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β-catenin and PI3Kδ inhibition expands precursor Th17 cells with heightened stemness and antitumor activity

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ICOS costimulation generates Th17 cells with durable memory responses to tumor. Herein, we found that ICOS induces PI3K/p110δ/Akt and Wnt/β-catenin pathways in Th17 cells. Coinhibiting PI3Kδ and β-catenin altered the biological fate of Th17 cells. Th17 cells inhibited of both pathways expressed less RORγt, which, in turn, reduced their ability to secrete IL-17. Unexpectedly, these cells were more effective (than uninhibited cells) at regressing tumor when infused into mice, leading to long-term curative responses. PI3Kδ inhibition expanded precursor Th17 cells with a central memory phenotype that expressed nominal regulatory properties (low FoxP3), while β-catenin inhibition enhanced Th17 multifunctionality in vivo. Remarkably, upon TCR restimulation, RORγt and IL-17 rebounded in Th17 cells treated with PI3Kδ and β-catenin inhibitors. Moreover, these cells regained β-catenin, Tcf7, and Akt expression, licensing them to secrete heightened IL-2, persist, and eradicate solid tumors without help from endogenous NK and CD8 T cells. This finding shines a light on ways to repurpose FDA-approved drugs to augment T cell–based cancer immunotherapies.

Introduction

Recent breakthroughs in adoptive cell transfer (ACT) therapies have generated excitement for Th17 cells as effective agents for clearing tumors. Th17 cells are defined as a CD4 helper T cell subset that secretes IL-17A (1–4). Th17 cell development is controlled by transcription factor RORγt (5); cell function is maintained via IL-23 signaling (6). These cells display an effector memory phenotype, as indicated by nominal CD62L expression; however, in contrast to other CD4 subsets, Th17 cells exhibit stemness, as manifested by multipotency in vivo (3). Several lines of evidence point to Th17 stemness properties analogous to those of hematopoietic stem cells (HSCs). Th17 cells express high levels of *Tcf7* and *Lef1*, downstream target genes of the Wnt/β-catenin pathway, which is associated with self-renewal potential of HSCs (7). Th17 cell multipotency is evidenced by the ability of Th17 cells to self-renew and gives rise to Th1 progeny. (3). Unlike Th1 cells, Th17 cells are resistant to apoptosis and preserve a stem memory molecular signature. Th17 stemness offers a potential explanation for superior tumor regression in vivo compared with Th1 cells (3, 8).

Th17 cells require T cell receptor (TCR) signaling and costimulation for activation. We reported that the inducible costimulator (ICOS) is crucial for human Th17 generation and bolsters the cells' antitumor activity (9). The work herein recapitulates and extends those findings using *murine* Th17 programmed cells expanded with ICOS agonist, which mediate potent antitumor immunity in vivo (vide infra). Notably, ICOS induces Wnt/β-catenin and phosphoinositide 3-kinase (PI3K)/p110δ (PI3Kδ) pathways in Th17 cells to a greater extent than CD28. Yet, it is unclear if these pathways are responsible for regulating antitumor Th17 cell immunity.

Several biological properties of these two (ICOS-induced) pathways hint that they may be involved in supporting antitumor Th17 cell activity. PI3Kδ signaling augments innate and adaptive immune responses

(10). In particular, PI3Kδ regulates T cell cytokine production during primary and secondary immune responses in mice and humans (11). Thus, we posit that ablating this pathway would compromise antitumor Th17 activity and cytokine production. However, PI3Kδ cannot solely be considered in isolation, as it works in tandem with the Wnt/β-catenin pathway to promote HSC self-renewal (7). Crucial for T cell durability, the Wnt/β-catenin pathway tunes cell survival and lineage fate decisions (12). In HSCs, the pathway promotes self-renewal and sustains an undifferentiated state. However, constitutive β-catenin activation alone unexpectedly induced HSC apoptosis (7). Only upon simultaneous activation of the PI3Kδ/Akt and Wnt/β-catenin pathways did HSCs demonstrate long-term expansion and self-renewal (7). Thus, we suspect both ICOS-induced pathways augment antitumor Th17 memory.

We posited that ICOS-activated Th17 cells maintain antitumor effectiveness via mechanisms involving sustenance of stemness by these two pathways. To test this idea, PI3Kδ and β-catenin were inhibited in Th17 cells using a pharmaceutical approach: idelalisib (CAL-101) to block p110δ and indomethacin (Indo) to inhibit β-catenin. We initially anticipated that inhibiting these pathways would enervate cellular antitumor activity; our results directly contradicted this expectation. ICOS-stimulated Th17 cells treated in vitro with CAL-101 plus Indo mediated a *more* potent tumor response when infused into mice. Mechanistically, p110δ inhibition in vitro armed precursor Th17 cells with a central memory phenotype and attenuated regulatory properties, while β-catenin inhibition enhanced cell function long term. As these small-molecule drugs — already FDA approved — augment T cell–mediated immunity, this work has broad clinical implications for various types of cancer immunotherapeutics.

Results

ICOS signaling augments antitumor Th17 cell immunity. Th17 cells are superior to Th1 cells at regressing melanoma when infused into mice (1–3). In addition, human CAR+Th17 cells stimulated with ICOS possess potent antitumor activity in vivo compared with those stimulated with CD28 (9). We recapitulated these findings in a syngeneic mouse model of B16F10 melanoma using TCR transgenic TRP-1 CD45.2+CD4+ T cells programmed toward a Th17 phenotype and expanded for 7 days with CD28 or ICOS (via agonist on either αCD3 beads or TRP-1 peptide–pulsed splenocytes). These mice have a MHC II–restricted TCR on their CD4+ T cells that recognizes tyrosinase-related protein 1 (TRP-1) on melanoma (1). ICOS costimulation improved the antitumor activity of donor TRP-1 Th17 cells compared with those stimulated with CD28 (Figure 1A). By either (a) increasing the number of Th17 cells infused into mice or (b) treating mice with smaller tumors, CD28-stimulated Th17 cells could mediate durable responses in mice, yet treatment with ICOS-stimulated Th17 cells was more effective (data not shown). Donor CD45.2+ Th17 cells persisted in multiple organs (lymph nodes, lung, and spleen) of surviving CD45.1+ recipient mice long term (200 days after transfer) if they were originally stimulated with ICOS (Figure 1B). Two hundred days after transfer, donor Th17 cells originally primed with ICOS secreted more effector cytokines (IFN-γ, IL-21, and IL-17A) upon ex vivo TCR activation with TRP-1 peptide than Th17 cells primed with CD28 (Figure 1C). Th17 cells activated with ICOS mediated long-term tumor protection against melanoma rechallenge (Figure 1D). Thus, our data reveal that ICOS signaling propagates Th17 cells that evince superior antitumor activity.

ICOS confers a distinct Th17 molecular profile. We hypothesized that the striking differences in Th17 cell–mediated antitumor activity induced by ICOS stimulation would be reflected in a distinct Th17 gene signature. Indeed, ICOS priming induces, to a greater extent than CD28, genes implicated in bolstering Th17 fate. First, ICOS increased the expression of *RORC* (Figure 2A), a master transcription factor for Th17 cell differentiation (5). We confirmed enhanced RORγt expression by flow cytometry (Figure 2B). Second, ICOS signaling enhanced *cMAF,* a Th17 cell–associated transcription factor that triggers IL-21 production (13). *IL21* gene expression was also increased (Figure 2A). Further, ICOS induced *Cpt1a*, a mitochondrial gene that plays a role in supporting CD8+ T cell memory (14). Interestingly, ICOS activation induced (to a greater extent than the CD28 stimulation) transcription factors *Lef1* and *Tcf7* in Th17 cells (Figure 2A). Importantly, these genes regulate the Wnt/β-catenin pathway expressed at high levels in HSCs — and are found in stem memory CD8 and Th17 cells (3, 15). These data reveal that ICOS induces pathways possibly important for supporting memory Th17 cells.

ICOS induces Wnt/β-catenin and PI3K/p110δ signaling pathways in Th17 cells. We suspected that ICOS signaling promotes stemness in memory Th17 cells. To test this idea, we stimulated TRP-1 Th17 cells with tyrosinase peptide and an ICOS or CD28 agonist and then measured proteins in the Wnt/β-catenin and PI3 Kinase/Akt pathways. ICOS stimulation induced β-catenin and phosphoAkt
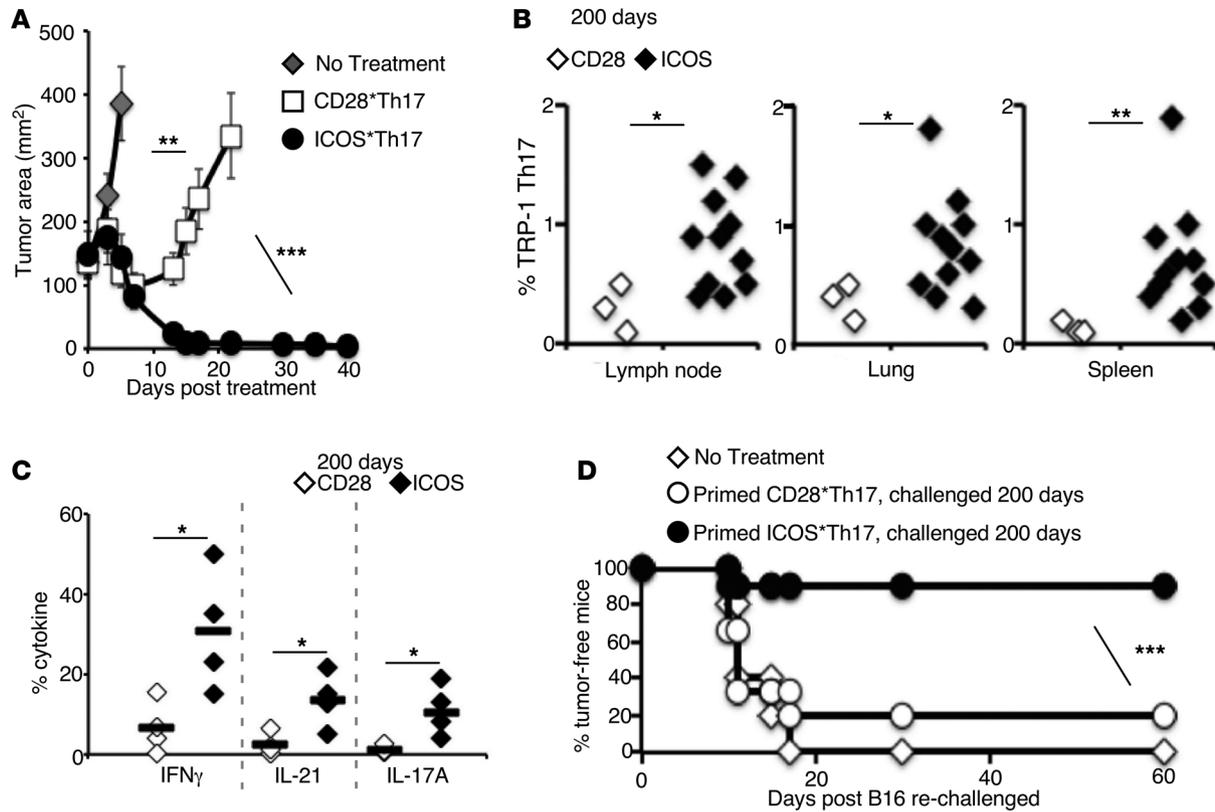
Figure 1. ICOS but not CD28 stimulation generates memory Th17 cells with superior antitumor activity. (**A**) Th17 cells costimulated with ICOS regress melanoma to a greater extent than CD28-stimulated Th17 cells. C57BL/6 mice bearing large subcutaneously B16F10 melanoma tumors established for 10 days received nonmyeloablative 5 Gy total body irradiation. One day later, mice received an ACT treatment regimen consisting of the adoptive transfer of 1×10^6 cultured tumor-reactive TRP-1 CD4+ T cells programmed to a Th17 phenotype (with IL-β, IL-21, IL-6, TGF-β, anti-IL-4, and anti-IFN-γ) and expanded for 7 days with beads coated with CD3 agonist and with either a CD28 or ICOS agonist. Tumor growth was measured every 2 to 3 days; n = 8 mice/group. Data (mean ± SEM) are representative of 2 independent experiments. Mean tumor areas compared by 1-way ANOVA with multiple comparisons; $**P < 0.01$, $***P < 0.001$. (**B**) Th17 cells primed with ICOS but not CD28 persist in vivo for 200 days. Quantification of tumor-specific (Vβ14+CD45.2+) TRP-1 Th17 cells expanded with ICOS versus CD28 from lymph nodes, lungs, and spleen of CD45.1+ recipient mice 200 days after transfer. Percentage frequencies compared by Student's t test; $*P < 0.05$, $**P < 0.01$. (**C**) ICOS-stimulated CD45.2+ Th17 cells secrete more IFN-γ, IL-17A, and IL-21 than CD28-stimulated Th17 cells when reactivated ex vivo. Donor CD45.2+ Th17 cells were isolated from CD45.1+ recipient mouse spleens 200 days after transfer and then reactivated with irradiated spenotyces pulsed with TRP-1 peptide. IFN-γ, IL-21, and IL-17A production was then measured using flow cytometry. Percentage frequencies compared by Student's t test; $*P < 0.05$. (**D**) Th17 cells stimulated with ICOS possess a durable memory response to tumors after rechallenge with B16F10 tumor. 200 days after initial transfer, surviving mice previously treated with TRP-1 Th17 cells activated with ICOS or CD28 were rechallenged with B16F10 and monitored for tumor burden compared with mice not previously treated (i.e., no treatment). Mantel-Cox curves compared by log-rank test, $***P < 0.001$.

