observed in comparison to the earlier time point, or when compared with TCR^{hi} T cells isolated from the spleen of naïve and tumor-bearing mice (Fig. 3A and B). Interestingly, we noted a loss of

Figure 2. T-cell avidity determines the magnitude of response to B16-tumor derived antigen. A, CFSE-labeled $CD8^+CD44^{lo}$ TCR^{hi} and unsorted TCR^{lo} Tg T cells were transferred into naïve B6 mice on day 0. The following day, mice were challenged s.c. with 1×10^5 B16 tumor cells. Twenty days after tumor challenge, the adoptively transferred T cells in B16 tumors were analyzed by testing for CD8, Thy1.1, CD44, and IFN-γ expression. Top right, TCR^{hi} T cells from draining lymph nodes were similarly analyzed. **, $P < 0.0001$. B, mice were treated as in A except for receiving a s.c. TRP-2–pulsed BMDC vaccine 15 days after adoptive T-cell transfer. C, TCR^{hi} T cells were injected i.v. and B16 tumor cells were injected s.c. into naïve B6 mice. Mice were vaccinated with TRP-2–pulsed BMDCs 9 days after tumor challenge. Tumor size was monitored. Data are representative of 3 separate experiments.
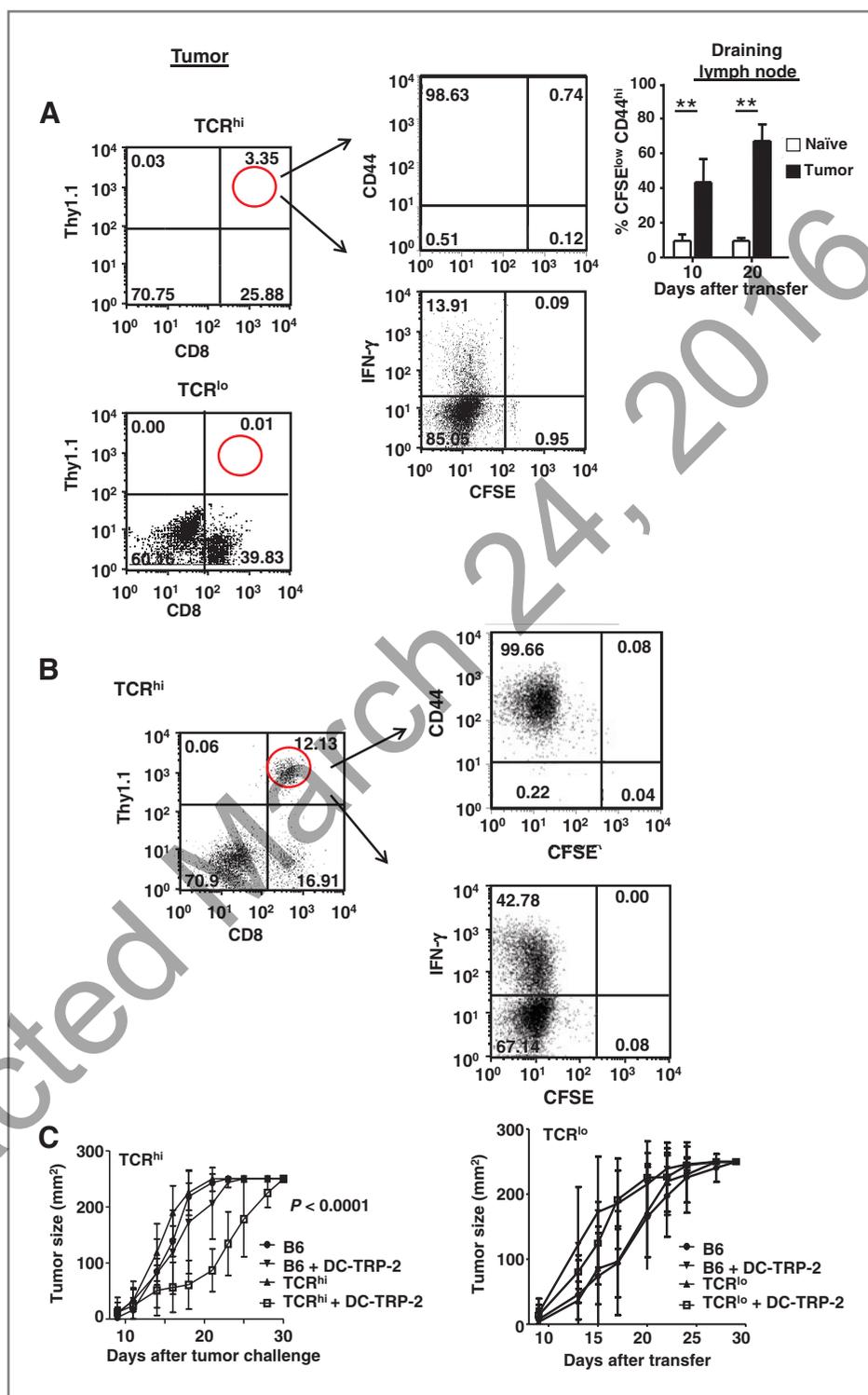
