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The Inducible Costimulator Augments Tc17 Cell Responses to Self and Tumor Tissue

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The inducible costimulator (ICOS) plays a key role in the development of Th17 cells, but its role in the development and antitumor activity of IL-17–producing CD8+ T cells (Tc17) remains unknown. We found that ICOS costimulation was important for the functional maintenance, but not differentiation, of Tc17 cells in vitro. Blocking the ICOS pathway using an antagonist mAb or by using recipient mice genetically deficient in the ICOS ligand reduced the antitumor activity of adoptively transferred Tc17 cells. Conversely, activating Tc17 cells with an ICOS agonist in vitro enhanced their capacity to eradicate melanoma and induce autoimmune vitiligo when infused into mice. However, ICOS stimulation did not augment the antitumor activity of IL-2 expanded T cells. Additional investigation revealed that ICOS stimulation not only increased IL-2Rα, CXCR3, and IL-23R expression on Tc17 cells, but also dampened their expression of suppressive molecule CD39. Although Tc17 cells activated with an ICOS agonist cosecreted heightened IL-17A, IL-9, and IFN-γ, their therapeutic effectiveness was critically dependent on IFN-γ production. Depletion of IL-17A and IL-9 had little impact on antitumor Tc17 cells activated with an ICOS agonist. Collectively, our work reveals that the ICOS pathway potentiates the antitumor activity of adoptively transferred Tc17 cells. This work has major implications for the design of vaccine, Ab and cell-based therapies for autoimmune, infectious disease, and cancer.

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Interleukin-17–producing CD8+ T cells (Tc17) have been identified in both mice and humans (1–3). Compared with classical CTLs, Tc17 cells mediate a less cytotoxic effector function toward antigenic targets, because of their diminished capacity to secrete IFN-γ and granzyme B in vitro (4). Yet in vivo, Tc17 cells play a role in exacerbating autoimmune diseases, such as multiple sclerosis, diabetes, colitis, and psoriasis (5–10). Tc17 cells also protect the host against lethal influenza challenge (2) and mediate tumor regression (11). Interestingly, IL-17A secretion by CD8+ T cells has recently been reported to support Th17 cell–mediated autoimmune encephalomyelitis (12), suggesting that Th17 and Tc17 cells cooperate to amplify immune responses to self tissue. Although Tc17 cells regulate immune responses to self, foreign, and tumor tissue, the cues that control their noncytotoxic and pathogenic states remain unclear.

Unlike Th17 cells, less is known about the role of Tc17 cells in disease pathogenesis. However, the cytokines that control IL-17–producing CD4+ and CD8+ T cell differentiation and maintenance appear to be similar. TGF-β1 and IL-6 differentiate naïve CD8+ T cells into Tc17 cells, as evidenced by their induction of ROR-γt and IL-17A, whereas IL-23 maintains their function and phenotype in long-term cultures (13–15). Although the cytokines that regulate Tc17 cell development are known, the specific costimulatory pathways that impact their differentiation, expansion, and maintenance remain to be fully elucidated.

Ag-specific and costimulatory signals from APCs are needed for the activation and function of T cells (16). The costimulatory molecule CD28 is constitutively expressed on lymphocytes, whereas the costimulatory protein ICOS (also known as CD278) is inducibly expressed on activated T cells and on a small cohort of resting regulatory T cells (Tregs) and Th17 cells (17–19). In addition to CD28, signaling via ICOS is required for optimal cytokine secretion, because both costimulatory molecules are essential for optimal IL-17A secretion by murine Th17 cells (20). In contrast with murine Th17 cells, however, ICOS augments the expansion and function of human Th17 cells, whereas CD28 abrogates their function (18, 21). In a murine model, it has been reported that ICOS costimulation is not required for the differentiation of naïve CD4+ T cells toward a Th17 phenotype. Rather, ICOS costimulation is required for IL-23–driven expansion of already differentiated Th17 cells (19). Specifically, ICOS amplifies Th17 responses by inducing transcription factor c-MAF, transactivating IL-21 production, and upregulating IL-23R.
expression (19). Although ICOS is important for regulating Th17 generation, the role of ICOS costimulation in controlling Tc17 differentiation, expansion, and function remains to be identified.

We report that ICOS is important for supporting the maintenance, but not the differentiation, of memory Tc17 cells. In vivo, blockade of the ICOS–ICOS ligand (ICOSL) pathway impaired their capacity to eradicate melanoma and induce autoimmune vitiligo in mice. Conversely, activating Tc17 cells in vitro with an ICOS agonist augmented their capacity to mount immunity to self/tumor in vivo in an IFN-γ-dependent manner. ICOS stimulation not only increased IL-2Rα, IL-7Rα, and IL-23R expression on Tc17 cell, but also heightened their in vivo cytotoxicity and dampened their expression of suppressive/coinhibitory molecule CD39. Collectively, these data reveal that ICOS augments Tc17 responses to self and tumor tissue.

Materials and Methods

Mice and tumor lines

To study the role of ICOS in tumor therapy with Tc17 cells, we used the peml-1 model of adoptive immunotherapy against the poorly immunogenic B16F10 melanoma. peml-1, C57Bl/6 (B6), ICOS-deficient (ICOS−/−), and ICOSL-deficient (ICOSL−/−) mice (Jackson Laboratory) were housed and bred in the Medical University of South Carolina (MUSC) vivarium. Institutional Animal Care and Use Committee at the MUSC approved the animal work. B16F10 tumors were obtained from the laboratory of Dr. Nicholas Restifo.

