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\textsuperscript{hi} 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\textsuperscript{hi}) that bears higher avidity T cells and spontaneously developed autoimmunity. In contrast to TCR\textsuperscript{lo} T cells, which were ignorant of tumor-derived antigen, TCR\textsuperscript{hi} T cells initially delayed subcutaneous B16 melanoma tumor growth. However, persistence in the tumor microenvironment resulted in reduced IFN-\gamma production and CD107a (Lamp2a) mobilization, hallmarks of T-cell tolerization. IFN-\gamma expression by TCR\textsuperscript{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\textsuperscript{hi} T cells and enhanced their antitumor activity. In addition, TADCs tolerized TCR\textsuperscript{lo} T cells but not TCR\textsuperscript{hi} 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\textsuperscript{+} 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\textsuperscript{+} 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).

Adaptive immunotherapy with ex vivo expanded tumor-infiltrating lymphocytes (TIL) has achieved objective clinical responses in a significant fraction of patients with metastatic
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-2180-188) but differed in their functional avidity, we generated 2 lines of TRP-2–specific TCR transgenic (Tg) mice—TCRlo and TCRhi. Our previous studies showed that despite infiltrating B16 melanoma tumors and remaining reactive against TRP-2, the lower avidity TCRlo 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 TCRhi 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 (TCRlo mice) bears a TCR transgene that recognizes an H-2Kb–restricted epitope of TRP-2180-188 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-2180-188 TCR Tg mice (TCRhi mice)**

Vα5 and Vβ7 TCR chain usage by a TRP-2180-188 peptide–specific CD8+ T cell clone (clone 24; ref. 26) was identified by spectratype analysis (27) and were subsequently cloned and sequenced. TCRlo mice were generated by similar method as described for TCRhi mice (25). In some experiments, TCRlo mice were crossed to IFN-γ–deficient background (gift, Dr. Robert Wiltrout, NCI, Frederick, MD) to generate IFN-γ−/− TCRhi 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-2180-188 (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 clone–type–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-H2Kb 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-2180-188. After incubation, plates were washed and processed as previously described (19).

**51Cr Release assay**

The 51Cr 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 51Cr 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 TCRhi-Thy1.1+ mice were dispersed into a single-cell suspension. CD8+CD44lo 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-carboxyfluoresceindiacetate succinimidyl ester (CFSE) as previously described (25), and 2.0 × 106 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 (auxiliary, 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-2Kb) expression on tumor cells was
analyzed by immunostaining of enzymatically digested tumors, gating on CD44<sup>+</sup>CD45<sup>+</sup> tumor cells.

**Adoptive transfer of transgenic T cells to treat subcutaneous B16 tumor**

A total of 2.5 × 10<sup>6</sup> antigen-specific CD8<sup>+</sup> lymph node T cells from TCR<sup>hi</sup> or TCR<sup>lo</sup> Rag<sup>−/−</sup> 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<sup>5</sup>). Three, 7, and 11 days after tumor challenge, mice received an intravenous injection of in vitro generated TRP-2–specific effector cells (1 × 10<sup>5</sup>). Effector cells were generated as described above for the <sup>51</sup>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<sup>2</sup> and tumor size was recorded as 250 mm<sup>2</sup> 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<sup>+</sup>/CD317<sup>+</sup>.

For the in vitro tolerance assay, naïve TCR<sup>hi</sup> or TCR<sup>lo</sup> Thy1.1 cells were cocultured for 72 hours with TRP-2 antigen-pulsed B16 TADCs (CD11c<sup>+</sup>/B220<sup>−</sup>/BST2<sup>−</sup>) isolated from subcutaneous B16 tumors. The T cells were re-stimulated 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 [<sup>3</sup>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**

TCR<sup>hi</sup> T cells display higher functional avidity than TCR<sup>lo</sup> T cells

Our previous studies showed that TRP-2-specific TCR<sup>lo</sup> 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 TCR<sup>lo</sup> mice were derived and displayed greater IFN-γ expression at indicated antigen doses (Supplementary Fig. S1). We observed a striking phenotypic difference in TCR<sup>hi</sup> mice compared with TCR<sup>lo</sup> mice (Fig. 1A): TCR<sup>hi</sup> mice spontaneously developed autoimmune depigmentation as initially revealed by diminished pigmentation of the ear at weaning. Over time, progressive vitiligo-like
depigmentation was observed in the coat hair of TCRhi mice, which was rarely observed in TCRlo mice (Fig. 1A). Consistent with these observations, we detected T cells in the skin of TCRhi mice, but not in the skin of TCRlo mice (Supplementary Fig. S2).