responsiveness in TCR^{hi} T cells isolated from the spleen of tumor-bearing mice relative to those isolated from spleens of naïve mice. Total GrB expression remained consistent throughout the course of the experiments (Fig. 3C), indicative of prior activation of the TILs. These data show that high-avidity TCR^{hi} T cells gradually lost their effector functions during tumor progression, which is in contrast to our previously published findings showing that lower avidity TCR^{lo} TILs retain effector function within the TME, despite their inability to retard B16 tumor growth (25).
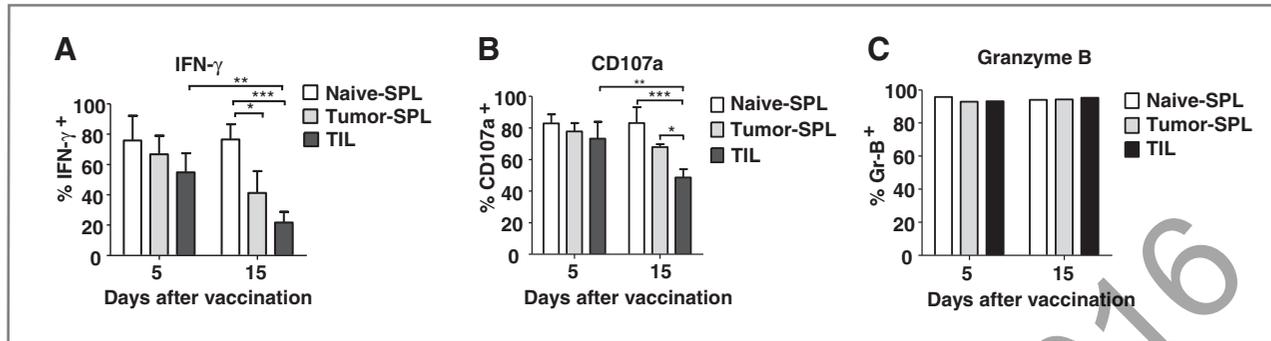
Figure 3. TCR^{hi} T cells infiltrating subcutaneous B16 tumors become tolerized. CD8^{+}CD44^{lo}Thy1.1^{+} TCR^{hi} T cells were adoptively transferred into wild-type (WT) B6 mice. The following day, mice were challenged s.c. with B16 tumor cells. Nine days later, mice were vaccinated with TRP-2–pulsed BMDCs. At the indicated time after vaccination, mice were euthanized and splenocytes (SPL) or TILs were analyzed for IFN-γ expression (A), CD107a mobilization (B), and Granzyme B (Gr-B) expression (C). A and B, data from 3 experiments were combined (mean ± SD). C, data are presented from 1 of 2 identical experiments using 3 mice per group, pooled together for the assay. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

