Differential PI3Kδ signaling in CD4+ 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+ T cell subsets (Treg and Tconv). In this study we show how a functional dichotomy in Class IA PI3K isoforms in these two subsets of CD4+ T cells be exploited to target Treg while leaving Tconv intact. Studies employing isoform-specific PI3K inhibitors and a PI3Kδ-deficient mouse strain revealed that PI3Kα and PI3Kβ were functionally redundant with PI3Kδ in Tconv. Conversely, PI3Kδ was functionally critical in Treg, acting there to control TCR signaling, cell proliferation and survival. Notably, in a murine model of lung cancer, co-administration of a PI3Kδ-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 anti-tumor efficacy. Overall, our results offer a mechanistic rationale to employ PI3Kδ inhibitors to selectively target Treg and improve cancer immunotherapy.
**Introduction:**

Decreasing the numbers and/or function of regulatory T-cells (Tregs) 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 [5].

Understanding the signaling pathways regulating conventional T-cells (Tconvs) and Tregs activation, function and survival can help to design drugs that could selectively modulates 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 [6-11]. Furthermore, it was recently reported that inhibition of the PI3Kδ isoform results in a decrease in Tregs number and delayed tumor growth in animal models [12]. 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 cell regulation. The Class IA PI3K family consists of a heterodimeric complex of 110-kD catalytic subunits, p110α, β, or δ, with a regulatory subunit (p85α, p55α, p50α, p85β, or p55γ) [7, 13, 14]. The PI3K 110α and β subunits are ubiquitously expressed while p110δ expression is restricted to hematopoietic cells [7]. 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 anti-apoptotic 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 anti-apoptotic member of
the Bcl-2 family [2], is critical for survival of Treg cells, 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 (GSK3α/GSK3β) 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]. GSK3β 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-3β that inhibits its activity [5, 6]. 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 PI3Kδ isoform, in Tconvs PI3Kδ is necessary, and in contrast to the situation in Tregs, PI3Kα and β provide a redundant pathway to PI3Kδ, ensuring the presence of alternative pathways to provide a robust effector function when 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 PI3Kδ 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.
Material and Methods:

**Animals:** C57BL/6 female 6-8-week-old mice were purchased from Jackson Laboratory (Bar Harbor, ME). P110delta-PI3K-D910A (kinase-dead) PI3Kδ KO mice was purchased from Charles River, Wilmington, MA 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 (San Jose, CA).

**PI3K inhibitors:** A66 (PI3Kα inhibitor; IC₅₀ of 32nM) [15, 16], TGX-221 (PI3Kβ inhibitor; IC₅₀ of 5nM) [16, 17], and CAL-101 (PI3Kδ inhibitor; IC₅₀ of 2.5nM)[18], GDC-0941 (PAN PI3K inhibitor) [19] were purchased from Selleckchem, (Houston, TX) The IC₅₀ for each isoform is listed in **(Supplementary Table 1)**.

**Western Blotting (WB):**

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 (1:1000 dilutions) overnight at 4°C and incubated with secondary antibodies (1:5000 dilution) for one hour at room temperature. Chemiluminescence was performed with Pierce reagents (Rockford, IL).
T-cell Stimulation Flow cytometry analysis and in vitro CellTrace™ Viole T-cell Proliferation (VCT-Viole T-cell Trace) assay:

Sorted Tregs and Tconvs were labeled with CellTrace™ Viole T-cell Proliferation stain (VCT) according to the manufacturer’s protocol (Life Technologies, NY). Cells were stimulated with and without inhibitors in the presence of 10μg/mL plate-bound anti-CD3, 2.5μg/mL soluble anti-CD28, and 100 IU/mL IL-2. For negative control (non-stimulated), cells were cultured in 100 IU/mL IL-2. After 3 days, cells were stained with fixable live/dead cell stain (Life Technologies, NY), fixed and permeabilized using the mouse Foxp3 buffer kit according to the manufacturer’s instructions (BD Bioscience, San Jose, CA) 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 Prof. T.C. Wu, Johns Hopkins University, Department of Pathology, and Baltimore, MD [20]. 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 manuscript [21]. 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 subcutaneously (s.c.) with 70,000 TC-1 tumor cells and monitored for development of tumors. On day 10-12, when tumor size reached 3-5mm in diameter, mice were treated with a single intraperitoneal (i.p.) injection of A66, TGX-221, or CAL-101 at 2 or 10mg/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 day 10-12, when tumor size reached 3-5mm in diameter, spleens and tumors from the pre-treatment group of animals were used to examine the T-cell population before starting the treatment (baseline). Animals were treated with either CAL-101 (10mg/kg) or 10% DMSO in water (vehicle) starting on the 12th day following TC-1 implantation for the next 14 days with three-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 day 10-12 after tumor implantation when tumor diameter reached 3-5 mm. For therapeutic experiments vaccine was given weekly throughout the experiment. CAL-101 treatment was provided on the day when tumor size reached 3-4mm 5-6 day 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 was monitored. Tumors were measured every 3–4 days using digital calipers, and tumor volume was calculated using the formula \( V = \frac{W^2L}{2} \), 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\(^3\). For immunology experiments mice were treated similarly and were sacrificed three 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 regulate TCR signaling and proliferation of regulatory T-cells and Conventional T-cells

