Adoptive Transfer of Tc1 or Tc17 Cells Elicits Antitumor Immunity against Established Melanoma through Distinct Mechanisms

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Adoptive Transfer of Tc1 or Tc17 Cells Elicits Antitumor Immunity against Established Melanoma through Distinct Mechanisms

Yu Yu,* Hyun-II Cho,* Dapeng Wang,* Kane Kaosaard,* Claudio Anasetti,*†§ and Xue-Zhong Yu*†§

Adoptive cell transfer (ACT) of ex vivo–activated autologous tumor-reactive T cells is currently one of the most promising approaches for cancer immunotherapy. Recent studies provided some evidence that IL-17–producing CD8+ (Tc17) cells may exhibit potent antitumor activity, but the specific mechanisms have not been completely defined. In this study, we used a murine melanoma lung-metastasis model and tested the therapeutic effects of gp100-specific polarized type I CD8+ cytotoxic T (Tc1) or Tc17 cells combined with autologous bone marrow transplantation after total body irradiation. Bone marrow transplantation combined with ACT of antitumor (gp100-specific) Tc17 cells significantly suppressed the growth of established melanoma, whereas Tc1 cells induced long-term tumor regression. After ACT, Tc1 cells maintained their phenotype to produce IFN-γ, but not IL-17. However, although Tc17 cells largely preserved their ability to produce IL-17, a subset secreted IFN-γ, indicating the plasticity of Tc17 cells in vivo. Furthermore, after ACT, the Tc17 cells had a long-lived effector T cell phenotype (CD127+KLRG-1low) as compared with Tc1 cells. Mechanistically, Tc1 cells mediated antitumor immunity primarily through the direct effect of IFN-γ on tumor cells. In contrast, despite the fact that some Tc17 cells also secreted IFN-γ, Tc17-mediated antitumor immunity was independent of the direct effects of IFN-γ on the tumor. Nevertheless, IFN-γ played a critical role by creating a microenvironment that promoted Tc17-mediated antitumor activity. Taken together, these studies demonstrate that both Tc1 and Tc17 cells can mediate effective antitumor immunity through distinct effector mechanisms, but Tc1 cells are superior to Tc17 cells in mediating tumor regression. The Journal of Immunology, 2013, 190: 1873–1881.

C D4 and CD8 T lymphocytes can be classified into distinct types of effector cells based on their cytokine-secretion profiles after Ag stimulation (1–4). Type I CD8+ cytotoxic T (Tc1) cells secrete IFN-γ and kill tumor targets by either perforin- or Fas-mediated mechanisms, whereas type II CD8+ cytotoxic T cells secrete IL-4, IL-5, and IL-10, and kill tumor targets predominantly through the perforin pathway. IL-17–producing CD8+ T (Tc17) cells secrete IL-17A, IL-17F, IL-21, and IL-22, and also possess killing activity that can result in antitumor responses (4, 5). Although the contribution of adoptively transferred Th1 and Tc1 cells in antitumor responses has been clearly established, the role of IL-17–producing CD4+ T (Th17) and Tc17 cells remains controversial (5–7). After skewing primed naive CD4 T cells toward a Th17 phenotype, IL-17 was shown to induce Th1-type chemokines (8), recruiting effector cells to the tumor microenvironment. Conversely, Th17 can promote IL-6–mediated Stat3 activation, generating a protumorigenic environment (9, 10). One study showed that tumor-specific Th17 cells exhibited stronger therapeutic efficacy than Th1 cells upon adoptive transfer, and were converted into effective IFN-γ-producing cells (5) that promoted the expansion, differentiation, and homing of tumor-specific CD8+ T cells into the tumor microenvironment (11). In addition, adoptive transfer of tumor-reactive Tc17 cells also reduced the volume of established tumors whereas differentiating into long-lasting IFN-γ-secreting cells (4). Therefore, IL-17 and IFN-γ produced by T cells in the tumor microenvironment may determine whether these cytokines negatively or positively may affect tumor growth.