T cell generation

Transgenic peml-1 TCR or B6 or ICOS−/− CD8+ splenocytes were cultured in IL-2–expanding conditions or in IL-17–polarizing conditions, as described elsewhere (11), using 1 μM hgp10025–33 (KYPQRNQDNL). In brief, peml-1 TCR transgenic or peml-1 cells were cultured with recombinant human (rh)IL-2 (100 IU/ml; National Institutes of Health). Tc17 cells were polarized using rhIL-6 (5 ng/ml; National Cancer Institute preclinical repository), rhTGF-β1 (10 ng/ml; BD Pharmingen) plus anti-mouse IFN-γ and anti-mouse IL-4 (10 μg/ml; BD Pharmingen). rhIL-2 (50 IU/ml; National Institutes of Health) was added on the second day of culture. Cells were cultured for 6 days unless otherwise indicated. For secondary stimulation, the cells were restimulated with irradiated splenocytes coated with CD3 agonist and IL-23 (20 ng/ml; R&D Systems) for an additional 5 d. B6 or ICOS−/− CD8+ T cells were cocultured with irradiated splenocytes and anti-CD3 (1 μg/ml; clone 145-2C11; Biolegend), or with or without Th17 polarization. In some experiments, cells were treated with a soluble ICOS agonist Ab (20 μg/ml; clone C93/8.4A; Biolegend), ICOSL blocker (20 μg/ml; clone HK5.3; Biolegend), or a control Ab on days 2, 4, and 6 of culture.

Adoptive cell transfer and vitiligo score

Adoptive transfer experiments have been described previously (22). In brief, recipient B6 mice were given 3 × 105 B16F10 melanoma tumor cells s.c. on day 0. The mice were then irradiated with 5 or 6 Gv total body irradiation (TBI), as indicated in the figure legends, 6 h before CD8+ T cell transfer. Mice received i.v. 1 × 105 peml-1 CD8+ T cells that were in vitro vaccinated, in conjunction with bolus rhIL-2 (was administered to mice i.p. once or twice daily at 3.6 μg/dose for a total of 4–6 doses). Vaccination involved coculturing the CD8+ T cells with peptide-pulsed B6 splenocytes and 1 μM hgp10025–33 peptide for 6 h as in Ref. (22). In some experiments, mice were given 0.1 mg neutralizing Ab i.p. every other day for a total of five treatments. Anti–IL-9 (9C1), anti–IL-17 (17F3), and anti–IFN-γ (XMGI.1.2) were purchased from BioXCell. Experiments were performed in a blinded, randomized fashion, and tumor measurements were taken over time. Vitiligo on treated mice was scored on a scale of 0 to 5: 0, no vitiligo (wild-type [WT]); 1, depigmentation detected; 2, >10% depigmentation; 3, >25% depigmentation; 4, >50% depigmentation; 5, >75% depigmentation. Two different investigators who were unaware of the treatment groups 5 wk after adoptive cell transfer scored mice. In one set of experiments, ciprofloxacin (50 mg/ml for 1–2 wk; Bayer) was added to the drinking water 2 d before irradiation.

Secreted cytokine quantification

ELISA. ELISAs were performed according to manufacturer’s protocol (Duoset ELISA; R&D Systems or Biolegend) on supernatants from day 4 cultures. The absorbance values of the supernatants were obtained at 450 nm using a Multiskan FC plate reader (ThermoScientific) and the tested cytokines were quantified.

Flow cytometry. Data were acquired on a BD FACSCalibur or FACSVerse (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR). Abs specific for ICOS (CD278), IL-2Rα (CD25), IL-23R, CD44, CD62L, ROR-γt, T-bet, Bcl-1, CD28, PD-1, CTLA-4, CCRX3, and CCR6 were purchased from Biolegend, and Abs specific for IFN-γ, IL-17A, IL-10, CD26, CD39, CD73, Vβ13, and IL-7Rα (CD127) were purchased from BD Pharmingen.

In vivo CTL assay

Tc17 cells from peml-1 mice were sorted based on ICOS expression using a Dako MoFlo cell sorter (Beckman Coulter). A total of 1 × 106 in vitro–vaccinated cells (22) were transferred into irradiated (5 Gy; 4 h before cell transfer) B6 mice that were given 3 × 105 B16F10 tumor s.c. 5 d before transfer. All animals were given bolus IL-2 (i.p. daily at 3.6 g/dose; every 24 h, for a total of 4 doses). Ten d after adoptive T cell transfer, 2 × 106 target cells were i.v. transferred to assess cytolytic function. To generate the target cells, we split the spleens into pieces in 2 ml of medium (RPMI supplemented with 10% FCS). Tc17 cells and tumor tissues were pulsed with 1 μM OVA peptide (SIINFEKL) for 45 min at 37°C and then labeled with a low amount (0.25 μM) of CFSE (Molecular Probes), for use as an internal control, and the second fraction was incubated with 1 μM hgp10025–33 (KYPQRNQDNL) and then labeled with a high level (2.5 μM) of CFSE. An equal number of cells from both fractions were labeled and then delivered simultaneously. Mice were euthanized after 12 h of incubation, and the spleens were harvested and single-cell suspensions made. Lymphocytes were isolated using Lymphocyte Separation Media (Mediatech), fixed, and analyzed by flow cytometric analysis. The percentage of specific lysis was calculated as (1 – ratio of untreated recipients / ratio of activated CD8+ T cell recipient) × 100, where the ratio is %CFSElow/%CFSEhigh.

Statistical analysis methods

Comparison of average levels across groups or conditions was done using either a two-sample t test or an ANOVA model. Several of the conditions in the tumor growth experiments resulted in prolonged periods of no tumor growth or reduced tumor area from day zero, and therefore were analyzed in two parts. First, the minimum tumor size over the course of the experiment for each mouse was noted, and the day of the first increase in tumor size, post minimum, was determined for each mouse. Kaplan-Meier methods were then used to analyze the results of time to first tumor growth past minimum, and log rank tests were used to compare results across groups. The second part of the analysis quantified the tumor growth rates once tumor growth has started (postminimum area) and analyzed on the log scale in a mixed-effects linear regression model. Comparing their respective slopes, which were generated by various linear combinations of the model coefficients, we compared tumor growth rates across the groups. For all the hypothesis tests, p < 0.05 is considered statistically significant.

