

Full Title:

Enhanced therapeutic efficacy and memory of tumor specific CD8 T cells by *ex-vivo* PI3K- δ inhibition

Authors:

Rasha Abu Eid^{1,2*}, Shamim Ahmad^{2*}, Yuan Lin^{2,3}, Mason Webb², Zuzana Berrong², Rajeev Shrimali^{2,4}, Takumi Kumai², Sudha Ananth², Paulo C. Rodriguez², Esteban Celis², John Janik², Mikayel Mkrtichyan^{2,5}, and Samir N Khleif²

¹The University of Aberdeen Dental School and Hospital, The Institute of Medicine, Medical Sciences & Nutrition, Aberdeen, Scotland, United Kingdom

²Georgia Cancer Center, Augusta University (previously Georgia Regents University), Augusta, Georgia, United States

³La Jolla Institute for Allergy and Immunology, Athena Circle, La Jolla, California, United States.

⁴Peloton Therapeutics, Dallas, Texas, United States

⁵Five Prime Therapeutics, San Francisco, California, United States

* RAE and SA contributed equally to this work.

Running Title: PI3K- δ inhibition enhances efficacy and memory CD8 T-cells

Key Words: memory, Akt, PI3K, proliferation, CD8+ T-cells, adoptive cell transfer

Financial support: This work was supported by Georgia Cancer Center, Augusta University (S. Khleif)

Conflict of Interest: The authors declare that there are no conflicts of interest in the authorship or publication of this manuscript

Corresponding author:

Samir N. Khleif
Georgia Cancer Center, Augusta University (previously Georgia Regents University)
1120 15th Street, CN-2101A
Augusta, GA 30912, USA.
Tel: (706) 721-0570
Fax: (706) 721-8787
E-mail: SKHLEIF@gru.edu

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 anti-tumor 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 of *ex vivo* PI3K- δ inhibition in CD8⁺ T cells destined for adoptive transfer, enhancing their survival and also the anti-tumor 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.

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 (T_{EM}) and central (T_{CM}) memory T-cells 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 to 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 anti-tumor 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 anti-tumor therapeutic ability of adoptively transferred CD8⁺ T-cells in animal models compared to non-treated 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 anti-tumor 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 were mainly attributed to infections by *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 anti-viral CD8⁺ T-cells.

Our findings suggest a strategy that enhances the anti-tumor 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* anti-tumor 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 (V β 13) 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, 4-6 week old WT female mice were used. (Mice were purchased from Jackson Laboratory and housed under pathogen-free conditions according. All procedures were carried out in accordance with Institutional Animal Care and Use Committee (IACUC).

B16 cell line was purchased from American Type Culture Collection (ATCC) (Manassas, VA) which routinely authenticate and test 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 IC₅₀ of 3nM for p110 α , 33nM for p110 β , 3nM for p110 δ and 75nM for p110 γ . GDC-0941 was used *in vitro* at 11, 33, 99 and 279nM concentrations, ensuring inhibition of all three Class I isoforms. A66 is a selective p110 α inhibitor with IC₅₀ of 32nM for the p110 α , 236nM for PI4K β , 462nM for C2 β and >1.25 μ M for p110 δ . A66 was used *in vitro* at 32, 96 and 288nM concentrations, ensuring selectivity to PI3K α . TGX-221 is a highly selective PI3K β inhibitor with IC₅₀ of 5 μ M for p110 α , 5nM for p100 β , 0.1 μ M for p100 δ and > 10 μ M for p110 γ . In *in vitro* experiments,

TGX-221 was used at 5, 15 and 45nM to ensure selectivity. CAL-101 is a selective PI3K δ inhibitor with IC₅₀ of 820nM for p110 α , 565nM for p110 β , 2.5nM for p110 δ and 89nM for p110 γ . This inhibitor was tested *in vitro* at 0.28, 0.83, 2.5, 7.5, 22.5, 67.5, and 202.5nM to maximize the drug's specificity.

The gp100₂₅₋₃₃ 9-mer peptide (KVPRNQDWL) (ANASPEC) was used for *in vitro* activation of pMel-1 splenocytes at 1 μ M as described (10).

For *in vivo* experiments, the vaccine was prepared using the same gp100₂₅₋₃₃ peptide and administered at 100 μ g per mouse in combination with PADRE at 10 μ g and Quil-A at 25 μ g per mouse.

Lymphodepletion of mice was achieved using a combination of 250mg/kg cyclophosphamide (Sigma) and 50mg/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 gp100₂₅₋₃₃ peptide at 1 μ M (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 IL-2 (100U/ml) (Peprotech) at 37°C with 5% CO₂. 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 48-72 hours.

On Days 7, 14 and 21, cells were re-stimulated with gp100₂₅₋₃₃ at 1 μ M using feeder cells (irradiated WT splenocytes, 4000 Rads) at 1:1 ratio using the same culture conditions.

TCR stimulation and co-stimulation

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 μ g/ml) and cultured in activation media (IL2 (100U/ml) and anti-CD28 (2.5 μ g/ml)) in the presence or absence of PI3K inhibitors for 72 hrs.

Proliferation assay and phenotyping of CD8⁺ T-cells

Cells were labeled with 5 μ M 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 (Tree Star).

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-V β 13, 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⁻), V β 13⁺CD8⁺ T-cells.

For intracellular staining, cells were stained with the fixable near infra-red Live/Dead viability stain (Life Technologies, Invitrogen), and fixed, permeabilized and stained with APC labeled anti-CD8, V450 labeled anti-V β 13, 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), V β 13⁺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 co-incubated (at 1:1 ratio) with 1 μ M gp100₂₅₋₃₃ pulsed irradiated splenocytes (4000 Rads) for 24 hours using the same culture conditions. Supernatants were collected and the level of IL-2, TNF- α and IFN- γ 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 250mg/kg cyclophosphamide and 50mg/kg fludarabine (CyFlu). On Day 8, gp100 activated CD8⁺ T-cells from pMel-1 mice cultured in the presence or absence of CAL-101 (202.5nM, 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 (gp100₂₅₋₃₃ 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 1.5cm³ according to institutional regulations.

