Cancer Research

Enhanced Therapeutic Efficacy and Memory of Tumor-Specific CD8 T Cells by *Ex Vivo* PI3K-δ Inhibition

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Abstract

Inhibition of specific Akt isoforms in $CD8^+$ T cells promotes favored differentiation into memory versus effector cells, the former of which are superior in mediating antitumor immunity. In this study, we investigated the role of upstream PI3K isoforms in $CD8^+$ T-cell differentiation and assessed the potential use of PI3K isoform–specific inhibitors to favorably condition $CD8^+$ T cells for adoptive cell therapy. The phenotype and proliferative ability of tumor antigen–specific CD8+ T cells was assessed in the presence of PI3K-α, -β, or -δ inhibitors. Inhibition of PI3K-δ, but not PI3K-α or PI3K-β, delayed terminal differentiation of $CD8^+$ T cells and maintained the memory phenotype, thus enhancing their proliferative ability and survival while maintaining their cytokine and granzyme B production ability. This effect was preserved *in vivo* after *ex vivo* PI3K-δ inhibition in $CD8^+$ T cells destined for adoptive transfer, enhancing their survival and also the antitumor therapeutic activity of a tumor-specific peptide vaccine. Our results outline a mechanism by which inhibitions of a single PI3K isoform can enhance the proliferative potential, function, and survival of $CD8^+$ T cells, with potential clinical implications for adoptive cell transfer and vaccine-based immunotherapies. *Cancer Res; 77(15); 4135–45. ©2017 AACR.*

Introduction

$CD8^+$ T-cell response comprises effector and memory T cells (1, 2). Memory T cells possess enhanced proliferative ability, greater functionality, and better longevity than effector cells. Different subsets of CD8 memory T cells, including effector memory (T_{EM}) and central memory T cells (T_{CM}), represent different stages of the CD8 differentiation spectrum (2, 3). T_{CM} are an earlier stage of differentiation and therefore possess superior qualities, enabling them to better fight microbial challenges and mediate therapeutic antitumor immunity when compared with T_{EM}, which in turn are superior to the terminally differentiated effector cells (4–7).

The differentiation of T cell is under the control of the PI3K/Akt pathway (1). Akt activation was found to regulate the effector/memory $CD8^+$ T-cell differentiation (8). In fact, Akt inhibition was reported to augment antitumor immune responses by enhancing the expansion of $CD8^+$ T cells with memory characteristics (9). Also, we have reported that inhibition of specific Akt isoforms, Akt1 and Akt2, delays the terminal differentiation of $CD8^+$ T cells while enhancing the T_{CM} phenotype. Targeting these specific Akt isoforms therefore enhanced the proliferative ability, longevity, and cytokine production in $CD8^+$ T cells (10).

We therefore investigated the potential role of specific upstream PI3K isoforms in the regulation of the $CD8^+$ T-cell differentiation with the aim of exploring the potential use of specific PI3K isoforms inhibitors to condition $CD8^+$ T cells for adoptive cell transfer for better therapeutic outcome.

We found that the inhibition of only PI3K-δ, but not PI3K-α or PI3K-β, delays the terminal differentiation of $CD8^+$ T-cell and maintains the memory phenotype thus enhancing their proliferative ability and survival while maintaining their cytokine and granzyme B production ability. We further demonstrated the *ex vivo* PI3K-δ inhibition enhances antitumor therapeutic ability of adoptively transferred $CD8^+$ T cells in animal models compared with nontreated $CD8^+$ T cells.

In cancer immunotherapy, it is important to maintain a CD8 that is antigen specific, highly cytotoxic, renewable, highly proliferative, and in earlier stages of differentiation to delay exhaustion. Here, we report that *ex vivo* inhibition of a single isoform, PI3K-δ, in $CD8^+$ T cells enhances their proliferation, cytokine production, and subsequently their antitumor therapeutic ability and delays their exhaustion.

This discovery has important clinical implications. Recently, clinical trials using the PI3K-δ inhibitor idelalisib (Zydelig, CAL-101) to treat malignancies were put on hold (11) and new guidelines for its prescription were introduced. This was due to the increased number of deaths in the idelalisib (Zydelig, CAL-101) group, which was mainly attributed to infections by

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P jirovecii and cytomegalovirus, in addition to respiratory events possibly caused by infection (12). Our discovery provides a plausible explanation, where the use of PI3K-δ inhibitors delays the later stages of CD8 differentiation, which are thought to be the most potent against opportunistic viral infections. Hence, their systematic administration deprives the body of the most potent antiviral $CD8^+$ T cells.

Our findings suggest a strategy that enhances the antitumor therapeutic efficiency of adoptive cell transfer while avoiding the adverse effects of the systemic administration of PI3K-δ inhibitors. We show that *ex vivo* PI3K-δ inhibition delays terminal differentiation, maintains memory phenotypes, prolongs the life span, and enhances the expansion of tumor specific $CD8^+$ T cells without affecting their cytotoxic activity. This translates into an enhanced *in vivo* antitumor therapeutic ability and therefore holds great clinical implications for the use of these inhibitors as immune modulators in a safe and effective approach.

Materials and Methods

Mice and reagents

pMel-1 mice (B6.Cg-*Thy1*^{a}/Cy Tg(TcraTcrb)8Rest/J) used for *in vitro* experiments carry a rearranged T-cell receptor transgene (Vβ13) specific for gp100 (13). For feeder cells and *in vitro* experiments, female C57BL/6(H-2b) wild-type (WT) mice were used. For *in vivo* experiments, 4- to 6-week-old WT female mice were used. Mice were purchased from The Jackson Laboratory and housed under pathogen-free conditions. All procedures were carried out in accordance with Institutional Animal Care and Use Committee.

The B16 cell line was purchased from ATCC, which routinely authenticates and tests cell lines (for mycoplasma, by the Hoechst stain, PCR, and the standard culture test). These cells were used in experiments after two to three passages from thawing (between 2014 and 2015). B16 was authenticated and tested for mouse parvovirus (MPV) and mouse hepatitis virus (MHV) using PCR at Augusta University. All tests were negative.

Inhibitors were purchased from Selleckchem. GDC-0941 is a pan-PI3K inhibitor with IC_{50} of 3 nmol/L for p110α, 33 nmol/L for p110β, 3 nmol/L for p110δ, and 75 nmol/L for p110γ. GDC-0941 was used *in vitro* at 11, 33, 99, and 279 nmol/L concentrations, ensuring inhibition of all three class 1 isoforms. A66 is a selective p110α inhibitor with IC_{50} of 32 nmol/L for the p110α, 236 nmol/L for PI4Kβ, 462 nmol/L for C2β, and >1.25 μmol/L for p110δ. A66 was used *in vitro* at 32, 96, and 288 nmol/L concentrations, ensuring selectivity to PI3Kα. TGX-221 is a highly selective PI3Kβ inhibitor with IC_{50} of 5 μmol/L for p110α, 5 nmol/L for p100β, 0.1 μmol/L for p100δ, and > 10 μmol/L for p110γ. In *in vitro* experiments, TGX-221 was used at 5, 15, and 45 nmol/L to ensure selectivity. CAL-101 is a selective PI3Kδ inhibitor with IC_{50} of 820 nmol/L for p110α, 565 nmol/L for p110β, 2.5 nmol/L for p110δ, and 89 nmol/L for p110γ. This inhibitor was tested *in vitro* at 0.28, 0.83, 2.5, 7.5, 22.5, 67.5, and 202.5 nmol/L to maximize the drug's specificity.

