

# Differential PI3K $\delta$ Signaling in CD4<sup>+</sup> T-cell Subsets Enables Selective Targeting of T Regulatory Cells to Enhance Cancer Immunotherapy

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## Abstract

To modulate T-cell function for cancer therapy, one challenge is to selectively attenuate regulatory but not conventional CD4<sup>+</sup> T-cell subsets [regulatory T cell (Treg) and conventional T cell (Tconv)]. In this study, we show how a functional dichotomy in Class IA PI3K isoforms in these two subsets of CD4<sup>+</sup> T cells can be exploited to target Treg while leaving Tconv intact. Studies employing isoform-specific PI3K inhibitors and a PI3K $\delta$ -deficient mouse strain revealed that PI3K $\alpha$  and PI3K $\beta$  were functionally redundant with PI3K $\delta$  in Tconv. Conversely, PI3K $\delta$  was function-

ally critical in Treg, acting there to control T-cell receptor signaling, cell proliferation, and survival. Notably, in a murine model of lung cancer, coadministration of a PI3K $\delta$ -specific inhibitor with a tumor-specific vaccine decreased numbers of suppressive Treg and increased numbers of vaccine-induced CD8 T cells within the tumor microenvironment, eliciting potent antitumor efficacy. Overall, our results offer a mechanistic rationale to employ PI3K $\delta$  inhibitors to selectively target Treg and improve cancer immunotherapy. *Cancer Res*; 77(8); 1892–904. ©2017 AACR.

## Introduction

Decreasing the numbers and/or function of regulatory T cells (Treg) is needed to produce better therapeutic outcomes for cancer patients. The ideal Treg-targeting approach should be selective to maintain specifically in the frame of cancer immune therapy for which maintenance of a potent effector arm of the immune system is vital. Several approaches have been tested to deplete or inactivate Tregs (1–4), but these strategies do not provide selective inhibition of Tregs and may result in a decrease of effector T cells (4).

Understanding the signaling pathways regulating conventional T cells (Tconv) and Tregs activation, function and survival can help to design drugs that could selectively modulate Tregs and Tconvs. PI3K-Akt is an important pathway involved in signaling downstream of the T-cell receptor (TCR) and regulates proliferation, cell metabolism, and cell growth, and as we have previously shown, it is differentially regulated in these CD4 T-cell subsets (5–10). Furthermore, it was recently reported that inhibition of the PI3K $\delta$  isoform results in a decrease in Tregs number and delayed tumor growth in animal models (11).

However, the role of the PI3K isoforms in the regulation of Tregs and Tconvs is not fully understood. Here, we hypothesized that there may be a differential role of Class IA PI3K isoforms in Tconv and Treg regulation.

The Class IA PI3K family consists of a heterodimeric complex of 110-kD catalytic subunits, p110 $\alpha$ ,  $\beta$ , or  $\delta$ , with a regulatory subunit (p85 $\alpha$ , p55 $\alpha$ , p50 $\alpha$ , p85 $\beta$ , or p55 $\gamma$ ; refs. 6, 12, 13). The PI3K 110 $\alpha$  and  $\beta$  subunits are ubiquitously expressed while p110 $\delta$  expression is restricted to hematopoietic cells (6). Cell death by apoptosis is a major regulator of hematopoietic cell homeostasis. B-cell lymphoma 2 (BCL-2) family proteins, which have either pro- or antiapoptotic activities, have been studied intensively for the past decade owing to their importance in the regulation of apoptosis (1). Myeloid cell leukemia 1 (Mcl-1), an antiapoptotic member of the Bcl-2 family (2), is critical for survival of Tregs, and it has been shown that the loss of this antiapoptotic protein caused fatal autoimmunity (3). Mcl-1 is tightly regulated by glycogen synthase kinase. The glycogen synthase kinase 3 (GSK-3 $\alpha$ /GSK-3 $\beta$ ) is a ubiquitously expressed serine/threonine kinase and regulates a wide variety of functions, including metabolism, cell proliferation, cell differentiation, and apoptosis, and is reported to be required for Mcl-1 degradation (4). GSK-3 $\beta$  is a downstream target of PI3K-Akt signaling pathway and usually remains active in cells; however, PI3K-induced activation of Akt results in the phosphorylation (S9) of GSK-3 $\beta$  that inhibits its activity (4, 5). The differential role of Class IA PI3K isoform in regulating the survival and apoptosis of Tregs and Tconvs has not been elucidated yet.

Here, we report that Class IA PI3K isoforms play different roles in Tregs and Tconvs. We found that in contrast to Tregs that are primarily dependent on the PI3K $\delta$  isoform, in Tconvs, PI3K $\delta$  is necessary, and in contrast to the situation in Tregs, PI3K $\alpha$  and  $\beta$  provide a redundant pathway to PI3K $\delta$ , ensuring the presence of alternative pathways to provide a robust effector function when

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**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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needed to eliminate any assault on the immune system. We have also evaluated the translation of these findings *in vivo* on therapeutic efficacy by assessing the effect of treatment with PI3K $\delta$  inhibitor on the antitumor immune response. Our findings clarify the role of the PI3K isoforms in CD4 T-cell TCR signaling and offer a further understanding of the development of selective strategies for the modulation of different CD4 T-cell subsets in the frame of immunotherapy.

## Materials and Methods

### Animals

C57BL/6 female 6- to 8-week-old mice were purchased from The Jackson Laboratory. P110delta-PI3K-D910A (kinase-dead) PI3K $\delta$  KO mice were purchased from Charles River Laboratories and bred under pathogen-free conditions in the Augusta University animal facility. Foxp3-GFP mice were housed and bred under pathogen-free conditions in the Augusta University animal facility. All procedures were carried out in accordance with approved institutional animal protocols.

### Antibodies

All fluorophore-labeled antibodies were purchased from BD Biosciences.

### PI3K inhibitors

A66 (PI3K $\alpha$  inhibitor; IC<sub>50</sub> of 32 nmol/L; refs. 14, 15), TGX-221 (PI3K $\beta$  inhibitor; IC<sub>50</sub> of 5 nmol/L; refs. 15, 16), CAL-101 (PI3K $\delta$  inhibitor; IC<sub>50</sub> of 2.5 nmol/L; ref. 17), and GDC-0941 (PAN PI3K inhibitor; ref. 18) were purchased from Selleckchem. The IC<sub>50</sub> for each isoform is listed in Supplementary Table S1.

### Western blotting

Total protein lysates were collected in RIPA buffer. Forty micrograms of lysates were run on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were probed with primary antibodies (1:1,000 dilutions) overnight at 4°C and incubated with secondary antibodies (1:5,000 dilution) for 1 hour at room temperature. Chemiluminescence was performed with Pierce reagents.

### T-cell stimulation flow cytometry analysis and *in vitro* proliferation (violet cell trace) assay

Sorted Tregs and Tconvs were labeled with violet cell trace stain according to the manufacturer's protocol (Life Technologies). Cells were stimulated with and without inhibitors in the presence of 10  $\mu$ g/mL plate-bound anti-CD3, 2.5  $\mu$ g/mL soluble anti-CD28, and 100 IU/mL IL2. For negative control (nonstimulated), cells were cultured in 100 IU/mL IL2. After 3 days, cells were stained with fixable live/dead cell stain (Life Technologies), fixed and permeabilized using the mouse Foxp3 buffer Kit according to the manufacturer's instructions (BD Bioscience) and stained with anti-CD4-FITC and anti-Foxp3-AlexaFluor 647. For pAkt (S473) and pS6 (S244) analysis, the signal was amplified using a biotin-conjugated donkey anti-rabbit antibody (BD Biosciences) and streptavidin-PE. After staining, cells were acquired on a LSRII SORP flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

### Tumor cell lines

TC-1 cell line was a gift from Professor T.C. Wu (Department of Pathology, Johns Hopkins University, Baltimore, MD; ref. 19).

Briefly, TC-1 tumor cell line was generated from lung epithelial cells immortalized with HPV16 E6 and E7. Cell line authentication was carried out by morphology and flow cytometry 4 months prior to the first submission of the article (20). Cell lines used in this study were routinely tested for contamination by PCR. All cell culture stocks were stored in liquid nitrogen. The growth of tumors formed from these cells is enhanced by Tregs.

### *In vivo* experiments

C57BL/6 mice were injected s.c. with 70,000 TC-1 tumor cells and monitored for development of tumors. On days 10 to 12, when tumor size reached 3 to 5 mm in diameter, mice were treated with a single intraperitoneal injection of A66, TGX-221, or CAL-101 at 2 or 10 mg/kg. The control group received 10% DMSO in water (vehicle). Mice were then sacrificed 3 or 6 days after treatment. Splenocytes were harvested, stained for CD3, CD4, CD8, and Foxp3, and analyzed by flow cytometry (LSRII SORP).