Adoptive cell transfer (ACT) of ex vivo–activated autologous tumor-reactive T cells is currently one of the most promising approaches for the treatment of patients with advanced melanoma (12–14). Therapeutic efficiency mediated by ACT is dependent, in part, on the ability of tumor Ag-specific T cells to persist and to retain their antitumor activity in vivo. ACT in combination with nonmyeloablative lymphoid depletion has emerged as one of the most effective antitumor therapies. The mechanisms involved in this model of immunotherapy include the elimination of regulatory T cells and the increased cytokine-dependent homeostatic proliferation of effector T cells (12, 15). The improved effectiveness of ACT after nonmyeloablative lymphodepletion provides the rational basis for the evaluation of more intensive conditioning regimens such as a myeloablative regimen in conjunction with bone marrow (BM) transplantation (BMT). In fact, more recent studies have indicated that hematopoietic stem cells promote the expansion and function of antitumor CD8+ T cells after ACT into tumor-bearing hosts (16, 17).

In this study, we used a murine melanoma model to assess the therapeutic effects of tumor-specific, polarized Tc1 or Tc17 cells.
combined with autologous BMT after total body irradiation (TBI). Although ACT with tumor-specific Tc1 or Tc17 cells induced significant regression of established large tumors, Tc1 cells showed higher therapeutic efficacy than Tc17 cells. Furthermore, our results show that Tc1 and Tc17 cells mediated their antitumor effects through distinct mechanisms.

**Materials and Methods**

**Animals**

C57BL/6 (B6) (H-2b) mice were purchased from the National Cancer Institute/Charles River program. Pmel-1 mice (B6 background) were purchased from The Jackson Laboratory. All animals were housed in the American Association for Laboratory Animal Care-accredited Animal Resource Center at Moffitt Cancer Center. All experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee.

**Tumor cells**

The luciferase-transduced B16-F10 cell line, a weakly immunogenic melanoma cell derived from B6 mice, was purchased from Xenogen Corporation (Berkeley, CA). The B16–IFN-γRDN tumor cell line expressing high levels of dominant-negative IFN-γ receptor (IFN-γRDN), which renders the cells unresponsive to the effects of this cytokine, was established as described previously (18). The EL4 cell line was obtained from the American Type Culture Collection (Manassas, VA).

**Spleen and lung cell preparation**

Spleens were collected from mice, and single-cell suspensions were prepared, washed twice in PBS, and resuspended in RPMI 1640 (Life Technologies), supplemented with 2 mM pyruvate, 100 μM penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated FCS (Life Technologies). To prepare single-cell suspension of lung parenchyma, lung tissues were minced finely and incubated in RPMI 1640 with 5% FCS, penicillin/streptomycin, 10 mM HEPES, 50 μM 2-ME, 20 mM l-glutamine containing 20 μM collagenase D (Sigma, St. Louis, MO), and 1 μg/ml DNAse (Sigma). After incubation for 60 min at 37°C, cells were collected by centrifugation. The cell pellet was suspended in 40% Percoll and layered onto 80% Percoll. The cells at the interphase were collected for further analysis.

**Generation of tumor-specific Tc1 and Tc17 cells**

Tc1 cells recognizing hgp100 25–33 were generated using the methods described previously with slight modifications (19). In brief, splenocytes from Pmel-1 transgenic mice were stimulated with the hgp25–33 peptide (1 μg/ml), recombinant mouse IL-12 (10 ng/ml; R&D Systems), and recombinant mouse IFN-γ (1000 U/ml; R&D Systems). Tc17 cells were generated by methods described previously (4). Splenocytes from Pmel-1 mice were stimulated with the hgp25–33 peptide (1 μg/ml), recombinant human TGF-β (5 ng/ml; R&D Systems), and anti-IFN-γ Abs (10 ng/ml; Bioxcell). Three days after peptide stimulation, the cell cultures were expanded with recombinant mouse IL-2. Cell phenotypes were confirmed on day 4 by intracellular cytokine staining for IFN-γ and IL-17.