Results

ICOS supports the maintenance, but not differentiation, of Tc17 cells

ICOS is critical for the generation of follicular helper, Th17, and Tregs (17–19). Moreover, there is a reduced population of effector memory CD4+ and CD8+ T cells in ICOS−/− mice and humans, because ICOS signaling is important for sustaining their survival (23, 24). However, it remains unknown how ICOS regulates the differentiation of naive CD8+ T cells into IL-17–producing Tc17 cells and whether ICOS regulates their long-term function and memory phenotype. We hypothesized that ICOS was important for the differentiation of naive CD8+ T cells toward a Tc17 phenotype and subsequently for sustaining their function and effector memory profile after secondary expansion with IL-23. First, to determine the role of ICOS in these processes, we sorted naive CD8+ CD62LhiCD44hi T cells from WT and ICOS−/− C57BL/6 mice with a flow sorter, programmed them to a Tc17 phenotype (with TGF-β, IL-6, anti–IFN-γ, and anti–IL-4), and then activated them with irradiated APCs cultured with a CD3 agonist (scheme; Fig. 1). In contrast with our hypothesis, after 5 d of in vitro expansion, we found that WT and ICOS−/− Tc17 cells expressed equally high levels of ROR-γt (the master transcription factor for
ICOS does not regulate Tc17 differentiation. As shown in the schematic, naive CD8\(^+\)CD62L\(^-\)CD44\(^+\) T cells were sorted with the MoFlo instrument from either WT or ICOS\(^{−/−}\) mice. These cells were then activated with irradiated C57BL6 splenocytes coated with a CD3 agonist (1 \(\mu\)g/ml, clone I45-2C11 mAb; Biolegend) and programmed toward a Tc17 phenotype with IL-6, TGF-\(\beta\), anti–IL-4, and anti–IFN-\(\gamma\) as described in Materials and Methods. IL-17A and IFN-\(\gamma\) production by these cells were assessed 5 d after primary expansion. Representative flow plots (A) and mean (B) are shown (mean \(\pm\) SEM, \(n = 3\)). (C and D) The central and effector memory phenotype of Tc17 cells from WT and ICOS\(^{−/−}\) mice were determined 5 d after their expansion via their CD44 and CD62L expression by flow cytometry. Tc17 cells from ICOS\(^{−/−}\) mice contain a higher frequency of lymphocytes with an effector memory-like phenotype (mean \(\pm\) SEM, \(n = 3\)). (E) After 5 d of expansion, the expression of IL-23R was assayed on Tc17 cells from WT and ICOS\(^{−/−}\) mice (\(n = 4\)). Student \(t\) test was performed on the combination of three to four experiments. *\(p < 0.05\).

FIGURE 1. ICOS does not regulate Tc17 differentiation. As shown in the schematic, naive CD8\(^+\)CD62L\(^-\)CD44\(^+\) T cells were sorted with the MoFlo instrument from either WT or ICOS\(^{−/−}\) mice. These cells were then activated with irradiated C57BL6 splenocytes coated with a CD3 agonist (1 \(\mu\)g/ml, clone I45-2C11 mAb; Biolegend) and programmed toward a Tc17 phenotype with IL-6, TGF-\(\beta\), anti–IL-4, and anti–IFN-\(\gamma\) as described in Materials and Methods. IL-17A and IFN-\(\gamma\) production by these cells were assessed 5 d after primary expansion. Representative flow plots (A) and mean (B) are shown (mean \(\pm\) SEM, \(n = 3\)). (C and D) The central and effector memory phenotype of Tc17 cells from WT and ICOS\(^{−/−}\) mice were determined 5 d after their expansion via their CD44 and CD62L expression by flow cytometry. Tc17 cells from ICOS\(^{−/−}\) mice contain a higher frequency of lymphocytes with an effector memory-like phenotype (mean \(\pm\) SEM, \(n = 3\)). (E) After 5 d of expansion, the expression of IL-23R was assayed on Tc17 cells from WT and ICOS\(^{−/−}\) mice (\(n = 4\)). Student \(t\) test was performed on the combination of three to four experiments. *\(p < 0.05\).

Th17 and Tc17 cells [25, 26]; data not shown) and secreted similarly high amounts of IL-17A, but very little IFN-\(\gamma\) (Fig. 1A, 1B). Our findings with Tc17 cells are in alignment with work by Bauquet and coinvestigators (19) with Th17 cells, who found that naive CD4\(^+\) T cells from ICOS\(^{−/−}\) mice expressed comparable ROR-\(\gamma\) (data not shown) and IL-17A (Supplemental Fig. 1A) as WT CD4\(^+\) T cells when initially differentiated to a Th17 phenotype.

Next, we assessed the role of ICOS in regulating the memory-like profile of Tc17 cells in vitro, which may affect the cells’ in vivo persistence and effector function. The central memory phenotype of Tc17 cells was identified as CD44\(^Hi\)CD62L\(^Lo\) T cells, whereas their effector memory phenotype was discerned by CD44\(^Hi\)CD62L\(^Hi\) expression. We detected a significantly lower frequency of effector memory cells in ICOS\(^{−/−}\) Tc17 (45%) compared with WT Tc17 (63%) cells (Fig. 1C, 1D). There was also a slightly (but not significantly) higher frequency of naive (23 versus 14%) and central memory cells (28 versus 20%) from ICOS\(^{−/−}\) versus WT Tc17 cells. These phenotypic data are displayed representatively in dot plots (Fig. 1C) and as an average of three separate experiments in Fig. 1D. Similar to Tc17 cells, a reduced pool of effector memory cells was detected from Th17 cells deficient in the ICOS receptor compared with WT counterparts (Supplemental Fig. 1B). These in vitro data suggest that ICOS costimulation is important for supporting the generation of effector memory Tc17 cells.

ICOS augments the generation of IL-17A/IFN-\(\gamma\)Tc17 cells upon IL-23 restimulation

ICOS induces IL-23R expression on differentiated Th17 cells (18, 19) and is important for supporting their long-term function (19). Therefore, we hypothesized that ICOS also induces IL-23R on Tc17 cells and that the IL-23/IL-23R pathway is important in fostering Tc17 function. To test this idea, we assessed IL-23R expression on differentiated WT and ICOS\(^{−/−}\) Tc17 or Th17 cells. Similar to ICOS\(^{−/−}\) Th17 cells (Supplemental Fig. 1C), we consistently found that ICOS\(^{−/−}\) Tc17 cells expressed less IL-23R on their cell surface than WT counterparts (mean fluorescent intensity of IL-23R\(^{+}\) cells that are either WT (2747 \(\pm\) 143) or ICOS\(^{−/−}\) Tc17 cells (820 \(\pm\) 84; \(p < 0.05\); Fig. 1E). Collectively, these data reveal that ICOS signaling is important for inducing IL-23R on Tc17 cells.