Alternatively, mice injected with TC-1 were monitored for development of tumor. On days 10 to 12, when tumor size reached 3 to 5 mm in diameter, spleens and tumors from the pretreatment group of animals were used to examine the T-cell population before starting the treatment (baseline). Animals were treated with either CAL-101 (10 mg/kg) or 10% DMSO in water (vehicle) starting on the 12th day following TC-1 implantation for the next 14 days with 3-day interval. All groups were euthanized on day 26 of TC-1 implantation for evaluation of the T-cell subsets, and their proliferation was measured by Ki67 expression.

### Tumor implantation and treatment

For both therapeutic and immunology experiments, 70,000 TC-1 cells were inoculated s.c. on day 0. The growth of tumors formed from these cells is enhanced by Tregs. Vaccine was given weekly s.c. starting on days 10 to 12 after tumor implantation when tumor diameter reached 3 to 5 mm. For therapeutic experiments, vaccine was given weekly throughout the experiment. CAL-101 treatment was provided on the day when tumor size reached 3 to 4 mm 5 to 6 days before vaccination. CAL-101 was given every third day for entire duration of treatment. Total of four groups, (i) no treatment, (ii) CAL-101, (iii) vaccine, and (iv) combination of vaccine with CAL-10 ( $n = 5$  for each group), of mice were utilized in these experiments. In these studies, tumor growth and survival were monitored. Tumors were measured every 3 to 4 days using digital calipers, and tumor volume was calculated using the formula  $V = (W^2 \times L)/2$ , whereby  $V$  is the volume,  $L$  is the length (longer diameter), and  $W$  is width (shorter diameter). Mice were sacrificed when moribund or if tumor volume reached 1.5 cm<sup>3</sup>. For immunology experiments, mice were treated similarly and were sacrificed 3 days after the second immunization, which ensured no significant differences existed in tumor size between different groups. Tumors were harvested for analysis of tumor-infiltrating cells.

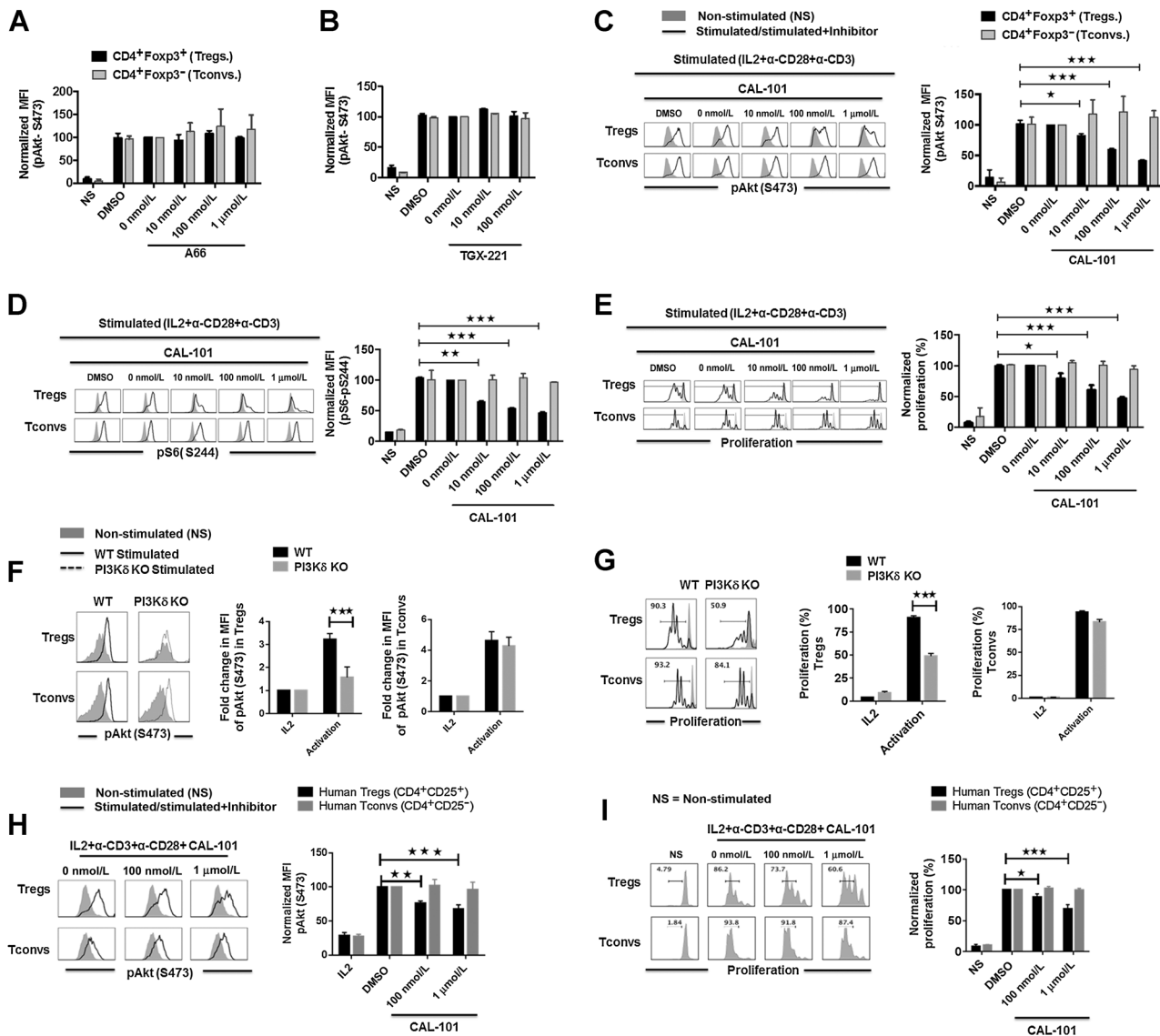
## Results

### Class IA PI3K isoform differentially regulates TCR signaling and proliferation of Tregs and Tconvs

We recently reported that several inhibitors that target PI3K and its downstream effector, Akt, selectively inhibit the *in vitro* proliferation of human and murine Treg when compared with Tconv. This selective decrease in Treg proliferation provided us with a potential strategy to modulate the Treg/Tconv balance

*in vivo* (9). However, the exact mechanism of PI3K/Akt regulation in these subtypes has not been fully elucidated. The differential sensitivity of Tregs and Tconvs could be an outcome of disparate expression level of the PI3K isoforms in Tregs and Tconvs. We therefore checked the expression level of different PI3K isoforms in Tregs and Tconvs. We found no significant differences in protein expression patterns of the PI3K subunits, p85 $\alpha$ , p110 $\alpha$ , p110 $\beta$ , or p110 $\delta$ , between Tregs and Tconvs (Supplementary Fig. S1). To further explore the role of the PI3K isoforms in Tconvs and Tregs, we examined the effect of titrated concentrations of specific PI3K isoform inhibitors on

the downstream signaling of this pathway in these CD4 T-cell subpopulations. FACS-sorted Tregs and Tconvs were activated in the presence or absence of specific inhibitors of PI3K $\alpha$  (A66), PI3K $\beta$  (TGX-221), or PI3K $\delta$  (CAL101). The phosphorylation status of the downstream Akt was measured by flow cytometry (Supplementary Fig. S2A and S2B). The range of used concentrations was selected based on the previously published IC<sub>50</sub> of these inhibitors (Supplementary Table S1; refs. 14, 15, 17). We did not detect any remarkable inhibition of pAkt (S473) in either Tregs or Tconvs at all the tested concentrations of either A66 or TGX-221 (Fig. 1A and B; Supplementary Fig. S3A



**Figure 1.**

Tregs are more sensitive to PI3K $\delta$  inhibition *in vitro*. FACS-sorted Tregs and Tconvs from WT B16 (foxp3-GFP) mice (**A-E**), WT B16 and PI3K $\delta$  KO mice (**F** and **G**), and human PBMC (**H** and **I**) were plated on anti-CD3-coated plates and cultured in activation media (IL2 and anti-CD28) without inhibitors (DMSO, 0 nmol/L) and with inhibitors for 72 hours. For negative control (NS, nonstimulated), cells were left in media containing IL2 for 72 hours. Intracellular phosphorylation of Akt (S473), S6 (S244) (**D**), and proliferation (**E**) in live gated cells was measured by flow cytometry of Tregs and Tconvs treated with A66 (PI3K $\alpha$  inhibitor), TGX-221 (PI3K $\beta$  inhibitor), and CAL-101 (PI3K $\delta$  inhibitor) and normalized for three independent experiments. Intracellular level of pAkt (S473) and proliferation in live gated cells was measured by flow cytometry of Tregs and Tconvs from WT B16 and PI3K $\delta$  KO mice (**F** and **G**), and human Tregs and Tconvs treated with CAL-101 (PI3K $\delta$  inhibitor; **H** and **I**), respectively.

and S3B). In contrast, inhibition of the PI3K $\delta$  isoform with CAL-101 significantly mitigated the phosphorylation of Akt (S473) and Akt (T308) in Tregs but had no effect on Tconvs as shown in Fig. 1C and Supplementary Fig. S4, respectively. The effect was also reflected further downstream of the PI3K-Akt pathway, where the phosphorylation status of ribosomal protein (rp) S6 (pS244) in Tregs treated with CAL-101 was significantly inhibited (Fig. 1D).