IFN-γ expression by TCR^{hi} T cells is critical for their antitumor immunity

One consequence of IFN-γ expression by TILs is the upregulation of MHC-I expression by tumor cells, which may affect susceptibility of tumor cells to T-cell–mediated lysis. Given the progressive loss of IFN-γ expression by TCR^{hi} T cells, we next tested whether this loss of antigen responsiveness by TCR^{hi} T cells correlated with a decrease in MHC-I expression by B16 tumor cells. While infiltration by both TCR[hi] and TCR^{lo} T cells led to an increase in MHC-I expression by B16 tumor cells (Supplementary Fig. S6), we noted that there was a progressive decrease in MHC-I expression by B16 tumor cells that paralleled the loss of IFN-γ expression by TCR^{hi} T cells (Fig. 4A, $P = 0.002$). To confirm the connection between IFN-γ

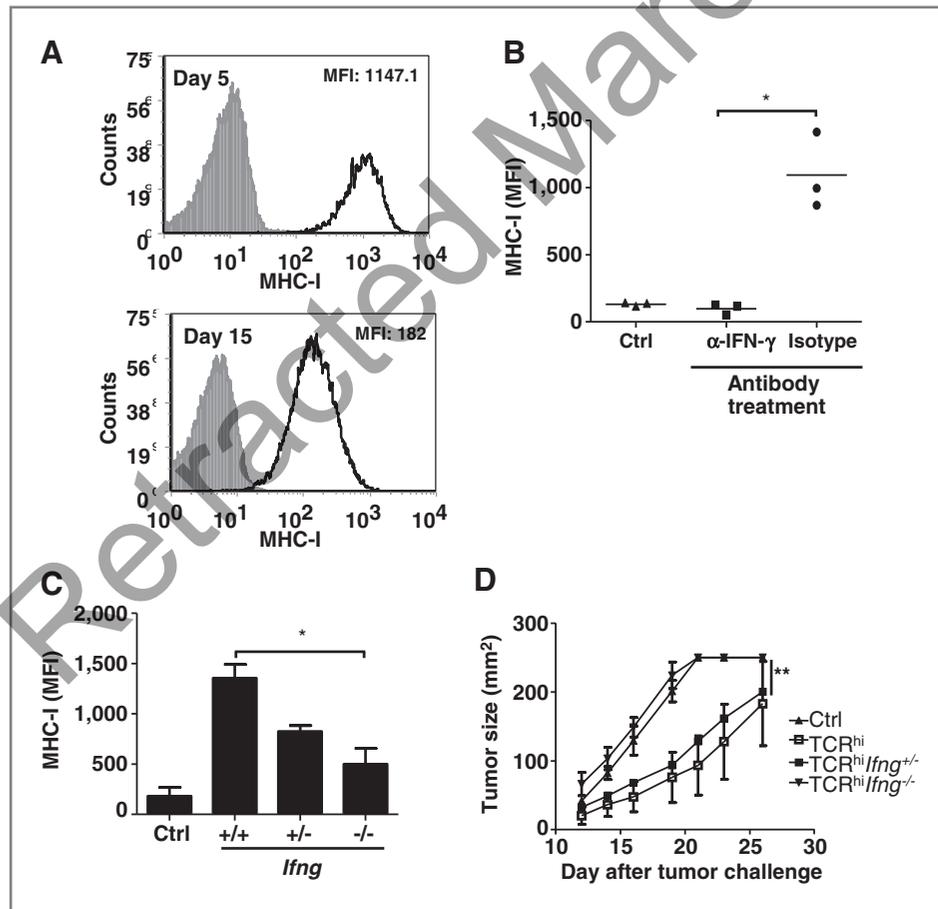**Figure 4.** IFN-γ expression by TCR^{hi} T cells is critical for antitumor immunity. A, TCR^{hi} T cells were injected i.v. and B16 tumor cells were injected s.c. into naïve B6 mice. Mice were vaccinated with TRP-2-pulsed BMDCs 9 days after tumor challenge. Five and 15 days after DC vaccination, subcutaneous B16 tumors were excised. MHC-I (H-2K[b]) expression on tumor cells was analyzed by gating on CD44^{+}CD45^{−} tumor cells. B, mice were challenged with tumor and treated as in A. Anti-IFN-γ antibody or an isotype control antibody was delivered i.p. on days 9 and 13 after tumor challenge. On day 15, MHC-I expression on tumor cells was analyzed by FACS staining. Some tumor-bearing mice received no T-cell transfer or DC vaccine (Ctrl). Data are representative of 3 independent experiments (mean ± SD). *, $P < 0.005$. C + D, TCR[hi] Tg mice were backcrossed to the *Ifng*-deficient background and T cells from these mice were transferred into 9-day tumor-bearing B6 mice. C, MHC-I expression on tumor cells was compared. D, tumor growth was monitored. *, $P < 0.05$; **, $P < 0.0001$. Representative of 3 separate experiments, 4 to 5 mice per group.

expression and increases in MHC-I expression on tumor cells, we blocked IFN-γ using an IFN-γ neutralizing antibody. We observed that upregulation of MHC-I on tumor cells was significantly reduced following treatment with TCR^{hi} T cells in combination with IFN-γ blockade (Fig. 4B).