Statistics

Statistical parameters (average values, SD, significant differences between groups) were calculated using Microsoft Excel and GraphPad Prism. Statistical significance between groups

was determined by paired *t* test or one-way ANOVA with *post hoc* Tukey's 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 μ M gp100₂₅₋₃₃.

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 (Figure 1A). Unlike non-treated cells, the higher percentage of memory CD8⁺ T-cells (both T_{CM} and T_{EM}) was maintained after the second and third stimulations (Figure 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 non-treated cells after each stimulation. In Fact, Non-treated 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 lead to a significantly higher

percentage of T_{EM} in GDC treated cells following the third stimulation in comparison to the non-treated 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 non-treated 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.

Since 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 three days of the first stimulation, the proliferation and expansion of CD8⁺ T-cells treated with GDC was only slightly inhibited (Figure 1B), which is expected given the role PI3K plays in the proliferation of T-cells. At the highest concentration tested (279nM) 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 non-treated cells (Figure 1B) Non-treated 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 (Figure 1C).

We next measured IL-2 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 IL-2 secretion after re-encountering the antigen (Figure 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 IFN- γ and TNF- α and the level of Granzyme B production.

CD8⁺ T-cells were re-stimulated on days 7 and 14 (stim2 and 3) and the level of IFN- γ and TNF- α production after 24 hours was assessed. After the second and third stimulations, GDC-treated and non-treated cells produced high and comparable levels of IFN- γ and TNF- α in response to antigen re-encounter (Figure 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 to CD8⁺ T-cell that were not treated with PI3K inhibitors .

The maintained levels of IFN- γ and TNF- α 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 non-treated cells (Figure 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 to non-treated cells (Figure 2A and Supplemental Figure 1). As expected, due to memory recall, this effect was less prominent after the second and third stimulations when

compared to 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}) (Figure 2A and Supplemental Figure 1), 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 isoforms inhibitors. While we found that the inhibition of PI3K- δ marginally inhibited the proliferation of CD8⁺ T-cells compared to cells treated with PI3K- α and PI3K- β inhibitors (Figure 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) (Figure 2C). We also found that treatment of CD8⁺ T-cells with the PI3K- δ inhibitor maintained high expression levels of CD62L (Figure 3A) and high secretion levels of IL-2 (Figure 3B and Supplemental Figure 2), consistent with the enhanced proliferative ability of the memory CD8⁺ T-cells. These high levels of CD62L expression and IL-2 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 TNF- α and IFN γ secretion (Figure 4A and Supplemental Figure 2) and Granzyme B (Figure 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 to knocking down PI3K α or β (Supplemental Figure 3A). Furthermore, the proliferation of CD8⁺ T-cells and their ability to produce Granzyme B was not affected by the knock down of any of the isoforms (Supplemental Figure 3A).

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 (Supplemental Figure 3B). 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 - β were inhibited (Supplemental Figure 3B).

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 anti-tumor 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 anti-tumor 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 assessed. Similar to what is presented above, treated cells consisted of a 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) (Figure 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 anti-tumor 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 (Figure 5B and 5C).

Furthermore, the combination of ACT of CAL-101 treated CD8⁺ T-cells with the vaccine greatly prolonged the animal survival (Figure 5D). Similar results were obtained when treatment was started at a later date with larger tumors (Supplemental Figure 4).

These data clearly demonstrate the superior anti-tumor 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 mediation of therapeutic antitumor immunity when compared to terminally differentiated effector cells. T_{CM} cells are by far superior to T_{EM} cells, due to their greater proliferative capacity upon antigen re-encounter. 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 and proliferative ability upon re-encountering 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 IL-2 secretion. We further demonstrate that

CD8⁺ T-cells treated specifically with an inhibitor of PI3K- δ greatly enhance their anti-tumor 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 IL-2 (3). Here, we further show that PI3K- δ inhibition maintains a higher level of IL-2 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 et al (2008) 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, IFN γ 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 anti-tumor 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 since memory CD8⁺ T-cells respond to antigen re-encounter 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 anti-

tumor 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 (Supplemental Figure 5). 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). Our data in this manuscript 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 anti-viral 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 to WT mice (Supplemental Figure 6). 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 anti-tumor 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.

Acknowledgements

The authors would like to thank Dr Frank Ward for his advice on experimental design.

References

1. Kim EH, Suresh M. Role of PI3K/Akt signaling in memory CD8 T cell differentiation. *Front Immunol.* 2013;4:20.
2. Klebanoff CA, Gattinoni L, Restifo NP. CD8+ T-cell memory in tumor immunology and immunotherapy. *Immunol Rev.* 2006;211:214-24.
3. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature.* 1999;401:708-12.
4. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *Proc Natl Acad Sci U S A.* 2005;102:9571-6.
5. Roberts AD, Ely KH, Woodland DL. Differential contributions of central and effector memory T cells to recall responses. *J Exp Med.* 2005;202:123-33.
6. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol.* 2003;4:225-34.
7. Wu F, Zhang W, Shao H, Bo H, Shen H, Li J, et al. Human effector T cells derived from central memory cells rather than CD8T cells modified by tumor-specific TCR gene transfer possess superior traits for adoptive immunotherapy. *Cancer Lett.* 2013.