The $gp100_{25–33}$ 9-mer peptide (KVPRNQDWL; ANASPEC) was used for *in vitro* activation of pMel-1 splenocytes at 1 μmol/L as described (10).

For *in vivo* experiments, the vaccine was prepared using the same $gp100_{25–33}$ peptide and administered at 100 μg per mouse in combination with PADRE at 10 μg and Quil-A at 25 μg per mouse.

Lymphodepletetion of mice was achieved using a combination of 250 mg/kg cyclophosphamide (Sigma) and 50 mg/kg fludarabine (Selleckchem).

In vitro activation of $CD8^+$ T cells

Tumor antigen–specific CD8$^+$ T cells. $CD8^+$ T cells from pMel-1 mice were activated *in vitro* as described (10). Briefly, homogenized pMel-1 splenocytes were stimulated with $gp100_{25–33}$ peptide at 1 μmol/L (day 0). Cells were cultured in RPMI-1640 (Lonza) supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), 0.1% β-mercaptoethanol (Life Technologies, Invitrogen), and IL2 (100 U/ml; Peprotech) at 37°C with 5% CO_2. pMel-1 cells were cultured with or without different PI3K inhibitors. The concentration of the inhibitors was maintained throughout the culture by changing the media every 48 to 72 hours.

On days 7, 14, and 21, cells were restimulated with $gp100_{25–33}$ at 1 μmol/L using feeder cells (irradiated WT splenocytes, 4,000 Rads) at 1:1 ratio using the same culture conditions.

T-cell receptor stimulation and costimulation. Viable $CD8^+$ T cells from WT mice were sorted using FACS ARIA II (BD Biosciences; purity > 99%). Cells were activated on anti-CD3–coated plates (10 μg/mL) and cultured in activation media [IL2 (100 U/mL) and anti-CD28 (2.5 μg/mL)] in the presence or absence of PI3K inhibitors for 72 hours.

Proliferation assay and phenotyping of CD8$^+$ T cells. Cells were labeled with 5 μmol/L Violet Cell Trace (VCT) proliferation dye (Life Technologies, Invitrogen) prior to their stimulation (day 0). Proliferation of $CD8^+$ T cells was assessed via VCT dye dilution (day 3) using LSRII SORP with HTS Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo-10 (TreeStar).

Cultured cells were harvested on days 3, 7, 14, and 21 to assess their phenotype. Cells were stained with the following surface marker antibodies (BD Biosciences): APC-Cy7 labeled anti-CD8, FITC labeled anti-Vβ13, PE labeled anti-CD62L, APC labeled anti-CD44, PE-CF594 labeled anti-CD127, APC labeled anti-KLRG-1, in addition to the viability stain 7AAD. All analyses were performed on viable ($7AAD^-$), $Vβ13^+CD8^+$ T cells.

For intracellular staining, cells were stained with the fixable near-infrared Live/Dead viability stain (Life Technologies, Invitrogen), and fixed, permeabilized, and stained with APC-labeled anti-CD8, V450 labeled anti-Vβ13, PE-labeled anti-CD62L and PE-CF594–labeled anti-CD44 (BD Biosciences), and FITC-labeled granzyme B (Biolegend). The analyses were performed on viable (live/dead negative), $Vβ13^+CD8^+$ T cells.

Cytometric bead array

Using the stimulation protocol above, $CD8^+$ T cells were harvested on day 7 after the first and second stimulation. Viable (trypan blue negative) cells were coincubated (at 1:1 ratio) with 1 μmol/L $gp100_{25–33}$–pulsated irradiated splenocytes (4,000 Rads) for 24 hours using the same culture conditions. Supernatants were collected and the level of IL2, TNFα, and IFNγ was assessed using the mouse Th1/Th2/Th17 Cytokine Kit BD Cytometric Bead Array (CBA) kit. Cytokine levels were collected using an LSRII SORP with HTS flow cytometer (BD Biosciences) and analyzed using the FCAP Array Software v3.0 (BD Biosciences).

In vivo tumor treatment

C57BL/6 female mice were implanted with 400,000 B16 cells/mouse subcutaneously (s.c.) in the right flank on day 0 (B16 expresses gp100 antigen). On day 7, mice were lymphodepleted by s.c. injection of a cocktail of 250 mg/kg cyclophosphamide and 50 mg/kg fludarabine (CyFlu). On day 8, gp100-activated $CD8^+$ T cells from pMel-1 mice cultured in the presence or absence of CAL-101 (202.5 nmol/L, for 7 days as described above) were adoptively transferred intravenously (i.v.; 1 million cells per mouse). The appropriate groups were vaccinated with gp100 peptide vaccine ($gp100_{25–33}$ with PADRE and Quil A) on days 8, 15, and 22. The vaccine doses represented stimulations 2, 3, and 4 of $CD8^+$ T cells. Animal survival and tumor growth were monitored and animals were sacrificed upon tumor ulceration or reaching the volume of 1.5 cm^3 according to institutional regulations.

Statistical analysis

Statistical parameters (average values, SD, and significant differences between groups) were calculated using Microsoft Excel and GraphPad Prism. Statistical significance between groups was determined by a paired *t* test or one-way ANOVA with *post hoc* Tukey multiple comparison test ($P < 0.05$ was considered statistically significant).

Results

PI3K inhibition enhances the proliferative ability and survival of $CD8^+$ T cells by preserving the memory phenotype

Memory $CD8^+$ T cells are superior mediators of antitumor immunity than effector cells due to their greater proliferative ability (4–7, 10). Many T-cell functions are regulated by the PI3K/Akt pathway (14, 15). To test the role of PI3K in the differentiation and proliferation of $CD8^+$ T cells, we tested the effect of the pan-PI3K inhibitor GDC-0941 (GDC) on stimulated pMel-1 $CD8^+$ T cells activated with 1 μmol/L $gp100_{25–33}$.

After 3 days of stimulation, we found that GDC-treated cells consisted of a high percentage of T_{CM} cells ($CD62L^{hi}CD44^{hi}$) in addition to T_{EM} cells ($CD62L^{lo}CD44^{hi}$), while the majority of non–GDC-treated cells were T_{EM}. This was observed at all concentrations used (Fig. 1A). Unlike nontreated cells, the higher percentage of memory $CD8^+$ T cells (both T_{CM} and T_{EM}) was maintained after the second and third stimulations (Fig. 1A). As expected, the percentage of T_{CM} in GDC-treated cells decreased following the second and third stimulations due to the memory recall of T_{CM} following multiple stimulations, leading to the differentiation of T_{CM} into T_{EM} and effector cells. However, the memory phenotype was significantly higher than the nontreated cells after each stimulation. In fact, nontreated cells virtually lost all the T_{CM} phenotype after the second stimulation and started losing their T_{EM} following the third stimulation in favor of the terminally differentiated phenotype ($CD62L^{lo}CD44^{lo}$). This led to a significantly higher percentage of T_{EM} in GDC-treated cells following the third stimulation in comparison with the nontreated cells.