To gain a more complete understanding of the differential role of Class IA PI3K isoform in Tregs and Tconvs, we tested the effect of specific PI3K isoform inhibition on the proliferation of these T-cell subtypes. Using Class IA isoform-specific inhibitors, we found that inhibiting PI3K $\alpha$  or PI3K $\beta$  has no effect on either Treg or Tconv proliferation as shown in Supplementary Fig. S5A and S5B, respectively. On the other hand, PI3K $\delta$  inhibition led to a significant abrogation of Treg proliferation in a concentration-dependent manner, without affecting Tconv proliferation (Fig. 1E). The findings were further confirmed using Tregs and Tconvs from mice with PI3K $\delta$  inactive kinase (PI3K $\delta$  KO). As expected, we found that the level of phospho-Akt (S473) in Tregs obtained from PI3K $\delta$  KO mice (Fig. 1F) and their proliferation ability under TCR/IL2 stimulation (Fig. 1G) were significantly lower compared with Tregs from wild-type (WT) mice. Furthermore, there were no significant differences in Tconvs obtained from the two mice strains.

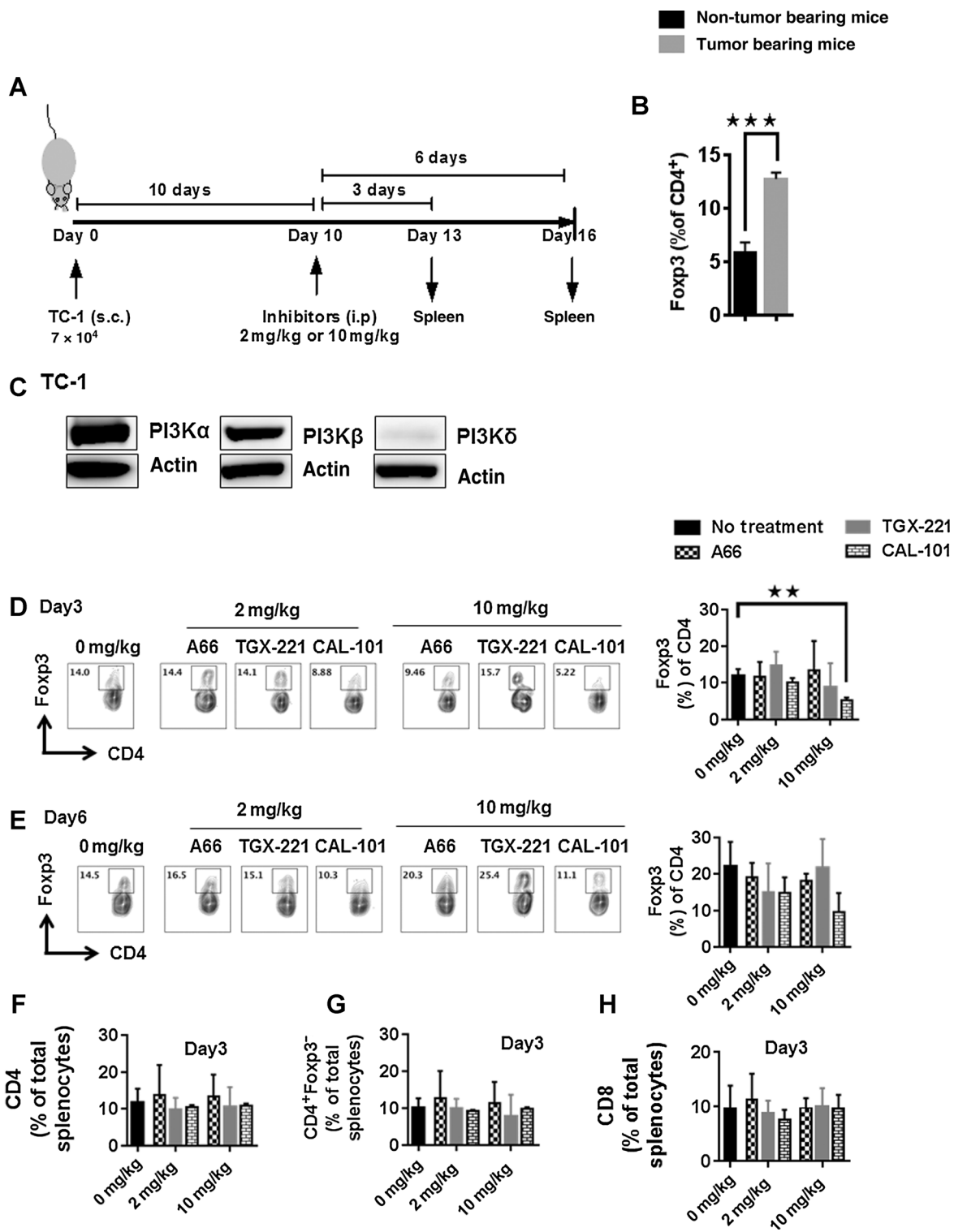
These data demonstrate that TCR signaling in Tregs is exclusively dependent on PI3K $\delta$  but not on PI3K $\alpha$  or PI3K $\beta$ , whereas in Tconvs, no specific isoform was found to be dominant in TCR downstream signaling.

To further validate these findings for therapeutic approaches in immunotherapy, we tested the effect of PI3K isoform inhibitors on human CD4 T cell. Human Tregs and Tconvs were fractionated from peripheral blood mononuclear cells (PBMC) by FACS. Tregs were sorted based on expression of CD4<sup>+</sup>CD25<sup>HI</sup>, whereas Tconvs were identified as CD4<sup>+</sup>CD25<sup>-</sup>. We first tested the effect of PI3K $\delta$  isoform inhibitors on the level of phosphorylated Akt (pAkt-S473) in Treg and Tconv and on the proliferation of these two cell types. We tested the effect of the PI3K $\delta$  inhibitor after stimulation with anti-CD3/anti-CD28 in a 3-day culture in media containing 100 IU/mL of IL2, with titrated amounts of CAL-101. Similar to the effect on mouse cells, we found that PI3K $\delta$  inhibition led to a significant decreased level of pAkt in Tregs but not Tconv and significant abrogation of Treg proliferation, without affecting Tconvs, in a concentration-dependent manner (Fig. 1H and I). Based on the reproducibility of the data in human cells, we believe that using specific PI3K $\delta$  inhibitor would be a reasonable approach to enhance cancer immunotherapy.

#### PI3K isoform differentially regulates the frequency of T-cell subsets in tumor-bearing mice

After demonstrating that the inhibition of PI3K $\delta$  isoform but not PI3K $\alpha$  or  $\beta$  impairs TCR signaling in Tregs and not Tconvs *in vitro*, we next tested whether this specificity is translated in an *in vivo* model. We selected TC-1 (19) syngeneic mouse model, which is a Treg-dependent tumor model (21), and the frequency of Tregs increases significantly in tumor-bearing mice compared with nontumor mice (Fig. 2B). In addition, TC-1 expresses PI3K $\alpha$  and PI3K $\beta$ , but do not express detectable levels of p110 $\delta$  (Fig. 2C). Consequently, specific