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 to Tconv. This selective decrease in Treg proliferation provided us with a potential strategy to modulate the Treg/Tconv balance in vivo[10]. 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, p85α, p110α, p110β, or p110δ, between Tregs and Tconvs (Supplementary Figure 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 PI3Kα (A66), PI3Kβ (TGX-221), or PI3Kδ (CAL101). The phosphorylation status of the downstream Akt was measured by flow cytometry (Supplementary Figure S2A and S2B). The range of used concentrations was selected based on the previously published IC$_{50}$ of these inhibitors [15, 16, 18] (Supplementary Table 1). 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 (Figure 1A, 1B and Supplementary Figure S3A, Supplementary S3B). In contrast, inhibition of the PI3Kδ 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 Figure 1C and Supplementary Figure 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 (Figure 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 PI3Kα or PI3Kβ has no effect on either Tregs or Tconvs proliferation as shown in Supplementary figure Figure S5 A & B, respectively. On the other hand, PI3Kδ inhibition led to a significant abrogation of Tregs proliferation in a concentration-dependent manner, without affecting Tconvs proliferation (Figure 1E). The findings were further confirmed using Tregs and Tconvs from mice with PI3Kδ inactive kinase (PI3Kδ KO). As expected, we found that the level of phospho-Akt (S473) in Tregs obtained from PI3Kδ KO mice (Figure 1F) and their proliferation ability under TCR/IL-2 stimulation (Figure 1G) were significantly lower compared to Tregs from WT. Furthermore, there was no significant differences in Tconvs obtained from the two mice strains. These data demonstrate that TCR signaling in Tregs is exclusively dependent on PI3Kδ but not on PI3Kα or PI3Kβ, while in Tconvs, no specific isoform was found to be dominant in TCR downstream signaling.

To further validate these finding for therapeutic approached in immunotherapy, we tested the effect of PI3K isoforms inhibitors on human CD4 T-cell. Human Tregs and Tconvs were fractionated from PBMCs by fluorescence-activated cell sorting. Tregs were sorted based on expression of CD4^+CD25^{HI}, whereas Tconvs were identified as CD4^+CD25^{-}. We first tested the
effect of PI3Kδ isoform inhibitors on the level of phosphorylated Akt (pAkt-S473) in Tregs and Tconvs and on the proliferation of these two cell types. We tested the effect of the PI3Kδ 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 PI3Kδ inhibition led to a significant decreased level of pAkt in Tregs but not Tconvs and significant abrogation of Tregs proliferation, without affecting Tconvs, in a concentration-dependent manner (Figure 1H and 1I). Based on the reproducibility of the data in human, we believe that using specific PI3Kδ inhibitor would be a reasonable approach to enhance cancer immunotherapy.