While multiple cell populations could deliver IFN-γ to increase tumor MHC-I expression, we determined the role of TCR^{hi} T-cell–derived IFN-γ by crossing TCR^{hi} mice onto the IFN-γ–deficient background. Using T cells from these mice for adoptive transfer into B16 tumor–bearing mice, we noted that $Ifng^{-/-}$TCR^{hi} T cells were unable to upregulate MHC-I expression on tumor cells as efficiently as WT TCR^{hi} T cells (Fig. 4C). Loss of IFN-γ production by TCR^{hi} T cells also resulted in the loss of their ability to slow B16 tumor growth (Fig. 4D). Taken together, these data indicate that IFN-γ expression by TCR^{hi} T cells was critical for their antitumor activity and their ability to enhance MHC-I expression on their tumor targets.

PD-1 blockade improves TCR^{hi} T-cell responses and reduced tumor burden

PD-1 is an inhibitory receptor expressed on activated T cells (23). We and others have reported that PD-1 blockade prevents T-cell exhaustion and tolerization, which confers improved immunity to tumors (24, 31). To determine whether PD-1 ligation contributes to TCR^{hi} T-cell tolerization, we used an anti-PD-1 antibody to block PD-1–mediated signals. As shown in Supplementary Fig. S7A and S7B, PD-1 blockade improved T-cell responses and prevented tolerization of TCR^{hi} TILs. In addition, we also observed restoration of responsiveness of TCR^{hi} T cells in the spleen of tumor-bearing mice. This retention of T-cell responsiveness was associated with reduced tumor burden (Supplementary Fig. S7C). While these findings implicate a role for PD-1 in tolerization of TCR^{hi} T cells, we cannot rule out the possibility that PD-1 blockade also targets another effector cell population,

including natural killer cells (32) and/or endogenous B16-reactive T cells.

B16 tumor–associated dendritic cells tolerize TCR^{hi} but not TCR^{lo} T cells

Given the differential tolerization of TCR^{hi} and TCR^{lo} T cells infiltrating B16 tumors, we tested the possibility that B16 TADCs may preferentially tolerize TCR^{hi} T cells. We recently identified a population of plasmacytoid-like TADCs with immunosuppressive function in both human and murine tumors, including B16 melanoma (31). These TADCs were CD11c^{lo}/B220$^+$/BST2 (CD317)$^+$ (Supplementary Fig. S8). To test the tolerogenic ability of the B16 TADCs, TCR Tg T cells were cocultured with TRP-2 peptide–pulsed TADCs for 4 days before re-isolation and subsequent restimulation with TRP-2–pulsed splenic antigen-presenting cells (APC). TCR^{hi} T cells initially cultured with B16 TADCs did not proliferate (Fig. 5A) and had reduced IFN-γ production (Fig. 5B) in response to secondary antigenic stimulation, whereas marked proliferative and cytokine responses were observed when splenic plasmacytoid DCs (pDC) isolated using an identical approach were used as APCs for the primary stimulation. In contrast, TCR^{lo} T cells initially cultured with either B16 TADCs or splenic pDCs displayed robust proliferative and IFN-γ responses after secondary stimulation (Fig. 5A and B). Furthermore, TADCs from $Foxo3^{-/-}$ mice did not tolerize TCR^{hi} T cells (Supplementary Fig. S9), which is consistent with our recent finding that FOXO3 may program TADCs to become tolerogenic (31). Here, we further showed that T-cell avidity also contributes T-cell tolerance. Taken together, these data indicate that only TCR^{hi} T cells were tolerized by B16 TADCs, consistent with our observations on tolerance induction following TCR^{hi} T-cell infiltration into B16 tumors.