(Figure 2C) in Th17 cells to a greater extent than CD28 stimulation. Likewise, TRP-1 Th17 cells deficient in ICOS expressed less β-catenin, phosphoAkt, and p110δ (a PI3 kinase subunit expressed in T cells, ref. 11) when stimulated with an ICOS agonist than WT controls (Figure 2, D and E). Although ICOS$^{-/-}$TRP-1 Th17 cells did not express ICOS, they did express CD28 (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.90547DS1). Yet, CD28 stimulation did not rescue the diminished β-catenin, phosphoAkt, and p110δ in ICOS$^{-/-}$ Th17 cells. ICOS$^{-/-}$Th17 cells expressed comparable CD69 compared with WT cells, suggesting that their activation status was not compromised (Supplemental Figure 1B). Our data show that ICOS induces β-catenin and P13Kδ signaling in Th17 cells to a greater degree than CD28 stimulation.

As Th17 cells are multifunctional, we tested if ICOS signaling bolsters their ability to secrete many cytokines at once. Compared with WT Th17 cells, ICOS$^{-/-}$Th17 cells produced fewer cytokines (IL-17A, IL-17F, IL-22, CCL20, IL-21, IL-4, and IL-10) (Supplemental Figure 1C and Supplemental Figure 2). However, ICOS$^{-/-}$Th17 cells secreted more IFN-γ than WT Th17 cells (Supplemental Figure 2). Thus, ICOS potentiates Th17 multifunctionality — a marker of stemness (3). In light of these findings, we next sought to determine the as-yet-unclear means by which these pathways contribute to the function of antitumor Th17 cells.
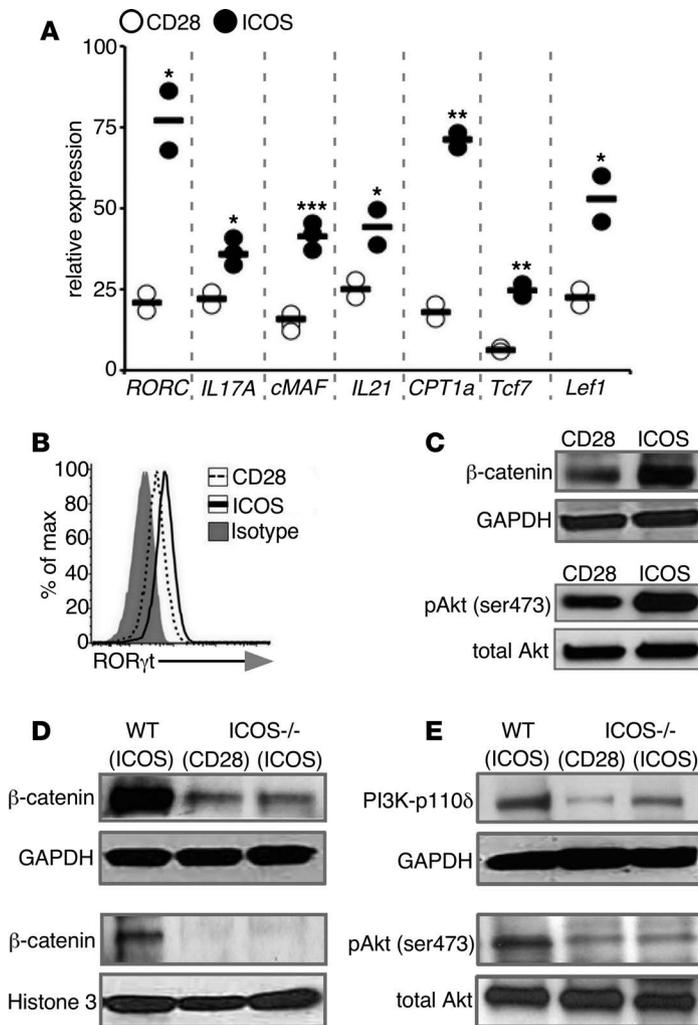
Figure 2. ICOS stimulation induces β-catenin and PI3K/Akt/p110δ in Th17 cells. (**A**) ICOS confers a distinct gene expression profile in Th17 cells. Relative expression of Th17-associated and stemness genes *RORC, IL17A, cMAF, IL21, CPT1a, Tcf7,* and *Lef1* in TRP-1 Th17 cells stimulated with ICOS or CD28, assayed via qPCR. Analysis performed on listed transcripts relative to β-actin, compared by Student's *t* test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (**B**) ICOS induces RORγt in Th17 cells to a greater extent than CD28 signaling. Representative histogram of RORγt in TRP-1 Th17 cells stimulated with an ICOS (solid line) or CD28 (dashed line) agonist (day 8). (**C**) ICOS induces β-catenin and P13K/Akt in Th17 cells. Western blot analysis of β-catenin and phosphoAkt (serine 473) expression in day 8–expanded Th17 cells. (**D** and **E**) ICOS$^{-/-}$Th17 cells express low levels of β-catenin and p110δ/Akt proteins compared with WT Th17 cells. (**D**) Western blot analysis of nuclear (top) and cytoplasmic (bottom) β-catenin expression in WT versus ICOS$^{-/-}$Th17 cells stimulated with an ICOS or CD28 agonist (day 8). (**E**) Western blot analysis of cytoplasmic PI3K-p110δ (top) and phosphoAkt (serine 473) (bottom) in WT TRP-1 Th17 cells (stimulated with an ICOS or CD28 agonist. Representative of 2 or 3 separate experiments.

β-Catenin and p110δ coinhibition alters Th1 and Th17 function. HSCs require induction of both PI3K and Wnt pathways for self-renewal and expansion (7); we anticipated that a similar mechanism operates through ICOS signaling support of Th17 cells. Testing this hypothesis involved in vitro pharmacological suppression of the implicated pathways: (a) the PI3Kδ pathway using CAL-101 (a specific p110δ subunit small-molecule inhibitor, refs. 16, 17) or using Ly294002 (a pan inhibitor of PI3 kinase activity, ref. 18, that inhibits p110α, p110β, and p110δ subunits) and (b) the Wnt/β-catenin pathway using Indo (a COX1/COX2 inhibitor that suppresses β-catenin, ref. 19). These drugs markedly altered the cytokine profile normally observed in ICOS-primed Th17 cells. P110δ inhibition with CAL-101 reduced IL-17A, IFN-γ, and IL-22 secretion but overall did not affect IL-2 or TNF-α production compared with untreated Th17 cells (Figure 3, A and B). Reduced Th17 function by Ly294002 treatment was also observed (Supplemental Figure 3).

β-Catenin inhibition with Indo did not impair IL-17A, IFN-γ, or IL-2 secretion by ICOS-stimulated Th17 cells but increased IL-22 and TNF-α (Figure 3, A and B). Simultaneous inhibition of both pathways, however, nearly abolished IL-17A, IL-22, and IFN-γ production (Figure 3, A and B). Interestingly, ICOS-stimulated Th17 cells possessed an enhanced capacity to secrete TNF-α and IL-2 when treated with both inhibitors (Figure 3, A and B), whereas neither treatment alone affected IL-2. A similar pattern was observed by using Indo plus Ly294002 (Supplemental Figure 3). Although Th17 cell growth was slightly reduced and the amount of late apoptotic cells (PI$^+$AnnexinV$^+$) was slightly increased (data not shown), we found that Th17 cells could expand in the presence of both drugs in vitro (Figure 3C). Treatment with either CAL-101 or Indo alone did not impair the overall yield of Th17 cells expanded in vitro for 1 week (Figure 3C).

We next determined how p110δ and β-catenin inhibition regulates core master transcription factors RORγt, cMaf, and STAT3 (20), which promote Th17 cell generation. As shown in Figure 3D, in vitro p110δ inhibition with CAL-101 or coinhibition of p110δ with β-catenin (with CAL-101 plus Indo) substantially decreased RORγt in ICOS-activated Th17 cells. Similarly, cMaf decreased in these cells after treatment with CAL-101 or with Indo plus CAL-101. Finally, STAT3 was inhibited in Th17 cells after treatment CAL-101 or with Indo plus CAL-10. We confirmed that treatment with CAL-101 alone or treatment with Indo plus CAL-101 blunts RORγt and STAT3 protein via Western blot analysis (Figure 3E). Interestingly, Indo treatment alone did not alter the expression of these transcription factors. Our data reveal that p110δ, but not β-catenin, inhibitors reduce Th17 transcription factors in vitro.