PI3K isoform inhibitors were tested in the TC-1 syngeneic mouse tumor model. Mice were treated with a single dose of the inhibitors 10 days after tumor implantation (Fig. 2A). Mice were sacrificed 3 or 6 days after treatment, and the level of splenic Tregs was assessed. We found that inhibition of PI3K $\delta$  with CAL-101 led to a dose-dependent inhibition of Tregs in the spleen with significant reduction at 10 mg/kg on day 3 after treatment (Fig. 2D). In contrast, inhibition of PI3K $\alpha$  or  $\beta$  had no significant effects on splenic Tregs with either a 2 mg/kg or 10 mg/kg dose on day 3 (Fig. 2D) and day 6 (Fig. 2E) after treatment. Considering the short half-life of CAL101 (~1 hour; ref. 22), not surprisingly, the level of Tregs returned to control levels on day 6 after treatment (Fig. 2E). Importantly, we found that inhibition of PI3K $\alpha$ ,  $\beta$ , or  $\delta$  with either a 2 mg/kg or 10 mg/kg dose of inhibitors had no significant effects on splenic CD4<sup>+</sup>, CD4<sup>+</sup>Foxp3<sup>-</sup>, or CD8<sup>+</sup> cells (Fig. 2F-H) on day 3 after treatment. The level of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was also not affected on day 6 by any of the PI3K inhibitors at either dose (data not shown). This indicates that PI3K $\delta$ , but not PI3K $\alpha$  or PI3K $\beta$ , inhibition reduces the number of Tregs and spares other T-cell subsets. Consistent with *in vitro* findings, we found that *in vivo* Treg is dependent on PI3K $\delta$  exclusively but not on PI3K $\alpha$  or PI3K $\beta$ , whereas in Tconvs, no specific isoform was found to be functionally dominant. We next tested the impact of continuous treatment of CAL-101 (10 mg/kg) on splenic T-cell population in TC-1 model. TC-1-injected (s.c.) mice were monitored for development of tumor. On day 12, when tumor size reached 3 to 5 mm in diameter, spleens from one group of animals were used to examine the percentage of Tregs (base line) before starting the treatment. Animals were treated with either CAL-101 or vehicle every 3 days with total of five doses (Supplementary Fig. S6A). Animals were sacrificed on day 26 after tumor implantation. As expected, we observed that there was an increase in Treg population in vehicle-treated animals on day 26 compared with baseline (Supplementary Fig. S6B). However, the continuous administration of CAL-101 at 3-day intervals significantly decreased the percentage of Tregs in CAL-101-treated mice compared with vehicle-treated animals (Supplementary Fig. S6B). We also evaluated the effects of CAL-101 on effector T-cell subsets including CD4<sup>+</sup> and CD8<sup>+</sup> cells. We did not find any effects of CAL101 on percentage of CD4<sup>+</sup>Foxp3<sup>-</sup> (Supplementary Fig. S6C) and CD3<sup>+</sup>CD8<sup>+</sup> (Supplementary Fig. S6D) cells. These observations suggest a schedule for CAL-101 administration for keeping the Tregs at significantly low level without affecting the other T-cell populations. Thus, our data demonstrate that CAL-101 treatment selectively impaired the survival signal in Tregs *in vitro* that could be correlated with decreasing their number in CAL-101-treated animals *in vivo*. As CAL-101 treatment selectively impaired proliferation of Tregs *in vitro*, we investigated whether their proliferative capacity was reduced *in vivo* as well. We performed analyses of Ki67 expression in response to CAL-101 administration. Tregs from CAL-101-treated animals showed a decreased proliferative response as assessed by Ki67 staining (Supplementary Fig. S6E). The proliferation potential of effector T cells (CD4<sup>+</sup> and CD8<sup>+</sup>) from CAL-101-treated animals did not show any impaired proliferative response (Supplementary Fig. S6F and S6G). This suggests that the selective reduction of Tregs in CAL-101-treated animals could be a consequence of impaired survival signals as well as inhibition of proliferation.



**Figure 2.** Class IA PI3K isoforms differentially regulate T-cell subsets *in vivo*. C57BL/6 mice were injected s.c. with 70,000 TC-1 cells. On day 10, all mice developed visible tumors of equal size. **A**, Mice were grouped and injected i.p. with either 2 mg/kg or 10 mg/kg CAL-101, A66, or TGX-221 dissolved in 10% of DMSO. **B**, Percentage of Tregs in tumor-bearing mice on day 10 compared with nontumor-bearing mice. **C**, Protein lysates were prepared from TC-1 cells, separated by SDS-PAGE, and immunoblotted with Class IA isoform-specific antibodies; actin was used as loading control. The percentage of Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>; **D**) on day 3 of treatment, Tregs (CD4<sup>+</sup>Foxp3<sup>+</sup>; **E**) on day 6 of treatment, CD4<sup>+</sup> cells on day 3 of treatment (**F**), CD4<sup>+</sup> Foxp3<sup>-</sup> cells on day 3 of treatment (**G**), and CD8<sup>+</sup> cells on day 3 of treatment (**H**) was analyzed by flow cytometry. The percentage of cells was normalized and presented as mean  $\pm$  SD. Representative data from two independent experiments are shown (3 to 5 mice for each group per experiment). Statistical significance was determined by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ ).

### PI3K $\alpha$ and PI3K $\beta$ provide a redundant pathway to PI3K $\delta$ in Tconvs for TCR signaling and proliferation

Because our data demonstrate that downstream TCR signaling and proliferation of Tregs are dependent on PI3K $\delta$ , while none of the inhibitors alone showed any effect on activation and proliferation in Tconvs, we next investigated whether the PI3K isoforms provide functional redundancy in Tconvs. To examine this, we tested whether the inhibition of all Class IA PI3K isoforms using the pan inhibitor GDC (GDC-0941) affects downstream TCR signaling. We found that the pan inhibition of PI3K isoforms with GDC significantly mitigated the phosphorylation of Akt (S473) in Tconvs as shown in Fig. 3A. The effect was also reflected downstream of the PI3K-Akt pathway, where the phosphorylation status of rp S6 (Fig. 3B) and proliferation (Fig. 3C) in Tconvs treated with GDC was significantly inhibited.

To examine the role of each PI3K isoform in Tconvs, we tested whether the simultaneous inhibition of any two PI3K isoforms affects downstream TCR signaling. We treated Tconvs with PI3K inhibitors specifically targeting two isoforms at a time: PI3K $\alpha$  and PI3K $\beta$  (A66+TGX-221), PI3K $\alpha$  and PI3K $\delta$  (A66+CAL101), and PI3K $\beta$  and PI3K $\delta$  (TGX-221+CAL101). We found that combining inhibition of both PI3K $\alpha$  and PI3K $\beta$  did not affect the phosphorylation of Akt and S6 (Fig. 3D and E). As expected, there was no effect on proliferation of Tconvs either (Fig. 3F). These findings show that PI3K $\delta$  is sufficient for TCR signaling and proliferation of Tconvs. On the other hand, combining PI3K $\delta$  inhibitor with either PI3K $\alpha$  or PI3K $\beta$  inhibitor significantly reduced the levels of pAkt and pS6 (Fig. 3D and E) and inhibited proliferation of Tconvs (Fig. 3F). We further confirmed our data using T cells lacking PI3K $\delta$  obtained from PI3K $\delta$  KO mice. As expected, we found that although proliferation of Tconvs from WT mice was not affected in the presence of PI3K $\alpha$  or PI3K $\beta$  inhibitors (Fig. 1E and F), proliferation of Tconvs from PI3K $\delta$  KO mice was significantly inhibited in the presence of either of these inhibitors (Fig. 3G). Further, targeting both PI3K $\alpha$  and PI3K $\beta$  together, using A66 and TGX-221, did not inhibit the proliferation of Tconvs from WT mice. However, combined inhibition of PI3K $\alpha$  and PI3K $\beta$  (A66+TGX-221) significantly mitigated the proliferation of Tconvs from PI3K $\delta$  KO mice (Fig. 3G).

Together, these findings show that PI3K $\delta$  is sufficient for TCR signaling and proliferation of Tconvs. However, in contrast to Tregs, in Tconvs, PI3K $\alpha$  and PI3K $\beta$  combined can compensate for the absence of PI3K $\delta$ .

### Class IA PI3K isoform differentially regulates the survival of Tregs through GSK-3 $\beta$ and Mcl-1 signaling pathway

Differential role of Class IA PI3K for regulation of TCR survival signaling in conventional CD4<sup>+</sup>Foxp3<sup>-</sup> cells and regulatory CD4<sup>+</sup>Foxp3<sup>+</sup> cells has not been explored. Because the inhibition of individual PI3K isoforms does not affect Tconvs, and inhibition of only PI3K $\delta$  decreases the frequency of Tregs in tumor-bearing mice, we next tested whether their survival is affected in the presence of isoform-specific inhibitors *in vitro*. Cells were activated *in vitro* with and without inhibitors for 72 hours, and the viability of Tregs and Tconvs was analyzed after treatments with different PI3K inhibitors using fixable live/dead cell stain (Life Technologies). Tregs and Tconvs showed no sign of reduced survival when PI3K $\alpha$  or PI3K $\beta$  was inhibited (Fig. 4A and B); however, in contrast to Tconvs, the inhibition of PI3K $\delta$  with CAL-101 significantly decreased the survival of Tregs (Fig. 4C). Interestingly, the survival of Tconvs was not

affected in the presence of any of the PI3K isoform-specific inhibitors. These results indicated that Treg survival is dependent on PI3K $\delta$  and accordingly sensitive to its inhibition. To further confirm this, we tested whether the inhibition of PI3K $\delta$  induces apoptosis in Tregs. Using Annexin V binding assay, we found that frequency of Annexin V-positive (apoptotic) cells increased with increasing concentrations of CAL-101 (Supplementary Fig. S7A).

