Dendritic Cells in Irradiated Mice Trigger the Functional Plasticity and Antitumor Activity of Adoptively Transferred Tc17 Cells via IL12 Signaling

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Abstract

Purpose: The adoptive cell transfer (ACT) of CD8+ T cells is a promising treatment for advanced malignancies. Lymphodepletion before ACT enhances IFNγ+CD8+ T cell (Tc0–)–mediated tumor regression. Yet, how lymphodepletion regulates the function and antitumor activity of IL17A+CD8+ T cells (Tc17) is unknown.

Experimental Design: To address this question, pmel-1 CD8+ T cells were polarized to secrete either IL17A or IFNγ. These subsets were then infused into mice with B16F10 melanoma that were lymphoreplete [no total body irradiation (TBI)], or lymphodepleted with nonmyeloablative (9 Gy) or myeloablative (9 Gy with hematopoietic stem cell transplantation) TBI. The activation of innate immune cells and function of donor T-cell subsets were monitored in recipient mice.

Results: Tc17 cells regress melanoma in myeloablated mice to a greater extent than in lymphoreplete or nonlymphodepleted mice. TBI induced functional plasticity in Tc17 cells, causing conversion from IL17A to IFNγ producers. Additional investigation revealed that Tc17 plasticity and antitumor activity were mediated by IL12 secreted by irradiated host dendritic cells (DC). Neutralization of endogenous IL12 reduced the antitumor activity of Tc17 cells in myeloablated mice, whereas ex vivo priming with IL12 enhanced their capacity to regress melanoma in nonmyeloablated animals. This, coupled with exogenous administration of low-dose IL12, obviated the need for host preconditioning, creating curative responses in nonirradiated mice.

Conclusions: Our findings indicate that TBI-induced IL12 augments Tc17 cell–mediated tumor immunity and underlie the substantial implications of in vitro preparation of antitumor Tc17 cells with IL12 in the design of T-cell immunotherapies.

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

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Introduction

The tumor microenvironment is rife with immune cells and secreted factors that hinder antitumor T-cell function (1–3), necessitating lymphodepletion with total body irradiation (TBI) before transfer of tumor-infiltrating or gene-engineered T cells to improve treatment outcomes in patients with cancer (4). Lymphodepletion augments the function of transferred CD8+ T cells by depleting immunosuppressive host cells, such as myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), and other cells that compete for homeostatic γ-chain cytokines such as natural killer cells and non–tumor-specific T cells (5–7). Lymphodepletion also enhances the antitumor activity of CD8+ T cells by activating innate immune cells via microbial translocation, a process that promotes the liberation of gut microbes from the compromised bowel into the blood (8). Recent findings revealed that activated dendritic cells (DC) from lymphodepleted patients secrete proinflammatory cytokines IL12 and IL23, which further improve the function of human tumor-infiltrating CD8+ T cells (9). Preclinical work has demonstrated that lymphodepletion resets DC homeostasis and increases their capacity to secrete IL12, while reducing their ability to produce IL10, resulting in better T-cell priming and antitumor responses (10). Increasing the intensity of lymphodepletion from a nonlymphodepleted to a myeloablative regimen (which requires stem cell transplantation) further enhances the treatment efficacy of infused T cells, eliciting an objective response rate of >70% in melanoma patients (11). Although the role of lymphodepletion on IL2-expanded CD8+ T cells [Tc0–, i.e., classic IFNγ+CTls] is well studied, how and to what extent lymphodepletion regulates the plasticity, phenotype, and antitumor activity of IL17A-producing CD8+ T cells (Tc17) remains unclear. Given that murine and human IL17A-producing T cells have shown preclinical promise in adoptive cell transfer (ACT) therapies (12–14), it is important to understand how lymphodepletion regulates antitumor Tc17 cells.
Translational Relevance

The adoptive cell transfer (ACT) of ex vivo expanded tumor-reactive T cells is one of the most promising approaches for the treatment of patients with advanced malignancies, such as melanoma and leukemia. Increasing the intensity of lymphodepletion before ACT enhances CD8⁺ T cell–mediated tumor regression in mice and humans, with objective response rates in melanoma patients of >70% and curative responses of nearly 20%. Here, we report a mechanism underlying the effectiveness of lymphodepletion via total body irradiation (TBI). We show that TBI not only creates space for the infused tumor-reactive T cells to engraft, but also activates the innate immune system, thereby bolstering the functional plasticity of antitumor Tc17 cells. Proinflammatory cytokine IL12, but not IL23, secreted by dendritic cells (DC) augmented the antitumor activity of Tc17 cells in irradiated animals. Moreover, IL12 could be safely used in cultures in vitro and in vivo to recapitulate the effectiveness of TBI in nonirradiated mice infused with Tc17 cells. These data suggest that alternative regimens to chemotherapy or TBI may be used to bolster T cell–based therapies and safely treat patients with advanced malignancies.
with 1 μmol/L human gp10025–33 peptide using irradiated spleenocytes (1:10 pmel-1-irradiated spleenocytes) from C57BL/6 mice for 5 hours. Monensin (BioLegend) was added after 1 hour of stimulation with the peptide. After surface staining, intracellular staining with antibodies was performed according to the manufacturer's protocol using Fix and Perm buffers (BioLegend). Data were acquired on FACVerse or Accuri (BD Biosciences). All data were analyzed with the FlowJo software (TreeStar).