We therefore posited that after IL-23 restimulation, ICOS\(^{−/−}\) cells would display a weakened capacity to secrete IL-17A because of a reduced ability for IL-23 to bind and signal through the IL-23R. To address this idea, we reactivated 5-d ICOS\(^{−/−}\) and WT differentiated Tc17 cells with IL-23 and anti-CD3–coated APCs, and analyzed their capacity to secrete IL-17A and/or IFN-\(\gamma\) on day 10 (scheme; 2-degree expansion; Fig. 2). As a control, the role of ICOS on Th17 function was also tested. By flow cytometry, we found that ICOS\(^{−/−}\) Tc17 cells cosecreted less IL-17A and IFN-\(\gamma\) than their WT counterparts (Fig. 2A, 2B). Moreover, compared with type 17 CD8\(^+\) T cells, ICOS\(^−/−\)/CD4\(^+\) Th17 cells secreted even less IL-17A than WT cells (Supplemental Fig. 1D, 1E), as reported by Bauquet and coworkers (19). We next quantitated the amount of IL-17A and IFN-\(\gamma\) produced by WT versus ICOS\(^{−/−}\) Tc17 cells via ELISA analysis. Although WT and ICOS\(^{−/−}\) Tc17 cells secreted comparable amounts of IL-17 and IFN-\(\gamma\) during their primary expansion (Fig. 2C), ICOS\(^{−/−}\) Tc17 cells secreted less IL-17A and IFN-\(\gamma\) than WT Tc17 cells when reactivated with IL-23 during their secondary expansion (Fig. 2D). ICOS\(^{−/−}\) Tc17 cells also expressed slightly reduced (not significant) levels of the ROR-\(\gamma\), CD107A, granzyme B, and antiapoptotic Bcl-2 molecule compared with WT Tc17 cells (Fig. 2E). Further, ICOS\(^{−/−}\) Tc17 cells expressed slightly higher T-bet levels (a transcription factor for Tc1 cells) (27) and secreted significantly less IL-10 than WT Tc17 cells, which we expected, because ICOS promotes IL-10 secretion by T cells (28). Collectively, our data show that ICOS,
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FIGURE 2. ICOS regulates the effector function of Tc17 cells. WT or ICOS−/− Tc17 cells were restimulated with irradiated splenocytes coated with CD3 agonist and IL-23 for an additional 5 d. The ability of already differentiated Tc17 cells to produce IL-17A and IFN-γ after restimulation with IL-23 was detected by (A) flow cytometry, and (B) the percentage of cells producing IL-17A and/or IFN-γ was graphically represented. The cells ability to secrete IL-17A and IFN-γ after (C) primary and (D) secondary stimulation was also analyzed by ELISA. (E) ROR-γt, T-bet, IL-10, granzyme B, CD107A, and Bcl-2 expression were analyzed on restimulated Tc17 cells from WT and ICOS−/− mice via flow cytometry.

CD39 is elevated, whereas CXCR3 is reduced, on ICOS−/− Tc17 cells

Next, we sought to determine whether there existed a difference in the expression of costimulatory and coinhibitory molecules on ICOS−/− Tc17 cells, because these molecules are critical for regulating T cell activation and expansion (30–32). We found that ICOS−/− Tc17 cells expressed comparable amounts of CD28, CD73, CD26, PD-1, and CTLA-4 on their cell surface as WT cells (Fig. 3B). These data may explain, at least in part, how Tc17 cells that lack ICOS are still able to expand as effectively as their WT counterparts (Fig. 3A).

We then sought to investigate whether ICOS regulates the expression of chemokine receptors on CCR6 and CXCR3, as well as immunosuppressive ectoenzymes CD39 and CD73 on Tc17 cells. Although ICOS signaling did not alter CCR6 on Tc17 cells, we observed an ∼20% reduction of CXCR3 on ICOS−/− Tc17 cells compared with WT cells (Fig. 3C, upper panel). Moreover, CD39, but not CD73, was expressed at a higher level on ICOS−/− Tc17 cells (Fig. 3C, lower panel). Our data reveal that ICOS signaling partially regulates chemokine receptors and ectoenzyme molecules on Tc17 cells, which may impact their migration to inflamed tissue and immune tolerance mediated by CD39/CD73-induced adenosine (33, 34). But it remains unclear whether ICOS plays a role in Tc17 cell–mediated immunity to self and tumor tissue.

Ab blockade of ICOS impairs the antitumor activity of infused Tc17 cells

Given that Tc17 cells mediate robust regression of melanoma in mice (11, 35), we wanted to determine the role of the ICOS/ICOSL pathway on Tc17-mediated tumor immunity. We hypothesized that blockade of the pathway would impair the antitumor activity of Tc17 cells in vivo. To test this idea, we programmed melanoma-specific pmel-1 transgenic CD8+ T cells toward a Tc17 phenotype, expanded them with a hgp10025–33 peptide, and then cultured them in the presence of an ICOSL blocking mAb (clone...
The ICOS signal is induced by interactions with its partner, the ICOSL (CD275), a molecule expressed on APCs, such as B cells and dendritic cells (16, 36). We found that Ag-presenting CD11c+ cells from the spleens of irradiated mice expressed higher levels of ICOSL on their cell surface (Supplemental Fig. 2D). Irradiated mice treated with ciprofloxacin, which ablates microbes liberated from lymphodepletion, induce ICOSL on APCs. Given these findings and that lymphodepletion augments the function and antitumor activity of transferred T cells in patients (37), we surmised that the antitumor/self-activity of Tc17 cells would be compromised in ICOSL−/− mice lymphodepleted with 5 Gy TBI. To test this concept, we infused pmel-1 Tc17 cells into 5 Gy irradiated WT or ICOSL−/− C57BL6 mice bearing established B16F10 melanoma. Indeed, Tc17 cells were less effective in mediating tumor immunity (p < 0.001; Fig. 4A) and autoimmune vitiligo (p < 0.05; Fig. 4B) when infused into ICOSL−/− mice compared with WT hosts. Collectively, these data support the notion that engaging ICOS via its ligand (upregulated by lymphodepletion) is important for potentiating the Tc17 cell–mediated immunity to self and tumor tissue.