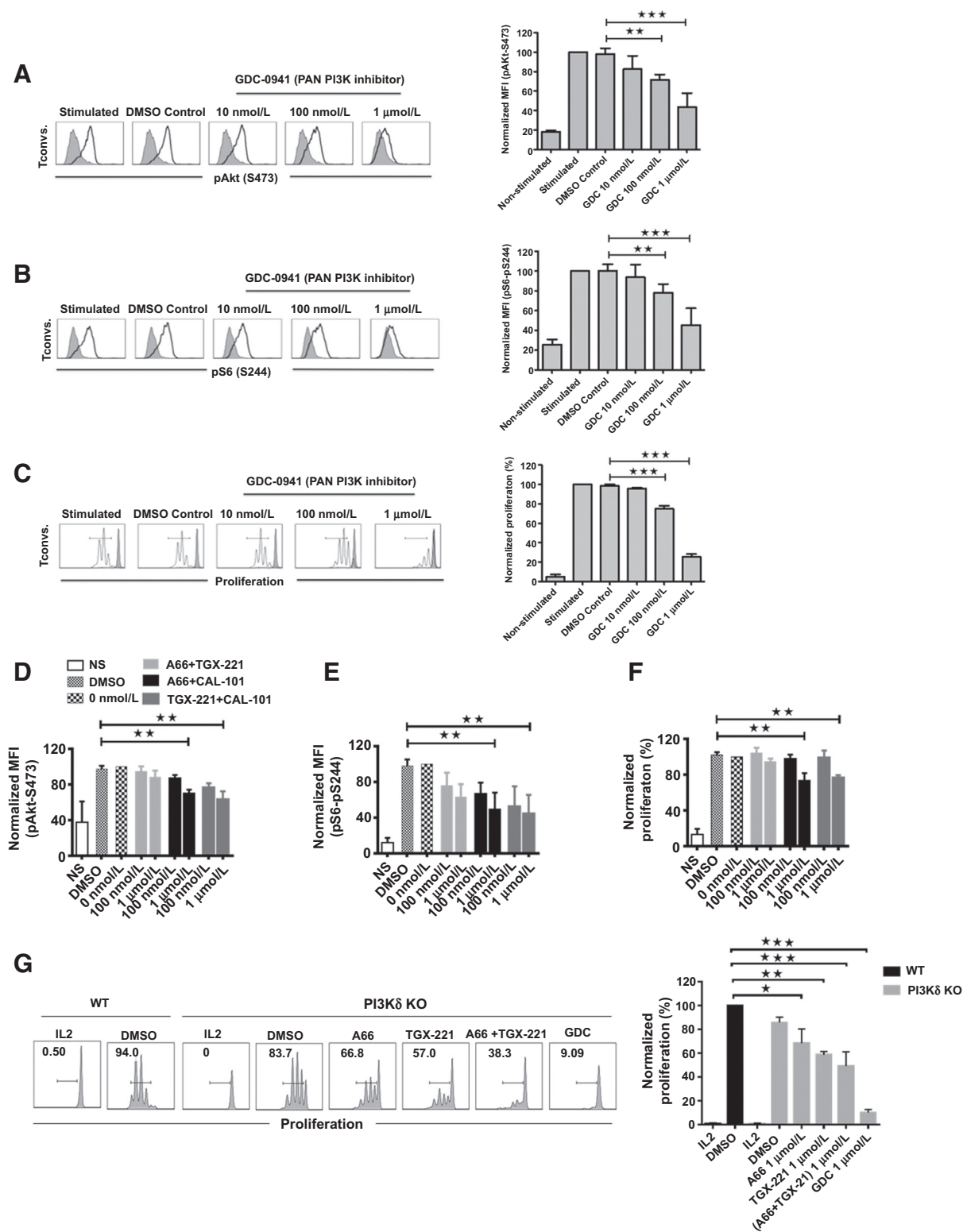
To understand the molecular mechanism by which PI3K $\delta$  inhibition selectively induces apoptosis, leading to reduced survival of Tregs, we next evaluated the effect of specific PI3K isoform inhibition on the antiapoptotic protein Mcl-1, which is critical for the survival of T cells including Tregs (23) and is rapidly degraded when cells undergo apoptosis in response to various stimuli (24–27). Mcl-1 can sequester direct activator BH3-only proteins, such as Bim, Bad, and Puma, and prevent them from activating Bak. In the absence of Mcl-1, Bak forms pore in the outer mitochondrial membrane to release cytochrome c, activate caspases, and induce apoptosis (Fig. 4H).

Accordingly, we tested the expression of Mcl-1 in Tregs and Tconvs treated with specific PI3K isoform inhibitors. We found that inhibition of PI3K $\alpha$  or PI3K $\beta$  does not affect the expression of Mcl-1 levels in Tregs or Tconvs (Fig. 4D). Conversely, the inhibition of PI3K $\delta$  by CAL-101 remarkably decreases the expression of Mcl-1 in Tregs but has no effect on Tconvs (Fig. 4E). These observations further indicate that PI3K $\delta$  selectively regulates the survival of Tregs through the Mcl-1 pathway. Because the survival of Tconvs was not affected with either of isoform-specific inhibitor, therefore, as expected, we did not find any changes in expression of Mcl-1.

It has been reported that degradation of antiapoptotic protein Mcl-1 is controlled by GSK-3 (27), and the expression of constitutively active GSK-3 decreases Mcl-1 expression (27). GSK-3 $\beta$  is a downstream target of PI3K-Akt signaling pathway and usually remains active in cells; however, PI3K-induced activation of Akt results in the phosphorylation (S9) of GSK-3 $\beta$  that inhibits its activity (28, 29) as shown in Fig. 4A. To further dissect the mechanism, we checked the effect of PI3K inhibitors on the phosphorylation levels of GSK-3 $\beta$ . Treatment of cells with titrated concentrations of PI3K $\alpha$  inhibitor (A66) or PI3K $\beta$  inhibitor (TGX-221) did not block the S9 phosphorylation of GSK-3 $\beta$  (Fig. 4F). However, when T cells were treated with PI3K $\delta$  inhibitor, the S9 phosphorylation of GSK-3 $\beta$  was blocked only in Tregs (Fig. 4G), leading to activation of GSK-3 $\beta$ , which correlates with the decreased Mcl-1 expression (Fig. 4E) and survival (Fig. 4C) in Tregs. These results demonstrate that contrary to Tconv, PI3K $\delta$  is crucial for the regulation of GSK-3 $\beta$ - and Mcl-1-dependent survival of Tregs. On the other hand, PI3K $\alpha$  and PI3K $\beta$  as single isoforms are not crucial in the regulation of either Tconv or Tregs.

### PI3K $\delta$ is required for the GSK-3 $\beta$ - and Mcl-1-dependent survival pathway in Tconvs, and is compensated by PI3K $\alpha$ and PI3K $\beta$

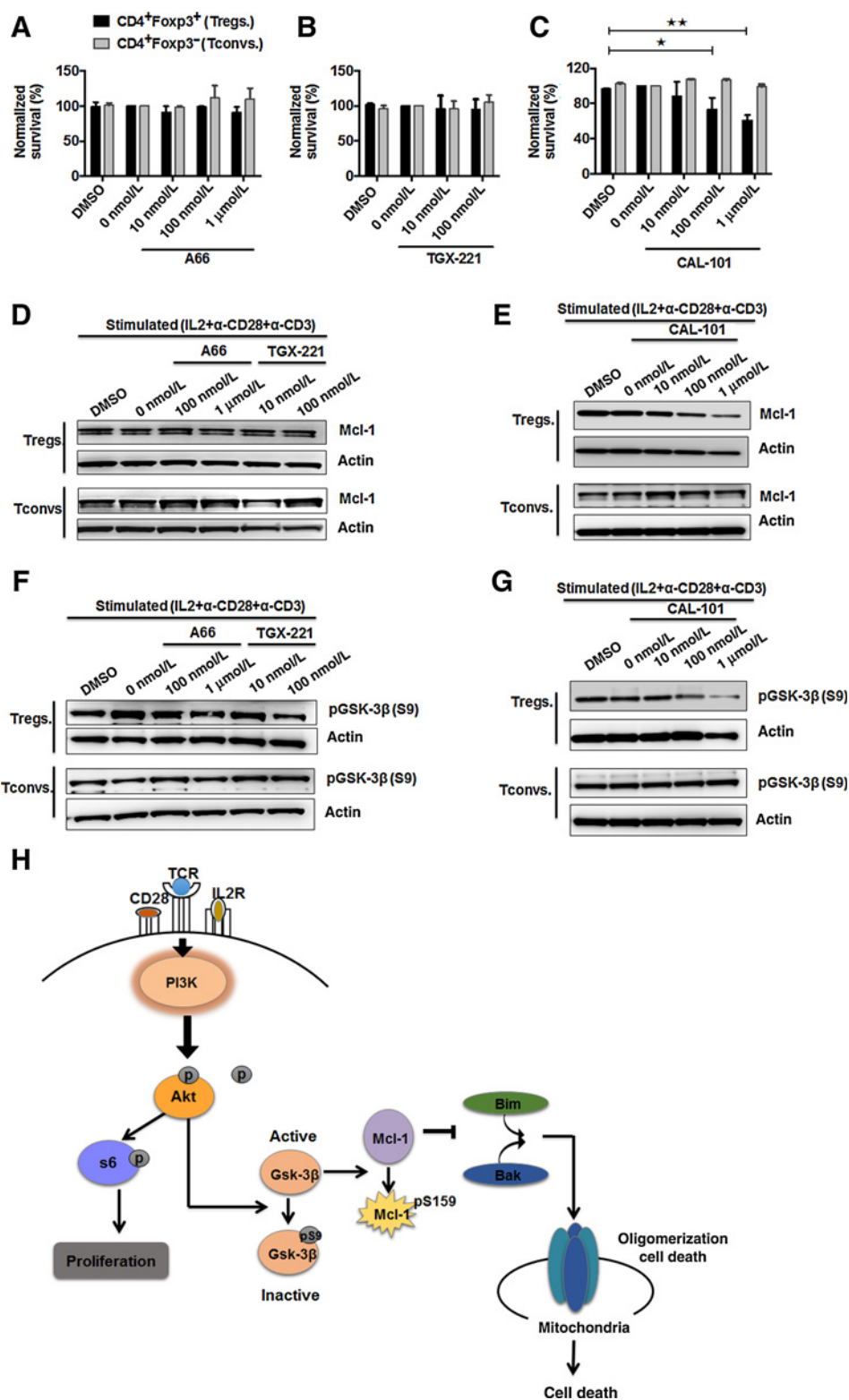
We have shown that inhibition of PI3K $\delta$  selectively activates GSK-3 $\beta$  in Tregs, which favors the degradation of antiapoptotic protein Mcl-1 leading to the decreased survival of Tregs, and had no effect on Tconvs. To understand the role of PI3K isoforms in the regulation of survival in Tconvs, we first examine the effect of pan PI3K inhibitor. We found that the survival of Tconvs is dependent on pan Class IA PI3K isoforms