ICOS stimulation enhances antitumor Th1 cell activity to a greater degree than CD28 stimulation (Supplemental Figure 4A). However, ICOS-stimulated Th1 cells were less effective at
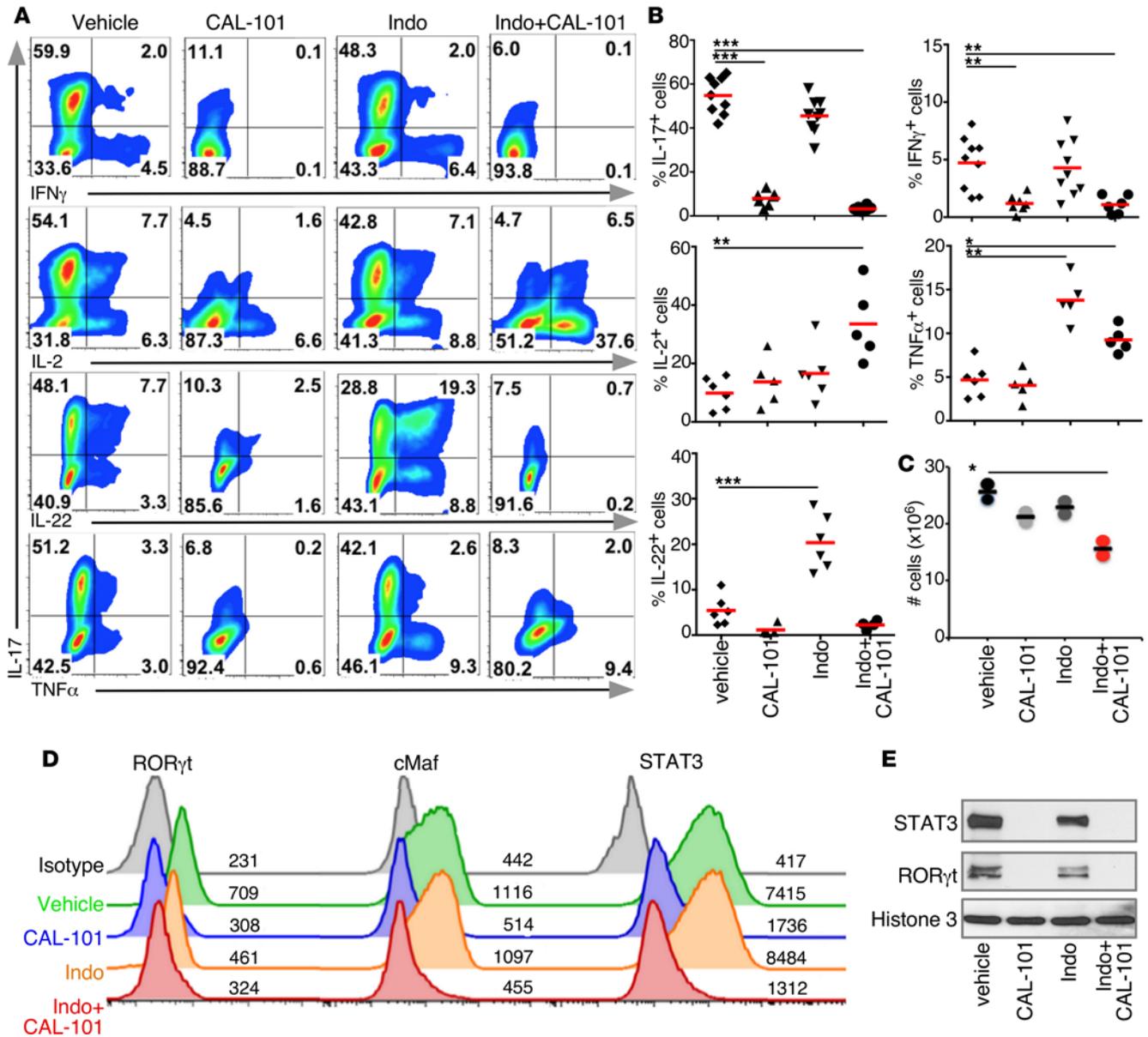
Figure 3. β-Catenin and PI3Kδ inhibition alters the cytokine and transcription factor profile in ICOS-activated Th17 cells. (A and B) PI3Kδ but not β-catenin inhibition suppresses IL-17A and IFN-γ secretion by ICOS-activated Th17 cells. **(A)** Representative FACS plot of cytokine production (IL-17A, IFN-γ, IL-2, TNF-α, IL-22) by TRP-1 CD4+ cells polarized toward a Th17 phenotype, costimulated with ICOS agonist for 8 days (vehicle) and expanded in the presence of specific PI3Kδ subunit inhibitor (CAL-101, 10 μM) and/or specific β-catenin inhibitor (indomethacin [Indo], 60 μM). **(B)** Cytokine production (IL-17A, IFN-γ, IL-2, TNF-α, IL-22). **(C)** Blockade of both pathways slightly impairs Th17 cell expansion. Absolute number (×10^6) of TRP-1 Th17 cells in the absence (vehicle) or presence of CAL-101 and/or Indo, on day 7. Values represent of at least 3 independent experiments. Data in **B** and **C** were compared by Student's t test; *P < 0.05, **P < 0.01, ***P < 0.001. **(D and E)** Inhibition of p110δ by CAL-101 decreases RORγt, cMaf, and STAT3 expression induced in ICOS-activated Th17 cells. **(D)** Representative histograms showing expression of transcription factors (RORγt, cMaf, STAT3), and **(E)** Western Blot analysis of nuclear RORγt and STAT3 expression in TRP-1 Th17 cells in the absence (control) or presence of inhibitors, as indicated (day 8).

regressing tumor than Th17 cells stimulated with ICOS when infused into mice. Like Th17 cells activated with ICOS, we suspected that p110δ and β-catenin inhibition would impair the function of ICOS-stimulated Th1 cells. Indeed, CAL-101 plus Indo treatment or CAL-101 treatment alone impaired Th1 cell function, as these cells secreted less IFN-γ and granzyme B than Indo-treated or untreated Th1 cells (Supplemental Figure 4, B and C). While CAL-101 treatment ablated RORγt, cMAF, and STAT3 in ICOS-stimulated Th17 cells (Figure 3D), T-bet in ICOS-stimulated Th1 cells was reduced slightly (yet substantially) by CAL-101 and Indo treatment (Supplemental Figure 4D).
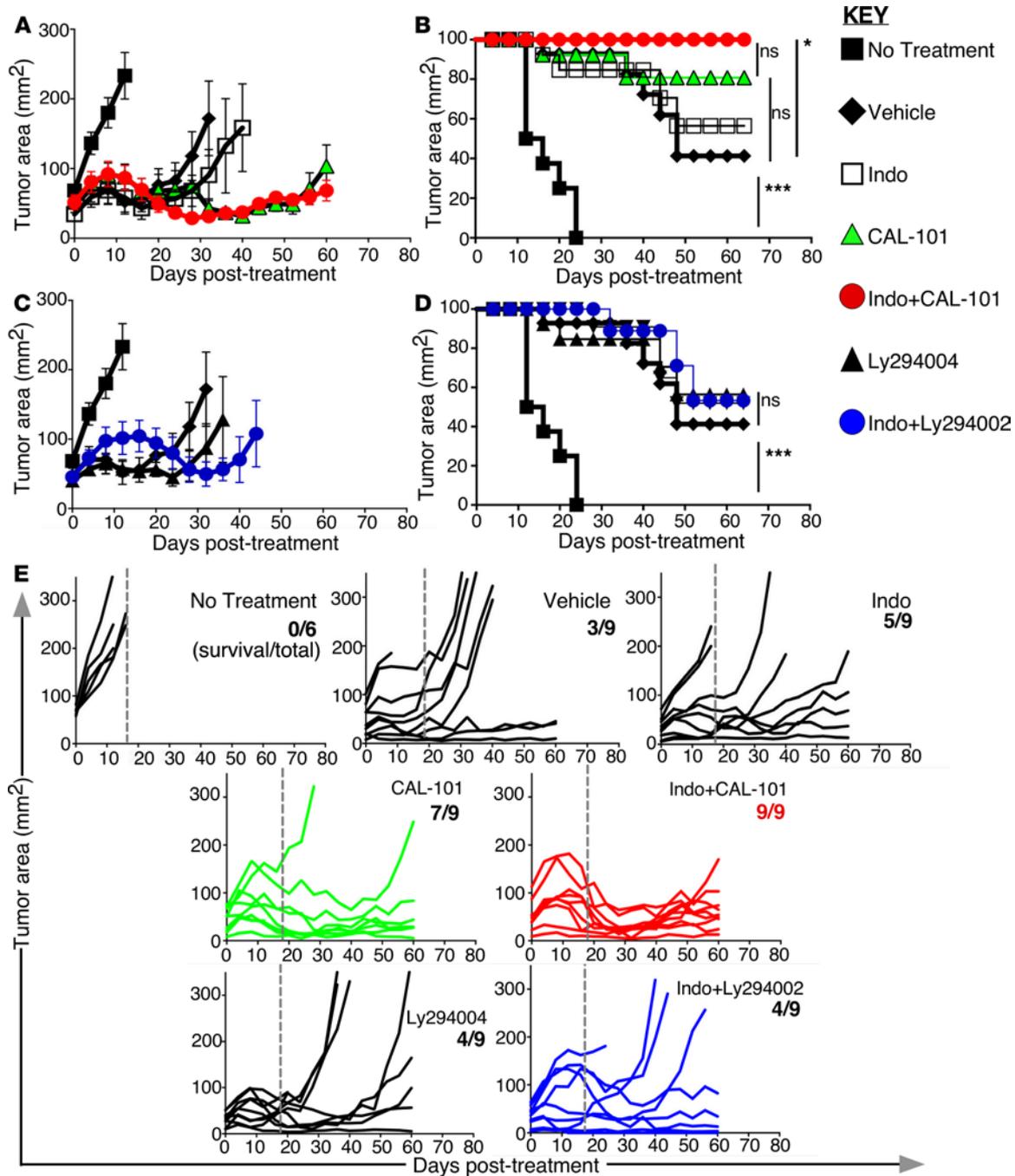
Figure 4. β-Catenin and p110δ inhibition augments antitumor Th17 immunity. (**A** and **B**) Treating ICOS-activated Th17 cells with indomethacin (Indo) plus CAL-101 improves their capacity to regress tumors. (**A**) Average tumor growth curve. (**B**) Survival after transfer of 0.75×10^6 TRP-1 CD4$^+$ T cells polarized toward a Th17 phenotype were expanded with TRP-1 peptide and ICOS agonist and/or primed in vitro with CAL-101 or Indo. Percentage survival of mice; n = 6–9 mice/group. Mantel-Cox curves compared by log-rank test; *$P < 0.05$, ***$P < 0.001$. (**C–E**) Pan inhibition of PI3 kinases with Ly294002 does not replace the effectiveness of CAL-101. (**C**) Average tumor growth curve. (**D**) Survival after transfer of 0.75×10^6 TRP-1 Th17 cells stimulated with TRP-1 peptide and ICOS agonist and expanded in vitro with Ly294002 and/or Indo. Percentage survival by Mantel-Cox curves, compared by log-rank test; ***$P < 0.001$. (**E**) Individual tumor growth curves after transfer of 0.75×10^6 TRP-1 ICOS-activated Th17 cells expanded in vitro with inhibitors, as indicated. Cells were transferred into irradiated (5-Gy total body irradiation) mice bearing established B16F10 melanomas. Tumor growth was measured every 2 to 3 days; n = 9 mice/group. Representative of 2 independent experiments. NT, no treatment.

We next addressed how dual p110δ and β-catenin inhibition affects the fate of CD28-stimulated Th17 cells. CD28 stimulation induces β-catenin and p110δ to a lesser extent in Th17 cells compared with ICOS-stimulated Th17 cells (Figure 2). Although Th17 cells stimulated with CD28 secrete less IL-17 and nominal IFN-γ, we suspected that inhibiting these pathways (CAL-101 alone or CAL-101 plus Indo) would

impair their function. Indeed, these drugs reduced their ability to secrete IL-17A (Supplemental Figure 5, A and B). Conversely, IL-17A was not affected by Indo therapy alone. Interestingly, RORγt and cMAF (relative to ICOS-stimulated Th17 cells) was reduced to a lesser extent in CD28-stimulated Th17 cells treated with CAL-101 and Indo (Supplemental Figure 5C).