**PI3K isoform differentially regulate the frequency of T-cell subsets in tumor bearing mice**

After demonstrating that the inhibition of PI3Kδ isoform but not PI3Kα or β 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 [20] syngeneic mouse model which is a Treg-dependent tumor model [22], and the frequency of Tregs increases significantly in tumor bearing mice compared to non-tumor mice (Figure 2B). In addition, TC-1 expresses PI3Kα and PI3Kβ, but do not express detectable levels of p110δ (Figure 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 (Figure 2A). Mice were sacrificed 3 or 6 days after treatment and the level of splenic Tregs was assessed. We found that inhibition of PI3Kδ with CAL-101 led to a dose dependent inhibition of Tregs in the spleen with significant reduction at 10mg/Kg on day 3 after treatment (Figure 2D). In contrast, inhibition of PI3Kα or β had no significant effects on splenic Tregs with either a 2mg/kg or 10 mg/kg dose on day 3.
(Figure 2D) and day 6 (Figure 2E) after treatment. Considering the short half-life of CAL101 (~1 hour [23]), not surprisingly, the level of Tregs returned to control levels on day 6 after treatment (Figure 2E). Importantly, we found that inhibition of PI3Kα, β or δ with either a 2mg/kg or 10mg/kg dose of inhibitors had no significant effects on splenic CD4+, CD4+Foxp3− or CD8+ cells (Figure 2F, 2G and 2H) on day 3 after treatment. The level of CD4+ and CD8+ T-cells was also not affected on day 6 by any of the PI3K inhibitors at either dose (data not shown). This indicates that PI3Kδ, but not PI3Kα or PI3Kβ inhibition reduces the number of Tregs and spares other T-cell subsets. Consistent with in vitro findings, we found that, in vivo Tregs is dependent on PI3Kδ exclusively but not on PI3Kα or PI3Kβ, while in Tcons, no specific isoform was found to be functionally dominant. We next tested the impact of continuous treatment of CAL-101 (10mg/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 3-5mm 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 total of five doses (Supplementary Figure S6A). Animals were sacrificed on day 26 after tumor implantation. As expected we observed that there was an increase in Tregs population in vehicle treated animals on day 26 compared to base-line (Supplementary Figure S6B). However, the continuous administration of CAL-101 at three day intervals significantly decreased the percentage of Tregs in CAL-101 treated mice compared to vehicle treated animals (Supplementary Figure S6B). We also evaluated the effects of CAL-101 on effector T-cell subsets including CD4+ and CD8+ cells. We did not find any effects of CAL101 on percentage of CD4+Foxp3− (Supplementary Figure S6C) and CD3+CD8+ (Supplementary Figure 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 Treg cells 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 Treg cells 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. Treg cells from CAL-101 treated animals showed a decreased proliferative response as assessed by Ki67 staining (Supplementary Figure S6E). The proliferation potential of effector T-cells (CD4+ and CD8+) from CAL-101 treated animals did not show any impaired proliferative response (Supplementary Figure S6F & S6G). This suggests that the selective reduction of Treg cells in CAL-101 treated animals could be a consequence of impaired survival signals as well as inhibition of proliferation.

**PI3Kα and PI3Kβ provides a redundant pathway to PI3Kδ in Tconvs for TCR signaling and proliferation**

Since our data demonstrate that downstream TCR signaling and proliferation of Tregs are dependent on PI3Kδ, 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 Figure 3A. The effect was also reflected downstream of the PI3K-Akt pathway, where the phosphorylation status of ribosomal protein S6
(Figure 3B) and proliferation (Figure 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 2 isoforms at a time: PI3Kα and PI3Kβ (A66+TGX-221), PI3Kα and PI3Kδ (A66+CAL101), and PI3Kβ and PI3Kδ (TGX-221+CAL101). We found that combining inhibition of both PI3Kα and PI3Kβ did not affect the phosphorylation of Akt and S6 (Figure 3D & E). As expected, there was no effect on proliferation of Tconvs either (Figure 3F). These findings show that PI3Kδ is sufficient for TCR signaling and proliferation of Tconvs. On the other hand, combining PI3Kδ inhibitor with either PI3Kα or PI3Kβ inhibitor significantly reduced the levels of pAkt and pS6 (Figure 3D & E) and inhibited proliferation of Tconvs (Figure 3F). We further confirmed our data using T-cells lacking PI3Kδ obtained from PI3Kδ KO mice. As expected, we found that while proliferation of Tconvs from WT mice were not affected in presence of PI3Kα or PI3Kβ inhibitors (Figure 1E and 1F), proliferation of Tconvs from PI3Kδ KO mice was significantly inhibited in presence of either of these inhibitors (Figure 3G). Further, targeting both PI3Kα and PI3Kβ together, using A66 and TGX-221, did not inhibit the proliferation of Tconvs from WT mice. However, combined inhibition of PI3Kα and PI3Kβ (A66+TGX-221) significantly mitigated the proliferation of Tconvs from PI3Kδ KO mice (Figure 3G).