On the basis of the above findings that TADCs preferentially tolerized TCR^{hi} T cells *in vitro*, we next sought to determine whether depletion of TADCs *in vivo* would prevent TCR^{hi} T-cell
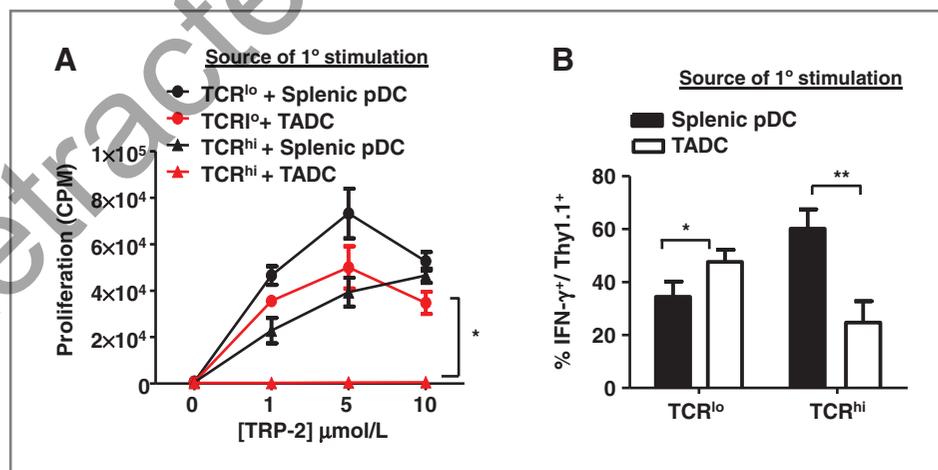

Figure 5. B16 TADCs tolerize TCR^{hi} T cells but not TCR^{lo} T cells *in vitro*. TADCs were isolated from 15- to 20-day-old subcutaneous B16 tumors for use in the tolerization assay. Naïve TCR Tg T cells were cocultured for 4 days *in vitro* with TRP-2 peptide and B16 TADCs or, as a positive control, wild-type (WT) splenic plasmacytoid DCs (pDC) isolated using an identical protocol. TCR Tg T cells were re-isolated and tested for secondary stimulation with peptide-pulsed splenic APCs and proliferation was monitored by measuring [^{3}H] thymidine incorporation (A) and IFN-γ production by intracellular staining (B). A, *, $P = 0.0001$ each point 1, 5, 10; B, *, $P < 0.05$; **, $P < 0.005$.

tolerization and promote tumor regression. Administration of an anti-CD317 antibody 1 week before T-cell transfer and vaccination, which was previously reported to deplete pDCs (30) and prostatic TADCs (31), depleted approximately 80% of TADCs in B16 melanoma tumors (Supplementary Fig. S10). Administration of anti-CD317 alone was not sufficient to alter tumor growth (Supplementary Fig. S11A), consistent with our observation that B16 tumor cells do not express CD317 (Supplementary Fig. S11B) and delivery of tumor-specific T cells is required for enhancing immunity to B16. Following TADC depletion, the frequency of IFN-γ–secreting TCR^{hi} T cells from B16 tumors was significantly higher than those isolated from mice injected with a control antibody (Fig. 6A). CD107a mobilization was slightly but not significantly increased after TADC depletion (Fig. 6B), suggesting that the reduction of TADCs in TME at least partially prevented tolerization of TCR^{hi} TILs. Consistent with the restoration of IFN-γ production, we noted significant upregulation of MHC-I on tumor cell after the depletion of TADCs (Fig. 6C). Moreover, B16 tumor burden was reduced in TADC-depleted mice compared with those in control antibody–treated mice (Fig. 6D). Taken together, these data show that selective tolerization of TCR^{hi} T cells may ultimately contribute to their loss of tumor growth control.

Discussion

Using TCR Tg T cells that recognize the same tumor/self-antigen but display different avidity, we provide direct evidence that while higher avidity CD8$^+$ T cells induce autoimmunity, they also display superior antitumor activity. However,

higher avidity T cells are more susceptible to tolerization in the tumor microenvironment.

In the current study, we show that higher avidity CD8$^+$ T cells from TCR^{hi} Tg mice had a small but detectable population of cells that were "antigen-experienced" and these cells persisted in the periphery as putative auto-reactive T cells, which is supported by our observation that TCR^{hi} mice develop spontaneous autoimmune depigmentation. Recognition of cognate antigen in the periphery can be regulated by either the avidity of responding T cells and/or antigen dose (33). Consistent with this idea, TCR^{hi} T cell proliferation was more vigorous in response to tumor-derived antigen than endogenous antigen. This is presumably due to higher levels of TRP-2 antigen presentation in the tumor-draining lymph nodes. However, providing improved antigen priming through peptide-pulsed DC vaccination was capable of augmenting both TCR^{hi} and TCR^{lo} T cell responses and promoting tumor infiltration by both populations.