8. Kim EH, Sullivan JA, Plisch EH, Tejera MM, Jatzek A, Choi KY, et al. Signal integration by Akt regulates CD8 T cell effector and memory differentiation. *J Immunol*. 2012;188:4305-14.
9. Crompton JG, Sukumar M, Roychoudhuri R, Clever D, Gros A, Eil R, et al. Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. *Cancer Res*. 2014.
10. Abu Eid R, Friedman KM, Mkrtychyan M, Walens A, King W, Janik J, et al. Akt1 and -2 inhibition diminishes terminal differentiation and enhances central memory CD8+ T-cell proliferation and survival. *Oncoimmunology*. 2015;4:e1005448.
11. Food-and-Drug-Administration. FDA Alerts Healthcare Professionals About Clinical Trials with Zydelig (idelalisib) in Combination with other Cancer Medicines. 2016 [cited; Available from:
12. Medicines-and-Healthcare-products-Regulatory-Agency. Idelalisib (Zydelig): interim measures following signal of serious infection and deaths related to infection found in clinical trials; 2016.
13. Zhou S, Tao H, Zhen Z, Chen H, Chen G, Yang Y. Depletion of CD4+ CD25+ regulatory T cells promotes CCL21-mediated antitumor immunity. *PLoS One*. 2013;8:e73952.
14. Finlay D, Cantrell D. Phosphoinositide 3-kinase and the mammalian target of rapamycin pathways control T cell migration. *Ann N Y Acad Sci*. 2010;1183:149-57.
15. Kane LP, Weiss A. The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol Rev*. 2003;192:7-20.
16. van der Waart AB, van de Weem NM, Maas F, Kramer CS, Kester MG, Falkenburg JH, et al. Inhibition of Akt-signaling promotes the generation of superior tumor-reactive T cells for adoptive immunotherapy. *Blood*. 2014.

17. Li Q, Rao R, Vazzana J, Goedegebuure P, Odunsi K, Gillanders W, et al. Regulating mammalian target of rapamycin to tune vaccination-induced CD8(+) T cell responses for tumor immunity. *J Immunol.* 2012;188:3080-7.
18. Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, et al. mTOR regulates memory CD8 T-cell differentiation. *Nature.* 2009;460:108-12.
19. Mineharu Y, Kamran N, Lowenstein PR, Castro MG. Blockade of mTOR Signaling via Rapamycin Combined with Immunotherapy Augments Anti-glioma Cytotoxic and Memory T cells' Functions. *Mol Cancer Ther.* 2014.
20. Sinclair LV, Finlay D, Feijoo C, Cornish GH, Gray A, Ager A, et al. Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. *Nat Immunol.* 2008;9:513-21.
21. Ahmad S, Mkrtychyan M, Abu Eid R, Shrimali R, Doroodchi A, Khleif SN. Functional redundancy of PI3K isoforms in conventional T cells provides a selective Treg-targeting strategy through inhibition of PI3K-delta isoform. *Journal for ImmunoTherapy of Cancer.* 2014;2:O4.
22. Ali K, Soond DR, Pineiro R, Hagemann T, Pearce W, Lim EL, et al. Inactivation of PI(3)K p110delta breaks regulatory T-cell-mediated immune tolerance to cancer. *Nature.* 2014;510:407-11.
23. Ahmad S, Abu-Eid R, Shrimali RK, Webb M, Verma V, Doroodchi A, et al. Differential PI3Kdelta signaling in CD4+ T cell subsets enables selective targeting of T regulatory cells to enhance cancer immunotherapy. *Cancer Res.* 2017.
24. Pearce VQ, Bouabe H, MacQueen AR, Carbonaro V, Okkenhaug K. PI3Kdelta Regulates the Magnitude of CD8+ T Cell Responses after Challenge with *Listeria monocytogenes*. *J Immunol.* 2015;195:3206-17.

25. Gracias DT, Boesteanu AC, Fraietta JA, Hope JL, Carey AJ, Mueller YM, et al. Phosphatidylinositol 3-Kinase p110delta Isoform Regulates CD8+ T Cell Responses during Acute Viral and Intracellular Bacterial Infections. *J Immunol.* 2016;196:1186-98.

Figure Legends

Figure 1:

PI3K inhibition preserves the memory phenotype and enhances the proliferative ability of CD8+ T-cells. Non-fractionated splenocytes from pMel-1 mice were stained with violet cell trace (VCT) and activated with gp100₂₅₋₃₃ peptide (1 μ M) in the presence or absence of GDC-0941 (11, 33, 99 and 279nM). The cells were re-stimulated with gp100₂₅₋₃₃ on days 7 and 14 and their phenotype and proliferation assessed. Gated cells were viable (7AAD-) CD8+V β 13+.

A) In this representative example (left panel), 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 non-treated cells (36%) compared to 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 to only less than 0.01 and 64% in the non-treated 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 expand 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 panel shows a representative example of the CD62L expression. Bottom panel shows mean fluorescence intensity (MFI) for CD62L expression (data from at least 2 independent experiments). * $p < 0.05$, ** $p < 0.01$
- D) GDC-treated CD8⁺ T-cells secrete significantly higher levels of IL-2 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 IFN γ and TNF- α 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. Non-fractionated splenocytes from pMel-1 mice were stained with VCT and activated with gp100₂₅₋₃₃ peptide (1 μ M) in the presence or absence of A66 (32, 96 and 288nM), TGX-221 (5, 15 and 45nM) or CAL-101(0,28, 0.83, 2.5, 7.5, 22.5, 67.5 or 202.5nM). The cells were re-stimulated with gp100₂₅₋₃₃ on days 7, 14 and 21. Gated cells were viable (7AAD-)CD8⁺V β 13⁺.

- 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 non-treated cells, while CAL-101 treated cells have a significantly higher percentage of T_{CM}

(CD62L^{hi}CD44^{hi}) (32% compared to 4%, D3, stim1) and T_{EM} cells (CD62L^{lo}CD44^{hi}) (95% compared to 64%, D7, Stim3), and a lower percentage of terminally differentiated T-cells (CD62L^{lo}CD44^{lo}) (5% compared to 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 (day3).

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 . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3:

The inhibition of PI3K- δ , but not PI3K- α or PI3K- β , preserves high expression levels of CD62L and high secretion of IL-2 in CD8⁺ T-cells. Non-fractionated splenocytes from pMel-1 mice were stained with VCT and activated with gp100₂₅₋₃₃ peptide (1 μ M) in the presence or absence of A66 (32, 96 and 288nM), TGX-221 (5, 15 and 45nM) or CAL-101(0,28, 0.83, 2.5, 7.5, 22.5, 67.5 or 202.5nM). The cells were re-stimulated with gp100₂₅₋₃₃ on days 7, 14 and 21. Gated cells were viable (7AAD⁻)CD8⁺V β 13⁺.

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 secrete significantly higher levels of IL-2 following stimulation 3, which is consistent with their higher proliferative potential. Data normalized to GP100, * $p < 0.05$.