Together, these findings show that PI3Kδ is sufficient for TCR signaling and proliferation of Tconvs. However, in contrast to Tregs, in Tconvs, PI3Kα and PI3Kβ combined can compensate for the absence of PI3Kδ.
Class IA PI3K isoform differentially regulate the survival of Tregs through GSK-3β and Mcl-1 signaling pathway.

Differential role of Class IA PI3K for regulation of TCR survival signaling in conventional CD4+Foxp3- cells and regulatory CD4+Foxp3+ cells has not been explored. Since, the inhibition of individual PI3K isoforms does not affect Tconvs, and inhibition of only PI3Kδ 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 72hrs and the viability of Tregs and Tconvs was analyzed after treatments with different PI3K inhibitors using fixable live/dead cell stain (Life Technologies, NY). Tregs and Tconvs showed no sign of reduced survival when PI3Kα or PI3Kβ was inhibited (Figure 4A & 4B), however, in contrast to Tconvs, the inhibition of PI3Kδ with CAL-101 significantly decreased the survival of Tregs (Figure 4C). Interestingly, the survival of Tconvs was not effected in the presence of either of PI3K isoform specific inhibitor. These results indicated that Tregs survival is dependent on PI3Kδ and accordingly sensitive to its inhibition. To further confirm this we tested whether the inhibition of PI3Kδ 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 figure S7A). To understand the molecular mechanism by which PI3Kδ inhibition selectively induces apoptosis leading to reduced survival of Tregs, we next evaluated the effect of specific PI3K
isoform inhibition on the anti-apoptotic protein myeloid cell leukemia 1 (Mcl-1), which is critical for the survival of T-cells including Tregs [24] and is rapidly degraded when cells undergo apoptosis in response to various stimuli [25-28]. Mcl-1 can sequester direct activator BH3-only proteins, such as Bim, Bad and Puma and prevent them from activating Bak. In absence of Mcl-1, Bak forms pore in the outer mitochondrial membrane (OMM) to release cytochrome c, activate caspases and induce apoptosis (Figure 4H).

Accordingly, we tested the expression of Mcl-1 in Tregs and Tconvs treated with specific PI3K isoform inhibitors. We found that inhibition of PI3Kα or PI3Kβ does not affect the expression of Mcl-1 levels in Tregs or Tconvs (Figure 4D). Conversely, the inhibition of PI3Kδ by CAL-101 remarkably decreases the expression of Mcl-1 in Tregs but has no effect on Tconvs (Figure 4E). These observations further indicate that PI3Kδ selectively regulates the survival of Tregs through Mcl-1 pathway. Since, the survival of Tconvs were 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 anti-apoptotic protein Mcl-1 is controlled by glycogen synthase kinase 3 (GSK-3) [28] and the expression of constitutively active GSK-3 decreases Mcl-1 expression [28]. GSK3β is a downstream target of PI3k-Akt signaling pathway and usually remain active in cells, however PI3K induced activation of Akt results in the phosphorylation (S9) of GSK-3β that inhibits its activity [29, 30] as shown in Figure 4A. To further dissect the mechanism, we checked the effect of PI3K inhibitors on the phosphorylation levels of GSK-3β. Treatment of cells with titrated concentrations of PI3Kα inhibitor (A66) or PI3Kβ inhibitor (TGX-221) did not block the S9 phosphorylation of GSK-3β (Figure 4F). However, when T-cells were treated with PI3Kδ inhibitor the S9-phosphorylation of GSK-3β was blocked only in Tregs (Figure 4G), leading to activation of GSK-3β, which correlates with
the decreased Mcl-1 expression (Figure 4E) and survival (Figure 4C) in Tregs. These results demonstrate that contrary to Tconv, PI3Kδ is crucial for the regulation of GSK3β and Mcl-1 dependent survival of Tregs. On the other hand, PI3Kα and PI3Kβ as single isoforms are not crucial in the regulation of either Tconv or Tregs.