**FIGURE 4.** Tc17 cell–mediated tumor regression and autoimmune vitiligo is reduced in mice genetically deficient in the ICOSL. Ten million pmel-1 CD8+ Tc17 cells were infused into WT or ICOSL−/− mice bearing 10-d established B16F10 melanomas. Recipient mice were pretreated with 5 Gy TBI and then received in vitro–vaccinated Tc17 cells cultured with bolus IL-2. Tumor areas were calculated over time. Error bars indicate the SEM (n = 7 mice/group). Tc17 (IgG) compared with Tc17 (anti-ICOS) time to growth and slope of growth, p < 0.001, log rank test. All experiments were repeated twice.

Tc17 cells express ICOS and regress melanoma to a greater extent than classic CTLs

We and other investigators reported that Tc17 cells (which secrete IL-17A and nominal levels of IFN-γ; Fig. 5A) mediate superior melanoma regression than IL-2–programmed tumor-specific CD8+ T cells (IFN-γ–producing cells; Fig. 5A, IL-2–polarized CD8+ T cells [IL-2-P]), as shown in Fig. 5B and published elsewhere (11). To explain why Tc17 cells and IL-2–programmed CD8+ T cells mediate differential antitumor responses, we assessed their ICOS expression level, because it has been reported as a biomarker for enhanced survival in melanoma patients (38). Indeed, we found higher ICOS expression on Tc17 cells versus IL-2–programmed cells 5 d after their infusion (Fig. 5C, 5D). No difference in ICOS was detected on host CD8+ T cells from either treatment group (Fig. 5D). These data may explain why there is a higher frequency of Tc17 cells in the blood, tumor, draining lymph nodes (dLNs), and mesenteric lymph nodes compared to mice receiving IL-2–expanded CD8+ T cells (Fig. 5E). These data reveal that ICOS is preferentially expressed on transferred Tc17 cells in vivo, but whether ICOS expression augments their in vivo cytotoxicity is unknown.

**FIGURE 3.** ICOS−/− Tc17 cells are compromised in their ability to induce tumor in vivo. Naive CD4+ and CD8+ T cells (i.e., sorted by CD44−CD62L+) were isolated from WT or ICOS−/− mice, programmed toward a type 1 or type 17 phenotype, and activated with irradiated splenocytes that were coated with a CD3 agonist. (A) The expansion of Tc17 cells was monitored, and their relative yield on day 10 is shown. (B and C) ICOS, CD28, CD27, CD26, PD-1, CTLA4, CCR6, CXCR3, CD39, and CD73 expression on WT and ICOS−/− Tc17 cells as assayed by flow cytometry on day 10 postexpansion. Student t test was performed on the combination of two experiments. **p < 0.001. (D) Pmel-1 CD8+ Tc17 cells were expanded in the presence, or not, of an ICOS antagonist for 8 d; then 1 × 106 cells were infused into WT mice bearing 10-d established B16F10 melanomas. Recipient mice were pretreated with 5 Gy TBI and then received in vitro–vaccinated Tc17 cells cultured with bolus IL-2. Tumor areas were calculated over time. Error bars indicate the SEM (n = 7 mice/group). Tc17 (IgG) compared with Tc17 (anti-ICOS) time to growth and slope of growth, p < 0.001, log rank test. All experiments were repeated twice.

Tc17-mediated tumor regression is impaired when infused into ICOS−/− mice

The ICOS signal is induced by interactions with its partner, the ICOSL (CD275), a molecule expressed on APCs, such as B cells and dendritic cells (16, 36). We found that Ag-presenting CD11c+ cells from the spleens of irradiated mice expressed higher levels of ICOSL on their cell surface (Supplemental Fig. 2D). Irradiated mice treated with ciprofloxacin, which ablates microbes liberated from the radiation-injured gut (37), decreased the absolute number of CD11c+ cells expressing ICOSL and the number of infused T cells in the animal (Supplemental Fig. 2E). These data imply that, at least in part, microbes liberated from lymphodepletion induce ICOSL on APCs. Given these findings and that lymphodepletion augments the function and antitumor activity of transferred T cells in patients (37), we surmised that the antitumor/self-activity of Tc17 cells would be compromised in ICOSL−/− mice lymphodepleted with 5 Gy TBI. To test this concept, we infused pmel-1 Tc17 cells into 5 Gy irradiated WT or ICOSL−/− C57BL6 mice bearing established B16F10 melanoma. Indeed, Tc17 cells were less effective in mediating tumor immunity (p < 0.001; Fig. 4A) and autoimmune vitiligo (p < 0.05; Fig. 4B) when infused into ICOSL−/− mice compared with WT hosts. Collectively, these data support the notion that engaging ICOS via its ligand (upregulated by lymphodepletion) is important for potentiating the Tc17 cell–mediated immunity to self and tumor tissue.
ICOS AUGMENTS Tc17 CELL RESPONSES TO SELF AND TUMOR TISSUE