**Adoptive immunotherapy model**

Syngeneic B6 mice were injected i.v. with 5 × 10^5 luciferase-transduced B16F10 or IFN-γRDN melanoma cells to establish pulmonary metastases. Six days after tumor challenge, mice were conditioned with TBI (1200 cGy in split doses). Bone marrow was flushed from donor femurs and tibias with RPMI 1640, and passed through sterile mesh filters to obtain single-cell suspensions. BM cells were depleted of T cells with anti-Thy1.2 mAb plus low-toxicity rabbit complement (C6 Diagnostics). T cell-depleted (TCD-BM) cells, referred to as TCD-BM, were used for all immunotherapy experiments. On day 7 after tumor implantation, mice received ACT (i.v.) of 2 × 10^6 splenic T cells (Tc1 or Tc17). Health status of tumor-bearing mice was monitored daily, and tumor growth was measured at specific time points using in vivo bioluminescent imaging. For the IFN-γRDN tumor model, antitumor effects were evaluated by examination and measurements of tumor masses or by counting the number of tumor nodules in the lungs.

**Abs and flow cytometry**

The following Abs were used for cell-surface staining: anti–CD4-FITC or -allophycocyanin (L3T4), anti–CD8ε-FITC, -allophycocyanin, allopococyanin-Cy7, anti–CD45.1-FITC or -allophycocyanin (A20), anti–CD90.1-PE or allophycocyanin were purchased from eBioscience; anti–CD4-Pacific Blue (RM4-5) was purchased from BD Biosciences. Detection of biotinylated Abs was performed using allopococyanin-Cy7 or allophycocyanin conjugated to streptavidin (BD Biosciences). Intracellular staining was carried out using anti–IFN-γ-PE or Per-cp-5.5 (XM1G2; BD Biosciences), anti–IL-17-allophycocyanin (17B7; eBiosciences). Cells were analyzed on a LSR II (BD Biosciences). Data were analyzed using FlowJo (Tree Star, Ashland, OR). For intracellular cytokine staining, splenocytes from recipient mice at the time specified were stimulated in vitro with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), 500 ng/ml ionomycin (Sigma-Aldrich), and 1 μl Golgi Plugs (BD Biosciences), and incubated at 37°C for 4–5 h before staining, as described in our previous work (20).

**ELISPOT assays for IFN-γ and IL-17 secretion**

ELISPOTs were performed according to the manufacturer’s protocol (Mabtech). In brief, 96-well Multiscreen-IP plates (Millipore) were coated with 10 μg/ml rat anti-mouse IFN-γ mAb (AN-18) or IL-17 mAb (TC11-18H10) overnight at 4°C. After washing the plates, freshly isolated splenocytes were incubated with hgp100 peptide (1 μg/ml) at 37°C for 18–20 h in RPMI 1640 containing 10% FBS. In some ELISPOT assays, peptide-pulsed (10 μg/ml) or unpulsed EL4 cells were used as APCs. After washing the plates and removing the cells, biotinylated rat anti-mouse IFN-γ mAb (R4-6A2) or biotinylated rat anti-mouse IL-17a mAb (TC11-8H1.4) was added and incubated for 2 h at room temperature, followed by addition of streptavidin-HRP for 1–2 h at room temperature. Spot counting was done with an ELISPOT reader system (Autoimmune Diagnostika). Experiments were performed in duplicate or triplicate wells.

**Statistical analysis**

To compare the survival of tumor-bearing mice in different groups, the log-rank test was used to determine statistical significance. To compare T cell expansion or cytokine production, a paired Student t test was used. The p values <0.05 were considered statistically significant.

**Results**

**ACT with Tc1 or Tc17 cells effectively control established melanoma after myeloablative conditioning and syngeneic BMT**