We next characterized the phenotype of CD8+ T cells from TCRhi mice. We observed a distinct population of cells that were CD25+, CD44hi, and Ly6c− that was not observed in TCRlo mice, which instead displayed a uniformly naïve phenotype: CD62Llo (data not shown), CD25−, CD44lo, and Ly6c+ while displaying comparable levels of TCR (Supplementary Fig. S3A). This phenotype suggested that these were antigen-experienced TCRhi T cells, presumably responding to endogenous TRP-2 antigen.

To confirm that TCRhi T cells respond to endogenous antigen, we enriched the CD44hi 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 transgenic mice (25), only T cells from TCRlo mice transferred to WT mice showed detectable CFSE dilution. Among those cells that diluted CFSE, most were CD44hi (Supplementary Fig. S3B). These findings confirm that only TCRhi T cells responded to endogenous TRP-2 antigen in the absence of exogenous priming, presumably due to their elevated avidity.

We next conducted tetramer decay analysis to confirm that TCRhi T cells truly possess higher avidity than TCRlo T cells. This assay measures the binding strength of a soluble form of the TCR ligand, a TRP-2180/H-2Kb tetramer. The decay time to 50% maximal tetramer binding for the TCRhi T cells was 65 minutes that was not observed in TCRlo cells (Fig. 2C). This increased avidity resulted in increased responsiveness to TRP-2 Ag, including expression of IFN-γ and Granzyme B as well as CD107a mobilization, an indicator of CTL granule exocytosis. These observations were consistent with our previous studies examining TCRlo TILs (25). However, at later time points (e.g., 15 days after vaccination), a significant reduction in the frequency of TCRlo TILs expressing IFN-γ and mobilizing CD107a was observed in comparison to the earlier time point, or when compared with TCRhi T cells isolated from the spleen of naive and tumor-bearing mice (Fig. 3A and B). Interestingly, we noted a loss of prominent fraction of tumor-infiltrating TCRhi T cells displaying diminished CFSE levels and elevated CD44 expression. Approximately 15% of TCRlo T cells that infiltrate the B16 tumors expressed IFN-γ. When comparing CFSE dilution, TCRhi 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 TCRhi cells from tumor-bearing mice (22.6% vs. 5.7%, P < 0.0001). In contrast, in the absence of any exogenous stimulation, TCRlo 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 TCRhi T cells within the tumor and an elevated frequency of TCRhi T cells that expressed IFN-γ (Fig. 2B). This was again consistent with our previous report showing infiltration of TCRhi T cells into B16 tumors following DC vaccination (25).

Given our observation that TCRhi T cells generated more robust responses than TCRlo T cells to both endogenous and tumor-derived antigen, we next tested whether transfer of TCRhi 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 TCRhi T cells alone (Fig. 2C). However, transfer of TCRhi T cells in combination with a TRP-2–pulsed DC vaccine 9 days after tumor challenge significantly delayed tumor progression. This effect was not observed for TCRlo T cells (Fig. 2C; ref. 25). Similar results were obtained when treating s.c. B16 tumor cells with in vitro activated effector cells (Supplementary Fig. S4A). In addition, we also observed that s.c. B16 tumor growth was delayed in TCRhi Tg mice, but not in TCRlo Tg mice (Supplementary Fig. S4B). Taken together, these results indicate that TCRhi T cells generate superior antitumor activity compared with the lower avidity TCRlo T cells.

TCRhi T cells infiltrating subcutaneous B16 tumors are tolerated

Given that both in vivo and in vitro primed TCRhi T cells initially slowed subcutaneous B16 tumor growth, but all mice eventually developed progressive tumor growth, we hypothesized that TCRhi T cells may be progressively tolerized within the developing B16 TME. Therefore, we sequentially analyzed TCRhi T cell reactivity after tumor infiltration. As indicated in Fig. 3 and Supplementary Fig. S5, TCRhi 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 exocytosis. These observations were consistent with our previous studies examining TCRlo TILs (25). However, at later time points (e.g., 15 days after vaccination), a significant reduction in the frequency of TCRlo TILs expressing IFN-γ and mobilizing CD107a was observed in comparison to the earlier time point, or when compared with TCRhi T cells isolated from the spleen of naive and tumor-bearing mice (Fig. 3A and B). Interestingly, we noted a loss of
responsiveness in TCRhi 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 TCRhi T cells gradually lost their effector functions during tumor progression, which is in contrast to our previously published findings showing that lower avidity TCRlo TILs retain effector function within the TME, despite their inability to retard B16 tumor growth (25).