More importantly, our studies show that T cell avidity correlated with induction of tolerance, a significant obstacle to successful cancer immunotherapy (34). We show that unlike lower avidity TCR^{lo} T cells, the higher avidity TCR^{hi} T cells that persisted in the TME lost their ability to produce IFN-γ and to mobilize CD107a, hallmarks of tolerance. These data confirm our previous report showing loss of CTL function among tumor-specific CD8$^+$ T cells that infiltrate prostate tumors (21). Morgan and colleagues also reported that higher avidity, Flu-HA–specific T cells were more readily tolerized than lower avidity T cells with identical specificity (22). Thus, our findings
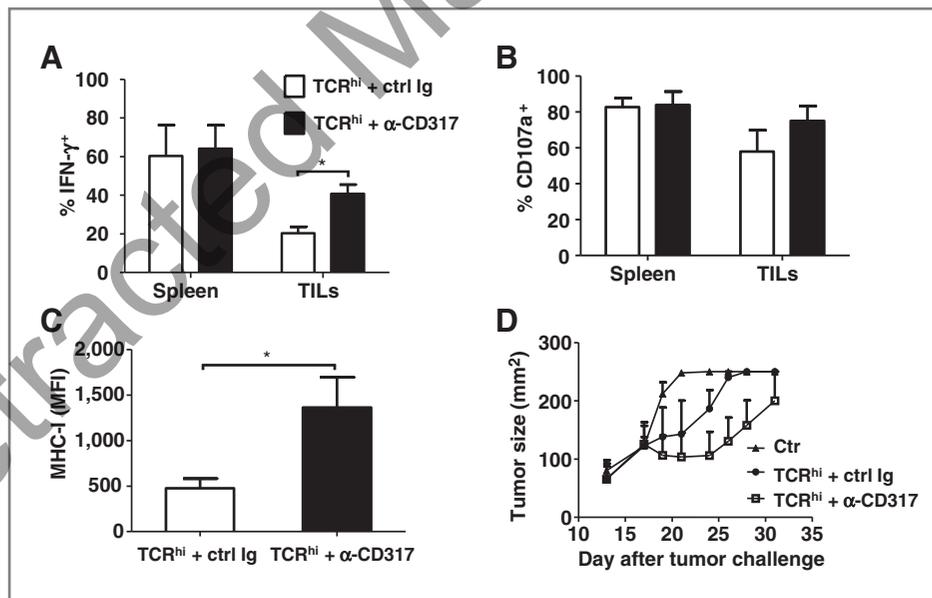

Figure 6. Depletion of B16 TADCs reduces tolerization of adoptively transferred TCR^{hi} T cells *in vivo*. Wild-type (WT) B6 mice were injected s.c. with 1×10^5 B16 tumor cells on day 0. At days 7, 8, and 15, mice were injected i.p. with anti-CD317 antibody. TCR^{hi} T cells were adoptively transferred into tumor-bearing mice on day 9 and mice were vaccinated with TRP-2–pulsed BMDCs the following day. A and B, mice were euthanized on day 21 and splenocytes or TILs were analyzed for IFN-γ expression (left) and CD107a mobilization (right). Data from 3 experiments were combined (mean ± SD). C, MHC-I (H-2K^b) expression on tumor cells was analyzed by gating on CD44$^+$CD45$^-$ cells. Data were pooled from 2 independent trials with 3 mice per group. *, $P < 0.05$. D, WT B6 mice were injected s.c. with 1×10^5 B16 tumor cells on day 0. At days 9, 10, 17, 21, and 24, mice were injected i.p with anti-CD317 antibody (0.5 mg/injection). TCR^{hi} T cells were adoptively transferred into tumor-bearing mice on day 12 and mice were vaccinated with TRP-2–pulsed BMDCs on day 13. Tumor size was monitored. Data are representative of 2 independent trials, with 4 to 5 mice per group.

show that avidity may be critical in determining the fates of TILs within TME.