It has been reported that both Tc1 and Tc17 cells promote antitumor immunity and increase the survival of mice with established s.c. B16 melanomas (4, 21). Given that the effectiveness of immunotherapy is enhanced by a lymphodepleting regimen and transfer of hematopoietic stem cells (17), we tested the hypothesis that the efficiency of adoptively transferred Tc1 or Tc17 cells would be enhanced by myeloablative TBI and syngeneic BMT. We injected B16-F10 cells i.v. to establish melanoma metastases in the lung. After tumor injection, the mice were separated into two groups: one group was sublethally irradiated on day 6 and received ACT using polarized pml-1 Tc1 or Tc17 cells on day 7; the other group was lethally irradiated on day 6 and received Tc1 or Tc17 ACT in combination with syngeneic TCD-BM on day 7. The antitumor effects mediated by either Tc1 or Tc17 cells were more evident in mice receiving myeloablative TBI/TCD-BM as compared with mice treated using the nonmyeloablative TBI regimen (Fig. 1A). To understand the mechanism accounting for the enhanced antitumor effect after myeloablative TBI/TCD-BM, we assessed the in vivo persistence of transferred Tc1 or Tc17 cells. The numbers of Tc1 or Tc17 cells recovered from the spleens of mice that received myeloablative TBI/TCD-BM were markedly higher as compared with mice that received the nonmyeloablative TBI regimen (Fig. 1B). Because infiltration of effector T cells into the tumors is critical for generating antitumor responses (22, 23), we analyzed the cellular composition in the lungs of the treated mice and found that higher numbers of Tc1 or Tc17 pml-1 cells infiltrated into the lungs of mice treated with myeloablative TCD-TBI as compared with the lungs of mice that received the nonmyeloablative TBI regimen (Fig. 1B). Supported by published data from others (15), we reason that the augmented proliferation...
and tumor infiltration of adoptively transferred pmel-1 Tc1 or Tc17 T cells were likely driven by both lymphodepletion and hematopoietic stem cells.

Characterization of Tc1 and Tc17 cells after ACT using myeloablative TCD-TBI

Because the myeloablative TBI/TCD-BMT more clearly promoted the therapeutic efficacy of ACT (Fig. 1A), we selected this regimen as the platform for the remainder of our studies. Tc1 and Tc17 cells were generated in vitro as described in Materials and Methods, and their phenotypes were confirmed before ACT (Fig. 2A). To directly compare the proliferation of Tc1 and Tc17 cells in vivo, we labeled polarized cells with CSFE and adoptively transferred them into lethally irradiated B16 tumor-bearing mice. We observed that almost 100% (99.6 ± 0.8%) Tc1 cells, but only 56% (56.3 ± 2.4%) Tc17 cells underwent through >3 cell division cycles, indicating that Tc1 cells proliferated substantially faster than Tc17 cells during the first 5 d after cell transfer (Fig. 2B). To monitor antitumor responses mediated by Tc1 and Tc17, we measured with ELISPO T ex vivo the frequency of Ag-specific CD8 T cells that produced IFN-γ and IL-17 (Fig. 2C, 2D). We found that Tc1 cells had an Ag-specific response exclusively in IFN-γ production that was significantly higher than Tc17 cells. In contrast, Tc17 cells had an Ag-specific response in IL-17, as well as IFN-γ production. These results indicate that after adoptive transfer, Tc1 cells have a stable phenotype, whereas some Tc17 cells can switch to a Tc1 phenotype, reflecting the inherent plasticity of Tc17 cells.

Tc1 cells are more effective than Tc17 cells in mediating antitumor responses

Using the treatment schedule shown in Fig. 3A, we further assessed the therapeutic efficacy of ACT using either Tc1 or Tc17 cells. Mice that received Tc1 ACT completely controlled tumor growth, resulting in long-term tumor-free survival (Fig. 3B, 3C). In contrast, Tc17 ACT, at the same dose, was only able to partially control tumor growth and significantly prolonged survival, but ultimately all mice succumbed to their disease. These experiments were repeated at least five times, with nearly identical results.

Antitumor effects of Tc1 cells, but not Tc17 cells, depend on intact IFN-γ signaling on the tumor