Because ICOS is expressed on Tc17 cells to a greater extent than IL-2–expanded cells and because ICOS<sup>high</sup> Tc17 cells lyse targets superior to other cells, we surmised that activating Tc17 cells with an ICOS agonist in vitro would augment their capacity to secrete cytokines and their therapeutic effectiveness in vivo. To test this question, melanoma-specific pmel-1 CD8<sup>+</sup> T cells were activated with hpg100<sub>25–33</sub> peptide with or without ICOS agonist Ab and then polarized toward a Tc17 subset. On day 3 after reactivation, we collected supernatant and assessed for the ability of the cells to secrete various cytokines. We found that the addition of ICOS agonists to the culture bolstered Tc17 function, as indicated by their increased ability to secrete IL-17A, IL-17A/F, IL-9, CCL20, IL-22, IFN-γ, TGF-α, and IL-10 (Fig. 7A). IL-2–expanded cells secreted more IFN-γ when activated with an ICOS agonist, but secreted nominal IL-17A, IL-17A/F, CCL20, and IL-22. Further, we investigated the transcription factor profile of these cells on day 7 after primary stimulation. As expected, IL-2–expanded cells (with or without the addition of the ICOS agonist) expressed significantly greater levels of T-bet, but nominal expression of ROR-γt by Western blot analysis, compared with Tc17 cells (data not shown). Conversely, Tc17 cells expressed high amounts of ROR-γt, but very little T-bet. ICOS agonist did enhance the ROR-γt expression on Tc17 cells. Additional investigation revealed that ICOS stimulation increased the coexpression of IL-7Rα and IL-2Rα on Tc17 cells, but not on IL-2–P (Fig. 7B), suggesting that in vitro ICOS-stimulated cells may have greater persistence.

To determine whether ICOS agonist treatment enhanced persistence in vivo of Tc17 cells compared with Tc9 cells, we transferred them in vivo ICOS agonist-stimulated pmel-1 Tc17 or IL-2–expanded CD8<sup>+</sup> T cells into a B16F10-bearing host. Consistently, we found that ICOS agonist treatment enhanced the persistence of Tc17 cells over IL-2–expanded cells (Fig. 7C, 7D). This was also demonstrated in the frequency of cells on day 21 after T cell infusion in both the spleen and the inguinal lymph node (Fig. 7E). Yet, similar frequencies of Tc17 and IL-2–primed cells stimulated with ICOS were detected in the tumor (data not shown).

Further, to examine whether the addition of ICOS agonist therapy would augment T cell–mediated tumor immunity, we treated pmel-1 Tc17 or IL-2–expanded CD8<sup>+</sup> T cells into a B16F10-bearing host. Consistently, we found that ICOS agonist treatment enhanced the persistence of Tc17 cells over IL-2–expanded cells (Fig. 7C, 7D). This was also demonstrated in the frequency of cells on day 21 after T cell infusion in both the spleen and the inguinal lymph node (Fig. 7E). Yet, similar frequencies of Tc17 and IL-2–primed cells stimulated with ICOS were detected in the tumor (data not shown).

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Tc17 cells engraft in melanoma-bearing mice to a greater extent when in vitro primed with an ICOS agonist (Fig. 8C). Yet, ICOS agonists did not augment the engraftment of IL-2–primed CD8+ T cells in mice (data not shown). We also found that Tc17 cells stimulated with an ICOS agonist expressed less KLRG-1 (an exhaustion marker) than Tc17 cells stimulated with an IgG control when in vitro primed with an ICOS agonist (Fig. 8C). Yet, ICOS signaling increases the expression of CD25, CD127, and IL-23R, which may help the infused Tc17 cells consume more cytokines, thereby bolstering their persistence.

Because ICOS ligation increases IL-17A, IL-9, and IFN-γ secretion by Tc17 cells, we sought to determine which cytokine(s) were responsible for mediating the enhanced antitumor immunity by this treatment. Thus, we blocked these cytokines in vivo using neutralizing Abs. Given the pronounced secretion of IL-17A by Tc17 cells stimulated with ICOS, we rationalized that IL-17A was responsible for enhancing the antitumor activity of ICOS-stimulated Tc17 cells. This hypothesis was based on previous reports showing that IL-17A−/−CD4+ Th17 cells were unable to eradicate large tumors, as well as their WT counterparts (39). In contrast with our hypothesis, we found that blocking IL-17A in vivo did not impair the antitumor response of ICOS-engaged Tc17 cells (Fig. 9 and Supplemental Fig. 3; p = 0.75). These data suggest that IL-17A plays a role as a priming factor needed in the survival of ICOS-activated Tc17 cells, but is not necessary for effector function and antitumor activity. We also found that IL-9 did not alter the antitumor response of ICOS-stimulated Tc17 cells, because its blockade did not impact treatment outcome (Fig. 9 and Supplemental Fig. 3; p = 0.96). This finding was unexpected because IL-9 was reported to augment Tc9 cell–mediated antitumor immunity (40, 41).

Because Th17 cells convert to IFN-γ producers when infused into an irradiated host and Muranski et al. (42) found that neutralizing IFN-γ impaired Th17-mediated tumor regression, we surmised that IFN-γ was responsible for tumor regression by ICOS-stimulated Tc17 cells. Indeed, we found that blocking IFN-γ dramatically retarded their ability to eradicate melanoma (Fig. 9 and Supplemental Fig. 3; p = 0.04). Collectively, our data suggest a temporal influence of IL-17A in the induction of ICOS+ CTL, and that the ICOS–ICOSL pathway is important for enhancing the cytotoxicity of Tc17 cells in an IFN-γ–dependent manner. Moreover, ICOS signaling increases the expression of CD25, CD127, and IL-23R, which may help the infused Tc17 cells consume more cytokines, thereby bolstering their persistence.

**Discussion**

Cytokines are not the only cues important in supporting the generation and function of Tc17 cells. We found that ICOS stimulation augments Tc17 cell responses to tumor/self tissue in a clinically relevant adoptive cell transfer therapy model of murine melanoma and autoimmune vitiligo. We identified that ICOS signaling is not needed for the in vitro differentiation of naive CD8+ T cells to a Tc17 subset. Rather, ICOS signaling is important for maintaining the multifunctionality of Tc17 cells in vitro. We also observed a reduced pool of effector-memory-like CD44hiCD62Llo Tc17 cells from ICOS<sup>−/−</sup> mice. Of clinical significance, we found that ICOS signaling was important for enhancing Tc17 cell–mediated tumor regression and autoimmunity in vivo, as Ab or genetic blockade of the ICOS pathway impaired their capacity to eradicate melanoma and induce autoimmune vitiligo in mice. Conversely, activating Tc17 cells in vitro with a soluble ICOS agonist further augmented their capacity to secrete multiple proinflammatory cytokines, eradicate tumors, and induce autoimmune vitiligo in vivo, which was found to be IFN-γ dependent.

