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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

METABOLIC REPROGRAMMING OF T CELLS TO OPTIMIZE ADOPTIVE T CELL THERAPY

by

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B.A., Southern Methodist University, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2019

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METABOLIC REPROGRAMMING OF T CELLS TO OPTIMIZE ADOPTIVE T CELL THERAPY

ALEXANDRA WALLER

ABSTRACT

The clinical efficacy of adoptive T cell therapies including CART therapy are limited by poor in vivo persistence and moderate anti-tumor efficacy. According to the literature, metabolism plays a critical role in the phenotypic state and fate of T cells during antigen-driven expansion. During different stages of a T cell life cycle, the predominant pathway used for metabolism changes. Naïve T rely on oxidative phosphorylation, but as the T cells becomes activated, their metabolic profile switches to become more reliant on glycolysis. Most T cells become terminally differentiated and become senescent once they have performed their cytotoxic function. A minority of the activated T cells gradually start to rely on oxidative phosphorylation once again and become memory T cells. Memory T cells can become either effector memory or central memory T cells. These memory T cells, specifically central memory T cells, are the key to T cells persistence during both ex vivo and in vivo expansion and following disappearance of the antigenic stimulus. Since the metabolic profile of the T cells plays a critical role in its differentiation state, we tested the hypothesis that inhibitors of intermediary metabolism could promote a metabolic profile that is more desirable for the optimal phenotype consistent with the memory phenotype that would favor persistence in spite of strong activation signals. The four inhibitors screened were: a PFKFB3 inhibitor,

an inhibitor of a key step in glycolysis; ibrutinib, an inhibitor of Bruton's tyrosine kinase; idelalisib, an inhibitor of PI3Kδ subunit; and duvelisib, an inhibitor of PI3Kδ and PI3K gamma subunits. To test this hypothesis, T cells were cultured with or without each compound and then the analysis included: phenotypic analysis by flow cytometry, quantitative analysis by counting cells with ethidium bromide acridine orange, and metabolic profiling by the Seahorse assay.

This study was conducted using T cells from a human healthy volunteer that were collected by apheresis. T cells were cultured in a G-Rex plate for 15 days with complete media supplemented with recombinant human IL-2 (30 U/mL). Cells were activated on day 1 and day 8 by the addition of anti-CD3/CD28 beads and test metabolic inhibitor compounds were added every 4 days.

T cells cultured with idelalisib, duvelisib, and ibrutinib had increased expansion (approximately50-fold: idelalisib/ duvelisib and 21-fold ibrutinib) when compared to control (cells with beads alone) with only 6-fold expansion. Phenotypic analysis performed using flow cytometry showed an increased percentage of CD27+ CD28+ in the CD8+ and CD4+ T cell cell populations in the idelalisib treated group and decreased number of senescent T cells that are double negative for CD27 and CD28. Consistent with our hypothesis, metabolic analysis showed that cells treated with idelalisib and duvelisib were more reliant on oxidative phosphorylation, rather than glycolysis as compared to the control cultures. Cells treated with duvelisib also showed an increased spare respiratory capacity (SRC), which is associated with more efficacious memory T cells.

The results of these studies show that metabolism plays a critical role in the long-term survival of T cells. We demonstrate that inhibiting intermediary metabolism, specifically inhibiting PI3K, favorably alters the metabolic state of the T cells leading to increased cell numbers and T cells with a phenotype consistent with enhanced ex vivo and in vivo proliferation and persistence.