Although CD8 T cells typically kill tumor targets via perforin/granzyme and CD95 ligand-dependent mechanisms, IFN-γ produced by these cells can also play an important role in achieving antitumor effects (16, 17). Because the Tc1 and some of the Tc17 cells produced IFN-γ after adoptive transfer (Fig. 2C, 2D), we investigated whether IFN-γ was required for the Tc1- or Tc17-mediated antitumor activity in vivo. To this end, we used a B16 tumor line transduced with a plasmid encoding an IFN-γRDN. The impairment of IFN-γR signaling on IFN-γRDN B16 cells was verified in our previously published work showing that IFN-γRDN B16 cells failed to upregulate MHC class I, MHC class II, and PD-L1 molecules upon IFN-γ stimulation (18). Using the same schedule as presented earlier for B16-F10 cells, polarized Tc1 or Tc17 cells were adoptively transferred into B6 mice that had established B16 IFN-γRDN lung tumors after myeloablative TBI and syngeneic TCD-BMT. In contrast with the findings using B16-F10 tumor cells (Fig. 3B, 3C), Tc1 cells were not able to achieve eradication of the B16 IFN-γRDN tumors, although they were able to prolong the survival of the tumor-bearing mice (Fig. 4A). Strikingly, ACT using Tc17 cells mediated complete regressions of the B16 IFN-γRDN tumors. These results indicate that the Tc1-mediated antitumor responses depended on functional IFN-γR on the tumor cells, which are not required for Tc17-mediated antitumor responses. Furthermore, the results also suggest that IFN-γ...
signals on the tumor cells limit the therapeutic efficacy of Tc17 cells.

In a parallel experiment, we assessed the effector function of Tc1 and Tc17 cells obtained from mice bearing B16 IFN-γRDN tumors after ACT/TCD-BMT. In these experiments, we evaluated the ability of the Tc1 and Tc17 cells to recognize B16-F10 and B16 IFN-γRDN tumors by IFN-γ and IL-17 ELISPOT assays. High numbers of gp100-specific IFN-γ–secreting cells, but few or no IL-17–secreting cells, were present in spleens of tumor-bearing mice that received Tc1 cells after stimulation with parental B16-F10 or IFN-γRDN tumor cells (Fig. 4B, 4C). Furthermore, the Tc1 responses increased when the parental B16-F10 cells were pre-treated with IFN-γ, indicating that IFN-γ augmented the capacity of the tumor cells and their potential to be recognized by Tc1 cells (Fig. 4C). Consistent with previous observations (Fig. 2C, 2D), a low frequency of IFN-γ–secreting cells and high frequency of tumor-reactive, IL-17–producing cells were detected in the spleens of tumor-bearing mice that received Tc17 cells (Fig. 4B, 4C). Similarly to the Tc1 response, Tc17 responses (measured by IL-17 secretion) were increased by pretreatment of the parental B16-F10 cells with IFN-γ (Fig. 4C). Lastly, splenocytes derived from both Tc1- and Tc17-treated mice were effective in recognizing the B16 IFN-γRDN tumor cells. Tc17-mediated antitumor activity depends on the effects of IFN-γ in the tumor environment

The data presented so far demonstrate that Tc1 cells mediate antitumor responses largely through the direct effects of IFN-γ and Tc17-mediated antitumor immunity responses than Tc17 cells. (A) Schema for treatment regimen of B16 melanoma. B6 mice were inoculated i.v. with luciferase-transduced B16-F10 melanoma cells. Six days later, tumor-bearing mice received 1200 cGy (split doses). One day after, Tc1 and Tc17 cells generated from Pmel-1/Thy1.1 mice were transferred into tumor-bearing mice together with TCD-BM cells from normal B6 mice. (B) Percentage survival of tumor-bearing mice was presented as pooled data from two replicate experiments combined. (C) Tumor growth was monitored by in vivo bioluminescent imaging (n = 4); one representative experiment from a total of five experiments is shown.
on the tumor cells, whereas IFN-γ signaling on tumor cells was not required for Tc17-mediated antitumor responses (Fig. 4A). Because Tc17 cells can produce both IL-17 and IFN-γ, and either cytokine can promote antitumor responses directly or indirectly, we assessed their potential roles by neutralizing either cytokine in vivo. In the B16-F10 parental tumor model, we found that neutralization of IFN-γ negatively affected the antitumor activity of Tc17 cells, whereas neutralization of IL-17A did not impair the Tc17-mediated antitumor effect (Fig. 4A), indicating that IFN-γ, but not IL-17A, contributed to Tc17-mediated antitumor activity. Because IFN-γ signaling on tumor cells was not required (Fig. 4A), we hypothesize that Tc17-mediated antitumor activity requires an indirect effect of IFN-γ. To test this hypothesis, we evaluated the impact of neutralization of IFN-γ or IL-17A on Tc17-mediated antitumor activity against IFN-γRDN B16 cells. Similar to parental B16 cells, neutralization of IFN-γ dramatically reduced Tc17-mediated antitumor activity against IFN-γRDN B16 cells, whereas neutralization of IL-17A did not have any effect (Fig. 5B). Given that IFN-γRDN B16 cells express 10-fold more IFN-γ than parental B16 tumor, we surmise that IFN-γ binds to IFN-γR on tumor cells, creating a microenvironment that promotes Tc17-mediated killing in an IL-17A–independent manner.