Our finding that activating Tc17 cells with an ICOS agonist in vitro augments their in vivo effectiveness has clinical
implications. Along with bolstering their cytotoxicity, the addition of an ICOS agonist to the Tc17 culture augmented their multifunctionality, as indicated by their increased ability to secrete IL-9, IL-17A, IL-17A/F, CCL20, IL-22, and IFN-γ. Despite the fact that these cells secreted numerous cytokines before transfer, it has been shown that Tc17 cells are plastic, because they are able to convert from a cell that mainly secretes IL-17A to one that mainly produces IFN-γ (43–46). Previous studies demonstrate that IL-17A and IFN-γ are both important for driving antitumor response of Th17 cells (42). In our work, we found that Tc17 cells stimulated with an ICOS agonist mediate tumor regression via their secretion of IFN-γ, despite the fact that very little of the master transcription factor T-bet is expressed and that only a nominal amount of IFN-γ is secreted by these cells on the day of transfer. Conversely, we found that blocking IL-17A did not impair the antitumor activity of ICOS-stimulated Tc17, even though ICOS dramatically increased IL-17A by these cells. However, it is important to appreciate that it is possible that neutralizing IL-17A in vivo did not sufficiently remove all of the IL-17A secreted by ICOS-stimulated Tc17 cells. Follow-up studies with IL-17A−/− Tc17 cells versus WT Tc17 cells will provide deeper insight into whether IL-17A contributes to augmenting the persistence and antitumor activity ICOS-activated Tc17 cells. Given that we also found that ICOS engagement increased IL-17A/F and IL-17F by Tc17 cells, it is also possible that these cytokines additionally contribute to their effectiveness. Experiments in our laboratory are ongoing to understand how these multiple cytokines contribute to the effectiveness of ICOS-activated Tc17 cells. Regardless, it is clear that Tc17 cells convert in vivo into IFN-γ–producing cells (11, 18, 47, 48) and that ICOS increases IFN-γ secretion by Tc17 cells, a type 1 cytokine with cytotoxic properties that impacted treatment outcome by ICOS-stimulated Tc17 cells.

We found that ICOS agonist greatly increased coexpression of IL-7Rα and IL-2Rα on Vβ13+ cells by flow cytometry (day 7). Recipient mice, bearing established B16F10 melanoma, were pretreated with 5 Gy TBI and then received 3 × 10⁶ pmel-1 in vitro–vaccinated nonpolarized IL-2-P + ICOS agonist or Tc17 + ICOS agonist cells in conjunction with bolus IL-2. (C and D) Blood was taken to assess persistence. (C) Representative plots from day 7 are shown, and (D) averages are graphed on the days indicated. Engraftment of adoptively transferred CD8 Vβ13+ nonpolarized or Tc17 cells + ICOS agonist in the blood on days 3 (*p = 0.0016), 7 (*p = 0.016), 14 (p = 0.0508), and 21 (NS) were examined (n = 3 mice/group), t test. (E) Surviving mice were sacrificed on day 21, and the inguinal lymph node, spleen, and tumors were harvested. Engraftment of Vb13+ CD8+ T cells in the spleen (*p = 0.0049), tumor (NS), and dLN (***p < 0.001) were assayed (n = 3 mice/group), t test. Data are the combination of two separate experiments.

FIGURE 7. Tc17 cells stimulated with an ICOS agonist in vitro secrete heightened levels of inflammatory cytokines, coexpress high receptor levels of IL-2 (CD25) and IL-7 (CD127), and persist better than Tc0 cells. Pmel-1 CD8+ T cells were primed in IL-17–polarizing conditions (Tc17) or not (IL-2-P), expanded with IL-2, and treated in vitro with an ICOS agonist or Ab control (20 μg/ml on days 2, 4, and 6 of culture). (A) Cytokine and chemokine secretion by reactivated IL-2–polarized or Tc17 cells was determined by ELISA (day 4). Student t test was performed, *p < 0.05, **p < 0.01. (B) In vitro expression of IL-7Rα and IL-2Rα on Vβ13+ cells by flow cytometry (day 7). Recipient mice, bearing established B16F10 melanoma, were pretreated with 5 Gy TBI and then received 3 × 10⁶ pmel-1 in vitro–vaccinated nonpolarized IL-2-P + ICOS agonist or Tc17 + ICOS agonist cells in conjunction with bolus IL-2. (C and D) Blood was taken to assess persistence. (C) Representative plots from day 7 are shown, and (D) averages are graphed on the days indicated. Engraftment of adoptively transferred CD8 Vβ13+ nonpolarized or Tc17 cells + ICOS agonist in the blood on days 3 (*p = 0.0016), 7 (*p = 0.016), 14 (p = 0.0508), and 21 (NS) were examined (n = 3 mice/group), t test. (E) Surviving mice were sacrificed on day 21, and the inguinal lymph node, spleen, and tumors were harvested. Engraftment of Vb13+ CD8+ T cells in the spleen (*p = 0.0049), tumor (NS), and dLN (***p < 0.001) were assayed (n = 3 mice/group), t test. Data are the combination of two separate experiments.
mice. Moreover, it has recently been reported that lymphodepletion induces APCs to secrete heightened IL-12 and IL-23 in patients with melanoma (50). Because ICOS induces IL-23R expression on T cells, it is possible that IL-23 (and perhaps IL-12) also augments anti-self/tumor immunity by ICOS-activated Tc17 cells in vivo.

It will be informative to evaluate how ICOS impacts the formation of long-lived memory Tc17 and Th17 cells in tumor immunity. Muranski, Gattinoni, Restifo, and coworkers (39, 51) found that Th17 cells have phenotypic and molecular signatures that are distinct from Th1 cells and that are reminiscent of CD8+ T cell with stem cell–like memory phenotype. Indeed, recent findings suggest that T cells with this phenotypic signature elicit robust and potent tumor immunity in murine and humanized mouse models of melanoma, ovarian cancer, and mesothelioma (39, 45).