Adoptive cell transfer

Tumor therapy was performed as described earlier (21). Briefly, 8-week-old mice were injected subcutaneously with 3 × 10^5 B16F10 tumor cells. Lymphopenia was induced by giving TBI at either a 5-Gy (nonmyeloablative) or 9-Gy (myeloablative) dose to tumor-bearing mice 1 day before pmel-1 CD8^+ T-cell transfer (day 8). HSCs, given to mice treated with 9-Gy TBI, were extracted from the bone marrow by lineage depletion with streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Dynal Biotech) against biotin-labeled antibodies (γδ T-cell receptor, αβ T-cell receptor, CD4, CD8a, NK1.1, Gr-1, B220, Ter-119, CD2, and CD11b; BD Biosciences) followed by a c-kit enrichment with CD117 Microbeads (Miltenyi Biotech) and cultured for 18 hours in 10% DMEM with 50 ng rmIL3, 500 ng rmIL6, and 500 ng rmCSF (PeproTech); 1 × 10^5 HSCs were administered 1 day after irradiation. The mice were treated via adoptive transfer of 1 × 10^7 in vitro activated pmel-1 T cells programmed as Tc0 or Tc17 cells (22). In other experiments, mice were lymphodepleted with a nonmyeloablative (5 Gy) preparative regimen 1 day before adoptive transfer and treated with Tc17 cells or Tc17 cells primed with IL12 or IL23. Serial tumor measurements were obtained and tumor area was calculated by multiplying the perpendicular diameters, and then plotted.

Tissue distribution analysis

For assays determining tissue distribution of transferred Vβ13^+ T-cell subsets, mice with B16F10 tumor that had received differing levels of TBI preconditioning and treated with either pmel-1 Tc0 or Tc17 cells were sacrificed 12 hours or 5 days after T-cell infusion. Spleens and tumor were harvested and processed by mechanical disruption. Tumors required 30-minute incubation with collagenase II at a concentration of 7.5 units/mL before tissue disruption. Single-cell suspensions from the organs were stained for transferred (pmel-1) cells and host myeloid cells.

In vivo cytokine neutralization

Neutralizing antibodies against murine IL-12p35 (Mmp35A1.6; eBioscience) or IL-23p19 (AF1619; R&D Systems) were used as previously published (13) or as prescribed on the website. Tumor-bearing C57BL/6 mice were treated via adoptive transfer of pmel-1 Tc17 cells. Immediately after transfer, mice were injected intraperitoneally with 100 μg neutralizing antibodies and repeated every other day for five cycles. The control group received isotype-matched antibody.

Results

Lymphodepletion augments the functional plasticity of antitumor Tc17 cells

A nonmyeloablative preparative regimen consisting of 5 Gy TBI before ACT of IFNγ”CD8^+” T cells (Tc0), vaccination with vaccinia virus hgp10025–33, and bolus IL2 induces regression of established B16F10 melanoma in mice (5). Increasing lymphodepletion to a myeloablative regimen requiring 9 Gy TBI and stem cell support augments ACT therapy without the need for vaccination (21). (Vaccine independence may be a key feature in the translation of these findings to humans, as vaccines for transferred T cells are not always available.) The mechanisms by which patient preconditioning with TBI enhances ACT treatment outcome is multifaceted, consisting of depleting (i) host lymphocytes that act as cytokine sinks (homeostatic cytokines are essential for the engraftment and expansion of infused T cells), (ii) suppressive Tregs and MDSCs within the tumor while (iii) comitobimately activating antigen-presenting cells (APC) of the innate immune system (8, 23). Although lymphodepletion augments the engraftment, function, and antitumor activity of infused Tc0 cells, it is unclear how and to what extent different intensities of host preconditioning affects the antitumor properties of IL17A-polarized CD8^+ T cells (Tc17), a lymphocyte subset that has shown promise in preclinical tumor models (14). We hypothesized that increasing the level of host preconditioning would augment the antitumor activity of Tc17 cells to a greater extent than Tc0 cells. To test this idea, we preconditioned mice bearing 7-day-old B16F10 melanoma tumor with either nonmyeloablative (5 Gy) or myeloablative (9 Gy plus 10^5 HSC transplantation) lymphodepletion regimens 1 day before transfer of pmel-1 Tc0 or Tc17 cells (pmel-1 cells: TCR Tg reactive against the tumor antigen gp100). Lymphopherete mice were used as a control (0 Gy). Mice were not vaccinated with hgp10025–33-expressing vaccinia virus, but were treated with bolus high-dose IL2. We found that adoptive transfer of pmel-1 CD8^+ T cells programmed to a Tc17 phenotype mediated greater regression of melanoma than pmel-1 Tc0 cells in all conditioning regimens (Fig. 1A–C). Moreover, a myeloablative regimen (9 Gy + HSC) was superior to nonmyeloablative preconditioning (5 Gy) in augmenting the antitumor activity of transferred pmel-1 Tc17 cells, resulting in curative responses in myeloablotted mice (9 Gy + HSC>5 Gy; Fig. 1B and C).

Given that pmel-1 Tc17 cells mediate superior antitumor responses in myeloablotted mice than Tc0 cells (Fig. 1C), we hypothesized that Tc17 cells traffic to the tumor more efficiently than Tc0 cells. We also posited that donor Tc17 cells prevailed at a higher ratio to remaining inhibitory host cells (i.e., host CD8, CD4, and NK cells) than donor Tc0 cells in myeloablotted mice. To test this concept, we evaluated the impact of a nonmyeloablative (5 Gy) versus myeloablative (9 Gy+HSC) preparative regimen on the engraftment of transferred pmel-1 Tc0 or Tc17 cells to the remaining host cells in the tumor of mice. The transgenic TCR marker Vβ13 was used to distinguish preactivated donor pmel-1 Tc0 or Tc17 cells from the host cells in mice that received TBI (Fig. 1D). As Wrzesinski and colleagues (21, 24) reported, we also found that transferred pmel-1 Tc0 prevailed at a higher ratio to remaining host CD8^+ T, CD4^+ T, and NK cells than donor pmel-1 Tc0 cells in myeloablotted mice. To test this concept, we evaluated the impact of a nonmyeloablative (5 Gy) versus myeloablative (9 Gy+HSC) preparative regimen on the engraftment of transferred pmel-1 Tc0 or Tc17 cells to the remaining host cells in the tumor of mice. The transgenic TCR marker Vβ13 was used to distinguish preactivated donor pmel-1 Tc0 or Tc17 cells from the host cells in mice that received TBI (Fig. 1D). As Wrzesinski and colleagues (21, 24) reported, we also found that transferred pmel-1 Tc0 prevailed at a higher ratio to remaining host CD8^+ T, CD4^+ T, and NK cells than donor pmel-1 Tc0 cells in myeloablotted mice (Fig. 1D and Supplementary Fig. S1). In contrast to our hypothesis, transferred pmel-1 Tc17 cells trafficked to the tumor at a comparable ratio against host CD8^+ T cells as pmel-1 Tc0 cells (Fig. 1D and E) in all regimens. Interestingly, only in the myeloablative setting (9 Gy + HSC) did transferred Tc17 cells reside in the tumor at a slightly higher ratio to host CD4^+ T and NK cells compared with transferred Tc0 cells (Supplementary Fig. S1). Because donor Tc17 cells prevail in the tumor over host elements as effectively as donor Tc0 cells at all
three levels of host preconditioning (0, 5, and 9 Gy + HSC), it remained unclear how escalating TBI doses enhanced the antitumor activity of pmel-1 Tc17 cells.