Distinct mechanisms of antitumor responses mediated by Tc1 and Tc17 cells

The data presented so far indicate that Tc1 cells had superior antitumor response through an IFN-γR signaling–dependent manner. In contrast, Tc17 cells had modest antitumor response against WT B16 but superior response against IFN-γRDN B16. Because some cytokines play important roles in T cell–mediated tumor progression (14, 24), we measured proliferation and cytokine production of transferred Tc1 and Tc17 cells in the spleen and lung of tumor-bearing recipients. Consistent with ELISPOT data in Fig. 2, Tc1 cells expressed higher levels of IFN-γ but little IL-17 in B16-bearing mice, whereas Tc17 cells expressed higher levels of IL-17 and low levels of IFN-γ, indicating that Tc1 cells had a more stable phenotype than Tc17 cells (Fig. 6A, left panels). Similar cytokine profiles were detected for transferred Tc1 or Tc17 cells in the recipients with IFN-γRDN B16 tumor (Fig. 6A, right panels). Interestingly, percentage IFN-γ+/IL-17+ (p = 0.07) and percentage IFN-γ+/IL-17+ cells (p = 0.03), in particular among transferred Tc17 cells, were significantly increased in the mice with IFN-γRDN B16 than those with WT B16 tumor (Fig. 6B). The data suggest that enhanced frequency of IFN-γ+/IL-17+ cells in the lungs might contribute to the superior activity of Tc17 cells against IFN-γRDN B16 tumor, given this double-positive pop-

FIGURE 5. Role of IFN-γ and IL-17 in Tc17-mediated antitumor effect. WT B6 mice (n = 4 per group) were given B16 (A) and IFN-γRDN B16 tumor (B) i.v. by tail vein. Seven days later, tumor-bearing mice were adoptively transferred with Tc1 and Tc17 cells in the combination of BM. Anti–IFN-γ mAb and anti–IL-17 mAb were administered i.p. on day 0 and continuously twice a week for 4 wk total. Overall survival was monitored. Corresponding groups of untreated tumor-bearing mice served as controls.
ulation was suggested to be the most functional subset among Th17 cells. TNF-α was expressed at low but similar levels by Tc1 and Tc17 cells (Fig. 6B), suggesting that TNF-α was not responsible for the differential ability of Tc1 and Tc17 cells in mediating antitumor responses against WT or IFN-γRDN B16.

To define the underlying mechanisms, we determined the expansion and migration of Tc1 and Tc17 cells by qualifying numbers of transferred cells in tumor-bearing mice. In the spleen, the numbers of Tc17 cells were significantly higher than Tc1 cells regardless of tumor type in days 14 and 28 after cell transfer (Fig. 6C, top panels). In the lung, the numbers of Tc1 and Tc17 cells were similar in the recipients with B16 tumor regardless of the time. However, the numbers of Tc17 cells were significantly higher than Tc1 cells in the recipients with IFN-γRDN B16 tumor in both time points (Fig. 6C, bottom panels). Moreover, the numbers of Tc17 cells were significantly higher in the recipients with WT tumor than those with IFN-γRDN B16 tumor in day 28, which may contribute to the superior activity of Tc17 cells against IFN-γRDN B16 tumor (Fig. 3).