Collectively, our work reveals that p110δ and β-catenin inhibition not only reduces the function of Th17 cells stimulated with ICOS, but also decreases the function of ICOS-stimulated Th1 cells and CD28-stimulated Th17 cells. However, coinhibition of these two pathways more profoundly influenced the function and transcriptional profile of ICOS-stimulated Th17 cells.

β-Catenin and p110δ inhibition in vitro augments donor Th17 tumor immunity in vivo. β-Catenin and p110δ inhibition reduces IL-17A secretion and ablates RORγt in Th17 cells in vitro. Thus, we posited that those cells would have diminished antitumor effectiveness in vivo. To test this idea, C57BL/6 mice were inoculated with melanoma B16F10 cancer cells, and the tumor grew for 12 days until ACT. One day before ACT, a nonmyeloablative lymphodepleting dose of 5 Gy total body irradiation was administered to mice as a host-preconditioning regimen to bolster cell therapy (21). The ICOS-stimulated TRP-1 Th17 cell load (0.75×10^6) used is insufficient to cure mice; tumor relapse approximately 30 days after transfer provides a treatment window to determine how priming with CAL-101 and/or Indo regulates Th17 antitumor activity.

Unexpectedly, adoptively transferred ICOS-stimulated Th17 cells mediated superior tumor regression if expanded in vitro in the presence of CAL-101 or CAL-101 plus Indo (Figure 4A). (We refer to ICOS-stimulated Th17 cells as "Th17 cells" in the remaining figures and throughout the text unless compared with CD28-stimulated Th17 cells). Moreover, dual-inhibited Th17 cell therapy also enhanced the overall survival of tumor-bearing mice (Figure 4B). Th17 cells primed solely with CAL-101 mediated tumor regression almost as potently as cells primed with both inhibitors (Figure 4A) but did not augment survival time of mice compared with control ICOS-stimulated Th17 cells (Figure 4B). Only when Th17 cells were primed with Indo plus CAL-101 did all mice ($n = 9$) survive over 64 days. Priming Th17 cells with Indo alone improved neither cell antitumor efficiency nor mouse survival upon ACT (Figure 4, A–D). Interestingly, CAL-101 and Ly294002 were not interchangeable in therapeutic effectiveness, even though both inhibit PI3Kδ activity (Figure 4, C and D). Th17 cells activated with ICOS and cultured in the presence of both CAL-101 and Indo demonstrated a far superior therapeutic index than cells treated with Ly294002 and Indo (Figure 4, C and D). While CAL-101 alone enhanced that antitumor activity of Th17 cells, Ly294002 alone was unable to affect treatment outcome. As shown in Figure 4E, the tumor growth curve of individual mice is displayed for all 7 groups.

We next assayed the engraftment and cytokine profile of inhibitor-treated donor Th17 cells in vivo. Six days after transfer, donor cells were detected in higher frequencies and absolute numbers in the spleen (Figure 5, A and B) when primed in vitro with Indo plus CAL-101. Moreover, these cells (and Th17 cells treated with Indo alone) produced far more IFN-γ, IL-2, and IL-17A upon ex vivo reactivation 64 days after transfer (Figure 5C). Our data indicate that treatment of ICOS-stimulated Th17 cells with p110δ plus β-catenin inhibitors paradoxically augments their function, persistence, and antitumor activity in vivo.

Th17 therapy outcome does not require host NK or CD8 T cells. Two weeks after ACT, we observed a marked but not significant increase in host NK cells in the tumors of mice treated with dual-inhibited Th17 cells (Figure 5D). Thus, we sought to determine if host NK or CD8+ T cells play a role in this therapy with dual-inhibited Th17 cells. Interestingly, we found that host NK and CD8 T cells may not contribute to treatment outcome, as antibody depleting them for the entire experiment (~2 months) did not compromise ACT therapy (Figure 5, G and H) compared with mice treated with an IgG control (Figure 5F). Mice depleted of host CD8 or NK cells experienced a profound antitumor response when infused with dual-inhibited Th17 cells. Curiously, not all mice (7 of 10) survived this therapy when treated with an IgG antibody control (Figure 5F), suggesting that depletion of host NK or CD8 T cells provided additional space for the donor cells to thrive, a concept we and others have previously explored with regard to why lymphodepletion augments ACT (21, 22). As expected, all mice succumbed to disease if they were not treated with T cells (Figure 5E). Our data may suggest that endogenous NK and CD8 T cells are not key for the potent antitumor immunity mounted in mice infused with a dual drug-treated Th17 cells.

As mice infused with naive TRP-1–transgenic CD4+ T cells have been reported to lyse tyrosinase-positive melanoma in a MHC II–restricted manner (23, 24), we suspected that our dual-drug inhibited TRP-1 Th17 cells may directly regress tumors in vivo; thus, depleting them would impair treatment outcome. To address this idea, we rechallenged mice with B16F10 melanoma that survived
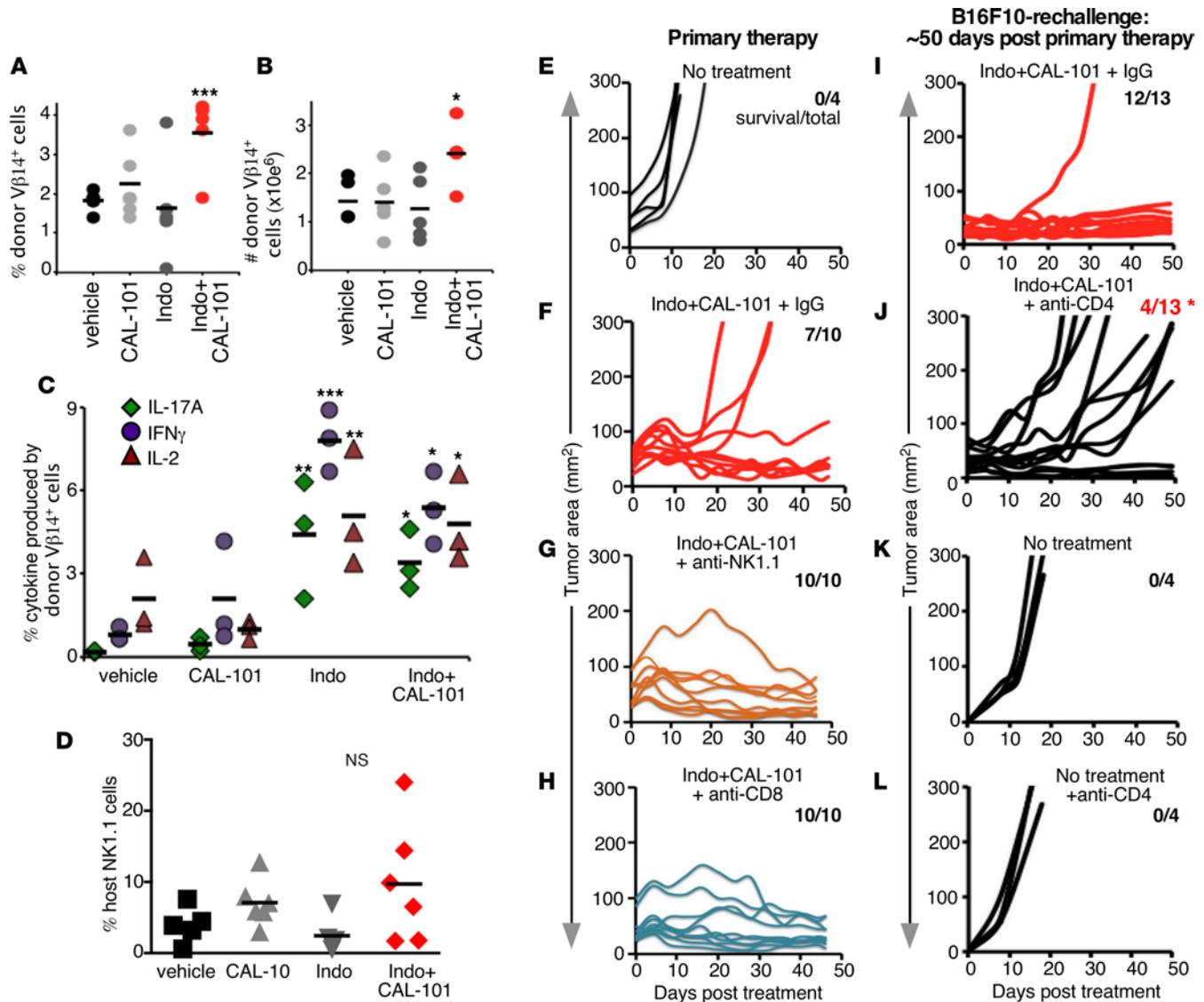
Figure 5. Dual-inhibited Th17 cells directly regress tumor and do not require host NK or CD8 T cells. (A–C) ICOS-activated Th17 cells cultured with indomethacin (Indo) plus CAL-101 engraft to a greater extent than cells subjected to other treatments in the spleen. (A) Percentage and (B) number (×10⁴) of TRP-1 Vβ14⁺CD45.2⁺ Th17 cells in the spleens of CD45.1⁺ recipient mice 6 days after transfer. (C) Donor Vβ14⁺CD45.2⁺ Th17 cells secrete IL-17A, IFN-γ, and IL-2 64 days after transfer in CD45.1⁺ recipient mice when primed with Indo or Indo plus CAL-101. Cytokine production in vivo of Th17 cells expanded in vitro with inhibitors, as indicated. Donor Vβ14⁺CD45.2⁺ cells were flow sorted from the spleens of CD45.1⁺ recipient mice and restimulated with PMA/ionomycin. Data represent mean ± SEM. Student's *t* test; *P < 0.05; **P < 0.01; ***P < 0.001. (D) NK cell frequency in the tumors of mice infused with various Th17 treatments. (E–H) Depletion of host NK or CD8 T cells in mice does not abrogate therapy. Individual tumor growth curves of mice treated with 0.75 × 10⁶ transferred TRP-1 Th17 cells expanded with TRP-1 peptide and ICOS agonist and primed in vitro with CAL-101 and Indo. Cells transferred into 5-Gy TBI mice bearing B16F10 melanomas. Mice were antibody depleted of host CD8 or NK cells (100 μg/mouse) twice weekly for the entire experiment, starting 2 days prior to ACT. As a control, mice given Th17 therapy were administered with an IgG isotype. (J) Depletion of donor cells in mice rechallenged with tumor impairs ACT. Surviving mice in (F–H) were rechallenged with B16F10 and then antibody depleted of host CD4 cells (100 μg/mouse) or with (I) an IgG control twice weekly for the first 2 weeks after rechallenge. Percentage survival; *n* = 13 mice/group. Mantel-Cox curves compared by log-rank test; *P < 0.05. As a control (K and L), naive mice were treated with an IgG isotype control (top right) or treated with a CD4-depleting antibody. Indo + CAL-101 + CD4 depletion is significant different that Indo + CAL-101 + IgG depletion (P < 0.05).

long term (~50 days) after ACT therapy with dual-inhibited Th17 cells (see Figure 5, F–H). Half of these mice were either treated with a CD4 antibody to deplete donor Th17 cells (and host CD4) or were treated with an IgG antibody control. As shown in Figure 5I, only 1 of 13 tumors grew in mice treated with an IgG antibody control. Importantly, the majority of these mice survived without tumor growth for an additional 50 days (~100 days total). In contrast, tumors grew in most animals (9 of 13 mice) treated with a CD4 antibody, suggesting that donor T cells were responsible for the durable antitumor responses (Figure 5J). Tumors grew rapidly in previously untreated "naive" mice, regardless of whether

they were CD4 depleted or not (Figure 5, K and L). Collectively, our work reveals that donor Th17 cells pharmaceutically coinhibited of p110δ and β-catenin can directly mediate profound antitumor activity against melanoma. However, the mechanisms underlying how coinhibition of p110δ and β-catenin potentiates Th17 cells to regress tumor and persist long term remains unknown.