We therefore sought to determine whether increasing TBI enhanced the function of the transferred Tc17 cells to a greater extent than Tc0 cells in vivo. To address this question, we measured IL17A and IFNγ secretion by infused pmel-1 Tc17 or Tc0 cells before (day −1) and after transfer (days 2 and 20) into mice given different TBI preconditioning regimens. Before transfer, pmel-1 Tc0 and Tc17 cells secreted high levels of IFNγ and IL17A, respectively, upon hgp10025 antigen restimulation (Fig. 1F, day −1). Furthermore, as previously reported (21, 24), increasing the level of lymphodepletion augmented the function of pmel-1 Tc0 cells in vivo, as indicated by their increased capacity to secrete IFNγ. On day 2 after transfer, IFNγ production by pmel-1 Tc0 cells was highest in mice given 9 Gy TBI (72%), followed by mice given 5 Gy TBI (61%) versus those not given TBI (54%). Pmel-1 Tc17 cells produced nominal IFNγ but ample IL17A 2 days after infusion into nonirradiated mice. Interestingly, the greatest functional plasticity of pmel-1 Tc17 cells was observed in those infused into mice pretreated with 9 Gy TBI (+HSC), as these cells dramatically converted from mainly IL17A producers (day before transfer) into those that cosecrete ample IL17A and IFNγ (25%) and IFNγ alone (31%) 2 days after infusion (Fig. 1F, day 2). By day 20, transferred pmel-1 Tc17 cells produced no IL17A but high amounts of IFNγ. To our surprise, by day 20, transferred pmel-1 Tc17 cells produced more IFNγ than pmel-1 Tc0 cells at any level of lymphodepletion (Fig. 1F). Our data show that increasing the intensity of host preconditioning rapidly converts Tc17 cells from IL17A to IFNγ producers in myeloablated mice, and Tc17 cells ultimately secrete more IFNγ than Tc0 cells. This improved Tc17 functionality may account for the pronounced tumor destruction observed in myeloablated mice compared with nonirradiated or nonmyeloablated mice.

Lymphodepletion enhances the activation and maturation of DCs

Because DCs are the APCs specialized for triggering T-cell responses (25), we hypothesized that an increase in the activation of host DCs following TBI was responsible for initiating the functional plasticity (Fig. 1F) and antitumor activity of pmel-1...
Tc17 cells in myeloablated mice (Fig. 1C). To test this idea, we first examined the fate of host DCs and macrophages in the spleens of B6 mice following host preconditioning with either a nonmyeloablative (5 Gy) or myeloablative (9 Gy + HSC) preparative regimens compared with host DCs and macrophages in lympho-epitope animals (0 Gy-control). Flow cytometry analysis showed that 12 hours after TBI, the frequency of host CD11c⁺CD11b⁺ DCs and CD11b⁺F4/80⁺ macrophages increased significantly in recipient spleens (Fig. 2A). Furthermore, increasing TBI intensity directly corresponded to greater splenic DC and macrophage density in mice (Fig. 2A). Yet, as anticipated, TBI reduced the overall cellularity of recipient spleens by 90.3% and 93.3% in mice 12 hours after given either a nonmyeloablative (5 Gy) or myeloablative regimen (9 Gy + HSC), respectively (Fig. 2B). The absolute number of host DCs (but not macrophages) declined slightly 12 hours following TBI (Fig. 2C), and by day 5 >95.0% of host DCs and macrophages in the spleen were eliminated in irradiated animals (not shown). The fast depletion of host DCs following TBI was unexpected given that Tc17 cells mediate durable tumor immunity at >50 days after infusion (Fig. 1C).

Because T-cell activation is induced by mature DCs in vivo (25), we next sought to determine whether host DCs might be differentially activated following various levels of lymphodepletion, but before their disappearance. As shown in Fig. 2D, 12 hours after Total cells

**Figure 2.**

TBI enhances the frequency and activation status of host DCs before their disappearance. A, frequency of CD11b⁺CD11c⁺ DCs and CD11b⁺F4/80⁺ macrophages in spleens of nonirradiated, 5-Gy irradiated, and 9-Gy irradiated mice. Splenocytes were isolated 12 hours after TBI. Data are representative of three independent experiments (n = 7 mice per group). B, total number of splenocytes in mice 12 hours after preconditioning with 0, 5, or 9 Gy TBI. C, absolute number of CD11b⁺CD11c⁺ DCs and CD11b⁺F4/80⁺ macrophages in spleens of nonirradiated, 5-Gy irradiated, and 9-Gy irradiated mice. D, mean fluorescent intensity (MFI) of H2-Aβ, CD86, CD80, ICOSL, 4-1BBL, OX40L, and CD70 on splenic macrophages and DCs from mice given increasingly higher levels of TBI. MFI of these molecules is detected 12 hours after TBI. *P < 0.05; **P < 0.01; ***P < 0.001, Student t test.
TBI, host DCs from irradiated mice (5 Gy and 9 Gy + HSC) dramatically increased their expression of MHC I (H2-D^d) and costimulatory ligands CD86, CD80, ICOS ligand (ICOSL), 4-1BB ligand (4-1BBL), OX40 ligand (OX40L), and CD70. To our surprise, only OX40L expression was increased in macrophages 12 hours after TBI. Collectively, our data suggest that TBI induces the activation of host DCs before their rapid elimination. This led us to question if TBI regulates the ability of DCs to secrete proinflammatory cytokines and determine their effects on transferred cells.