Higher levels of T cell proliferation in spleen likely contributed to the higher numbers of Tc17 cells accumulated in the lungs as compared with Tc1 cells. However, the ability of T cells in migration is also an important factor for effector T cell accumulation in lung. To this end, we measured chemokine receptors expressed on the cells present in lung. As expected, Tc1 cells expressed significantly higher levels of CXCR3 than Tc17 cells, whereas...
Thy 1.1+ population are shown from three to four mice per group. Data represent one of three experiments using similar settings. *p < 0.05, **p < 0.01, ***p < 0.001.

**Table I. Phenotype of Tc1 and Tc17 cells after ACT**

<table>
<thead>
<tr>
<th>B16</th>
<th>IFN-γ DN B16</th>
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<tbody>
<tr>
<td><strong>Spleen</strong></td>
<td><strong>Spleen</strong></td>
</tr>
<tr>
<td>Tc1</td>
<td>Tc17</td>
</tr>
<tr>
<td>CD27</td>
<td>85.7 ± 8.8</td>
</tr>
<tr>
<td>CD62L</td>
<td>40.6 ± 11.6</td>
</tr>
<tr>
<td>CD127</td>
<td>29.4 ± 3.3</td>
</tr>
<tr>
<td>KLRG1</td>
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<tr>
<td><strong>Lung</strong></td>
<td><strong>Lung</strong></td>
</tr>
<tr>
<td>Tc1</td>
<td>Tc17</td>
</tr>
<tr>
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<td>KLRG1</td>
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**Discussion**

The use of ACT with tumor-specific T cells has shown to be quite effective for the treatment of established melanoma both in animal models and in patients. However, eradication of established melanoma has not been consistently achieved when using T cells specific for naturally processed, tumor-associated Ags (e.g., gp100) (19, 26–30). In this study, we clearly demonstrate that a myeloablative conditioning plus syngeneic BMT dramatically increased the antitumor efficacy of ACT using gp100-specific Tc1 or Tc17 cells against established B16 lung tumors. Using a BMT platform, we further showed that gp100-specific Tc1 cells could eradicate established parental B16 melanoma, and that gp100-specific Tc17 cells could eradicate established IFN-γRDN B16 tumors. To our knowledge, this is the first report showing that the ACT with T cells specific for naturally processed, tumor-associated Ags completely cured established melanomas without the use of IL-2 or vaccination.

**Figure 7.** Phenotypes of transferred Tc1 and Tc17 cells in vivo. The experiment was set up as in Fig. 6. Leukocytes isolated from recipient lung on day 14 were stained for surface expression with Thy 1.1, CD8, CXCX3, CCR6, and CD107a. Percentages of CXCX3+ (A), CD127+ (B), and CD107a+ cells (C) on gated Thy 1.1+ population are shown from three to four mice per group. Data represent one of three experiments using similar settings. *p < 0.05, **p < 0.01, ***p < 0.001.
Using BMT as a platform, we found that Tc1 cells exhibited antitumor activity superior to Tc17 cells (Fig. 3B). Effective tumor rejection by adoptively transferred T cells is likely to rely on several direct and indirect immune effector mechanisms. First, both Tc1 and Tc17 effectors may directly eradicate tumor cells through cognate interactions involving perforin/granzyme-mediated lytic mechanisms (4, 28). As a result, tumor-associated Ags may be released and presented by host APCs that may enhance host immune responses at sites proximal and distal to tumor growth. Second, release of Tc1 and Tc17 cytokines, such as IFN-γ and TNF-α, have been shown to directly inhibit tumor cell growth (34–36), because they enhance Ag presentation through upregulation of MHC class I on both tumors and host APCs (37, 38), and increase the expression of effector molecules by T cells to facilitate antitumor responses and tumor rejection (39–41). Third, Tc1 and Tc17 effector cells can induce Ag nonspecific inflammatory responses that may indirectly aid in cytolytic and/or cytostatic antitumor effects. Local release of cytokines, such as Tc17-derived IFN-γ, TNF-α, IL-17 and IL-21, and Tc1-derived IFN-γ, have been shown to mediate the selective recruitment and localization of macrophages, NK cells, and granulocytes that may facilitate enhancement of tumor Ag presentation and inhibition of tumor growth (21, 42). In either case, use of discrete cytokines produced by Tc1 or Tc17 effector cells can mediate tumor rejection with distinct mechanisms and potentially affect the efficacy of antitumor responses and tumor regression.