Taken together, these data show that PI3K inhibition delays terminal differentiation and preserves a reservoir of memory cells (T_{CM} and T_{EM}), after several encounters with the antigen. The nontreated cells, on the other hand, lost their T_{CM} cells, and a significant percentage of the T_{EM} while simultaneously reaching terminal differentiation as evidenced by the significantly higher percentage of terminally differentiated effector $CD8^+$ T cells ($CD62L^{lo}CD44^{lo}$) after consecutive stimulations.

Because T_{CM} $CD8^+$ T cells possess a greater proliferative ability than T_{EM} (4–7), we assessed the proliferation and expansion of $CD8^+$ T cells under the effect of PI3K inhibitors. After 3 days of the first stimulation, the proliferation and expansion of $CD8^+$ T cells treated with GDC was only slightly inhibited (Fig. 1B), which is expected given the role PI3K plays in the proliferation of T cells. At the highest concentration tested (279 nmol/L), the inhibitor was found to be toxic and was therefore used at lower concentrations for the rest of the experiments. However, with further stimulation (stimulations 2 and 3), $CD8^+$ T cells treated with the PI3K inhibitor expanded at a significantly higher rate than nontreated cells (Fig. 1B) Nontreated cells lost the ability to expand following the third encounter with the antigen. These findings show that PI3K inhibition enhances the cell proliferation and survival of $CD8^+$ T cells, which correlates with their memory phenotype.

Correlating with the enhanced proliferation ability observed with the inhibitor treatment, $CD8^+$ T cells treated with GDC maintained high expression levels of CD62L (days 3, 7, 14, and 21), which is a marker associated with memory phenotype and with high proliferative potential (Fig. 1C).

We next measured IL2 secretion in $CD8^+$ T cells, a property that is diminished in terminally differentiated $CD8^+$ T cells. We found that $CD8^+$ T cells treated with GDC maintained a significantly high level of IL2 secretion after re-encountering the antigen (Fig. 1D).

Taken together, these data show that PI3K inhibition preserves the memory phenotype in antigen stimulated $CD8^+$ T cells, hence enhancing the proliferative potential and survival while delaying the terminal differentiation of these $CD8^+$ T cells.

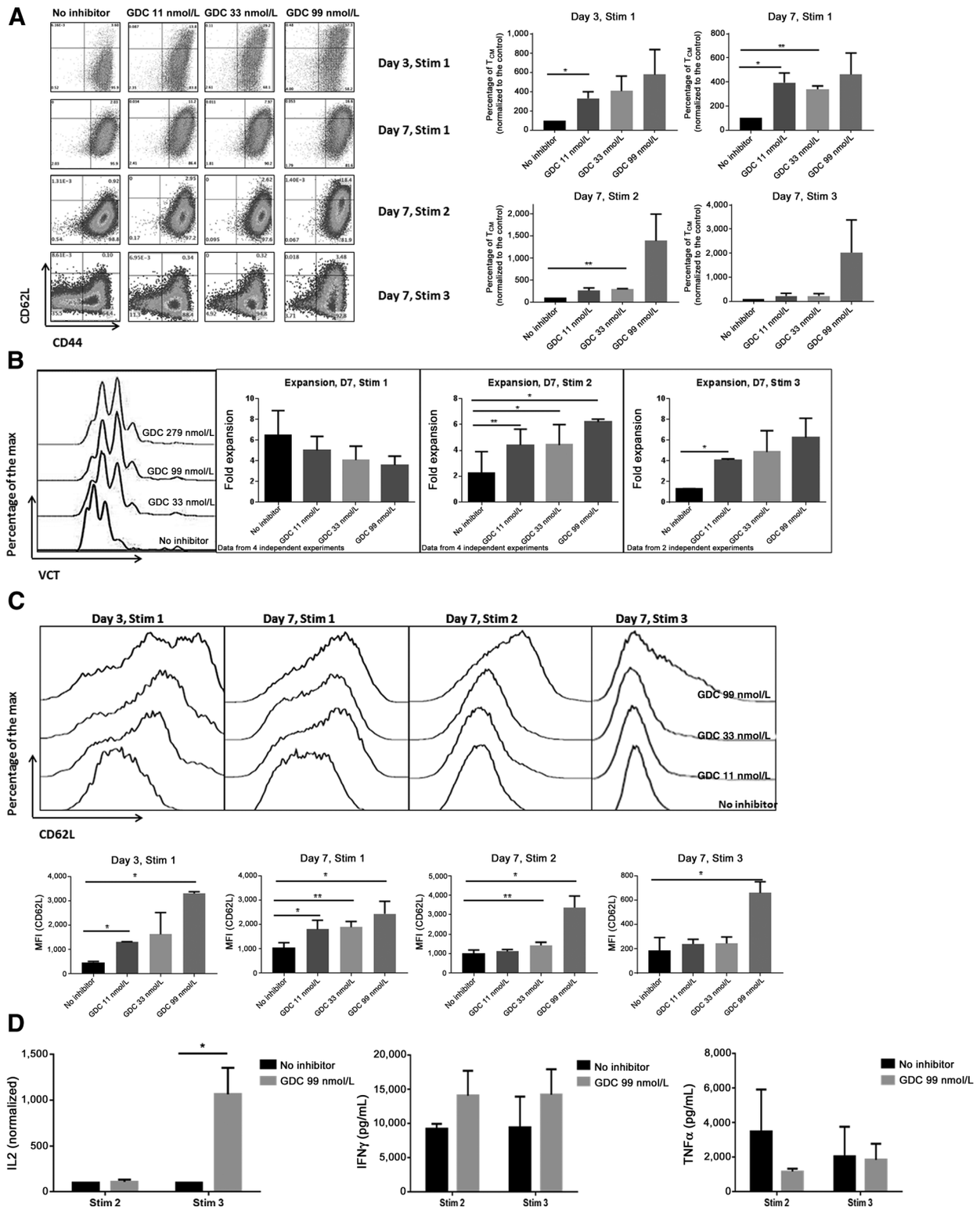
PI3K inhibition does not affect the ability of $CD8^+$ T cells to produce cytotoxic cytokines and granzyme B

We have demonstrated that PI3K inhibition enhances proliferation of $CD8^+$ T cells, preserves the T_{CM} phenotype, and delays terminal differentiation. Classically, effector functions were thought to peak at the effector state (2); however, more recent findings suggest that memory cells are superior in their cytotoxic abilities because of memory recall and proliferation potential, and therefore are superior options for ACT (7, 10, 16). To assess whether the function of the resultant T_{CM} cells is affected by the inhibition of PI3K, we measured their ability to secrete IFNγ and TNFα and the level of granzyme B production.

$CD8^+$ T cells were restimulated on days 7 and 14 (stim 2 and 3) and the level of IFNγ and TNFα production after 24 hours was assessed. After the second and third stimulations, GDC-treated and nontreated cells produced high and comparable levels of IFNγ and TNFα in response to antigen reencounter (Fig. 1D).

To further test the cytotoxic ability of the $CD8^+$ T cells treated with PI3K inhibitor, we assessed the level of granzyme B production by intracellular staining. As shown below, following the first and second stimulations, we found that the inhibition of PI3K does not affect the production of granzyme B when compared with $CD8^+$ T cells that were not treated with PI3K inhibitors.