Lymphodepletion enhances the function of host DCs
Preconditioning mice with 15 Gy TBI (with HSC) induces IL12 secretion by murine DCs (26) and Huang and colleagues (9) reported that increasing the intensity of irradiation augmented IL12 and IL23 secretion by human DCs in vitro. We therefore hypothesized that escalating TBI would also increase IL12 and IL23 secretion by irradiated host murine DCs, which, in turn, would augment the antitumor activity of transferred pmel-1 Tc17 cells in myeloablated hosts (Fig. 1C). As anticipated, splenic DCs isolated from myeloablated mice (9 Gy + HSC) secreted significantly more IL12 and IL23 1 day following TBI than DCs from nonirradiated mice (Fig. 3A). Moreover, the amount of IL12 and IL23 secreted by DCs was proportional to increased TBI. In contrast to DCs, macrophages secreted IL12 and IL23 due to TBI but to a markedly less amount that DCs (not shown). Our findings underscore that myeloablation augments the function of host DCs, as demonstrated by their heightened secretion of IL12 and IL23 compared with DCs from nonirradiated or non-myeloablated mice.

Lymphodepletion triggers changes in MDSC subsets
Pmel-1 T cells engineered to secrete IL12 eradicate melanoma when infused into mice (27). IL12 was found to trigger programmatic changes in dysfunctional MDSCs, in turn enhancing pmel-1 T cell–mediated tumor regression (28, 29). Specifically, Kerkar and colleagues (28) found that IL12 decreased monocyteic CD11b^+Ly6C^hiLy6G^lo cells (MDSC-M) but increased granulocytic CD11b^+Ly6C^loLy6G^hi cells (i.e., MDSC-G) in the tumor. Thus, as myeloablation (9 Gy + HSC) increases IL12 secretion by host DCs, we rationalized that this regimen altered the MDSC-G and MDSC-M cell composition in myeloablated mice compared with nonirradiated mice. Using flow cytometry, we examined the presence of these two MDSC subsets in the spleen of lymphodeplete (0 Gy), nonmyeloablated (5 Gy), or myeloablated (9 Gy + HSC) mice 12 hours after TBI. Indeed, the frequency and absolute number of splenic MDSC-G cells was increased in myeloablated mice (9 Gy + HSC) over MDSC-M cells compared with nonirradiated mice or in mice given a nonmyeloablate regime (Supplementary Fig. S2A and S2B). Yet, few of these cells remained in the spleen, blood, or tumor of recipient mice 5 days after TBI. Moreover, we found that TBI transiently induced MDSC-M but not MDSC-G cells to express a plethora of costimulatory molecules, including CD86, CD70, CD80, 4-1BBL, and ICOS ligand (Supplementary Fig. S3). Collectively, our data reveal that TBI alters the balance and activation status of DCs, macrophages, MDSC subsets in vivo, and causes host DCs from irradiated mice to secrete high quantities of IL12 and IL23.

TBI-induced IL12 augments the antitumor activity of transferred Tc17 cells
To determine whether the IL12 or IL23 secreted by DCs play a role in augmenting antitumor Tc17 activity in myeloablated mice, we first sought to determine how long activated DCs remain present and function in the irradiated animal. To monitor function, we measured when IL12p40 (a subunit shared by both IL12 and IL23; ref. 30) was most elevated in the serum of irradiated mice. Our objective was to use this kinetic information to identify when to deplete IL12 or IL23 to determine their putative role in Tc17 cell–mediated tumor immunity in myeloablated animals. Although both 5 Gy TBI and 9 Gy TBI decrease the absolute number of splenic CD11c^+DCs in mice by day 3 (Supplementary Fig. S4A, open diamond), a robust and transient increase in the absolute number of activated CD11c^+MHCII^+CD86^hi DCs was
observed in irradiated mice within 6 to 24 hours after TBI (Supplementary Fig. S4A, black square). Interestingly, the peak of CD11c<sup>+</sup>MHCII<sup>+</sup>CD86<sup>+</sup> DC activation was more rapid in mice given a myeloablative (9 Gy + HSC) regimen than in mice given a nonmyeloablative (5 Gy) regimen (6 hours vs. 24 hours, respectively; Supplementary Fig. S4A). Yet, as shown in Supplementary Fig. S4B, IL12p40 was detected within 6 to 12 hours in the serum of mice given either TBI regimen but was significantly lower in nonirradiated mice (0 Gy-basal is <100 pg/mL).

Moreover, significantly higher IL12p40 was detected in the serum of mice given a myeloablative preparative regimen than in the serum of mice from nonmyeloablative mice. Using this information, we next sought to determine whether TBI-induced IL12 or IL23 cytokines were responsible for the improved antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice. To do this, we neutralized IL12 or IL23 with antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice. To this end, we neutralized IL12 or IL23 cytokines were responsible for the improved antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice.