After ACT, Tc1 cells proliferated substantially faster than Tc17 cells during the first 5 d after cell transfer (Fig. 2B), but the numbers of Tc17 cells were significantly higher in spleen on days 14 and 28 regardless of tumor types (Fig. 6C). We interpret that Tc1 cells have superior potential to proliferate than Tc17 cells, but Tc17 cells have an advantage to survive as supported by our previously published observation (43). In WT B16 tumor, Tc1 cells were more efficient than Tc17 cells to mount antitumor responses against this poorly immunogenic melanoma (Figs. 1, 2). Because the comparable numbers of Tc1 and Tc17 cells were observed at the tumor site (lung) on days 14 and 28, we reason that the therapeutic efficacy of Tc1 and Tc17 cells against B16 tumor did not result from their differential expansion or infiltration in vivo. Instead, we propose that it is the quality of transferred T cells that dictates the therapeutic efficacy. Tc1 cells are known to have strong cytolytic activity against tumor targets, and this study demonstrated that IFN-γ signaling through tumor cells was required for Tc1-mediated antitumor activity (Fig. 4A). Consistent with recent reports showing that Tc17 cells have much weaker cytolytic activity (4, 21, 25), we also found that Tc1 cells expressed significantly higher levels of CD107a than Tc17 cells (Fig. 7C). Tc17 cells could produce IFN-γ after adoptive transfer in vivo, but the amount of IFN-γ or the frequency of IFN-γ-producing cells was lower than that observed in Tc1 cells. Because Tc17-mediated antitumor activity was also dependent on IFN-γ, we surmise that lower cytolytic activity and IFN-γ production by Tc17 cells likely accounted for their inferior therapeutic efficacy against B16 tumor as compared with Tc1 cells.

Strikingly and unexpectedly, we found that Tc17 cells were highly effective against IFN-γRDN melanoma (Fig. 4). It appears to be counterintuitive that neutralization of IFN-γ not only inhibited Tc17-mediated antitumor activity against parental B16, but against IFN-γRDN B16 cells as well. Because IFN-γRDN B16 cells express 10-fold more dominant-negative receptors that cannot mediate IFN-γ signaling, it is clear that Tc17-mediated antitumor activity is independent of receptor signaling on tumor cells. However, IFN-γRDN B16 cells could absorb a significant amount of IFN-γ. We surmise that a large number of IFN-γ/IFN-γR complexes created a microenvironment that promoted Tc17-mediated killing, which was independent of IL-17A because neutralization of this cytokine had no effect (Fig. 5). It is likely that the IFN-γ/IFN-γR complexes on the IFN-γRDN B16 cells serve as a rich source of IFN-γ for the tumor microenvironment, which promotes the antitumor effect. Although a precise mechanism remains to be further investigated, additional data (Figs. 6, 7) suggest that abundant IFN-γ accumulated at the tumor site might increase the recruitment of Tc17 cells through CXCR3 and the Tc17 activity by enhancing the IFN-γ/IL-17+ subset, which has strong antitumor activity (44). In addition, Tc17 cells have the phenotype of long-lived effector T cells (CD127<sup>hi</sup>KLRC1<sup>lo</sup>), which may also contribute to Tc17-mediated antitumor response against IFN-γRDN tumor. Other potential mechanisms may include other cytokines produced by Tc17 such as IL-21 and IL-22 could exert indirect antitumor response against IFN-γRDN tumor by recruiting innate immune cells such as macrophages and neutrophils.

Overall, this study leads to two novel and important findings: 1) the combination of BMT with TBI and adoptive transfer of tumor-specific Tc1 cells can cure established melanoma, which may represent a new strategy to treat patients with metastatic melanoma; 2) local accumulation of IFN-γ at the tumor site significantly contributes to T cell–mediated antitumor responses independent of IFN-γ signaling by tumor cells, which may have an implication that IFN-γ inside the tumor may act as an adjuvant to improve immunotherapy against established tumor.

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Disclosures
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