The maintained levels of IFNγ and TNFα secretion and Granzyme B production suggest that $CD8^+$ T cells treated with PI3K inhibitors maintain their cytotoxic functionality. Added to the marked increase in their proliferation potential and their



enhanced survival, $CD8^+$ T cells treated with PI3K inhibitors are potentially a superior option for ACT.

PI3K-δ is the isoform responsible for terminal differentiation of $CD8^+$ T cells

We have shown that PI3K inhibition in $CD8^+$ T cells delays their terminal differentiation, preserves T_{CM} cells, enhances their proliferative ability while maintaining their cytokine secretion ability and prolonging their survival. The role of specific PI3K isoforms (PI3K-α, PI3K-β, and PI3K-δ) in the development, proliferation, and function of $CD8^+$ T cells is not known. Using selective PI3K inhibitors, we next tested whether the inhibition of a single PI3K isoform would be sufficient to delay terminal differentiation of CD8.

When the phenotype of the cells was assessed after 3 days of the first stimulation, $CD8^+$ T cells treated with inhibitors specific for PI3K-α (A66) or PI3K-β (TGX-221), there were no differences in the phenotype of $CD8^+$ T cells from the nontreated cells (Fig. 2A). However, only when the PI3K-δ inhibitor (CAL-101) was used, $CD8^+$ T cells displayed a phenotype similar to that observed with pan-PI3K inhibition, where there was a higher percentage of T_{CM} cells when compared with nontreated cells (Fig. 2A; Supplementary Fig. S1). As expected, due to memory recall, this effect was less prominent after the second and third stimulations when compared with the first stimulation. Furthermore, the inhibition of PI3K-δ lead to a significantly lower percentage of terminally differentiated $CD8^+$ T cells ($CD62L^{lo}CD44^{lo}$; Fig. 2A; Supplementary Fig. S1), in particular after the third stimulation. These findings suggest that PI3K-δ is the isoform responsible for terminal differentiation of $CD8^+$ T cells and that its inhibition maintains $CD8^+$ T cells in earlier stages of differentiation (both T_{CM} and T_{EM}) even after several encounters with the antigen.

To test if the memory phenotype generated by the inhibition of PI3K-δ possesses an enhanced proliferative ability, we assessed the proliferation of $CD8^+$ T cells under same stimulation conditions using specific PI3K isoform inhibitors. While we found that the inhibition of PI3K-δ marginally inhibited the proliferation of $CD8^+$ T cells compared with cells treated with PI3K-α and PI3K-β inhibitors (Fig. 2B), the inhibition of PI3K-δ, but not PI3K-α or PI3K-β significantly enhanced the proliferative of $CD8^+$ T cells with further stimulations (days 7 and 14; Fig. 2C). We also found that treatment of $CD8^+$ T cells with the PI3K-δ inhibitor maintained high expression levels of CD62L (Fig. 3A) and high secretion levels of IL2 (Fig. 3B; Supplementary Fig. S2), consistent with the enhanced proliferative ability of the memory $CD8^+$ T cells. These high levels of CD62L expression and IL2 secretion were not observed when $CD8^+$ T cells were treated with either PI3K-α or PI3K-β inhibitors.

Treating $CD8^+$ T cells with the PI3K α, β, or δ isoform specific inhibitors did not affect the cells' ability to produce TNFα and IFNγ secretion (Fig. 4A; Supplementary Fig. S2) and granzyme B (Fig. 4B). This is important as it emphasizes that maintaining the cells in the early stages of differentiation does not affect their cytotoxic ability.

Similar results were observed when PI3K-δ was silenced in $CD8^+$ T cells, where the cells maintained a higher percentage of central memory phenotype in comparison with knocking down PI3K α or β (Supplementary Fig. S3A). Furthermore, the proliferation of $CD8^+$ T cells and their ability to produce granzyme B was not affected by the knockdown of any of the isoforms (Supplementary Fig. S3A).

These findings were replicated in human $CD8^+$ T cells, where the stimulation of purified human $CD8^+$ T cells from healthy human donors in the presence of the pan-PI3K inhibitor GDC resulted in maintaining a high level of CD62L without affecting the proliferation of the cells (Supplementary Fig. S3B). Furthermore, the inhibition of PI3K-δ in activated human $CD8^+$ T cells resulted in the maintenance of a high expression level of CD62L, which was not observed when PI3K-α or PI3K-β were inhibited (Supplementary Fig. S3B).

Taken together, our data demonstrate that PI3K-δ is responsible for the terminal differentiation of CD8 and the inhibition of PI3K-δ, but not PI3K-α or PI3K-β, preserves $CD8^+$ T cells in memory state, thus enhancing their proliferative potential, longevity, and survival without affecting their ability to produce cytokines and granzyme B.

The inhibition of PI3K-δ in $CD8^+$ T cells significantly enhances their antitumor therapeutic ability *in vivo*

We have shown that PI3K-δ inhibition delays the terminal differentiation of $CD8^+$ T cells and enhances their proliferative ability and survival without affecting their ability to produce cytokines and granzyme B. To test if these findings translate into enhanced therapeutic ability *in vivo*, we adoptively transferred tumor antigen–specific $CD8^+$ T cells treated with CAL-101 into tumor bearing mice and assessed their antitumor effect in combination with a tumor specific vaccine.

Briefly, pMel-1 cells activated with gp100 with or without CAL-101 were cultured for 7 days, and their phenotype was assessed. Similar to what is presented above, treated cells consisted of a

Figure 1.

PI3K inhibition preserves the memory phenotype and enhances the proliferative ability of $CD8^+$ T cells. Nonfractionated splenocytes from pMel-1 mice were stained with VCT and activated with $gp100_{25–33}$ peptide (1 μmol/L) in the presence or absence of GDC-0941 (11, 33, 99, and 279 nmol/L). The cells were restimulated with $gp100_{25–33}$ on days 7 and 14 and their phenotype and proliferation assessed. Gated cells were viable (7AAD-) $CD8^+V\beta13^+$. **A,** In this representative example (left), non–GDC-treated $CD8^+$ T cells are mainly T_{EM} cells ($CD62L^{lo}CD44^{hi}$; 96%), while GDC-treated cells have a high percentage (37% at the highest concentration) of T_{CM} phenotype ($CD62L^{hi}CD44^{hi}$). Terminally differentiated T cells ($CD62L^{lo}CD44^{lo}$) after the third stimulation are significantly higher in nontreated cells (36%) compared with only 4% with the highest GDC concentration. T_{CM} and T_{EM} were maintained with GDC treatment after the third stimulation (3.5 and 93%, respectively), compared with only less than 0.01 and 64% in the nontreated cells. The right panel shows bar graphs summarizing data from at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$. **B,** After 3 days of stimulation, the proliferation of $CD8^+$ T cells was inhibited in a dose-dependent manner by GDC-0941 (VCT dilution; far left). $CD8^+$ T cells treated with GDC expanded at a significantly high rate with further stimulations. *, $P < 0.05$; **, $P < 0.01$. **C,** PI3K inhibition by GDC-0941 maintains a high level of CD62L expression in $CD8^+$ T cells on day 3 and on day 7 after each stimulation with gp100. Top, a representative example of the CD62L expression. Bottom, mean florescence intensity (MFI) for CD62L expression (data from at least two independent experiments). *, $P < 0.05$; **, $P < 0.01$. **D,** GDC-treated $CD8^+$ T cells secrete significantly higher levels of IL2 following stimulation 3, which is consistent with their higher proliferative potential. Data normalized to GP100; *, $P < 0.05$. The ability of $CD8^+$ T cells to produce IFNγ and TNFα was not affected by PI3K inhibition using GDC-0941.