The impact of T-cell tolerization on antitumor immunity was amplified by the reduction of MHC expression by tumor cells, which correlated with loss of IFN-γ expression by TCR^{hi} T cells. Loss of MHC-I expression represents a major impediment to successful immunotherapy and is a well-described mechanism of immune escape in many cancer types (35). The correlation between TCR[hi] T cell-derived IFN-γ and increased MHC-I expression by B16 tumor cells was further supported by *in vivo* neutralization of IFN-γ as well as studies using IFN-γ–deficient TCR^{hi} T cells. These findings are in agreement with previous work implicating IFN-γ as an important factor for retention of antitumor immunity (35) and underscore the importance of maintaining T cell responsiveness for maintaining MHC expressing by tumors and tumor immunity. In separate studies, hydrodynamic delivery cDNA encoding IFN-γ only partially restored MHC-I expression by tumor cells and had minimal effect on restoring TCR[hi]-mediated antitumor activity (data was not shown). These observations are in agreement with the finding of Esumi and colleagues (36), suggesting that MHC-I expression is necessary but not sufficient for the induction of a host response to tumor and also imply that the source of IFN-γ may be critical, as well.

The mechanisms by which the tolerance induction occurs in tumors are only partially understood. It is widely accepted that the TME does not favor infiltrating tumor-specific T cells (37). In the present study, TADCs isolated from the B16 TME tolerized TCR[hi], but not TCR^{lo} T cells *in vitro*. Enhanced TCR[hi] T cell reactivity following depletion of B16 TADCs supports the idea that TADCs are at least one component of the TME responsible for tolerizing TCR^{hi} T cells. Incomplete depletion of TADCs may explain partial restoration of TCR[hi] T-cell function and partial enhancement of immunity to B16 tumor. However, we cannot rule out the possibility that anti-CD317 may deplete another cell population with immunosuppressive functions. Furthermore, TADCs isolated from B16 tumors growing in *Foxo3*$^{-/-}$ mice were not tolerogenic, which is consistent with our previous finding (31). It remains unclear whether other components of the TME, such as myeloid-deprived suppressor cells (MDSC; ref. 38), regulatory T (Treg) cells (39), macrophages (40), or mast cells (41), also contribute to the tolerization of infiltrating TCR^{hi} T cells. Similarly, blockade of PD-1 reduced tolerization of T cells and improved tumor immunity. As PD-1 has been associated with T cell exhaustion, it remains possible that the loss of T-cell function may be indicative of chronic T cell stimulation in the TME, a feature of T cell exhaustion. Strategies to eliminate TADCs or target their suppressive pathways such as FOXO3 or PD-1 may prevent high avidity T cell tolerization and thus enhance antitumor immunity.

Taken together, our findings show that while higher avidity T cells may initially confer better protection to tumors, they are more susceptible to tolerization in the TME. Many clinical trials are testing the use of transgenic TCRs for conferring melanoma specificity (42, 43). Specifically, an emphasis on higher avidity T cell populations has been proposed (44, 45). As T cell tolerance and exhaustion is also a feature of viral infections, these findings may also be applicable to anti-viral immune-based therapies (46). Therefore, caution should be exercised when selecting T cell populations for use in cancer immunotherapy. If low-avidity T cells are targeted, it will be necessary to optimize their effector function and overcome their reduced tumoricidal activity. However, identifying mechanisms that prevent or reverse tolerization and combat the potentially adverse results of autoimmune reactivity may also be acceptable alternatives for using higher avidity T cells. In addition, ongoing studies that elucidate the different signals transduced by these divergent T cell populations may further reveal novel therapeutic targets for maintaining durable and effective antitumor immunity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Zhu, V. Singh, V. Bronte, A.A. Hurwitz
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Study supervision: A.A. Hurwitz

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Retraction: High-Avidity T Cells Are Preferentially Tolerized in the Tumor Microenvironment

The editors and authors retract the article titled "High-Avidity T Cells Are Preferentially Tolerized in the Tumor Microenvironment," which was published in the January 15, 2013 issue of *Cancer Research* (1), based on the findings of data falsification regarding Fig. 5A. Following review by an NIH investigation committee, NIH found the author Stephanie Watkins was the sole individual responsible for the instances of research misconduct. None of the other authors were aware of the misconduct.

Six of the seven authors have agreed to this retraction and they are as follows:

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High-Avidity T Cells Are Preferentially Tolerized in the Tumor Microenvironment

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