P110δ and β-catenin inhibition propagate precursor Th17 cells. We next asked how p110δ and β-catenin inhibition modulate Th17 cells in vitro to lyse tumors in vivo. The drugs' regulation of cellular phenotype provided clues. We found that CAL-101 treatment (day 8) supported the generation of central memory–like CD44hiCD62LhiTh17 cells (Figure 6, A and F): precursor Th17 cells. Conversely, Ly294002 (total PI3K inhibitor) only slightly sustained CD62L+ cells in the culture (Figure 6, B and F). Only specific p110δ inhibition with CAL-101 strikingly promoted CD62L+ cells in these cultures. CAL-101–treated precursor Th17 cells expressed elevated CCR7 and CD28 compared with fully differentiated control Th17 cells (Figure 6C). They also expressed less CXCR3 (data not shown) and CCR6 (Figure 6C), classic effector memory markers (25). CAL-101 treatment dominated the Th17 profile, as those cells treated with both Indo and CAL-101 possessed a similar precursor phenotype (Figure 6, A and C). Conversely, Th17 cultures were mainly composed of effector memory cells when cultures were expanded in vitro with Indo alone or were untreated (Figure 6, A and C). Although CAL-101 treatment increased CD62L on Th1 cells by approximately 1.2-fold (Supplemental Figure 6, A and B), this therapy elevated CD62L by 18-fold more on Th17 cells (Figure 6A). Thus, p110δ inhibition propagates precursor Th17 cells.

Dual-inhibited Th17 cells express nominal Foxp3 in vitro. PI3K signaling maintains Treg stability (26, 27). Thus, we surmised that inhibiting PI3Kδ with CAL-101 or with Ly294002 would reduce FoxP3 in Th17 cells stimulated with ICOS. As reported previously (28), we posited that Indo would suppress FoxP3. Indeed, CAL-101 treatment decreased FoxP3 and CD25 expression on Th17 cells after primary and secondary stimulation (Figure 6D, compare top and bottom rows, and Figure 6G) in vitro. Surprisingly, those cells expressed more FoxP3 and CD25 when treated with Ly294002 (Figure 6, E and G). Concomitant Indo treatment suppressed the FoxP3 induction initiated by Ly294002 (Figure 6, E and G). Addition of Indo to CAL-101 therapy further decreased FoxP3 in primary and restimulated Th17 cells primed with ICOS to nearly undetectable amounts (Figure 6, D and G). Thus, treatment with Indo plus CAL-101 enhanced the ratio of central memory to suppressive cells by 50-fold in ICOS-stimulated Th17 cultures (Figure 6H).

We found that FoxP3 and CD25 were expressed at higher levels in Th17 cells when stimulated with CD28 compared with ICOS. More than 21% of Th17 cells coexpressed CD25 and FoxP3 when activated with CD28 (Supplemental Figure 7A, bottom row), while only a mere 3% of Th17 cells stimulated with ICOS coexpressed CD25 and FoxP3 (Figure 6D, top left). Although Indo plus CAL-101 treatment also abolished these suppressive molecules on CD28-activated Th17 cells, more than 5% of them coexpressed FoxP3 and CD25 (Supplemental Figure 7A, bottom row). Treatment with Indo plus CAL-101 also increased the ratio of central to suppressive cells in CD28-stimulated Th17 cultures in vitro (Supplemental Figure 7, A and B). However, this increase was only 10-fold more compared with >50-fold more in ICOS-stimulated Th17 cells primed with Indo plus CAL-101 (Supplemental Figure 7B compared with Figure 6H). Collectively, the enhancement of a central memory phenotype instead of a reduction of suppressive attributes in vitro, in part, offers an explanation for the robust antitumor capacity in vivo of ICOS-activated Th17 cells primed with p110δ and β-catenin inhibitors.

Dual-inhibited Th17 cells express little Foxp3 in vivo and persist in the tumor. While ICOS-activated Th17 cells treated with CAL-101 plus Indo have a central memory phenotype with nominal regulatory properties in vitro (Figure 6H), it remains unknown if this profile is maintained in vivo. To address this question, we monitored the phenotype and antitumor activity of these cells 2 weeks after ACT. A waterfall plot analysis and tumor weight on day 14 after ACT reiterated that Th17 cells treated with Indo plus CAL-101 in vitro regressed melanoma more efficiently in mice than in animals infused with Th17 cells treated with Indo or CAL-101 alone (Figure 7, A and B). Higher frequencies of these dual-inhibited cells were detected in the tumors and spleens of mice 2 weeks after transfer compared mice treated with control cells (Figure 7, C and D). These precursor-like Th17 cells acquired an effector memory phenotype in the tumors and spleens of mice (Figure 7C and Supplemental Figure 8). Importantly, donor Th17 cells primed with Indo plus CAL-101 retained a less regulatory phenotype in vivo, coexpressing nominal FoxP3 and CD25 (~2%) 2 weeks after ACT (Figure 7, E and F). In contrast, 10% of untreated donor cells possessed a Treg phenotype. Our data reveal that Th17 cells expanded with p110δ and β-catenin inhibitors persist, express nominal suppressive properties, and transition to an effector memory phenotype in vivo.
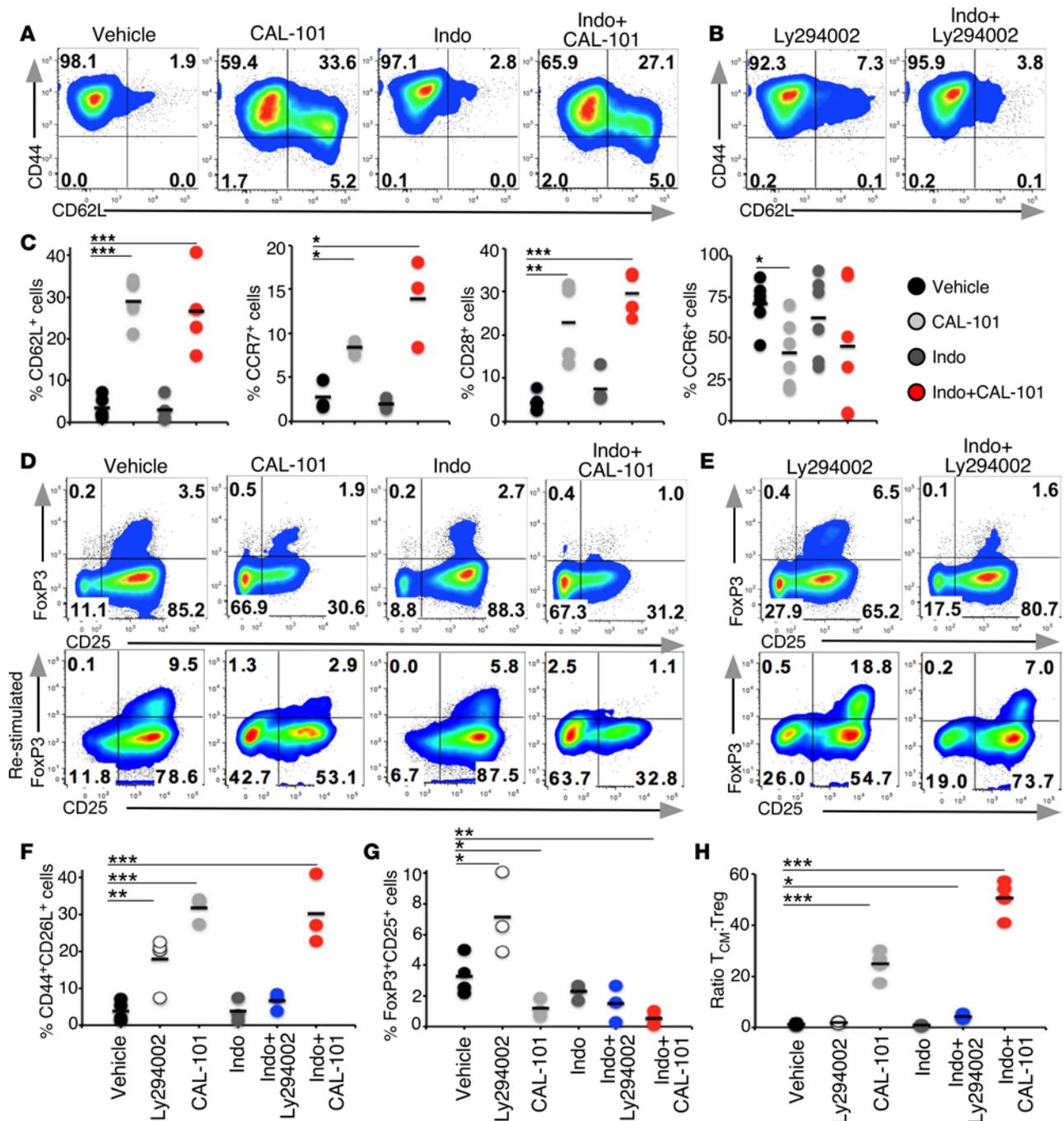
Figure 6. Pharmaceutical expansion of precursor Th17 cells. (**A–C**) PI3Kδ inhibition expands precursor Th17 cells with central memory properties. (**A** and **B**) Representative FACS plots showing memory phenotype on ICOS-costimulated Th17 cells expanded with distinct inhibitors as indicated (day 7). (**C**) Expression of central memory markers (CD62L, CCR7, CD28) and an effector memory marker (CCR6) on Th17 cells in the absence (control) or presence of inhibitors. (**D** and **E**) CAL-101 decreases FoxP3; Ly294002 supports FoxP3. Representative FACS plots showing FoxP3$^+$CD25^{hi} Tregs in Th17 cells expanded in the presence of distinct inhibitors, before and after secondary stimulation in vitro. (**F–H**) CAL-101 favors TCM over Tregs in Th17 cultures. (**F**) Quantification of CD44^{hi}CD62L^{hi} central memory T (T$_{CM}$) cells, (**G**) FoxP3$^+$CD25^{hi} Tregs, and (**H**) the ratio of T$_{CM}$ cells to Tregs in cultures and expanded with CAL-101 and/or indomethacin (Indo), on day 8 (gated on CD4$^+$, Vβ14$^+$ cells). Data represent mean ± SEM of at least 3 independent experiments. One-way ANOVA followed by Tukey HSD post-hoc test and Student's *t* test were applied. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Experiments in Figure 6 were performed concurrently, thus all data can be compared with one another.