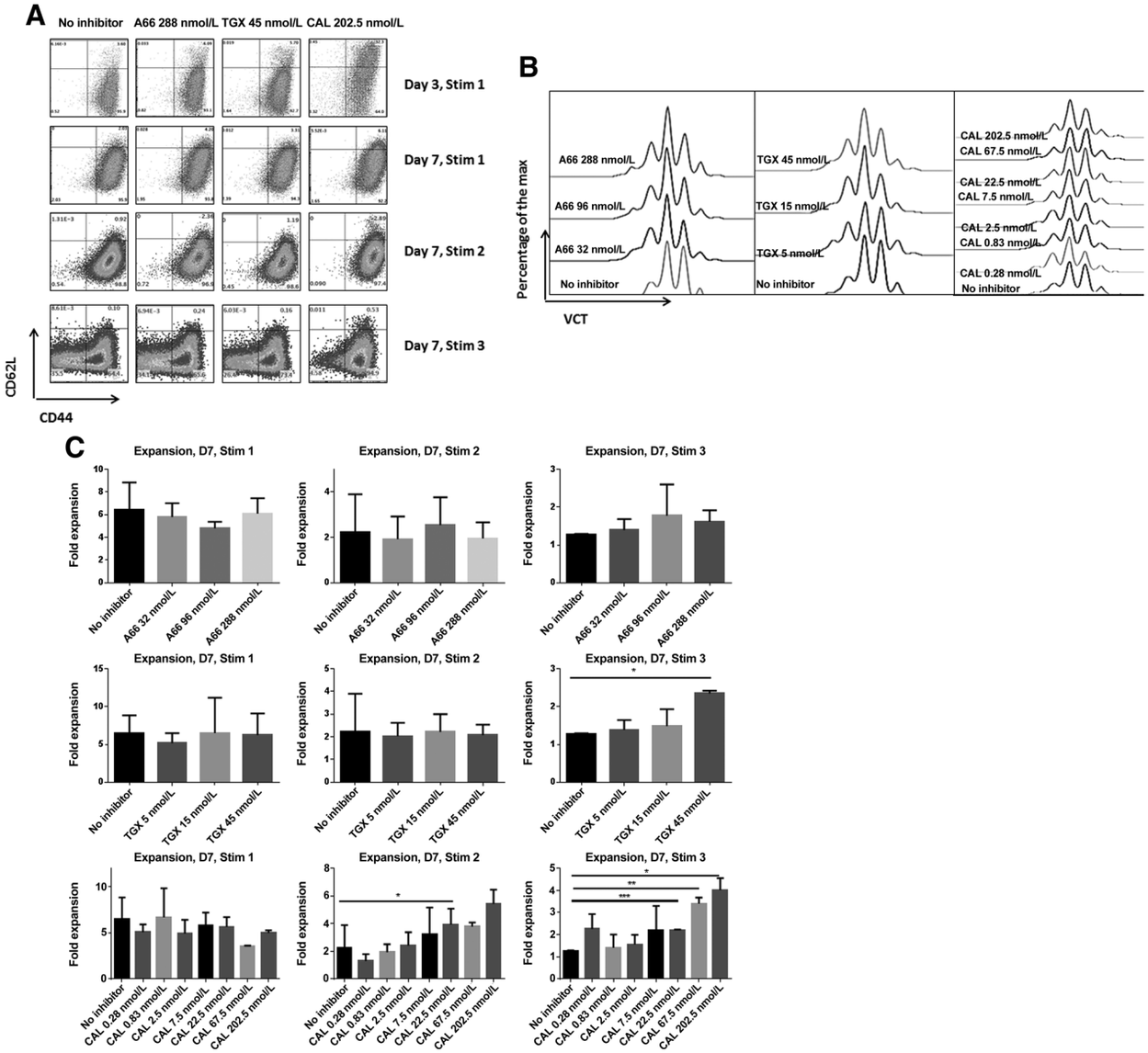


Figure 2.

The inhibition of PI3K-δ, but not PI3K-α or PI3K-β, preserves memory cells and enhances the proliferative ability of $CD8^+$ T cells. Nonfractionated splenocytes from pMel-1 mice were stained with VCT and activated with $gp100_{25–33}$ peptide (1 μmol/L) in the presence or absence of A66 (32, 96, and 288 nmol/L), TGX-221 (5, 15, and 45 nmol/L), or CAL-101 (0.28, 0.83, 2.5, 7.5, 22.5, 67.5, or 202.5 nmol/L). The cells were restimulated with $gp100_{25–33}$ on days 7, 14, and 21. Gated cells were viable (7AAD-)$CD8^+V\beta13^+$. **A,** PI3K-δ inhibition, but not PI3K-α or PI3K-β, preserves the memory phenotype. In this representative example, A66- and TGX-treated cells have a similar profile to nontreated cells, while CAL-101–treated cells have a significantly higher percentage of T_{CM} ($CD62L^{hi}CD44^{hi}$; 32% compared with 4%, D3, Stim 1) and T_{EM} cells ($CD62L^{lo}CD44^{hi}$; 95% compared with 64%, D7, Stim 3), and a lower percentage of terminally differentiated T cells ($CD62L^{lo}CD44^{lo}$; 5% compared with 36%, D7, Stim 3). **B,** The proliferation of $CD8^+$ T cells is marginally inhibited by PI3K-δ inhibition, but not PI3K-α or PI3K-β (day 3). **C,** The expansion of $CD8^+$ T cells treated with the inhibitor is significantly enhanced with further stimulations with PI3K-δ inhibition, but not PI3K-α or PI3K-β. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

large percentage of T_{CM}. These cells were adoptively transferred into tumor bearing, lymphodepleted mice in combination with gp100 vaccine (administered on days 8, 15, and 22 and corresponding to stimulations 2, 3, and 4; Fig. 5A).

Remarkably, the ACT of $CD8^+$ T cells that were activated *in vitro* in the presence of the PI3K-δ inhibitor CAL-101 greatly slowed down tumor growth in B16 tumor bearing mice. This effect was significantly enhanced when the ACT was combined with the gp100 peptide vaccine because the vaccine acted a second stimulation for the $CD8^+$ T cells, hence the more potent expansion, and the resulting antitumor effect. The enhanced therapeutic efficacy was much greater than any other single therapy, including the vaccine, the ACT of non–CAL-101-treated $CD8^+$ T cells or the combination of both (Fig. 5B and C).