**PI3Kδ is required for the GSK-3β and Mcl-1 dependent survival pathway in Tconvs, and is compensated by PI3Kα and PI3Kβ**

We have shown that inhibition of PI3Kδ selectively activates GSK-3β in Tregs, which favors the degradation of anti-apoptotic 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, survival pathway of Tconvs is dependent on PAN class IA PI3K isoforms (Figure 5A) inhibition of PAN PI3K activate GSK-3β in Tconvs which favors the degradation of anti-apoptotic protein Mcl-1 leading to the decreased survival of Tconvs (Figure 5B) and induced apoptosis (Supplementary figure S7C). We next investigated whether inhibition of any 2 PI3K isoforms affect survival of Tconvs. We found that combining inhibition of both PI3Kα and PI3Kβ did not induce apoptosis and did not affect the survival (Figure 5C and supplementary figure S7B). As expected, there was no effect on Mcl-1 and pGSK-3β (Figure 5D), which further provide evidences that, in 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 (Figure 5C), induces apoptosis (supplementary figure S7C), and decreased Mcl-1 protein with inhibited pGSK-3β of Tconvs (Figure 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 (Figure 5E).

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

We evaluated the anti-tumor 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 3-5mm in diameter. Tumor bearing mice received CAL-101 every third day and were treated with two doses of a peptide vaccine (Figure 6A). Tumor growth and survival were monitored. We found that CAL-101 in combination with vaccine lead to significant slowdown of tumor progression (Figure 6B) associated with prolonged survival (Figure 6C). These 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 3-5mm in diameter. Tumor bearing mice received CAL-101 every third day and were treated with two dose of an E7 peptide vaccine (Figure 6A). Mice were sacrificed and tumors were harvested for evaluation of tumor-infiltrating T-cell three 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+ cells) (Figure 6D). The infiltration of CD3+ T-cells was significantly
higher in the groups that received the vaccine treatment (vaccine alone or in combination) (Figure 6E). A similar effect was observed on tumor-infiltrating CD8+ 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 (Figure 6F) and more specifically, the antigen (E7) specific CD8 T-cells (Figure 6G).

Since 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 leading to the superior therapeutic efficacy of the combination treatment. We profiled the tumor-infiltrating CD4+ T-cells (both Tconvs and Tregs). While neither the vaccine alone nor its combination with CAL-101 affected the numbers of tumor-infiltrating Tconvs (Figure 6H), a significant decrease in Tregs was observed with CAL-101 and its combination with vaccine (Figure 6I).

Additionally, 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/Tregs ratio (Figure 6J) and E7 specific CD8/Tregs (Figure 6K) and Granzyme positive CD8 cells when compared to control groups.

These data demonstrate that targeting Tregs using Cal-101 enhances the antitumor therapeutic efficacy of vaccine treatments and exert effective anti-tumor 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 PI3Kδ 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 PI3Kα or PI3Kβ isoforms does not impair downstream signaling, proliferation or survival in either Tregs or Tconvs.

In contrast, inhibition of PI3Kδ 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 PI3Kδ.

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 PI3Kδ along with either PI3Kα or PI3Kβ. No such effect was observed after the combined PI3Kα and PI3Kβ inhibition. These data suggest that, similar to Tregs, PI3Kδ is sufficient for activation, proliferation and survival of Tconvs, but in contrast to Tregs, PI3Kα and PI3Kβ can compensate for the absence of PI3Kδ 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, since they provide essential help for both cytotoxic T-cell- and antibody-mediated responses. The naïve CD4+ 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 anti-apoptotic Mcl-1 protein, which is regulated by GSK3β. PI3K-Akt signaling is known to control the phosphorylation and inactivation of GSK3β [Figure 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-3β-mediated degradation of Mcl-1, reflecting the dichotomous control of these isoforms to survival. We found that inhibiting PI3Kδ is sufficient to lead to GSK-3β activation resulting in the subsequent degradation of Mcl-1 in Tregs, but not the inhibition of either PI3Kα or PI3Kβ. We further show that the inhibition of a single class IA PI3K isoform in Tconvs does not affect GSK3β, Mcl-1 and thus their survival. However, downstream survival signaling in Tconvs, GSK-3β phosphorylation and Mcl-1 degradation are significantly inhibited with the PAN PI3K inhibition or simultaneous inhibition of PI3Kδ along with either PI3Kα or PI3Kβ. No such effect was observed after the combined PI3Kα and PI3Kβ inhibition. These data are consistent with the effect of PI3K isoforms on the survival of Tconv and Tregs (figure 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 PI3Kδ isoform inhibitors showed significant decrease in Treg numbers and proliferation in vivo, but not when treated with PI3Kα or PI3Kβ. Furthermore, PI3Kδ inhibitors did not affect neither CD4+Foxp3- nor CD8 T-cells. Importantly, we also showed that these finding translated into therapeutic efficacy. We found
that inhibiting PI3Kδ 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 PI3Kδ and that such findings can have a major translational and clinical therapeutic impact. Accordingly, The differential regulation of the PI3K isoforms between Tregs and Tconvs presents a powerful approach to selectively mitigate Tregs and modulate CD4 T-cells. Targeting PI3Kδ can preferentially inhibit proliferation and induce cell death of Tregs while having no effects on Tconvs. Therefore, targeting PI3Kδ can be used as an immune-modulating mechanism in a variety of clinical settings to enhance anti-cancer immune therapy.