**Figure 3.**

Class IA PI3K isoforms have redundant functions in TCR-mediated activation and proliferation in conventional T cells. **A-F**, FACS-sorted Tconv (CD4<sup>+</sup> Foxp3<sup>-</sup>) treated with GDC-0941 (PAN PI3K inhibitor; **A-C**) or combinations of inhibitors, including A66 PI3K $\alpha$  inhibitor +TGX-221 PI3K $\beta$  inhibitor; A66 PI3K $\alpha$  inhibitor + CAL-101 PI3K $\delta$  inhibitor; and TGX-221 PI3K $\beta$  inhibitor+ CAL-101 PI3K $\delta$  inhibitor (**D-F**) for 72 hours were harvested, and pAkt (S473) (**A** and **D**), pS6 (S244) (**B** and **E**), and proliferation (**C** and **F**) were measured on live gated cells using flow cytometry and normalized for three independent experiments. **G**, FACS-sorted Tconv from PI3K $\delta$  KO mice were treated with A66 PI3K $\alpha$  inhibitor, TGX-221 PI3K $\beta$  inhibitor, A66 PI3K $\alpha$  inhibitor +TGX-221 PI3K $\beta$  inhibitor, and GDC-0941 (pan PI3K inhibitor), and proliferation was measured on live gated cells using flow cytometry and normalized for two independent experiments.



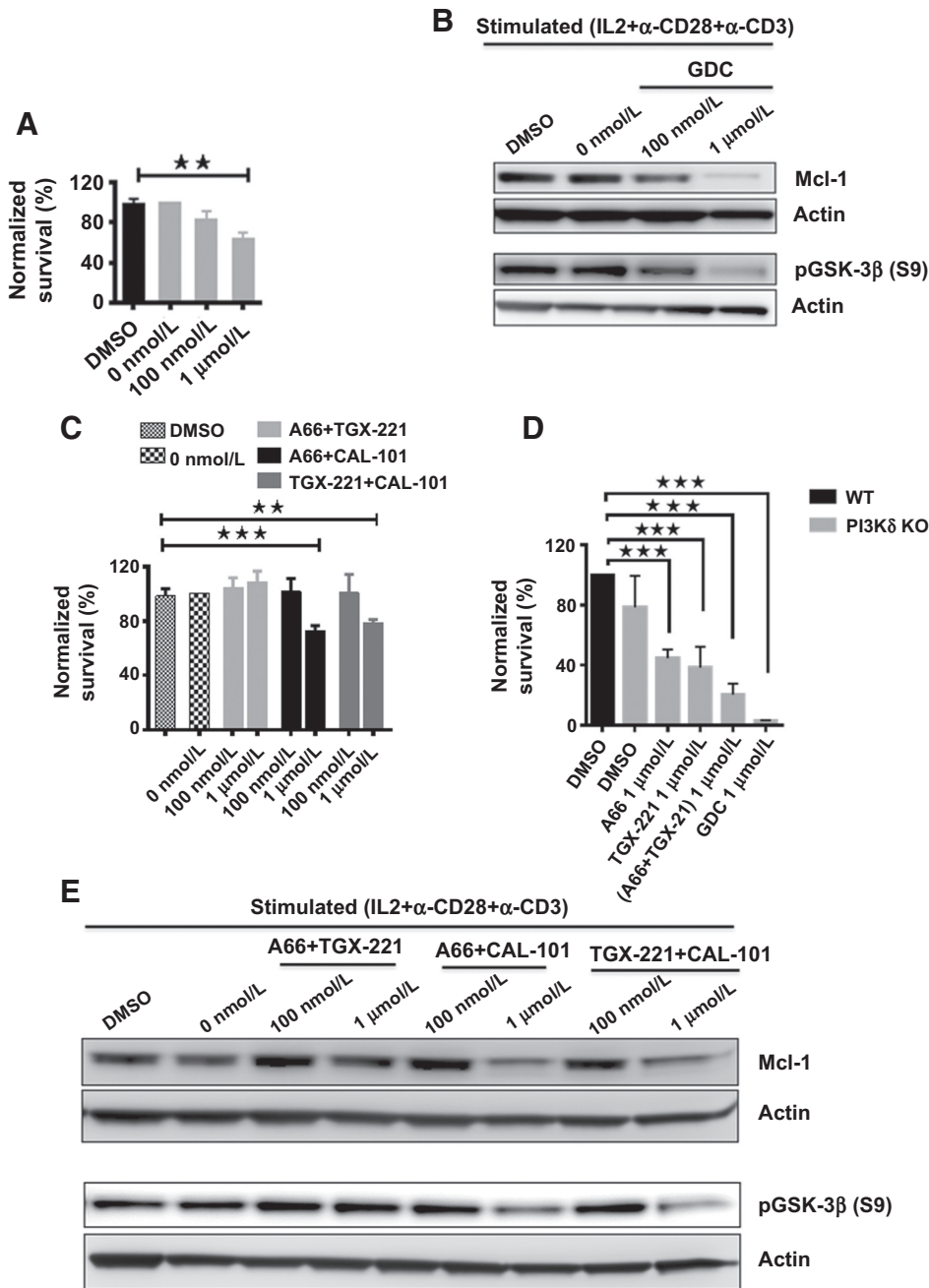


**Figure 4.** Inhibition of PI3Kδ affects GSK-3β- and Mcl-1-dependent survival of Tregs but not Tconvs. **A-C**, Cells were washed and stained for Live/Dead and fixed. Viability was measured by flow cytometry of Tregs and Tconvs treated with A66 (a PI3Kα inhibitor; **A**), TGX-221 (a PI3Kβ inhibitor; **B**), and CAL-101 (a PI3Kδ inhibitor; **C**). The results were normalized for three independent experiments. **D-G**, Cell lysates prepared on day 3 (72 hours) of treatment from Tregs and Tconvs were separated by SDS-PAGE and immunoblotted with specific antibodies (Mcl-1) or GSK-3β (pS9); actin was used as loading control. **H**, Schematic presentation showing the role of PI3K in regulation of proliferation, cell death, and survival.

(Fig. 5A). Inhibition of pan PI3K activates GSK-3β in Tconvs, which favors the degradation of antiapoptotic protein Mcl-1 (Fig. 5B), leading to the decreased survival of Tconvs and induced apoptosis (Supplementary Fig. S7C). We next investi-

gated whether inhibition of any two PI3K isoforms affects survival of Tconvs. We found that combining inhibition of both PI3Kα and PI3Kβ did not induce apoptosis and did not affect the survival (Fig. 5C; Supplementary Fig. S7B). As





**Figure 5.** Class IA PI3K isoforms compensate for GSK-3β- and Mcl-1-dependent survival of Tconvs. FACS-sorted Tregs and Tconvs from WT were plated on anti-CD3-coated plates and cultured in activation media (IL2 and anti-CD28) without inhibitors (DMSO, 0 nmol/L) and with inhibitors for 72 hours. Cells were washed and stained for Live/Dead and fixed. Viability was measured by flow cytometry of Tconvs treated with GDC-0941 (pan PI3K inhibitor; **A**) or combinations of inhibitors including A66 (a PI3Kα inhibitor) +TGX-221 (a PI3Kβ inhibitor), A66 (a PI3Kα inhibitor) + CAL-101 (a PI3Kδ inhibitor), TGX-221 (a PI3Kβ inhibitor) + CAL-101 (a PI3Kδ inhibitor; **C**). **D**, FACS-sorted CD4<sup>+</sup> T cells from PI3Kδ KO mice were activated with and without inhibitors and compared with activated CD4<sup>+</sup> T cells from WT. **B** and **E**, In a separate experiment, T-cell lysates prepared on day 3 (72 hours) of treatment from Tconvs were separated by SDS-PAGE and immunoblotted with specific antibodies (Mcl-1) or GSK-3β (pS9); actin was used as loading control.