Furthermore, the combination of ACT of CAL-101–treated $CD8^+$ T cells with the vaccine greatly prolonged the animal

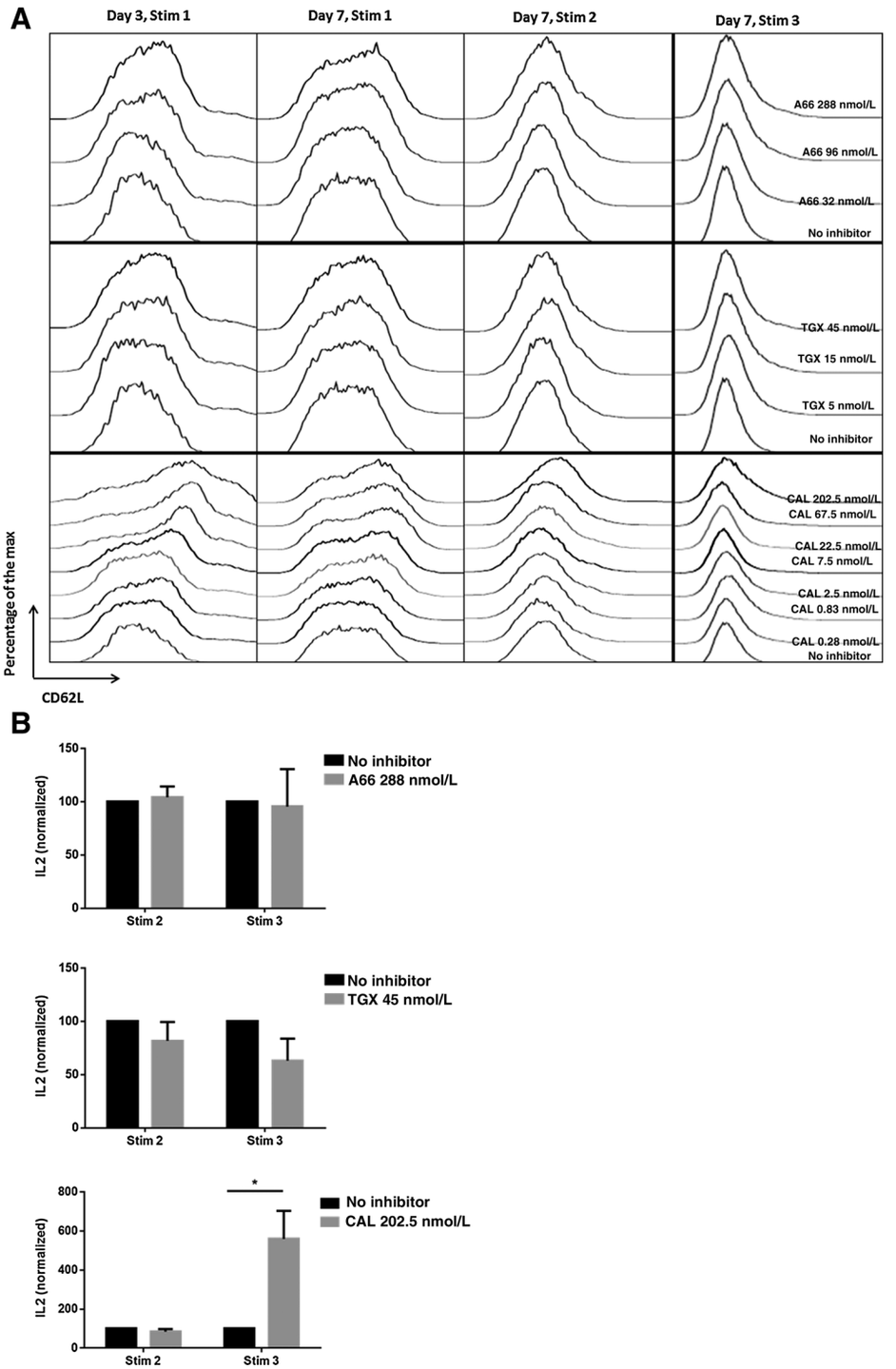


Figure 3.
The inhibition of PI3K-δ, but not PI3K-α or PI3K-β, preserves high expression levels of CD62L and high secretion of IL2 in $CD8^+$ T cells. Nonfractionated splenocytes from pMel-1 mice were stained with VCT and activated with $gp100_{25–33}$ peptide (1 μmol/L) in the presence or absence of A66 (32, 96, and 288 nmol/L), TGX-221 (5, 15, and 45 nmol/L), or CAL-101 (0.28, 0.83, 2.5, 7.5, 22.5, 67.5, or 202.5 nmol/L). The cells were restimulated with $gp100_{25–33}$ on days 7, 14, and 21. Gated cells were viable ($7AAD^-$)$CD8^+V\beta13^+$. **A,** PI3K-δ, but not PI3K-α or PI3K-β, maintains a high level of CD62L expression in $CD8^+$ T cells on day 3 and on day 7 after each stimulation with gp100. **B,** CAL-101–treated $CD8^+$ T cells secreted significantly higher levels of IL2 following stimulation 3, which is consistent with their higher proliferative potential. Data normalized to GP100; *, $P < 0.05$.

survival (Fig. 5D). Similar results were obtained when treatment was started at a later date with larger tumors (Supplementary Fig. S4).

These data clearly demonstrate the superior antitumor functionality of $CD8^+$ T cells treated with a PI3K-δ inhibitor. This can be attributed to the enhanced proliferative ability, longevity, survival, and maintenance of the memory phenotype.

Discussion

In response to antigen encounter, $CD8^+$ T-cell response comprises effector and memory T cells (1, 2). CD8 memory T cells include several subtypes, including T_{CM} and T_{EM} (2, 3). Memory cells represent earlier stages of differentiation and are superior in their cytotoxic ability against microbial challenges (4, 6) and