β-Catenin and Akt rebound in drug-treated Th17 cells regaining stemness after tumor antigen recognition. In agreement with Muranski and coworkers (3), we found that Th17 cells express β-catenin. Moreover, ICOS signaling induced β-catenin to a greater extent than CD28 (Figure 2D). As CAL-101 treatment expands ICOS-activated Th17 lymphocytes with a central memory profile, we posited that these cells would express more β-catenin than untreated cells. Indeed, we found elevated nuclear β-catenin in Th17 cells upon CAL-101 treatment, and,

as expected, Indo abolished β-catenin translocation to the nucleus (Figure 8A). Even though CAL-101–treated Th17 cells have higher β-catenin levels, their tumor-killing effectiveness in vivo was not as marked as cells treated with both drugs. This result is interesting given that Th17 cells treated with both small molecules did not express β-catenin before transfer (Figure 8A), which we and others (3, 12) directly associate β-catenin in T-cell therapy with better treatment outcome. As reported elsewhere (19, 29) and confirmed here, Indo suppressed nuclear β-catenin translocation (Figure 8A). Importantly, however, β-catenin ablation by Indo was temporary and reversible. Interestingly, we found that the relative expression of Wnt/β-catenin downstream target genes was elevated upon secondary stimulation of dual-treated Th17 cells in vitro, manifesting a 12-fold increase of *Tcf7*, a 4-fold increase of *Lef1*, and a 2-fold increase of *Ctnnb1* genes following treatment of Th17 cells with Indo and CAL-101 (Figure 8B). In alignment with our gene data, we found that Th17 cells treated with CAL-101 or with both Indo and CAL-101 expressed higher β-catenin, RORγt, STAT3 and Tcf7 protein levels by Western blot compared with control Th17 cells (vehicle) upon antigen-specific restimulation (Figure 8, C and D). Ly294002 also increased Tcf7, but not as extensively as CAL-101. Although CAL-101 inhibited p110δ activity, in turn blunting phosphoAkt (serine 473) (Supplemental Figure 9A), phosphoAkt (serine) recovered in Th17 cells (treated with CAL-101) when restimulated with TRP-1 antigen (Supplemental Figure 9B).

As p110δ-induced Akt, β-catenin, and RORγt rebounded in Th17 cells (primed with CAL-101 and/or Indo) upon peptide restimulation, we suspected they would regain their ability to secrete IL-17A. We also expected they would secrete IL-2, a cytokine produced by central memory T cells (30). Indeed, IL-17A production by these cells was restored (Figure 8E). Dual-inhibited Th17 cells secreted as much IL-I7A as control Th17 cells. Remarkably, Th17 cells treated with Indo or with Indo plus CAL-101 also secreted significantly more IL-2 than untreated Th17 cells or CAL-101–treated Th17 cells (Figure 8E). Our results provide evidence that β-catenin and PI3k/Akt signaling pathways rebound when infused into mice, licensing them to secrete IL-2 and persist. This idea is supported by our findings that donor Th17 cells (dual inhibited) secreted more IL-17A, IFN-γ, and IL-2 in vivo (Figure 5C).

P110δ and β-catenin inhibitors expand memory from differentiated Th17 cultures. Finally, we determined if p110δ and β-catenin inhibitors could be used to enrich and expand central memory cells from fully differentiated Th17 cultures. This question is important, as TILs from cancer patients are often fully differentiated or terminally exhausted, even after ACT or PD-1 blockade therapy (31–33). As numerous studies have shown that CD62L+ cells exert long-lived immunity, it is essential to find ways to preserve them in cancer immunotherapy (34–36). Thus, to test this concept, as detailed in Supplemental Figure 10A, CAL-101 and/or Indo were added to 1-week Th17 cell cultures, which express little CD62L and are classified as effector memory cells. These drugs were added to cultures either (a) with or (b) without peptide restimulation and expanded for an additional 5 days (12 days total). Interestingly, CAL-101 or CAL-101 plus Indo treatment supported the outgrowth of CD62L+ cells from these cultures (Supplemental Figure 10B). Conversely, very few central memory cells were detected in untreated Th17 cultures or those treated with Indo alone. Regardless of peptide reactivation, CAL-101–treated cells expressed 3-fold more CD62L than control cells on day 12 (Supplemental Figure 10, B and C). Our data imply that p110δ inhibitors can be used in vitro to enrich memory cells from differentiated cultures. This finding has exciting implications for the design of next-generation ACT clinical trials for cancer patients.

As visualized in Figure 9, our data reveal that p110δ and β-catenin inhibition paradoxically augments the antitumor activity of Th17 cells. We found that expansion of Th17 cells with CAL-101 and Indo in vitro generates lymphocytes with an augmented memory phenotype (enhanced CD62L expression) and reduced regulatory properties in vivo (less FoxP3 expression). Moreover, upon antigen recall, these precursor Th17 cells overexpressed genes and proteins of the PI3K/Wnt/β-catenin pathway (β-catenin and Tcf7), which, in turn, bolstered their multifunctionality and self-renewal, leading to enhanced persistence and tumor destruction when infused into mice. Our study underscores that FDA-approved small molecules can be repurposed to boost the antitumor properties of long-lived memory of T cells, a finding that may be applied to enhance various forms of cancer immunotherapy.

Discussion

ICOS signaling endows Th17 cells with a superior ability to regress tumors. ICOS-stimulated Th17 cells persist in vivo, self-renew, and mediate rapid recall responses against tumor rechallenge. Herein, we found that ICOS stimulation induced two survival pathways in Th17 cells: (a) PI3K/p110δ/Akt and (b) Wnt/β-catenin signaling pathways. Both pathways were reduced in Th17 cells deficient in ICOS, underscoring their putative role in regulating memory.
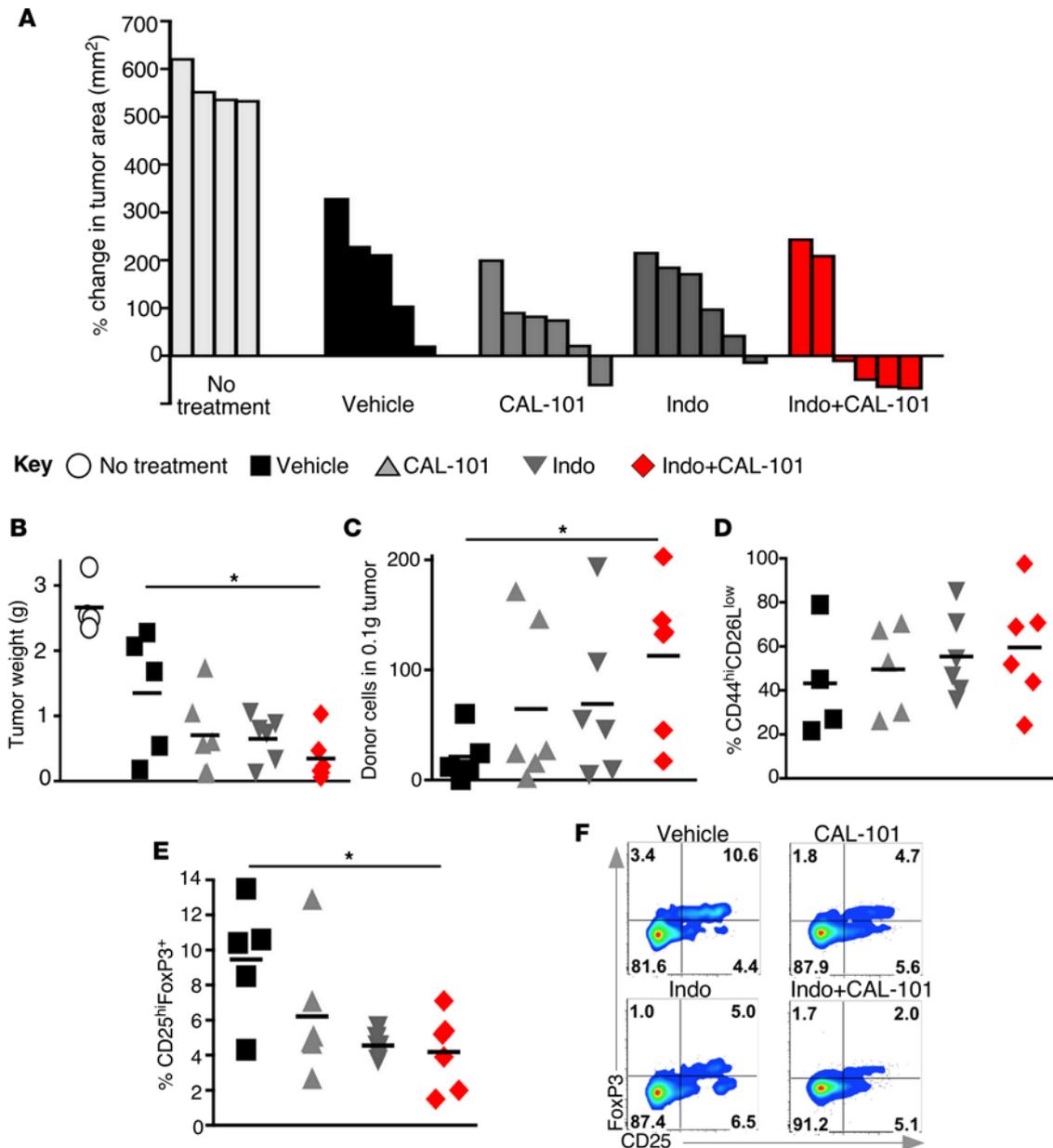
Figure 7. Th17 cells primed with both CAL-101 and Indo mediate robust antitumor activity by persisting in multiple organs with a diminished regulatory phenotype. (A and B) ICOS-activated Th17 cells primed with indomethacin (Indo) plus CAL-101 eradicate tumor more efficiently than nonprimed counterparts. **(A)** Waterfall plot showing the percentage change in tumor area. **(B)** Tumor weight in mice post various therapies, as indicated (day 14), primed with the inhibitors, as indicated (day 14). **(C)** Quantification of Vβ14+CD45.2+ donor cells per 0.1 g of tumor in CD45.1+ recipient mice. Vβ14+CD45.2+CD44hiCD62Llo effector memory T (T_{EM}) cells in the tumor 14 days after ACT, as indicated. **(D)** ICOS-activated Th17 cells expanded in vitro with Indo plus CAL-101 acquired an effector memory phenotype in the tumor upon ACT. **(E and F)** Donor Th17 cells stimulated with ICOS engraft better and express nominal FoxP3 in vivo, when primed in vitro with Indo and CAL-101. **(E)** Quantification of Vβ14+CD45.2+ donor cells expressing FoxP3 and CD25 in the spleen. Statistical significance analyzed using a Student's *t* test *$P < 0.05$. **(F)** Representative FACS plots showing donor Tregs in the spleen upon ACT of ICOS-stimulated Th17 cells expanded in the presence of inhibitors, as indicated.