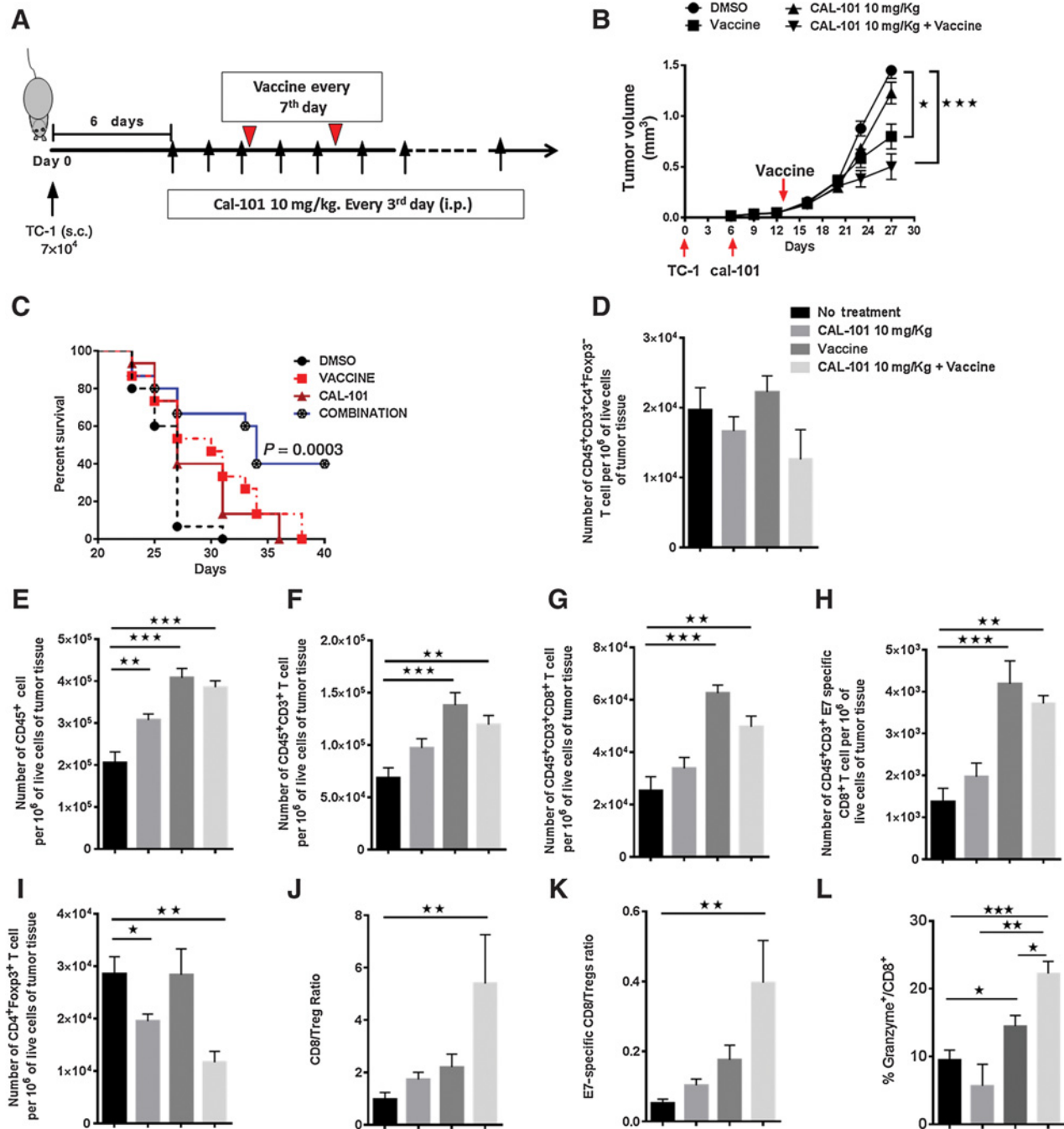
expected, there was no effect on Mcl-1 and pGSK-3β (Fig. 5D), which further provide evidences that, in the absence of PI3Kα and PI3Kβ, PI3Kδ is sufficient to regulate the survival of Tconvs.

On the other hand, combining PI3Kδ inhibitor with either PI3Kα or PI3Kβ inhibitor significantly reduced the survival (Fig. 5C), induced apoptosis (Supplementary Fig. S7C), and decreased Mcl-1protein with inhibited pGSK-3β of Tconvs (Fig. 5D).

Together, all these data suggest that PI3Kδ is sufficient for survival signaling in Tconvs; however, in contrast to Tregs, in Tconvs, PI3Kα and PI3Kβ combined can compensate for the absence of PI3Kδ (Fig. 5E).

**Inhibition of PI3Kδ enhances antigen immune response and synergizes with vaccine for better antitumor response**

We evaluated the antitumor therapeutic response of the addition of PI3Kδ inhibition to a tumor-specific peptide vaccine (E7) in TC-1 mouse tumor model. Tumor cells were implanted on day 0, and treatment was initiated when tumors reached 3 to 5 mm in diameter. Tumor-bearing mice received CAL-101 every third day and were treated with two doses of a peptide vaccine (Fig. 6A). Tumor growth and survival were monitored. We found that CAL-101 in combination with vaccine led to significant slowdown of tumor progression (Fig. 6B) associated with prolonged survival (Fig. 6C). These



**Figure 6.** Combination of vaccine with CAL-101 provides potent antitumor therapeutic efficacy. **A**, C57BL/6 mice ( $n = 5$ /group) were injected s.c. in the right flank with  $7 \times 10^4$  TC-1 cells. Mice from appropriate groups were injected weekly with vaccine (s.c.) or DMSO 5% as a control. CAL-101 was injected (i.p.) every third day starting on day 6 after tumor implantation throughout the experiment. **B**, Plots represent average tumor volumes of mice for each group. **C**, Kaplan-Meier plot of the overall survival. Statistical significance was determined by the log-rank (Mantel-Cox) test. C57BL/6 mice ( $n = 5$ /group) were injected with  $7 \times 10^4$  TC-1 cells and treated as for Fig. 4, and tumor-infiltrating hematopoietic cells (CD45<sup>+</sup>; **D**), T cells (CD45<sup>+</sup>CD3<sup>+</sup>; **E**), CD8 T cells (**F**), and antigen-specific CD8 T cells (**G**), CD4<sup>+</sup>Foxp3<sup>-</sup> (**H**), CD4<sup>+</sup>Foxp3<sup>+</sup> (**I**), ratio of CD8/Tregs (**J**), and ratio of antigen-specific CD8/Tregs (**K**) were analyzed in tumor homogenates by flow cytometry as described in Materials and Methods. The absolute numbers of tumor-infiltrating cells were standardized per  $10^6$  of total live gated cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$ .

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data demonstrate that the combinational treatment of vaccine with Cal-101 is a therapeutically potent strategy.

To understand the immune mechanisms leading to the therapeutic efficacy observed with the combination of CAL-101 and vaccine treatment, we next profiled the tumor-infiltrating immune cells. Tumor cells were implanted on day 0, and treatment was initiated when tumor reached 3 to 5 mm in diameter. Tumor-bearing mice received CAL-101 every third day and were treated with two doses of an E7 peptide vaccine (Fig. 6A). Mice were sacrificed and tumors were harvested for evaluation of tumor-infiltrating T-cell 3 days after the second vaccination. There were no significant differences in tumor volume between different groups at this stage. Administration of Cal-101, vaccine, and their combination significantly enhanced the numbers of tumor-infiltrating immune cells (CD45<sup>+</sup> cells; Fig. 6D). The infiltration of CD3<sup>+</sup> T cells was significantly higher in the groups that received the vaccine treatment (vaccine alone or in combination; Fig. 6E). A similar effect was observed on tumor-infiltrating CD8<sup>+</sup> T cells, where treatment with the vaccine alone or with its combination with Cal-101 significantly increased the total number of tumor-infiltrating CD8 T cells (Fig. 6F) and more specifically, the antigen (E7)-specific CD8 T cells (Fig. 6G).