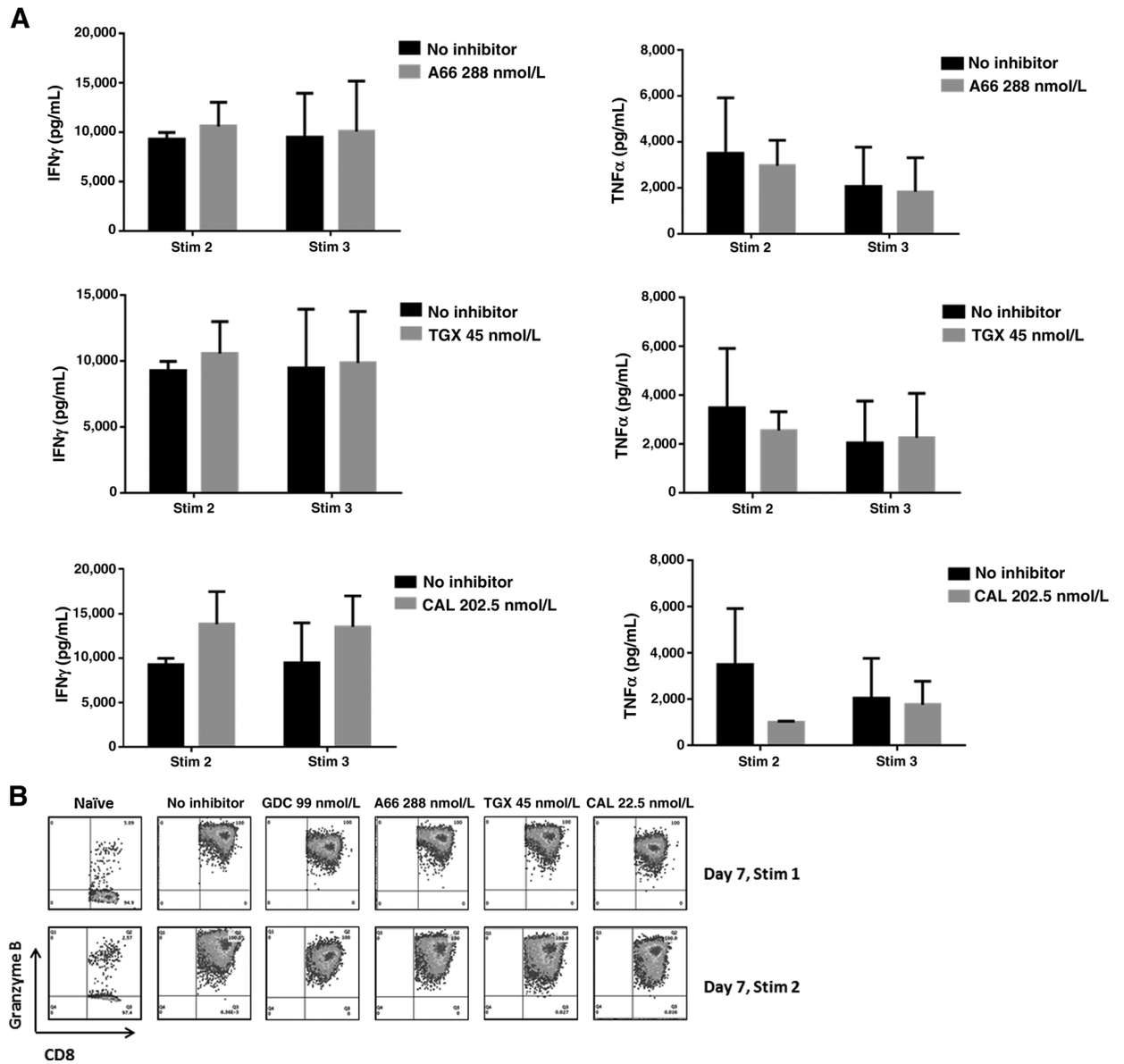


Figure 4.

PI3K inhibition does not affect the secretion of IFNγ and TNFα. $CD8^+$ T cells from pMel-1 mice were stimulated with $gp100_{25–33}$ peptide (1 μmol/L) in the presence or absence of GDC-0941 (99 nmol/L), A66 (288 nmol/L), TGX-221 (45 nmol/L), or CAL-101 (202.5 nmol/L). On days 7 and 14, $CD8^+$ T cells were restimulated with $gp100_{25–33}$ peptide and the IFNγ and TNFα levels in the supernatant assessed after 24 hours using CBA. Granzyme B expression was assessed on days 7 and 14. **A,** The ability of $CD8^+$ T cells to produce IFNγ and TNFα was not affected by the inhibition of specific PI3K isoforms. **B,** The ability of $CD8^+$ T cells to produce granzyme B was not affected by PI3K inhibition.

mediation of therapeutic antitumor immunity when compared with terminally differentiated effector cells. T_{CM} cells are by far superior to T_{EM} cells, due to their greater proliferative capacity upon antigen reencounter. T_{EM} in turn are superior to the terminally differentiated effector cells (4–7).

The PI3K/Akt pathway governs many T-cell functions, including proliferation, survival, migration, and metabolism (14, 15). The differentiation of CD8 cells into memory T cells is coordinated by PI3K/Akt signaling (1, 17, 18). Continuous activation of this pathway drives the terminal differentiation, while its inhibition (at the level of Akt or the downstream mTOR) enhances the quality of $CD8^+$ T cells by prompting a memory phenotype (8–10, 16–19).

Our group has reported that Akt1 and Akt2 isoforms are the specific drivers of terminal differentiation of $CD8^+$T cells and that their inhibition preserves a reservoir of highly proliferative and functionally superior memory $CD8^+$ T cells (10).

Here, we show, for the first time, that the PI3K-δ, but not PI3K-α or PI3K-β, drives the terminal differentiation of $CD8^+$T cells, and that the inhibition of PI3K-δ enhances their survival

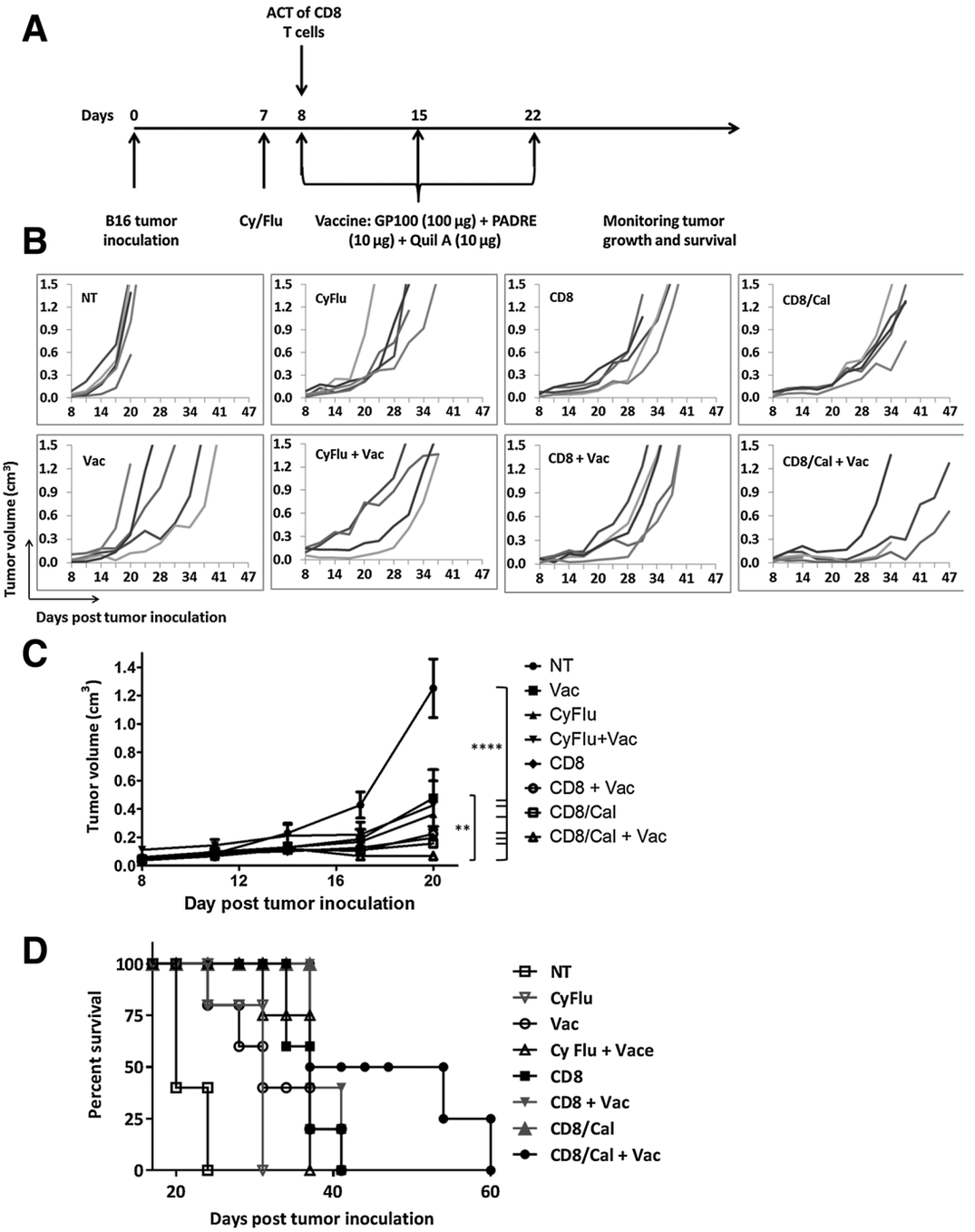


Figure 5.