Irradiated mice treated with a broad-spectrum antibiotic, ciprofloxacin, triggered and systemically liberated from the gut via TBI (37), dramatically decreased the absolute number of CD11chi cells expressing ICOSL. We likewise found a decrease in the number of donor pmel-1 T cells in the blood of these mice. Collectively, these data suggest that microbes might, at least in part, be responsible for regulating ICOSL expression on CD11chi APCs. Although the exact microbes responsible for regulating ICOSL expression on APCs is beyond the scope of this study, we are excited about the data because it leads us down a new and important line of study.

It has been reported that ICOSL expression on melanoma cells increases the Treg population in mice (52), but based on our findings, the expression of ICOSL on the tumor may also have a positive effect on Tc17 cells in vivo. Our studies did not investigate the expression of Foxp3, but we did see an increase of CD25 expression in Tc17 cells given the ICOS agonist. CD25 expression did not correlate with a regulatory phenotype, as these cells secreted more effector molecules and had increased antitumor potential in vivo.

It is interesting to consider the impact of coinhibitory blockade therapies for cancer. Many investigators use these therapies to induce robust lymphocyte activation and cytotoxicity to tumor Ags (53). Yet, melanoma patients treated with CTLA-4 blockade therapy (ipilimumab) do not merely have an increased frequency of functional tumor-specific T cells, but it has been reported that the patients who experience the best treatment outcome also have a greater frequency of ICOS+ T cells (54). Likewise, preclinical B16F10 melanoma models reveal that ICOS expression on T cells is vital for the effectiveness of CLTA-4 blockade therapy (55).

Given that ICOS and ICOSL mediate autoimmune via Tc17/Th17 cells and enhance tolerance by supporting Treg development (56), our data may suggest that this pathway bolsters the antitumor activity of Tc17 or Th17 cells in patients treated with ipilimumab. However, recent work by Fan et al. (57) revealed that this might not be the case. They found that combining CLTA-4 blocking therapy with a vaccine incorporating ICOSL augmented the antitumor activity of host type 1 CD8+ and CD4+ T cells. Interestingly, this combination therapy did not appear to potentiate the function of immune cells classically known to express ICOS, that is, Tregs, follicular helper T, Th17, or Tc17 cells (17–19). It is

**FIGURE 8.** ICOS-stimulated Tc17 cells mediate immunity to self and tumor tissue to a significantly greater extent than IL-2-expanded T cells stimulated with ICOS. (A) Recipient mice, bearing established B16F10 melanoma, were pretreated with 5 Gy TBI and then received 1 × 10^7 pmel-1 in vitro–vaccinated nonpolarized (IL-2-P), IL-2-P + ICOS agonist, Tc17, or Tc17 + ICOS agonist cells in conjunction with bolus IL-2. ICOS agonist or Ab control (20 μg/ml) was given during in vitro expansion on days 2, 4, and 6 of culture. Tumor areas were calculated over time. Error bars indicate the SEM (n = 7 mice/group). Comparing time to tumor growth of Tc17 + ICOS: p = 0.003, Tc17 with IL-2-P: p = 0.01, IL-2-P + ICOS with Tc17 + ICOS; p = 0.003, log rank test. (B) Vitiligo scores from two independent experiments in mice receiving pmel-1 Tc17 cells with or without ICOS agonist treatment. Circles indicate experiment 1 and triangles experiment 2. Student t test was performed on the combination of two experiments, p < 0.01. (C) The persistence of Tc17 cells (treated, or not, with ICOS agonist in vitro) in the spleen of tumor-bearing mice 7 d after T cell infusion. All experiments were repeated twice. Student t test was performed, *p = 0.0123.

**FIGURE 9.** ICOS-stimulated Tc17 cells mediate immunity in an IFN-γ (not IL-17)–dependent manner. Recipient mice treated with 6 Gy TBI bearing established B16F10 melanoma received 5 × 10^6 in vitro–vaccinated Tc17 pmel-1 cells treated during in vitro expansion with 20 μg/ml ICOS agonist on days 2, 4, and 6. Mice were then given 0.1 mg neutralizing Ab (IL-17A, IFN-γ, or IL-9) every other day for five treatments and IL-2 (50,000 IU) every day for the first 3 d after T cell transfer. Tumor measurements were recorded twice weekly. Average tumor curves are displayed. Error bars denote SEM (n = 10–13 mice/group). Comparing time to tumor growth of Tc17 + ICOS with no treatment: p = 0.01, Tc17 + ICOS with Tc17 + ICOS (IFN-γ depletion): p = 0.04, Tc17 + ICOS with Tc17 + ICOS (IL-9 depletion): p = 0.05, Tc17 + ICOS with Tc17 + ICOS (IL-9 depletion): p = 0.96, log-rank test. These experiments were repeated three times.
possible that CTLA-4 blockade induction of ICOS does impact Treg and Th17/Tc17 subsets, because of downstream signals induced by this CTLA-4 blockade or the tumor microenvironment. Such signals may drive Th17/Tc17 or Tregs to convert to a Th1-like cell. It is also possible that CTLA-4 blockade therapy induces signals that override ICOS-induced polarization of cells to a committed regulatory or type 17 phenotype. Regardless, the finding that ICOSL vaccines augment CTLA-4 blockade therapy is promising and has implications for cell-based therapies for cancer, particularly Th17 or Tc17 therapies.

In conclusion, our work reveals that ICOS augments the function and anti-self/tumor activity of Tc17 cells. Our findings are important for the design of next-generation cell therapies for cancer, such as those using genetically engineered T cells that recognize tumor using TCRs or chimeric Ag receptors (58–62). This work also suggests for the design of next-generation cell therapies for cancer, such as those using genetically engineered T cells that recognize tumor using TCRs or chimeric Ag receptors (58–62). This work also suggests that expanding redirected Tc17 with vaccines incorporating ICOSL could augment treatment outcome in cancer patients. Collectively, our work suggests that targeting this pathway may have therapeutic merit for patients with advanced diseases.

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Disclosures

C.M.P. holds a patent for the expansion of Th17 cells using ICOSL-expressing artificial APCs.

References