Furthermore, to determine whether IL12 promoted a shift in the T<sub>c17</sub> cultures that were primed with IL12 or IL23 to a more differentiated effector (CD62L<sub>lo</sub>CD44<sup>hi</sup>) memory phenotype (35). Indeed, T<sub>c17</sub> cells displayed an effector memory phenotype while T<sub>c0</sub> cells exhibited a central memory profile, as previously reported (13, 14). Interestingly, IL12 increased the frequency of central memory cells in T<sub>c0</sub> cultures but not T<sub>c17</sub> cultures (Fig. 4E). Collectively, our data reveal that priming T<sub>c17</sub> cells with IL12 increases their functional and transcriptional plasticity in vitro but does not alter their memory profile.

IL12 and IL23 distinctly regulate the functional plasticity, cytotoxicity, and transcription factor profile of adoptively transferred tumor-reactive T<sub>c17</sub> cells

Given that TBI-induced IL12 augments pmel-1 T<sub>c17</sub> cell antitumor activity in myeloablative mice (Fig. 3B), we surmised that if T<sub>c17</sub> cells were in vitro primed with IL12 that they would effectively regress melanoma in mice and that these mice would receive less lymphodepletion. We also posited that IL12 would bolster the functional plasticity of pmel-1 T<sub>c17</sub> cells in vitro, similar to that seen with pmel-1 T<sub>c17</sub> cells from myeloablative mice (9 Gy TBI + HSC; Fig. 1F, day 2). As expected, IL12-primed T<sub>c17</sub> cells were highly poly-functional, as 74% secreted IFNγ, 52% secreted IFNγ, and 41% cosecreted IL-17A plus IFNγ (Fig. 4A, T<sub>c17</sub> middle). Conversely, IL23-primed T<sub>c17</sub> cells maintained high IL-17A production (66%) and suppressed IFNγ (8% Fig. 4A, T<sub>c17</sub>-right bottom). In contrast to T<sub>c17</sub> cells, at this time point, neither IL12 nor IL23 priming enhanced the ability of pmel-1 T<sub>c0</sub> cells to cosecrete IL-17A and IFNγ (Fig. 4A, T<sub>c0</sub> panels). Our data reveal that IL12 triggers the functional plasticity of T<sub>c17</sub> cells in vitro, as demonstrated by their robust capacity to cosecrete IL-17A and IFNγ.

T<sub>c0</sub> cells are more cytotoxic than T<sub>c17</sub> cells in vitro (14). Thus, we next sought to determine how IL12 and IL23 regulate the cytotoxicity of T<sub>c0</sub> versus T<sub>c17</sub> cultures by measuring their release of granzyme B. We found that priming T<sub>c0</sub> cells with IL12 or IL23 only marginally increased the population of granzyme B and INFγ double producers (76% unprimed vs. 84% primed) and all T<sub>c0</sub> cultured cells secreted high amounts of granzyme B (Fig. 4B).

Unprimed T<sub>c17</sub> cells by contrast expressed low basal levels of granzyme B (20%), and priming with IL12 enhanced granzyme B secretion (38%) with the majority of these cells also producing INFγ (23% of 38%). Conversely, IL23 did not induce T<sub>c17</sub> cells to produce granzyme B compared with unprimed T<sub>c17</sub> cells (22% vs. 20%; Fig. 4B). Our data reveal that IL12 increases the cytotoxicity of T<sub>c17</sub> cells, as revealed by their marked gain in INFγ and granzyme B production.

Moreover, significantly higher IL12p40 was detected in the serum of mice given a myeloablative preparative regimen than in the serum of mice from nonmyeloablative mice. Using this information, we next sought to determine whether TBI-induced IL12 or IL23 cytokines were responsible for the improved antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice. To do this, we neutralized IL12 or IL23 with antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice. To this end, we neutralized IL12 or IL23 cytokines were responsible for the improved antitumor activity of the transferred pmel-1 T<sub>c17</sub> cells in myeloablative mice.

We next sought to investigate how IL12 and IL23 regulate the expression of transcription factors RORγ and T-bet in pmel-1 T<sub>c17</sub> cells. The transcription factor T-bet is important for sustaining IFNγ production by CD8<sup>+</sup> T cells while transcription factor RORγ is well known to promote IL17A secretion by T<sub>c17</sub> cells (32, 33). Using flow cytometry, we found that classic T<sub>c17</sub> cells expressed high levels of RORγ and nominal T-bet. Yet, IL12 priming decreased RORγ and increased T-bet expression in T<sub>c17</sub> cells while IL23 maintained RORγ (as shown by flow cytometry in Fig. 4C and in bar graph form from three different experiments in Fig. 4D). Conversely, T<sub>c0</sub> cells expressed nominal RORγ and T-bet. IL23 did not induce RORγ expression in T<sub>c0</sub> cells. However, IL12, but not IL23, significantly increased T-bet expression in T<sub>c0</sub> cells (Fig. 4C and D). Our finding is not surprising, given that IL12 induces T-bet in T cells (34). In summary, our data reveal that IL12 and IL23 differentially regulate RORγ and T-bet in antitumor T<sub>c17</sub> cells, which likely explains why they cosecrete INFγ and IL17A in myeloablative animals (Fig. 1F).

Given that IL12 increases T<sub>c17</sub> cytotoxicity, we wanted to determine whether IL12 promoted a shift in the T<sub>c17</sub> cultures from a less differentiated central (CD62L<sup>hi</sup>CD44<sup>lo</sup>) memory phenotype to a more differentiated effector (CD62L<sup>lo</sup>CD44<sup>hi</sup>) memory phenotype (35). Indeed, T<sub>c17</sub> cells displayed an effector memory phenotype while T<sub>c0</sub> cells exhibited a central memory profile, as previously reported (13, 14). Interestingly, IL12 increased the frequency of central memory cells in T<sub>c0</sub> cultures but not T<sub>c17</sub> cultures (Fig. 4E). Collectively, our data reveal that priming T<sub>c17</sub> cells with IL12 increases their functional and transcriptional plasticity in vitro but does not alter their memory profile.