The PI3K/Akt signaling pathway cooperates with the Wnt/β-catenin signaling pathway to support HSCs self-renewal (7). By extension we suspected that these pathways were important for sustaining Th17 fitness. A pharmacological approach to block both p110δ and β-catenin with CAL-101 and Indo, respectively, was developed to confirm this suspicion. Treatment of Th17 cells (ICOS stimulated) with these two inhibitors in vitro not only ablated phosphoAkt and β-catenin, but repressed RORγt and IL-17A secretion. Thus, we suspected that disruption of these factors would impair the cells' ability to clear tumors. We found the opposite to be true: ICOS-stimulated Th17 cells were far more effective at regressing melanoma in vivo when treated with both inhibitors in vitro.
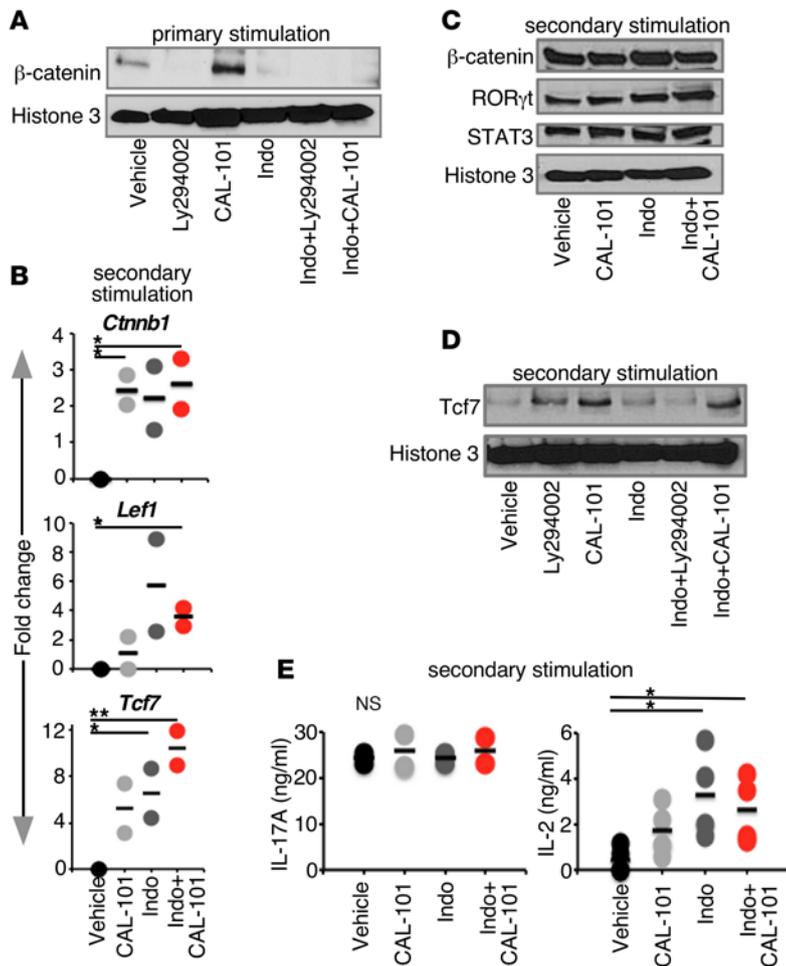
Figure 8. Wnt/β-catenin and cytokines rebound in dual-inhibited antitumor Th17 cells. (**A**) β-Catenin accumulates in the nuclei of ICOS-simulated Th17 cells expanded in the presence of CAL-101, whereas indomethacin (Indo) impairs β-catenin translocation to the nucleus. Western blot analysis of nuclear β-catenin and Histone-3 (loading control) expression in ICOS-stimulated Th17 cells expanded in the presence or not of CAL-101 and/or Indo (day 8). (**B**) Wnt/β-catenin pathway–associated genes are upregulated upon secondary stimulation with peptide in ICOS-activated Th17 cells primed with CAL-101 and/or Indo. Real-time PCR analysis of fold change of gene expression of *Tcf7*, *Lef1*, and *Ctnnb1* in Th17 cells expanded with inhibitors, as indicated and restimulated in vitro with anti-CD3 antibody (5 μg/ml for 1 hour). Student's *t* test; *$P < 0.05$, **$P < 0.01$. Fold change was calculated using the ΔΔCT method. (**C** and **D**) Western blot analysis of nuclear β-catenin, RORγt, STAT3, Tcf7, and Histone-3 (loading control) expression. Nuclear β-catenin, RORγt, and STAT3 are restored and Tcf7 expression is upregulated after in vitro restimulation of ICOS-activated Th17 cells primed in the presence of inhibitors, as indicated. (**E**) IL-17 rebounds and IL-2 is elevated in Th17 inhibited cells after secondary stimulation with tumor-specific tyrosinase peptide (2 days after reactivation, ELISA of IL-17A and IL-2). Representative of 3 independent experiments. Student's *t* test; *$P < 0.05$.

While counterintuitive — and initially perplexing — from a mechanistic perspective, the ability of drugs that block p110δ and β-catenin to render T cells more efficient tumor killers in vivo is exciting from a clinical standpoint — namely, it is exciting that the therapeutic effectiveness of ACT could be improved by simple treatments with readily available drugs. But this observation prompted the question of how the inhibitory drugs manage to improve treatment outcome, despite repressing pathways thought to be indispensable to Th17 antitumor capacity. We found that inhibition of p110δ with CAL-101 supported the generation of Th17 cells with a central memory phenotype possessing enhanced persistence and antitumor activity. Our data with CAL-101 are in line with recent findings that Akt inhibition by a drug called Akti-1/2 promotes the generation of central memory CD8^{+} T cells (37–39). These cells regressed tumors in mice. Tumor-infiltrating lymphocytes expanded ex vivo in the presence of Akti1/2 expressed enhanced fatty acid oxidation and augmented mitochondrial spare respiratory capacity (37). Thus, p110δ inhibition with CAL-101 may also augment Th17 cellular bioenergetics.

Th17 cells expressed less FoxP3 when treated with CAL-101, suggesting that this drug suppresses conversion to Tregs and sustains central memory–like Th17 cells. The significance of this finding is difficult to overstress: the phenotypic plasticity of Th17 cells in vivo means that they can convert to Tregs or Th1 cells with reduced antitumor properties (40). Any treatment, such as CAL-101, that can arrest the development of these poorly therapeutic phenotypes may enhance the antitumor activity of the cells. But not all pharmaceutics regulate Th17 cell biology equally. In contrast to CAL-101, treatment of Th17 cells with Ly294002 led to more CD25^{hi}FoxP3^{+} Tregs. The specificity of CAL-101 for the p110δ catalytic domain in PI3K is likely the crucial difference. We posit that inhibition of p110δ but not p110α or p110β isoforms in the PI3K pathway, for example, selectively blocks Akt signaling, in turn inhibiting Treg proliferation. Further, few Tregs existed in our ICOS-stimulated Th17 cultures when expanded in the presence of CAL-101 plus Indo in vitro, implicating an additive effect of these two drugs on suppressing regulatory elements. Notably, synergistic effects of the combination therapy on donor Th17 cells emerged, where only 2% could be identified as Tregs in vivo.

We have shown herein for the first time to our knowledge that coderepression of p110δ and β-catenin in Th17 cells unleashes their therapeutic potential. CAL-101 and Indo blocked Akt phosphorylation and β-catenin expression in Th17 cells, but only temporarily, as these pathways rebounded to a greater extent (compared with untreated cells) after secondary antigen recall responses. Th17 cells treated with both drugs expressed higher lev-
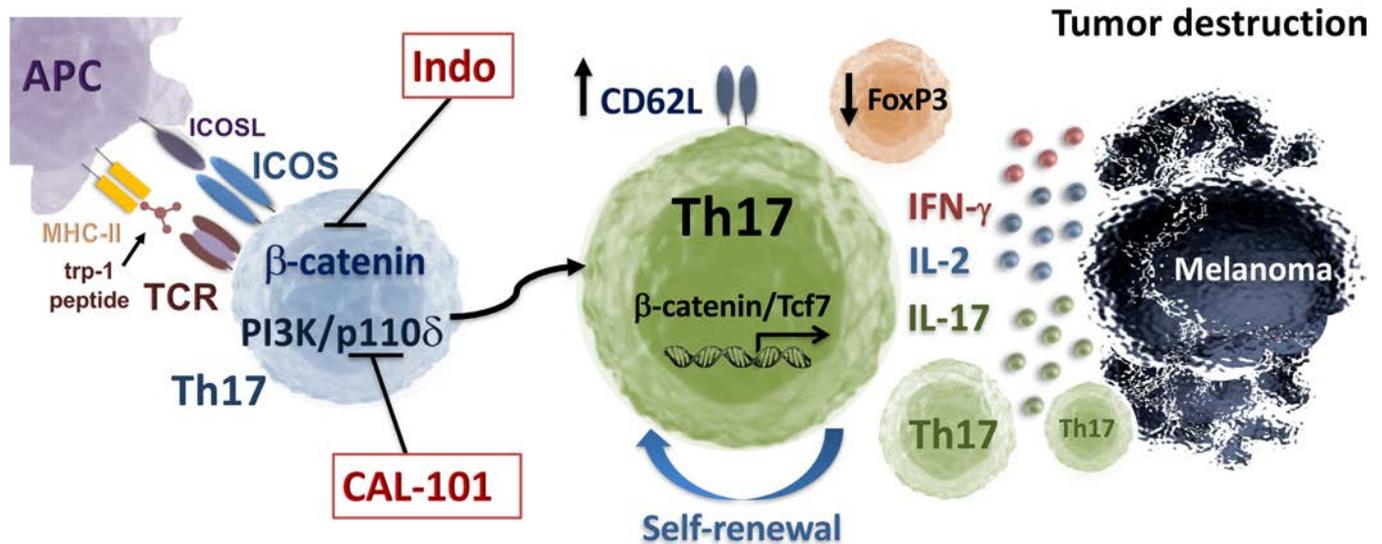
Figure 9. Illustration of how β-catenin and PI3Kδ coinhibition augments the antitumor activity and durable memory of Th17 cells. ICOS-activated Th17 cells expanded in the presence of CAL-101 and indomethacin (Indo) possess a central memory phenotype (i.e., elevated CD62L expression). There are fewer regulatory properties (decreased FoxP3 expression) in this culture. Moreover, upon antigen recall in vivo dual-inhibited Th17 cells are multipotent (producing many cytokines) and exhibit elevated nuclear β-catenin and Tcf7 and augmented persistence in the host. These cells possess self-renewal and durable memory responses to solid tumors, enabling them to directly lyse established malignancies without help from endogenous immune cells. This finding shines light on new ways to repurpose FDA-approved drugs to augment cancer immunotherapy.

els of Tcf7, a critical protein in the Wnt/β-catenin pathway responsible for promoting T cell self-renewal (3, 37, 41–44). Moreover, the treated Th17 cells reexpressed β-catenin and phosphoAkt upon restimulation in vivo. In addition, these cells persisted at higher frequencies, and their phenotype converted into an effector phenotype in the tumor. Additional in vitro studies revealed that inhibiting p110δ and β-catenin increased the ratio of memory to regulatory cells in less effective T cell cultures. Our data reveal that pharmaceutical drugs, used in combination, can enhance various types of cellular products for cancer immunotherapy.

Although Indo transiently ablates β-catenin in Th17 cells, we cannot rule out the fact that this COX2 inhibitor may influence other immune cells. For example, Indo reduces IL-23 secretion by APCs, which would likely affect Th17 function (45). For that matter, it is important to point out that our work with small molecules does not definitively address our original hypothesis that the ICOS-induced PI3K/Akt and Wnt/β-catenin signaling pathways synergize to augment Th17 cell–mediated tumor immunity. These drugs only regulated those pathways temporarily: perhaps yielding an unknown, yet desired, compensatory T cell programming pathway at the epigenetic level. In the future, it will be important to study how these pathways regulate T cell immunity by suppressing them transiently or permanently. We suspect that temporary-versus-permanent manipulations of these pathways will differentially affect antitumor T cell activity. Our laboratory is engaged in answering that very question using Th17 cells deficient in Tcf7, β-catenin, p110δ, or ICOS-YF (46, 47).