References


Figure Legends:

Figure 1. Regulatory T-cells are more sensitive to PI3Kδ inhibition in vitro. FACS-sorted Tregs and Tconvs from WT B16 (foxp3-GFP) mice (A-E), WT B16 and PI3Kδ KO mice (F & G) and Human PBMC (H&I) were plated on anti-CD3-coated plates and cultured in activation media (IL2 and anti-CD28) without inhibitors (DMSO, 0nM) and with inhibitors for 72 hrs. For negative control (Non-stimulated-NS) cells were left in media containing IL-2 for 72hrs. Intracellular phosphorylation of (A-C) Akt (S473) (D) S6 (S244) and (E) proliferation in live gated cells was measured by flow cytometry of Tregs and Tconvs treated with A66 (PI3Kα inhibitor), TGX-221 (PI3Kβ inhibitor), and CAL-101 (PI3Kδ 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 (F & G) WT B16 and PI3Kδ KO mice, (H & I) human Tregs and Tconvs treated with CAL-101 (PI3Kδ inhibitor) respectively.
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 2mg/kg or 10mg/kg CAL-101, A66, or TGX-221 dissolved in 10% of DMSO. (B) Percentage of Tregs in tumor bearing mice on day 10 compared to non-tumor bearing mice (C) Protein lysate 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 (D)Tregs (CD4⁺ Foxp3⁺) on day 3 of treatment (E) Tregs (CD4⁺ Foxp3⁺) on day 6 of treatment (F) CD4⁺ cells on day 3 of treatment and (G) CD4⁺ Foxp3⁻ cells on day 3 of treatment and (H) CD8⁺ cells on day 3 of treatment were analyzed by flow cytometry. The percentage of cells was normalized and presented as mean ± SD. Representative data from 2 independent experiments are shown (3-5 mice for each group per experiment). Statistical significance was determined by one-way ANOVA with Tukey’s multiple comparison test (*, p <0.05; **, p <0.01; ***, p < 0.001).

Figure 3. Class IA PI3K isoforms have redundant functions in TCR-mediated activation and proliferation in conventional T-cells. (A-F) FACS sorted Tconvs (CD4⁺ Foxp3⁻) (treated with (A-C) GDC-0941 (PAN PI3K inhibitor) or (D-F) combinations of inhibitors including A66 PI3Kα inhibitor + TGX-221 PI3Kβ inhibitor; A66 PI3Kα inhibitor + CAL-101 PI3Kδ inhibitor; and TGX-221 PI3Kβ inhibitor+ CAL-101 PI3Kδ inhibitor for 72 hours were harvested and (A & D) pAkt (S473), (B & E) pS6 (S244) and (C & F) proliferation were measured on live gated cells using flow cytometry and normalized for three independent experiments. (G) FACS sorted Tconvs from PI3Kδ KO mice were treated with A66 PI3Kα inhibitor, TGX-221 PI3Kβ
inhibitor, A66 PI3Kα inhibitor + TGX-221 PI3Kβ inhibitor and GDC-0941 (PAN PI3K inhibitor) and proliferation were 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 (A) A66 (a PI3Kα inhibitor), (B) TGX-221 (a PI3Kβ inhibitor), and (C) CAL-101 (a PI3Kδ inhibitor). The results normalized for three independent experiments. (D-G) Cell lysates prepared on day 3 (72hrs) 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.

**Figure 5. Class IA PI3K isoforms compensate for GSK-3β and Mcl-1 dependent survival of Tconvs.**

Cells were washed and stained for Live/Dead and fixed. Viability was measured by flow cytometry of Tconvs treated with (A) GDC-0941 (PAN PI3K inhibitor) or (C) 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. (B & D) In a separate experiment T-cell lysates prepared on day 3 (72hrs) 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.