IL12 priming augments the antitumor potential of T<sub>c17</sub> cells

Given that TBI induces host DCs to secrete high IL12 and IL23 levels in heavily pretreated mice (9 Gy TBI + HSC; Fig. 3A), we wished to assess whether priming T<sub>c17</sub> cells with IL12 or IL23 in vitro would augment their antitumor activity in mice preconditioned with a lower intensity and less toxic level of lymphodepletion (i.e., 5 Gy TBI). To address this idea, we transferred T<sub>c17</sub> cells that were primed with IL12 or IL23 in vitro into melanoma tumor-bearing mice conditioned with a nonmyeloablative (5 Gy TBI) preparative regimen. As an in vitro vaccination, pmel-1 subsets were restimulated with irradiated splenocytes presenting hgp100<sub>25-33</sub> peptide before transfer into recipient mice (22). T<sub>c17</sub> cells primed with IL23 did not induce significant tumor regression in the animals; in fact, following a period of relative stasis tumor area increased (Fig. 5A). In contrast, T<sub>c17</sub> cells primed with IL12-mediated tumor regression to a greater extent than unprimed or IL23-primed T<sub>c17</sub> cells (Fig. 5A). To determine whether priming T<sub>c17</sub> cells with IL12 affected their ability to engraft in the tumor or lymphoid tissues, we harvested spleens from preconditioned mice that had received T<sub>c17</sub> cells primed or not with IL12. As shown in Fig. 5B, we found that IL12 priming augmented trafficking of pmel-1 T<sub>c17</sub> cells to the tumor over host CD8<sup>+</sup>, CD4<sup>+</sup>, or NK cells in myeloablative mice. Interestingly, we
found that the combination of both host preconditioning with 5 Gy TBI and priming Tc17 cells with IL12 increased tumor infiltration of pmel-1 Tc17 cells over host cells to ratios similar or greater than those seen in the tumors of 9-Gy preconditioned hosts treated with Tc17 cells (Fig. 5B), but that IL12 priming did not increase the engraftment of Tc17 cells in the spleens of 5-Gy preconditioned hosts (Supplementary Fig. S5). These data reveal that priming Tc17 cells in vitro with IL12 augments their capacity to migrate to the tumor and regress melanoma in nonmyeloablated mice, in part, by increasing their ability to infiltrate the tumor to levels similar to that observed by Tc17 cells infused into myeloablated hosts. These data further underscore that TBI-induced IL12 potentiates the antitumor activity of Tc17 cells.

Exogenous IL12 therapy replaces a myeloablative preparative regimen

The robust antitumor response by IL12-primed Tc17 cells in nonmyeloablated hosts prompted us to question if a low dose of IL12 to nonirradiated animals would replace the prior need of preconditioning the host with TBI. To answer this question, we transferred Tc17 cells primed with IL12 into nonirradiated mice bearing B16F10 melanoma tumors and then treated them with or without exogenous IL12 (0.3 μg, injected subcutaneously) 1 and 7 days after T-cell transfer. We found that administration of exogenous IL12 bolstered the antitumor activity of IL12-primed Tc17 cells in nonirradiated animals (Fig. 6A). Importantly, this treatment mirrored the antitumor activity of Tc17 cells transferred into a myeloablated host (IL12-primed Tc17 cells plus exo. IL12 [open diamond; Fig. 6A] = Tc17 cells in 9-Gy conditions [open circle; Fig. 6B]). It is possible that this combination treatment in nonirradiated animals was potent because IL12-primed Tc17 cells migrated to the tumor effectively while exogenous IL12 collapses tumor stroma via Fas induction, as reported by Kerkar and colleagues (29). Interestingly, exogenous IL23 treatment (given at the same dose and time points as exogenous IL12) did not augment the antitumor activity of IL12-primed Tc17 cells (Fig. 6A), nor did IL23 alter tumor growth in mice not receiving cell therapy (not shown), despite its known role to promote the generation of pathogenic Tc17 cells (36). We also found that exogenous IL12 augmented the antitumor activity

**Figure 4.** IL12 and IL23 distinctly regulate the functional plasticity, cytotoxicity, and transcriptional profile of pmel-1 Tc0 and Tc17 cells. A, IL17A and IFNγ secretion by pmel-1 Tc0 and Tc17 cells primed with either IL12 or IL23 was analyzed on day 5 after expansion by flow cytometry. Results representative of five independent experiments with similar results. B, intracellular stain comparing IFNγ secretion with granzyme B production by pmel-1 Tc0 and Tc17 cells primed with IL12 or IL23 was analyzed on day 6 after expansion. Data are representative of four independent experiments. C, RORγt and T-bet transcription factor expression was analyzed day 5 in Tc0 and Tc17 cells primed with IL12 or IL23. Results representative of five independent experiments. D, percentage Tc0 and Tc17 cells expressing RORγt and/or T-bet after priming with IL12 or IL23 on day 5 as displayed in bar graph form. Data representative of three independent experiments; *, P < 0.05; **, P < 0.01; two-tailed t test, error bars represent standard error. E, memory phenotype of pmel-1 Tc0 and Tc17 cultures based on CD44 and CD62L expression as analyzed by flow cytometry. Data are representative of four independent experiments.
of IL12-primed Tc0 cells (Fig. 6C) but not to the extent achieved by IL12-primed Tc17 cells (Fig. 6A). Collectively, our data suggest that exogenous administration of IL12 obviates the need for a myeloablative preparative regimen to eradicate melanoma, if mice are treated with tumor-reactive IL12-primed Tc17 cells.