ICOS signaling promotes antitumor T cell memory responses. For instance, CTLA-4 blockade upregulates ICOS on T cells in mice and in human patients that experience durable survival (46–50). Activation of ICOS signaling in host T cells synergized with CTLA-4 blockade to regress tumors. In the context of adoptive immunotherapy, ICOS signaling enhances the antitumor activity of human and murine Th17 cells and Tc17 cells (9, 51). Moreover, introducing the ICOS domain into CARs enhanced cellular engraftment in vivo (52–54). In light of our work, we suspect that combining drugs or genetic strategies to regulate p110δ and β-catenin induced in T cells could augment various forms of cancer immunotherapy, including cytokine therapy, checkpoint modulators, and cellular therapy.

Collectively, our data indicate that priming Th17 cells with β-catenin and p110δ inhibitors during in vitro expansion profoundly armed them with unique therapeutic properties, such as enhanced stemness, diminished regulatory elements, and augmented multifunctionality in vivo (Figure 9). Our data demonstrate that small molecules, already FDA approved for human use, can be repurposed to enhance T cell memory for adoptive immunotherapy. It is likely that these drugs might also boost the antitumor activity of other T cell–based immunotherapies for cancer, such as vaccines and checkpoint modulators.

Methods

Study design. The objective of this study was to study the role of ICOS-induced pathways (Wnt/β-catenin and PI3K/p110δ) on the functional fate and antitumor activity of Th17 cells in male and female mice bearing large tumors and to provide insight into the role of these pathways in regulating treatment outcome. Laboratory investigations were performed with TCR transgenic TRP-1 CD4+ T cells programmed to a Th17 phenotype, ICOS activated, and inhibited of the Wnt/β-catenin and PI3K/p110δ pathways (discussed below) with FDA-approved drugs that inhibit β-catenin and p110δ.

Mice and tumor line. Six- to ten-week-old male TRP-1 TCR transgenic mice and eight-week-old female C57BL/6 mice were purchased from The Jackson Laboratory. Mice were housed in the Hollings Cancer Center vivarium and maintained in compliance with the Medical University of South Carolina Institutional Animal Care and Use Committee. The B16F10 tumor line was obtained from Nicholas Restifo at the National Cancer Institute, NIH, Bethesda, Maryland, USA.

Cell preparation and culture. Splenocytes were harvested from Vβ14+ TRP-1 TCR transgenic mice. Cells were cultured in RPMI-1640 medium with 10% FBS, 2 mmol/l L-glutamine, 1% Na pyruvate, 1% nonessential amino acids, 0.1% HEPES, 1% penicillin, 1% streptomycin, and 0.1% 2-β-mercaptoethanol (all from Sigma-Aldrich). TRP-1 splenocytes were activated with beads coated with anti-mouse CD3 (clone 145.2C11, BioLegend) and with CD28 agonist (clone 37.51, BioLegend) or ICOS agonist (clone 398.4A, BioLegend). On day 0, cells were polarized toward the Th17 phenotype using 10 ng/ml rhIL-1β, 100 ng/ml rmIL-6, 100 ng/ml rmIL-21, 30 ng/ml rmTGF-β, 10 μg/ml anti-mouse IL-4, and 10 μg/ml anti-mouse IFN-γ. T cells were split every day starting from day 3 and supplemented with 100 IU/ml rhIL-2 and 10 ng/ml rmIL-23 (R&D). Alternatively, on day 0, cells were polarized toward a Th1 phenotype using IL-12 (3.3 ng/ml; Peprotech), and anti-mouse IL-4. Th1 cells were also split every day starting from day 4 and supplemented with 100 IU/ml rhIL-2. Subsets were harvested on the days indicated and used for gene expression analysis (real-time PCR), protein expression analysis (Western blot), flow cytometry, or in vivo studies.

Th17 and Th1 cell expansion with β-catenin and p110δ inhibitors in vitro. TRP-1 splenocytes were activated with irradiated (10 Gy) C57BL/6 splenocytes pulsed with TRP-1 peptide (1 μM) added at a 5:1 ratio in 24-well plates (1 ml media containing 1.5×10^6 cells/well). TRP-1 splenocytes were costimulated with soluble ICOS agonist (clone 398.4A, BioLegend) and polarized toward Th17 phenotype, as describe above. For in vitro expansion from day 0 of culture, 10 μM Ly294002 (Cayman Chemical) or 10 μM CAL-101 (Selleckchem) and/or 60 μM Indo (Sigma-Aldrich) was added. In addition, dimethylosulfoxide 0.01% (Sigma-Aldrich) was added as a control. Th17 cultures were also supplemented with rhIL-2 (100 IU/ml) and rmIL-23 (10 ng/ml; R&D), while Th1 cells were only supplemented with rhIL-12 (100 IU/ml). Medium, including cytokines and inhibitors, was refreshed every 2 to 3 days. In some situations, fully differentiated Th17 cells were exposed to CAL-101 and/or Indo starting on day 7, and these cells were either restimulated or not with irradiated splenocytes that were TRP-1 peptide pulsed.

Real-time PCR. Total RNA isolation was performed using the Trizol method (Life Technologies) according to the manufacturer's protocol. RNA quantity and purity were assessed using a NanoDrop ND-1000. 1 μg total RNA was transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR was performed on the LightCycler480 machine following manufacturer's protocols (Roche). Probes utilized include *Rorc*, *IL-17*, *cMAF*, *IL-21*, *Cpt1a*, *Tcf-7*, *Lef1*, and *Ctnnb1*. Probes are commercially available (ABI). Relative gene expression was determined by the comparative CT method (45). β-Actin was used as a housekeeping gene.

Western blot analysis. Nuclear and cytoplasmic proteins were isolated by lysis using Nuclear and Cytoplasmic Extraction Reagent with Protease Phosphatase Inhibitor Cocktail (Thermo Scientific). Protein concentration was quantified using BSA Protein Assay (Thermo Scientific) according to the manufacturer's instructions. 10 to 30 μg of total protein was separated on a Mini-PROTEAN TGX, Any kD gel, followed by transfer onto PVDF membrane (Bio-Rad). The membranes were blocked with 5% BSA in TBS buffer containing 0.5% Tween20. The membranes were then incubated overnight at 4°C with the primary antibodies to β-catenin (BD Bioscience), p110δ and Tcf7 (both from Abcam), RORγt (Affymetrix), and phosphoAkt, total Akt, STAT3, Histone-3, and GAPDH (all from Cell Signaling) at the concentration recommended by the manufactures. Subsequently, membranes were washed and incubated for 1 hour at room temperature with HRP-conjugated goat antibodies to mouse or rabbit IgG (Cell Signaling). For detection of protein, the chemiluminescence method was used with Western ECL Blotting Substrate (Bio-Rad) followed by the X-ray film-based imaging method (ThermoScientific).

Flow cytometry analysis. Surface staining of Th17 cells was performed with anti-CD4-APCCy7 and anti-Vβ14-FITC (both from BD Biosciences); anti-CD62L-APC, anti-CD44-PerCPCy5.5, anti-CCR6-PE, anti-CD69-PECy7, anti-CCR7-PE, anti-CD28-PerCPCy5.5, anti-ICOS-PE, and anti-CD25-PECy7/FITC (all from BioLegend); and viability stain (Invitrogen) on day 8. For all intracellular staining of cytokines (anti-IL-17-PerCPCy5.5, anti-IFN-γ-v450, anti-IL-2-FITC, anti-IL-21-APC, anti-IL-22-PE, and anti-TNF-α-FITC; BioLegend) and transcription factors (anti-RORγt-APC, anti-cMaf-PerCPCy5.5, anti-STAT3-PE, anti-FoxP3-FITC/PE, and anti-T-bet-v450; eBiosciences), cells were stimulated for 4 to 5 hours with 50 ng/ml PMA and 750 ng/ml ionomycin (Sigma-Aldrich). After 1 hour, Monensin (BioLegend) was added per the manufacturer's instructions. After surface staining, intracellular staining for cytokines and transcription factors was performed using Fixation and Permeabilization buffers (BioLegend) and Foxp3 staining buffers (eBiosciences), respectively. Flow cytometry acquisition was performed on a FACSVerse or FACSAccuri (BD Bioscience). Data were analyzed with FlowJo software (TreeStar).

ELISA. On the days indicated, cytokine levels were measured using the mouse IL-17A, IFN-γ, IL-2, IL-9, IL-10, IL-21, IL-22, CCL20, and GM-CSF ELISA kits (R&D) following the manufacturer's protocol.

ACT. C57BL/6 mice were inoculated subcutaneously with 3×10^5 B16F10 melanoma cells. Ten to twelve days later, 1×10^6 TRP-1 CD4$^+$ T cells programmed toward the Th17 phenotype, costimulated with ICOS versus CD28 or 0.75×10^6 TRP-1 Th17 cells expanded in vitro for 8 days in the presence or absence of CAL-101 or Ly294002 and/or Indo, were transferred via tail vein injection. Recipient animals were sublethally irradiated (5 Gy) prior to ACT. Tumors were measured using calipers, and the perpendicular diameters were recorded. Experiments were repeated twice, with similar results.

Engraftment and persistence. A total of 1×10^6 Th17-polarized TRP-1 cells costimulated with ICOS versus CD28 (expressed on beads or irradiated feeders) or stimulated with ICOS and expended for 8 days in the presence or absence of CAL-101 and/or Indo were transferred into 5-Gy irradiated C57BL/6 mice bearing 10-day established tumors. Blood, spleens, lungs, inguinal lymph nodes, and tumors were collected at indicated days and homogenized. Cells were enumerated using trypan blue exclusion. Frequency of Vβ14$^+$CD4$^+$ cells was analyzed by flow cytometry. Surface staining of Th17 cells was performed with anti-CD4-APCCy7, anti-Vβ14-FITC, and anti-CD8-APC (all from BD Biosciences); anti-NK1.1-PE, anti-CD44-PerCPCy5.5, anti-CD62L-APC, anti-CD25-PECy7, and anti-PD-1-PerCPCy5.5 (all from BioLegend); and viability stain (Invitrogen). For cytokine secretion (anti-IL-17A-PerCPCy5.5, anti-IFN-γ-v450, anti-IL-2-PE) cells were stained according to the manufacturer's protocol using Fix and Perm buffers (BioLegend). The experiment with Th17 cells treated in vitro with drugs was performed 3 times with similar results.

Statistics. The data are presented as mean ± SEM and were analyzed using GraphPad Prism software. Comparisons between 2 groups were analyzed using a 2-tailed Student's *t* test with Welch's correction for parametric distribution or Mann-Whitney signed-rank test for nonparametric distribution. For comparisons between multiple groups, a 1-way ANOVA was performed followed by multiple comparisons. Statistical significance was also analyzed using a (unpaired) *t* test and 1-way ANOVA followed by Tukey multiple comparisons post-test (as indicated in the figure legends). Comparison of tumor survival was assessed by log-rank (Mantel-Cox) test. $P < 0.05$ was considered statistically significant, and $P < 0.01$ and $P < 0.001$ were considered highly significant.

Study approval. Studies were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina Animal Resource Center (no. 3039).

Author contributions

Acknowledgments

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