Figure 2. T-cell avidity determines the magnitude of response to B16-tumor derived antigen. A, CFSE-labeled CD8+ CD44lo TCRhi and unsorted TCRlo Tg T cells were transferred into naïve B6 mice on day 0. The following day, mice were challenged s.c. with 1 x 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, TCRhi 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, TCRhi 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.
IFN-γ expression by TCRβhi T cells is critical for their antitumor immunity

One consequence of IFN-γ expression by TILs is the up-regulation 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 TCRhi T cells, we next tested whether this loss of antigen responsiveness by TCRβhi T cells correlated with a decrease in MHC-I expression by B16 tumor cells. While infection by both TCRα TCRβhi 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 TCRβhi T cells (Fig. 4A, P = 0.002). To confirm the connection between IFN-γ...
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 TCRhi 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 TCRhi T-cell–derived IFN-γ by crossing TCRhi 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−/− TCRhi T cells were unable to upregulate MHC-I expression on tumor cells as efficiently as WT TCRhi T cells (Fig. 4C). Loss of IFN-γ production by TCRhi 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 TCRhi T cells was critical for their antitumor activity and their ability to enhance MHC-I expression on their tumor targets.

**PD-1 blockade improves TCRhi 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 TCRhi 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 TCRhi TILs. In addition, we also observed restoration of responsiveness of TCRhi 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 TCRhi 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 tolerate TCRhi but not TCRlo T cells**

Given the differential tolerization of TCRhi and TCRlo T cells infiltrating B16 tumors, we tested the possibility that B16 TADCs may preferentially tolerate TCRhi T cells. We recently identified a population of plasmacytoid-like TADC with immunosuppressive function in both human and murine tumors, including B16 melanoma (31). These TADCs were CD11cα/B220+/B220−/CD317+ (Supplementary Fig. S8). To test the tolerogenic ability of the B16 TADCs, TCR Tg T cells were cocultrated with TRP-2 peptide–pulsed TADCs for 4 days before re-isolation and subsequent restimulation with TRP-2–pulsed splenic antigen-presenting cells (APC). TCRhi 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, TCRlo 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 tolerate TCRhi 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 TCRhi T cells were tolerated by B16 TADCs, consistent with our observations on tolerance induction following TCRhi T-cell infiltration into B16 tumors.

On the basis of the above findings that TADCs preferentially tolerized TCRhi T cells in vitro, we next sought to determine whether depletion of TADCs in vivo would prevent TCRhi T-cell

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**Figure 5.** B16 TADCs tolerate TCRhi T cells but not TCRlo T cells in vitro. TADCs were isolated from 15- to 20-day-old subcutaneous B16 tumors for use in the tolerization assay. Naive TCR Tg T cells were cocultrated 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 [3H] 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.
Depletion of B16 TADCs reduces tolerization of adoptively transferred TCRhi T cells in vivo. Wild-type (WT) B6 mice were injected s.c. with 1 × 10⁷ B16 tumor cells on day 0. At days 7, 8, and 15, mice were injected i.p. with anti-CD317 antibody. TCRhi 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 3 mice per group.

Figure 6. Depletion of B16 TADCs reduces tolerization of adoptively transferred TCRhi T cells in vivo. Wild-type (WT) B6 mice were injected s.c. with 1 × 10⁷ B16 tumor cells on day 0. At days 7, 8, and 15, mice were injected i.p. with anti-CD317 antibody. TCRhi 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.

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 autoimmune munity, 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 TCRhi 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 TCRhi 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, TCRhi 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 TCRhi and TCRlo 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 TCRlo T cells, the higher avidity TCRhi 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
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 TCRhi 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 TCRhi 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 TCRhi 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-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 tolerated TCRhi, but not TCRlo T cells in vitro. Enhanced TCRhi T cell reactivity following depletion of B16 TADCs supports the idea that TADCs are at least one component of the TME responsible for tolerizing TCRhi T cells. Incomplete depletion of TADCs may explain partial restoration of TCR T-cell function and partial enhancement of immunity to B16 tumor. However, we cannot rule out the possibility that anti-CD3/CD28 may deplete another cell population with immunosuppressive functions. Furthermore, TADCs isolated from B16 tumors growing in Foxo3Δlox/lox mice were not tolerogenic, which is consistent with our previous finding (31). It remains unclear whether other components of the TME, such as myeloid-derived suppressor cells (MDSCs) (38), regulatory T (Treg) cells (39), macrophages (40), or mast cells (41), also contribute to the tolerization of infiltrating TCRhi 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
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References


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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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Reference

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