Figure 4:

PI3K inhibition does not affect the secretion of IFN γ and TNF- α . CD8 $^+$ T-cells from pMel-1 mice were stimulated with gp100₂₅₋₃₃ peptide (1 μ M) in the presence or absence of GDC-0941 (99 μ M), A66 (288nM), TGX-221 (45nM) or CAL-101 (202.5nM). On days 7 and 14, CD8 $^+$ T-cells were re-stimulated with gp100₂₅₋₃₃ peptide and the IFN γ and TNF- α 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 IFN γ and TNF- α 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.

Figure 5:

The inhibition of PI3K- δ in CD8 $^+$ T-cells significantly enhances their anti-tumor 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 day 8, 15 and 22. Animal survival and tumor growth was 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 non-treated CD8 (n=5), CD8 + Vac -ACT of non-treated 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 3-4 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 to all the other groups.
- C) Mean tumor volume for different groups shown in 5B. 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 to non-treated 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.

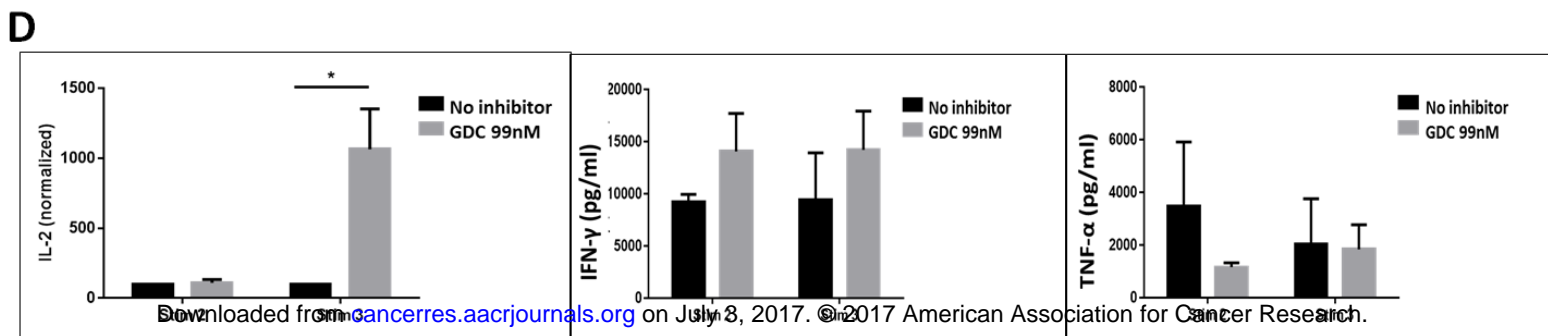
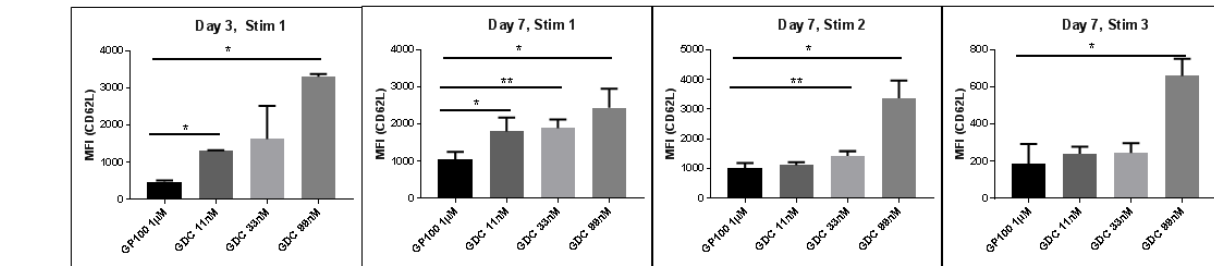
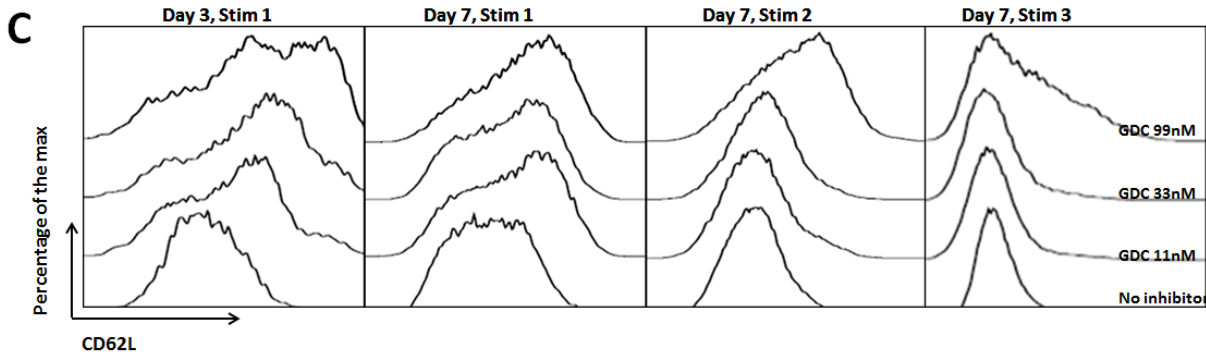
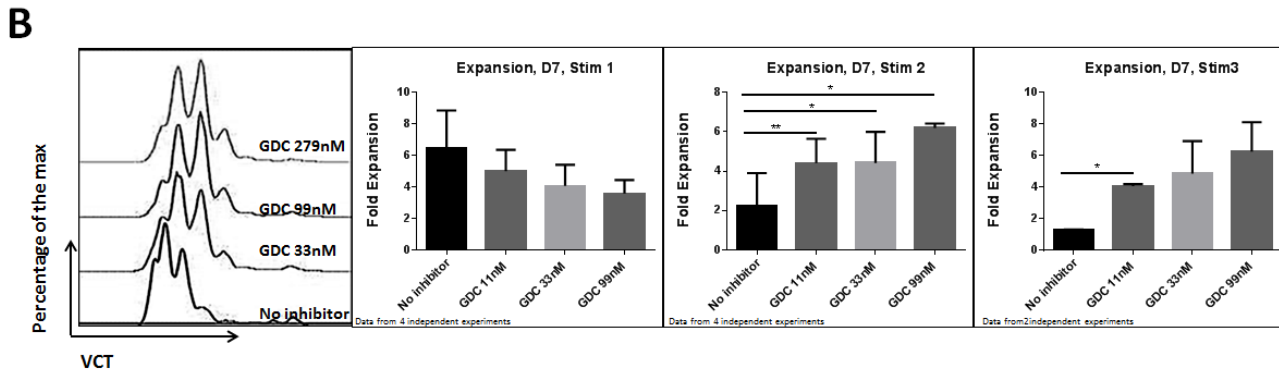
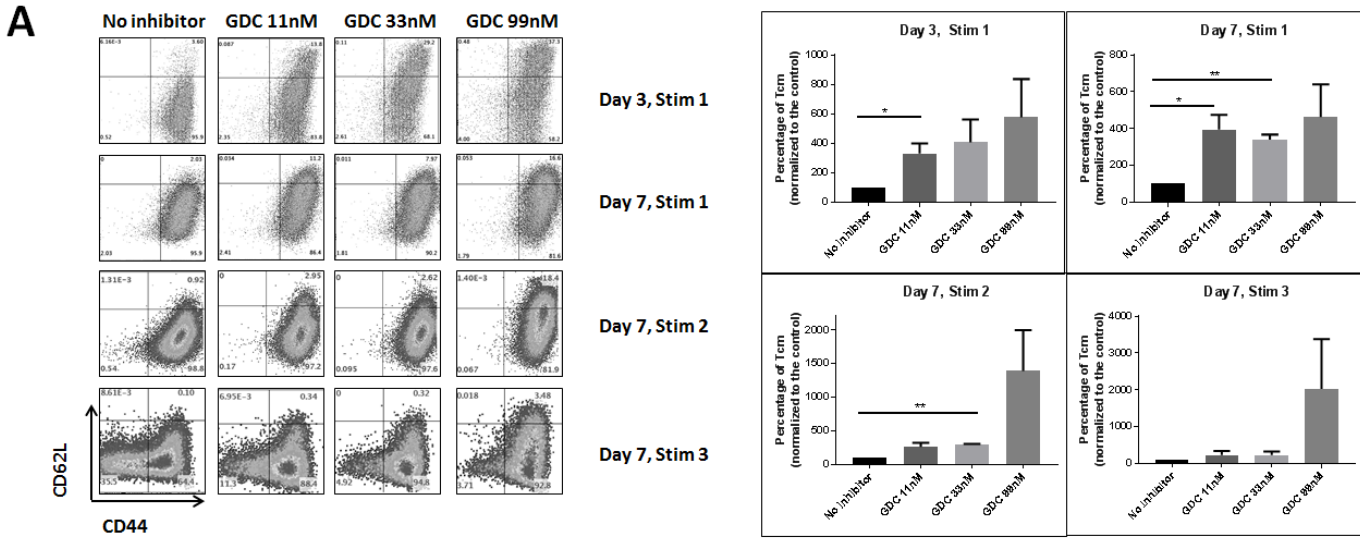
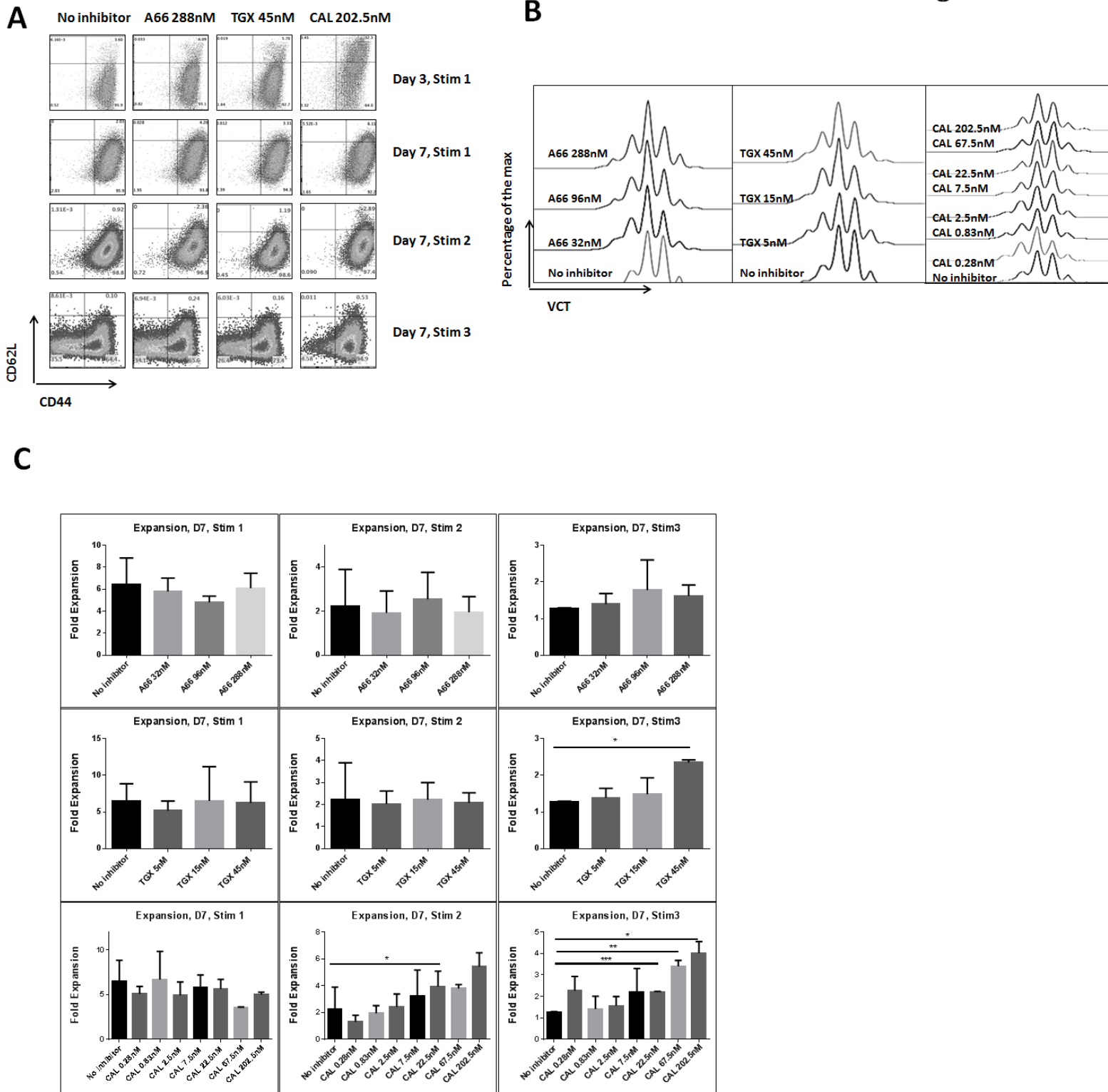
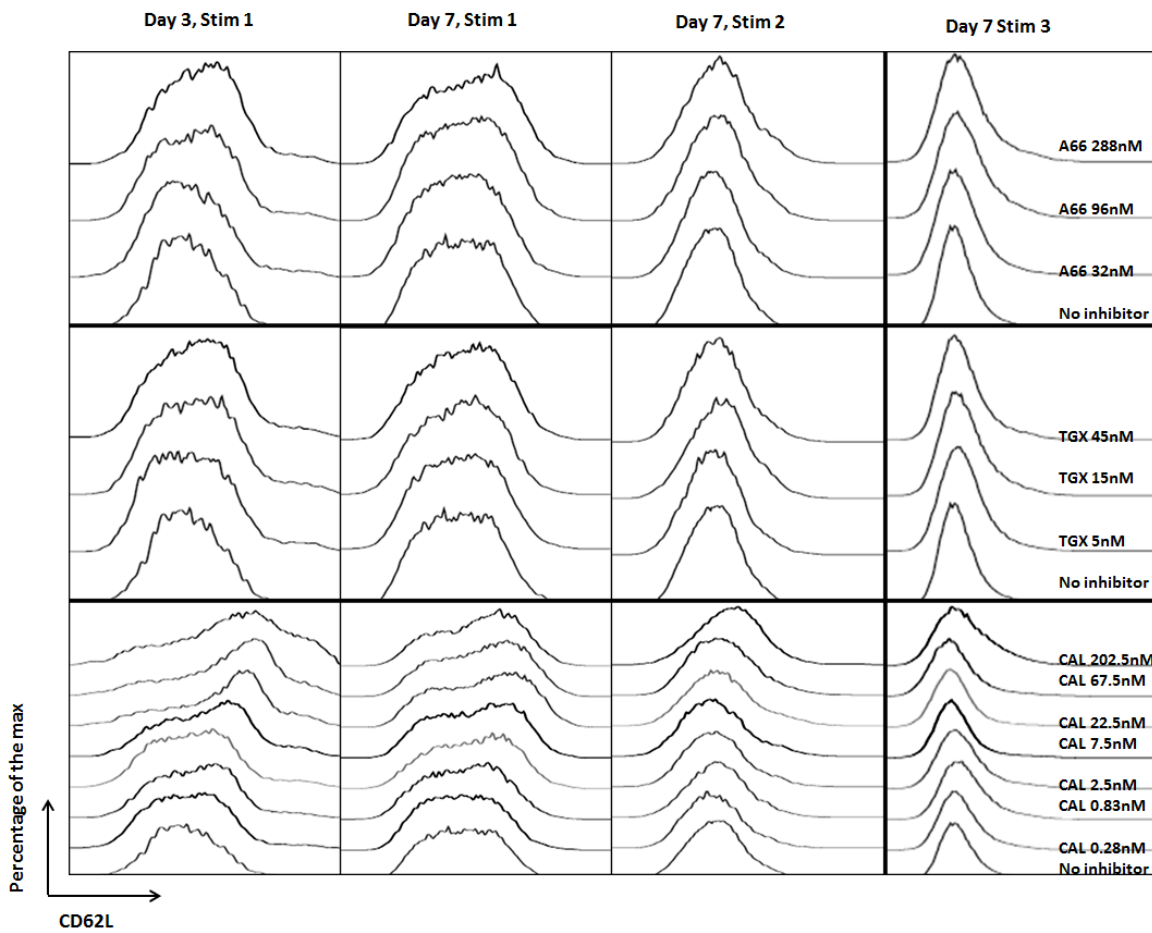