Figure 6. Combination of vaccine with CAL-101 provides potent anti-tumor therapeutic efficacy. A. C57BL/6 mice (n=5/group) were injected s.c. in the right flank with 7x10⁴ 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 Log-rank (Mantel-Cox) test.

C57BL/6 mice (n=5/group) were injected with 7x10⁴ TC-1 cells and treated as for Figure 4, and tumor-infiltrating (D) Hematopoietic cells (CD45+) (E) T-cells (CD45+CD3+) (F) CD8 T-cells and (G) antigen specific CD8 T-cells (H) CD4+Foxp3- (I) CD4+Foxp3+ (J) Ratio of CD8/Tregs (K) Ratio of antigen specific CD8/Tregs were analyzed in tumor homogenates by flow cytometry as described in Methods section. The absolute numbers of tumor-infiltrating cells were standardized per 10⁶ of total live gated cells. *p<0.05, **p<0.01, ***p<0.001.
Figure 7. A Model for the differential role of class 1A PI3K isoforms in modulating regulating the TCR signaling, proliferation and Survival of Tregs and Tconvs. PI3Kδ isoform is sufficient for TCR downstream signaling, proliferation and survival for either Tconvs or Tregs. In Tregs, however, PI3Kδ is a dominant isoform, where Tregs are fully dependent on PI3Kδ to regulate these properties as PI3Kα and PI3Kβ do not play any role in these biologic processes. On the other hand, in Tconvs, the two other isoforms, PI3Kα and PI3Kβ combined, provide redundant pathway to PI3Kδ 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 GSK3β controlling downstream degradation of the anti apoptotic protein Mcl-1 and downstream survival pathway.
Figure 3.

**A.** GDC-0941 (PAN PI3K inhibitor)

![Graph showing Akt phosphorylation](image)

**B.** GDC-0941 (PAN PI3K inhibitor)

![Graph showing pS6 phosphorylation](image)

**C.** GDC-0941 (PAN PI3K inhibitor)

![Graph showing proliferation](image)

**D.**

- NS
- A66+TGX-221
- DMSO
- A66+CAL-101
- 0nM
- TGX-221+CAL-101

![Normalized MFI (pAkt-S473)](image)

**E.**

![Normalized MFI (pS6-S244)](image)

**F.**

![Normalized Proliferation %](image)

**G.**

WT vs PI3Kδ KO

<table>
<thead>
<tr>
<th>Condition</th>
<th>WT</th>
<th>PI3Kδ KO</th>
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<tbody>
<tr>
<td>IL2</td>
<td>0.30</td>
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<tr>
<td>DMSO</td>
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<td>A66</td>
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<tr>
<td>GDC</td>
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![Proliferation](image)
Figure 4.

A. CD4+Foxp3+ (Tregs) and CD4+Foxp3− (Tconvs) normalized survival.

B. Normalized survival of A66 and TGX-221.

C. Normalized survival of CAL-101.

D. Stimulated (IL2+α-CD28+α-CD3) A66 and TGX-221.

E. Stimulated (IL2+α-CD28+α-CD3) CAL-101.

F. Stimulated (IL2+α-CD28+α-CD3) A66 and TGX-221.

G. Stimulated (IL2+α-CD28+α-CD3) CAL-101.

H. Flowchart showing the regulation of cell proliferation and death.
Figure 5.

A. Normalized Survival (%) for different treatments:

- DMSO
- 0nM
- 100nM
- 1μM

B. Stimulated (IL2+α-CD28+α-CD3) GDC:

- DMSO
- 0nM
- 100nM
- 1μM

- McI-1
- actin
- pGSK3β (S9)
- actin

C.

- DMSO
- A66+TGX-221
- 0nM
- A66+CAL-101
- TGX-221+CAL-101

D.

- WT
- PI3Kδ KO

E. Stimulated (IL2+α-CD28+α-CD3)

- DMSO
- A66+TGX-221
- A66+CAL-101
- TGX-221+CAL-101

- McI-1
- actin
- pGSK3β (S9)
- actin
Figure 7.
Differential PI3Kδ signaling in CD4+ T cell subsets enables selective targeting of T regulatory cells to enhance cancer immunotherapy

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