Figure 5.
Pmel-1 Tc17 cells primed with IL12 but not with IL23 traffic to the tumor and effectively mediate potent antitumor responses in vivo. A, 10 million pmel-1 Tc17 cells were primed with either IL12 or IL23 and then transferred into mice bearing established B16F10 melanoma. Pmel-1 Tc17 cells that were not primed with cytokines in the IL12 family served as a control. Recipient mice were irradiated with 5 Gy TBI 12 hours before adoptive transfer. No adjuvant vaccination and no IL2 support were administered. Data representative of two independent experiments and \( n = 8 \) per group; \(*, P < 0.05; **, P < 0.01; *** , P < 0.001\), one-way repeated measures ANOVA. B, relative infiltration of donor pmel-1 Tc17 cells compared with host CD8\(^+\) T cells, host CD4\(^+\) T cells, or host NK cells within the tumors of mice preconditioned with either 0, 5, or 9 Gy TBI (+HSC) receiving Tc17 cells or preconditioned with 0 or 5 Gy TBI and receiving IL12-primed Tc17 cells were combined, stained for donor V\(\beta\)13 to host CD8, CD4, and NK1.1, and analyzed by flow cytometry. Representative of three experiments.

Figure 6. Exogenous IL12 mediates durable antitumor immunity in nonirradiated mice infused with IL12-primed pmel-1 Tc17 cells. In all experiments using exogenous IL12 and IL23, these cytokines were given at 0.3 \( \mu \)g/mouse subcutaneously on days 1 and 7 following pmel-1 Tc17 cell transfer. A, 10 million IL12-primed Tc17 cells were adoptively transferred into nonirradiated tumor-bearing mice with or without exogenous IL2 or IL23 cytokine treatment after transfer. Mice receiving no treatment or only exogenous IL12 but no T cells were used as controls. B, 10 million Tc17 cells were transferred into either nonirradiated or lymphodepleted (9 Gy + HSC) tumor-bearing hosts. Mice receiving only myeloablative TBI with HSC were used as controls. C, 10 million IL12-primed Tc0 cells were transferred into nonirradiated tumor-bearing mice with or without exogenous IL12 treatment. Data representative of two independent experiments and \( n = 8 \) mice per group; \(*, P < 0.05; **, P < 0.01; *** , P < 0.001\), one-way repeated measures ANOVA.
Discussion

Host preconditioning with lymphodepletion augments the antitumor activity of infused IFNγ−CD8− T cells (Tc0) in mice and in humans with melanoma. Yet, the extent to which lymphodepletion affects the fate of antitumor IL17A−CD8+ T (Tc17) cells—an emerging subset showing great promise in ACT murine models—remains unknown. To address this, we polarized pmel-1 CD8+ T cells to secrete IL17A or IFNγ and infused them into melanoma-bearing mice that were either (i) not lymphodepleted (0 Gy TBI) or lymphodepleted with (ii) a nonmyeloablative (5 Gy TBI) or (iii) myeloablative (9 Gy TBI requiring HSC) preparative regimen. We found that Tc17 cells regressed melanoma in myeloablated mice to a greater extent than in lymphophage or nonmyeloablated mice (Fig. 1A–C). Moreover, Tc17 cells, but not Tc0 cells, mediated curative responses in myeloablated mice. Additional investigation revealed that Tc17 cells converted from mainly IL17A producers into IL12A−IFNγ− double producers 2 days after transfer into myeloablated mice and that these cells converted more rapidly in myeloablated mice than in lymphophage or nonmyeloablated mice (Fig. 1F). Interestingly, lymphodepletion triggered the innate immune system, as demonstrated by an increase in the number of DCs, granulocytic MDSCs, and macrophages before their disappearance (Fig. 2A and Supplementary Fig. S2). Among these cells, DCs showed significant increases in their costimulatory ligand expression after irradiation (Fig. 2D). Furthermore, they secreted the proinflammatory cytokines IL12 and IL23 (Fig. 3A). Interestingly, only IL12 secreted by TBI-activated host APCs that ultimately reconstituted our endogenous IL12, but not IL23, reduced the therapeutic efficacy of Tc17 cells in myeloablated mice (Fig. 3B). Conversely, priming Tc17 cells in vitro with exogenous IL12, but not IL23, enhanced their functional plasticity and capacity to regress melanoma in vivo (Figs. 4 and 5). Administration of low-dose IL12 to non-irradiated mice further potentiated the antitumor activity of IL12-primed Tc17 cells (Fig. 6), leading to long-term antitumor immunity in mice without the requisite for lymphodepletion.

Given that IL12 and IL23 enhance immune responses to tumors (29, 37, 38) and because we found that both IL12 and IL23 are induced in myeloablated mice (Fig. 3A), we suspected that these cytokines were important for enhancing Tc17 cell-mediated tumor immunity in myeloablated mice. As expected, blocking IL12 with an antibody that neutralizes IL12p35 impaired the antitumor activity of Tc17 cells in myeloablated mice. Unexpectedly, blocking IL23 with anti-IL23p19 did not impair treatment outcome in myeloablated mice infused with Tc17 cells. Although these data suggest that TBI-induced IL12, but not IL23, is responsible for augmenting Tc17-mediated tumor regression in myeloablated mice, we should proceed with caution with this interpretation as we only neutralized IL12 or IL23 for 1 week in mice following TBI. Thus, it is plausible that these cytokines were not sufficiently neutralized or that this approach did not remove all of the IL12 or IL23 secreted by activated host APCs that ultimately reconstituted the animal beyond the 3 days we assayed for these cytokines. Follow-up studies with recipients deficient in IL12 or IL23 may provide more insight into whether these cytokines contribute to the antitumor activity or persistence of Tc17 cells.