Because the vaccine-alone treatment and its combination with CAL-101 have similar effect on tumor infiltration of total and antigen-specific CD8T-cells, we explored the possible immune mechanisms that lead to the superior therapeutic efficacy of the combination treatment. We profiled the tumor-infiltrating CD4<sup>+</sup> T cells (both Tconvs and Tregs). While neither the vaccine alone nor its combination with CAL-101 affected the numbers of tumor-infiltrating Tconvs (Fig. 6H), a significant decrease in Tregs was observed with CAL-101 and its combination with vaccine (Fig. 6I).

In addition, as a result of the increase in CD8 T-cell and E7-specific CD8 T cells, the combination of CAL-101 and vaccine treatment showed a significant increase in CD8/Treg ratio (Fig. 6J) and E7-specific CD8/Treg (Fig. 6K) and granzyme-positive CD8 cells when compared with control groups.

These data demonstrate that targeting Tregs using Cal-101 enhances the antitumor therapeutic efficacy of vaccine treatments and exerts effective antitumor immune response through an increase of tumor-infiltrating granzyme-positive CD8 T cells, suggesting that this effect is predominantly facilitated by addition of Cal-101 to vaccine treatment.

Although the number of tumor-infiltrating CD8 T cells was significantly higher in all the groups that received the vaccine treatment, the therapeutic efficacy was only observed in combination with the PI3K $\delta$  inhibitor as a direct result of its inhibition of Tregs. This resulted in a significant reduction of the suppressive effect exerted by Tregs, enabling the CD8 T cells to effectively target the tumor cells.

## Discussion

Here, we have provided evidence that Tregs and Tconvs are differentially regulated by Class IA PI3K isoforms. We show that selective inhibition of PI3K $\alpha$  or PI3K $\beta$  isoforms does not impair downstream signaling, proliferation, or survival in either Tregs or Tconvs.

In contrast, inhibition of PI3K $\delta$  selectively affects downstream signaling of TCR in Tregs but not Tconvs, leading to inhibition of phosphorylation of Akt and S6 and abrogating their downstream biological effects on proliferation and survival. This indicates that

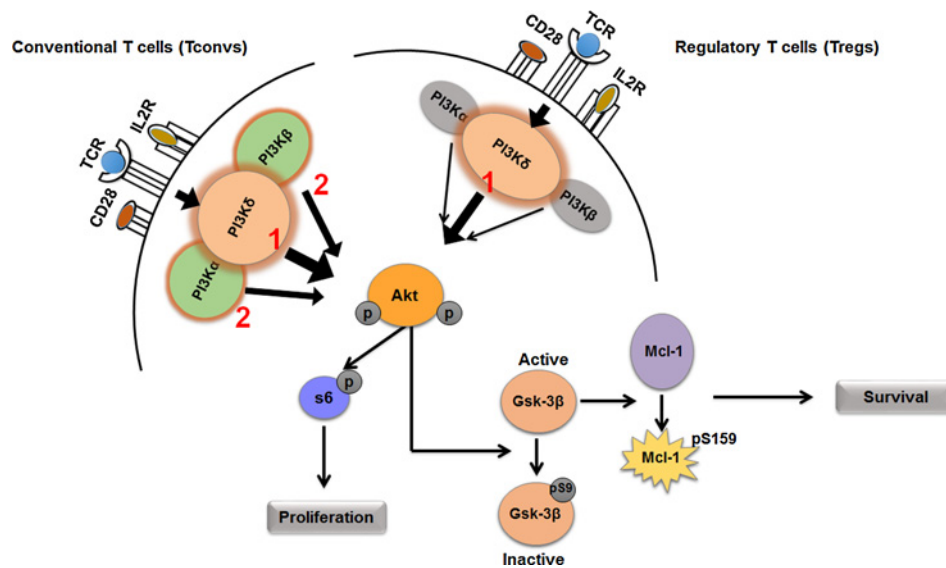
Treg TCR downstream signaling, proliferation, and survival are dominantly dependent on PI3K $\delta$ .

On the other hand, we show that none of the PI3K isoforms are dominant in Tconv, where the inhibition of any single isoform in Tconvs does not affect downstream pathway activation, proliferation, or survival. However, Tconvs are significantly suppressed with the simultaneous inhibition of PI3K $\delta$  along with either PI3K $\alpha$  or PI3K $\beta$ . No such effect was observed after the combined PI3K $\alpha$  and PI3K $\beta$  inhibition. These data suggest that, similar to Tregs, PI3K $\delta$  is sufficient for activation, proliferation, and survival of Tconvs, but in contrast to Tregs, PI3K $\alpha$  and PI3K $\beta$  can compensate for the absence of PI3K $\delta$  in Tconvs. Figure 7 illustrates the model of PI3K regulation of TCR signaling and downstream biologic outcome in Tregs and Tconv.

Naïve CD4 T cells have a central role in adaptive immunity, because they provide essential help for both cytotoxic T-cell- and antibody-mediated responses. The naïve CD4<sup>+</sup> T cells can differentiate into several lineages with distinct effector functions. We speculate that the compensatory mechanism of Class IA PI3K isoform could be required for naïve CD4 T cells to differentiate into different effector T cells.

We further dissected the downstream effect of these PI3K isoforms on the molecular mediators of Treg and Tconv cell survival pathways. T-cell survival is controlled by the antiapoptotic Mcl-1 protein, which is regulated by GSK-3 $\beta$ . PI3K-Akt signaling is known to control the phosphorylation and inactivation of GSK-3 $\beta$  (Fig. 5). Here, we show that Mcl-1 stability is differentially regulated by Class IA PI3K isoforms in Tregs and Tconvs, namely through the regulation of GSK-3 $\beta$ -mediated degradation of Mcl-1, reflecting the dichotomous control of these isoforms to survival. We found that inhibiting PI3K $\delta$  is sufficient to lead to GSK-3 $\beta$  activation, resulting in the subsequent degradation of Mcl-1 in Tregs, but not the inhibition of either PI3K $\alpha$  or PI3K $\beta$ . We further show that the inhibition of a single Class IA PI3K isoform in Tconvs does not affect GSK-3 $\beta$ , Mcl-1, and thus their survival. However, downstream survival signaling in Tconvs, GSK-3 $\beta$  phosphorylation, and Mcl-1 degradation are significantly inhibited with the pan PI3K inhibition or simultaneous inhibition of PI3K $\delta$  along with either PI3K $\alpha$  or PI3K $\beta$ . No such effect was observed after the combined PI3K $\alpha$  and PI3K $\beta$  inhibition. These data are consistent with the effect of PI3K isoforms on the survival of Tconv and Tregs (Fig. 7).

We further demonstrate that the *in vitro* understanding of the PI3K isoforms on the Tregs and Tconv cells also reflected *in vivo*. Animal treated with PI3K $\delta$  isoform inhibitors showed significant decrease in Treg numbers and proliferation *in vivo*, but not when treated with PI3K $\alpha$  or PI3K $\beta$ . Furthermore, PI3K $\delta$  inhibitors did not affect CD4<sup>+</sup>Foxp3<sup>-</sup> or CD8 T cells. Importantly, we also showed that these findings translated into therapeutic efficacy. We found that inhibiting PI3K $\delta$  with CAL101 synergistically enhanced the tumor suppression and survival effect of antigen-specific vaccine. While evaluating immunologic mechanisms responsible for this potent therapeutic outcome, we found that CAL-101 resulted in decreasing Tregs tumor infiltration resulting in significant increase of tumor-infiltrating antigen-specific CD8 T cells. Based on above, here we show the molecular reasons that Tregs but not Tconv are depended on PI3K $\delta$  and that such findings can have a major translational and clinical therapeutic impact. Accordingly, the differential



**Figure 7.**

A model for the differential role of Class 1A PI3K isoforms in regulating the TCR signaling, proliferation, and survival of Tregs and Tconvs. PI3K $\delta$  isoform is sufficient for TCR downstream signaling, proliferation, and survival for either Tconvs or Tregs. In Tregs, however, PI3K $\delta$  is a dominant isoform, where Tregs are fully dependent on PI3K $\delta$  to regulate these properties as PI3K $\alpha$  and PI3K $\beta$  do not play any role in these biological processes. On the other hand, in Tconvs, the two other isoforms, PI3K $\alpha$  and PI3K $\beta$  combined, provide redundant pathway to PI3K $\delta$  in the regulation of TCR signaling, proliferation, and survival. This dichotomy applies to downstream TCR signaling regulating pS6 phosphorylation controlling proliferation and applies to the phosphorylation of GSK-3 $\beta$  controlling downstream degradation of the antiapoptotic protein Mcl-1 and downstream survival pathway.

regulation of the PI3K isoforms between Tregs and Tconvs presents a powerful approach to selectively mitigate Tregs and modulate CD4 T cells. Targeting PI3K $\delta$  can preferentially inhibit proliferation and induce cell death of Tregs while having no effects on Tconvs. Therefore, targeting PI3K $\delta$  can be used as an immune-modulating mechanism in a variety of clinical settings to enhance anticancer immune therapy.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Study supervision:** S.N. Khleif

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