Figure 2



A



B

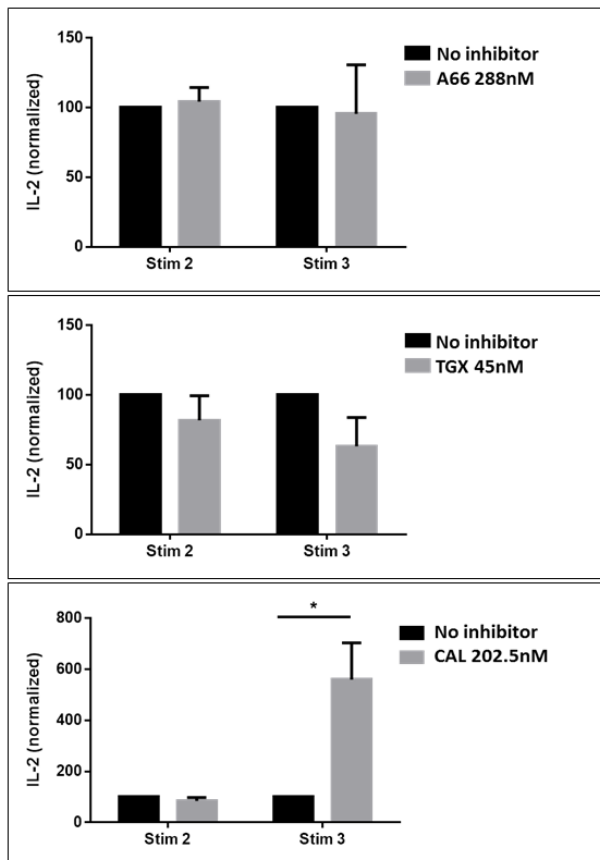
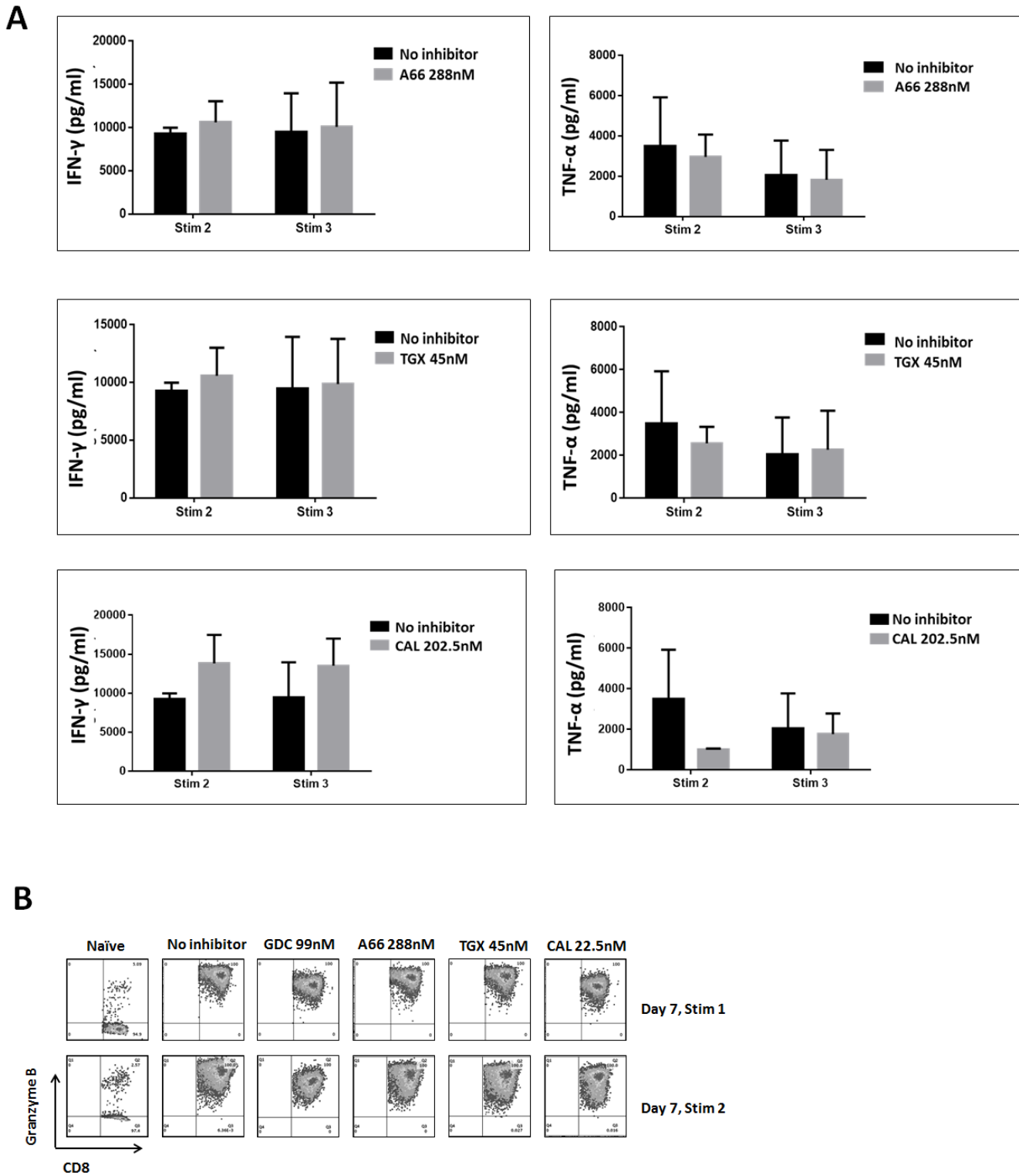
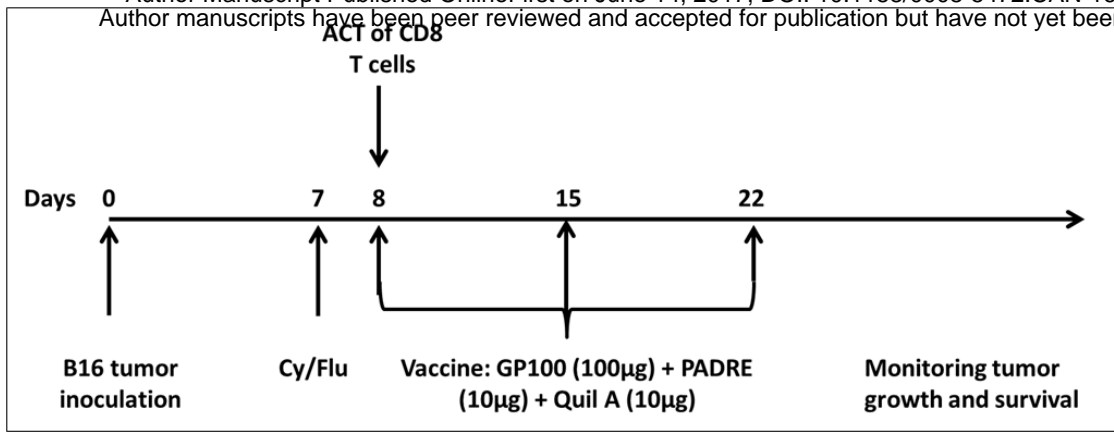