To understand whether TBI-induced IL12 augmented the functional plasticity and antitumor activity of Tc17 cells, we blocked IL12 with an antibody the binds the p35 subunit called anti-IL12p35 (Fig. 3B). While neutralizing IL12p35 with this antibody impaired the antitumor activity of Tc17 cells in myeloablated mice, it is worth noting that p35 is also a subunit of the cytokine IL35 (a cytokine in the IL12 family composed of p35 and EB3 subunits; ref. 39). Although our studies did not examine IL35 production in irradiated animals, future studies on the role of TBI induced IL35 would be interesting in the context of ACT therapy, particularly given that IL35 has been reported to promote cancer growth (40). Nevertheless, based on our findings, IL12p35 has a positive therapeutic effect on Tc17 cells in vivo (Fig. 3B). Indeed, IL12p35 has also been shown to be critical for inducing the secretion of IL18 in vivo, which can also be key for inducing IFNγ production by T cells (41–43). Thus, in future studies, it would be of interest to know whether blocking IL12p35 prevents IL18 secretion, thereby impairing IFNγ production by transferred Tc17 cells in myeloablated mice. Although it would be worthwhile to further delineate p35 for IL12, IL18, and IL35 in myeloablated mice, the difficulty due to overlap with these various cytokines is appreciated. However, considering our data where Tc17 cells primed in vitro with total recombinant IL12 drives the robust secretion of both IL17A and IFNγ (Fig. 4A), it should not be ruled out that the effect that is seen in vivo when blocking IL12 is also due to other downstream effects, such as lack of IL18. Although blocking cytokines in vivo is complex and can likely lead to unexpected immunologic outcomes, our findings imply that IL12 plays a key role in augmenting the functional plasticity and antitumor activity of Tc17 cells in vivo.

IL12 induces toxic side effects of humans, which has hampered its translation into the clinic (44). To circumvent these negative side effects of IL12 in vivo, we sought to use IL12 in pmel-1 Tc17 cultures to enhance their function and cytotoxicity in vitro and then wash out IL12 before infusing these cells into mice. Via this approach, we found that priming Tc17 cells with IL12 enhanced their function and cytotoxicity in vitro and in vivo (Fig. 4A and B) without inducing toxic side effects in the mice (Fig. 5A). Tc17 cells primed with IL12 greatly increased their IFNγ production (52%) compared with those primed with IL23 (8%) in Fig. 4A. Moreover, IL12-primed Tc17 cells secreted heightened amounts of granzyme B (Fig. 4B). This increase in classical Tc1 effectector molecules in Tc17 cells correlated with an induction of T-bet and suppression of RORγt (Fig. 4C and D). Along with modulating T-bet, IL12 has been reported to convert Tc17 cells to IL17A/IFNγ-double producers through epigenetic suppression of cytokine signaling 3 (SOCS3) gene promoters (45, 46). Thus, future studies that investigate the role of how TBI regulates SOCS3 as well as STAT signals in transferred Tc17 cells may shed light on their pronounced plasticity and antitumor activity in myeloblated mice.

There are three reported requirements for breaking tolerance to established melanoma with pmel-1 Tc0 cells: (i) preconditioning the host with lymphodepletion; (ii) transfer of effective T cells; and (iii) vaccination and administration of recombinant human IL2 (47). Yet, high doses of IL2 increase the generation of FoxP3+ Tregs in vivo, both in humans and mice (48). To circumvent Treg generation, we did not administer IL2 to mice infused with IL12 or IL23-primed Tc17 cells. IL12-primed Tc17 cells showed greater antitumor efficacy than IL23-primed and unprimed Tc17 cells, which we attribute to their poly-functional activity and enhanced capacity to traffic to the tumor over inhibitory host elements like Tregs in vivo (Fig. 5B). Tc17 cells primed with IL23 were not as effective at
killing the tumor. Collectively, our results demonstrated that IL12 plays a crucial role in enhancing the function, cytotoxicity, and tumor trafficking ability of pmel-1 Tc17 cells in vivo.

We demonstrated that mice infused with IL12-primed Tc17 cells that were also treated with a low dose of exogenous IL12 experienced durable antitumor responses without the normal need for host preconditioning with lymphodepletion (Fig. 6). However, these potent therapeutic results cannot be recapitulated by either (i) administration of exogenous IL23 given to mice infused with IL12-primed Tc17 cells or by (ii) exogenous IL12 given to mice infused with IL12-primed Tc0 cells. Our body of work herein underscore that IL12 is critical for potentiating the antitumor activity of Tc17 cells.

IL12 has been shown to play an important role in modulating the tumor microenvironment both by acting on the tumor stroma itself as well as triggering programmatic changes in dysfunctional tumor-derived myeloid-derived cells (28). Likewise, we found that TBI triggered programmatic changes in the myeloid-derived cell compartment of mice, including a robust yet transient induction of granulocytic MDSCs over monocytic MDSCs (Supplementary Fig. S2). In future studies, it will be interesting to determine the role of these distinct MDSC subsets in regulating T-cell subsets in our ACT model of melanoma. This line of investigation would be particularly interesting given that granulocytic MDSC secrete reactive oxygen species (ROS) and nominal nitric oxide (NO), whereas monocytic MDSC secrete NO but little ROS—both NO and ROS have been shown to regulate the biology of Th17 cells in tumor immunity and autoimmunity (49–51). Collectively, our data show that both environmental IL12 and the innate character-istics of Tc17 cells are required for a superior therapeutic result in nonirradiated animals. These data are important because they suggest that alternative regimens to chemotherapy or TBI, such as adjuvants that induce IL12 secreting DCs, may be used to safely treat patients with advanced disease and promote tumor regression comparable with that of nonmyeloablative preparative regimens. The relevance of our findings extends beyond melanoma and provides insight into mechanisms for improving the quality of T cells for passive and adoptive therapies that could treat a variety of cancers as well as infectious diseases in the clinic.

It is worth considering the impact of TBI on models of lymphodepletion, as many investigators use TBI to study lymphocyte engraftment and immunity to foreign, self, and tumor tissue (52, 53). Yet, the induction of homeostatic expansion of the T-cell compartment after TBI preconditioning cannot be viewed as simply expanding to fill empty space. We and others have reported that TBI induces a complex set of events, such as microbial translocation (8), which likely plays a role in inducing the secretion of IL12 and IL23 from activated DC, which, in turn, enhances the efficacy of adoptively transferred T cells, particularly Tc17 cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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