The inhibition of PI3K-δ in CD8$^+$ T cells significantly enhances their antitumor therapeutic ability *in vivo*. Mice were implanted with B16 in the right flank on day 0. On day 7, mice were lymphodepleted with CyFlu and on day 8, 1 million CD8$^+$ T cells from pMel-1 mice cultured in the presence or absence of CAL-101 were adoptively transferred. The appropriate groups were vaccinated with gp100/PADRE/Quil A vaccine on days 8, 15, and 22. Animal survival and tumor growth were monitored. NT, no treatment ($n = 5$); Vac, vaccine ($n = 5$); CyFlu, cycophosphamide/fludarabine ($n = 5$); CyFlu + Vac, cycophosphamide/fludarabine + vaccine ($n = 4$); CD8, ACT of nontreated CD8 ($n = 5$); CD8 + Vac, ACT of nontreated CD8 + vaccine ($n = 5$); CD8/Cal, ACT of CD8 treated with CAL-101 ($n = 5$); CD8/Cal + Vac, ACT of CD8 treated with CAL-101 + Vaccine ($n = 5$). All mice that received ACT were lymphodepleted with Cy/Flu. **A,** Treatment schedule. **B,** Tumor volumes of individual mice for each treatment measured every 3 to 4 days. The data clearly show that the combination of ACT of CAL-101–treated cells with the vaccine significantly slowed down tumor growth when compared with all the other groups. **C,** Mean tumor volume for different groups shown in **B**. Statistical analyses were performed between groups on day 20 (before any of the animals died). The combination of ACT of CAL-101–treated cells with the vaccine significantly slowed down tumor growth in comparison with nontreated mice and the vaccine alone. **, $P < 0.01$; ****, $P < 0.0001$. **D,** The Kaplan–Meier plot depicts overall survival. The combination of ACT of CAL-101–treated cells with the vaccine significantly prolonged survival.

and proliferative ability upon reencountering the antigen by preserving a high percentage of memory $CD8^+$ T cells. This occurs through enhancing the proliferative ability of $CD8^+$ T cells and maintaining a high CD62L expression level and IL2 secretion. We further demonstrate that $CD8^+$ T cells treated specifically with an inhibitor of PI3K-δ greatly enhance their antitumor therapeutic ability when adoptively transferred into tumor bearing mice.

As $CD8^+$ T cells differentiate from naïve to effector cells, they lose their ability to produce IL2 (3). Here, we further show that PI3K-δ inhibition maintains a higher level of IL2 secretion in $CD8^+$ T cells. Additionally, we show that the inhibition of PI3K-δ maintains a high level of CD62L, which is in agreement with the findings of Sinclair and colleagues (20) who showed that the proteolysis of CD62L is controlled by PI3K-δ (20). Classically, cytotoxic effector functions were thought to gradually increase from naïve through memory and peak at the effector state (2); however, more recent reports suggest that memory cells are superior in their cytotoxic abilities (7, 10, 16). This is in agreement with our findings, where PI3K-δ inhibition enhances the proliferative ability and longevity of $CD8^+$ T cells without affecting their cytotoxic functionality. We found that the inhibition of the PI3K-δ also does not affect the $CD8^+$ T cells' ability to secrete TNF, IFNγ, and granzyme B. Based on above, it is clear that treatment of $CD8^+$ T cells with PI3K-δ provides a superior quality T cells for more effective ACT.

Here, we further demonstrate that our *in vitro* findings translated to *in vivo* application where treating tumor specific $CD8^+$ T cells with PI3K-δ inhibitor greatly enhances their antitumor ability when adoptively transferred into tumor-bearing mice. This is not surprising given the enhanced proliferation, survival, and functionality of inhibitor treated cells *in vitro*. Furthermore, we also found that the therapeutic ability of these cells was greatly enhanced with the combination of a tumor specific vaccine because memory $CD8^+$ T cells respond to antigen reencounter by proliferating more robustly than exhausted effector $CD8^+$ T cells. Thus, the preservation of memory cells using only PI3K-δ inhibition produces an enhanced cytotoxic antitumor ability. This was demonstrated by a significant slowdown of tumor growth and a significant enhancement of animal survival.

Interestingly, we found that $CD8^+$ T-cell differentiation is controlled specifically through the PI3K-δ isoform that signals downstream through Akt1 and Akt2, the two Akt isoforms we had already shown to be responsible for the differentiation of $CD8^+$ T cells (10). We also found that in $CD8^+$ T cells, PI3K-α and PI3K-β play no role in proliferation and survival and that these two isoforms do not signal through Akt1 and Akt2 (Supplementary Fig. S5). Accordingly, PI3K-δ is the only isoform that controls these biologic functions in $CD8^+$ T cells.

Based on the above, our findings define a new and vital role for the PI3K-δ isoform in T-cell biology. We demonstrate that targeting PI3K-δ can modulate the differentiation of effector and memory $CD8^+$ T cells. This adds to the significant roles that PI3K-δ has in different T cells subsets; in particular, its definition as a key controller of the suppressive Tregs (21–23). This has important clinical implications for the use of PI3K-δ inhibitors to modulate both Tregs and $CD8^+$ T cells.

Clinical trials using PI3K-δ inhibitors have recently been put on hold due to a significant increase in the incidence of opportunistic infection (mostly CMV). Here, our data provide a plausible explanation for this increase. The later stages of CD8 differentiation are thought to be the most potent against CMV; it is therefore apparent that the use of PI3K-δ inhibitors delays the later stages of differentiation; hence, their systematic administration could deprive the body of the most potent antiviral $CD8^+$ T cells. We have shown this to be the case in PI3K-δ KO mice, where the percentage $CD8^+$ T cells at later stages of differentiation in response to antigen administration (in the form of a peptide vaccine) was significantly lower in KO mice in comparison with WT mice (Supplementary Fig. S6). This also explains the findings that PI3K-δ is required for the generation of an immediate effector response to viral and intracellular bacterial infections (24, 25).

In summary, we report that PI3K-δ inhibition, but not PI3K-α or PI3K-β, enhances the memory phenotype, improves $CD8^+$ T-cell survival, and enhances their proliferative potential while maintaining their ability to produce cytotoxic cytokines and granzyme B. These findings translate into antitumor therapeutic efficacy where the ACT of *ex vivo* PI3K-δ–treated $CD8^+$ T cells in an animal tumor model greatly slows down tumor growth and prolongs animal survival.

Agents with the ability to delay terminal differentiation of $CD8^+$ T cells without affecting their effector function and proliferation are needed. Here, we outline a strategy that enhances the memory phenotype, proliferative potential, and survival without affecting the effector function of $CD8^+$ T cells by targeting PI3K-δ. Our findings have significant clinical implications and strongly suggest the clinical use of PI3K-δ inhibitors as potent modulators of the immune response as part of different cancer immune therapy strategies.

Disclosure of Potential Conflicts of Interest

Y. Lin is a scientist at La Jolla Institute for Allergy and Immunology. M. Mkrtichyan is a scientist at FivePrime Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

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