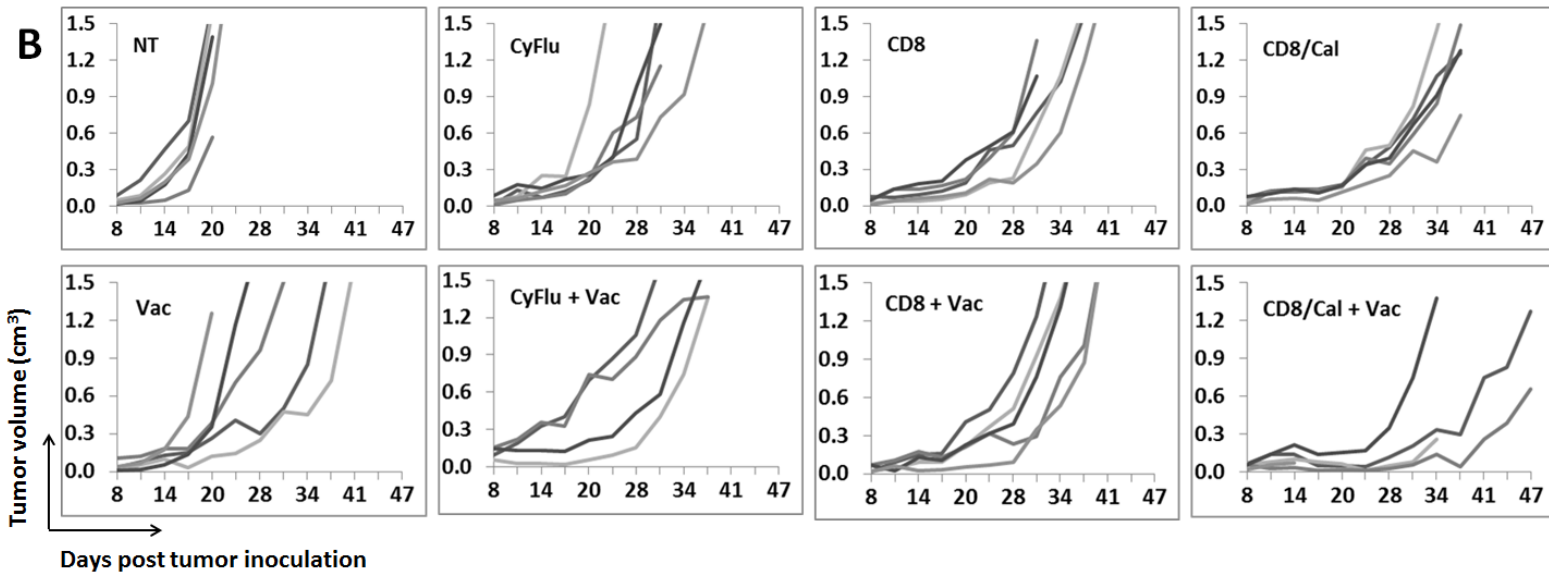
Figure 4



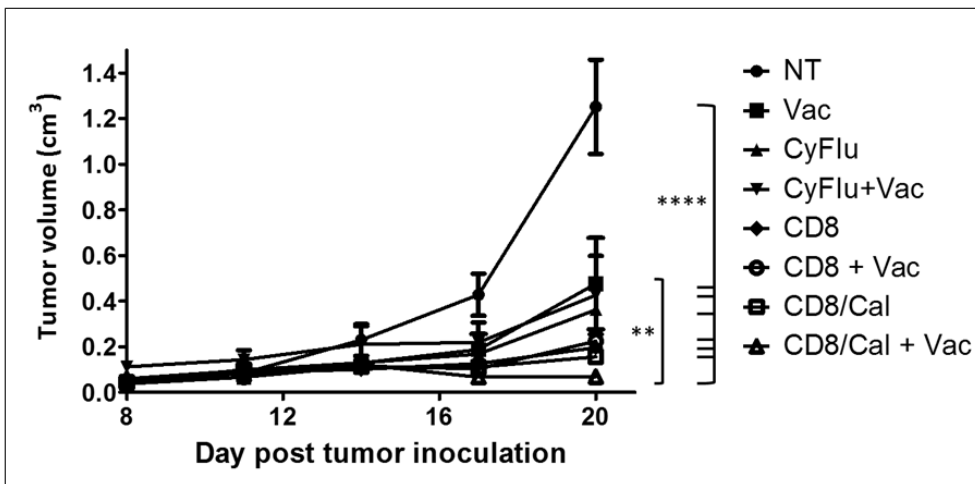
A



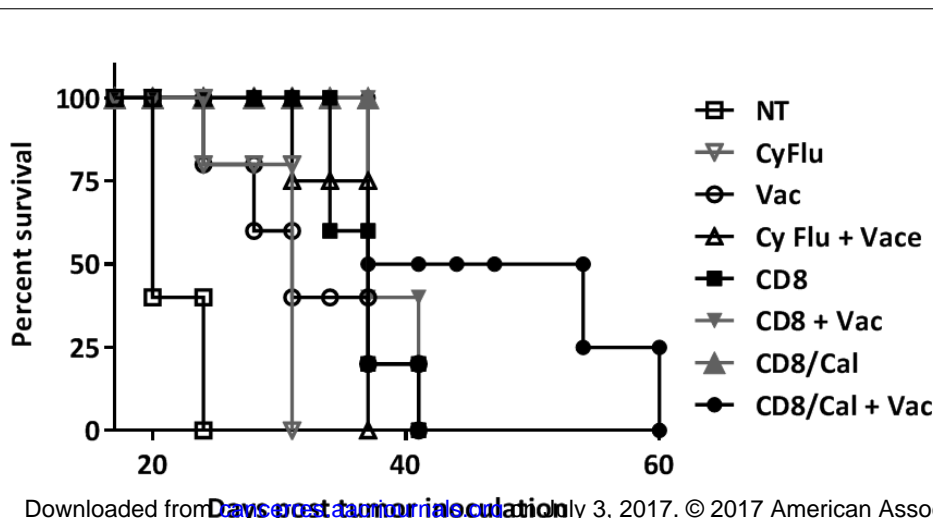
B



C



D



Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Enhanced therapeutic efficacy and memory of tumor specific CD8 T cells by *ex-vivo* PI3K- δ inhibition

Rasha Abu-Eid, Shamim Ahmad, Yuan Lin, et al.

Cancer Res Published OnlineFirst June 14, 2017.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-16-1925
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2017/06/14/0008-5472.CAN-16-1925.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.