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LIST OF ABBREVIATIONS

6AN……………………………………………………………6-aminonicotinamide

AF…………………………………………………………………………Alexa Fluor

AICD……………………………………………………… Activation induced cell death

AMPK……………………………………………………... 5’ AMP-activated protein kinase

ANOVA ……………………………………………………......... Analysis of variation

APC ……………………………………………………………......... Allophycocyanin

BCA ………………………………………………………… Bicinochoninic acid assay

BSA ……………………………………………………………. Bovine serum albumin

CART ………………………………………………Chimeric antigen receptor T cell

DMSO ……………………………………………………… ...Dimethyl sulfoxide

ECAR …………………………………………………….. Extracellular acidification rate

ECL ……………………………………………………. Enhanced chemiluminescence

F2,6P2 …………………………………………………. Fructose 2,6- bisphosphate

FBS ……………………………………………………………. Fetal Bovine Serum

FCCP……………………………… Trifluormethoxy carbonylcyanide phenylhydrazone

GDH ……………………………………………………... Glutamine dehydrogenase

HRP ………………………………………………………… Horseradish peroxidase

IL-2 ………………………………………………………………….. Interleukin-2

LKB1 ……………………………………………………………… Liver kinase B1

MHC ………………………………………………… Major histocompatibility complex

mTOR1 ………………………………………………… Mechanistic target of rapamycin 1

NFAT ……………………………………………………. Nuclear factor of activated T cells

OCR………………………………………………………… Oxygen consumption rate

PBMCs ……………………………………………… Peripheral blood mononuclear cells

PE ……………………………………………………………………….. Phycoerythrin

PERCP ………………………………………….. Peridinin Chlorophyll Protein Complex

PFK-1 ……………………………………………………….. 6-phosphofructo-1-kinase

PFKFB3 ……………………… 6 phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 3

PI3K ………………………………………………………... Phosphoinositide 3-kinase

PIP2 ……………………………………………..... Phosphatidylinositol 4,5- bisphosphate

PIP3 ………………………………………… Phosphatidylinositol (3,4,5) – trisphosphate

PLK-gamma ………..……………………………………... Polo-like kinase- gamma

SRC …………..…………………………………………… Spare refractory capacity

TBS-T ………………………………………………………….... Tris-buffered saline

TCR ……………………………………………………………………….. T cell receptor

INTRODUCTION

Field of Immunotherapy

An emerging field in medicine is immunotherapy, which involves modulating immune cells so that they can more effectively fight cancer and infections. A current limitation in this field is defining the optimal way to expand immune cells to ensure their growth and survival in vivo. To improve the technology of adoptive T cell therapy it is necessary to understand the process of T cell activation and how T cell proliferation is regulated.

Activation of T cells

As T cells are activated via engagement of the T cell receptor with peptide bound to a major histocompatibility complex (MHC) molecule in the context of a second costimulatory signal, they go through different phases of expansion
and effector differentiation, which utilize different metabolic pathways. Naïve T cells, which have not yet encountered an antigen, as well as memory T cells,
rely predominantly on oxidative phosphorylation for their energy needs. Oxidative phosphorylation leads to a higher amount of usable energy, but this energy is generated at a slower rate than that produced through glycolysis (Jones et al., 2017). Since, quiescent T cells, such as naïve and memory T cells are not actively dividing in response to a disease or infection, they do not require rapid availability of metabolic energy, as do activated effector T cells. When these naïve T cell recognize a foreign antigen complexed

to a MHC molecule, they are activated and undergo a rapid burst of expansion that requires quickly accessing metabolic energy stores, as well as generating metabolites that are required for lipid biosynthesis needed for cell division. Two sources of stimulation are required for complete activation. The first involves the T cell receptor (TCR), which is antigen specific, and the other is a co-stimulatory signal from CD28 (O'Sullivan & Pearce, 2015). When the TCR is engaged with the MHC the expression of the glucose transporters at the plasma membrane are upregulated and glycolytic flux is increased, which plays an important role in the phenotypic fate of the T cells (Hukelmann et al., 2016; Macintyre et al., 2014; Salmond, 2018). Activated T cells also upregulate the uptake of other sources of metabolic energy, such as amino acids, especially glutamine, which is converted to glutamate by glutamine dehydrogenase (GDH) through glutaminolysis (Klein Geltink, Kyle, & Pearce, 2018). Activated naïve T cells can differentiate into memory and effector T cell subsets. Less differentiated T cells, which mainly rely on oxidative in a quiescent state, have increased proliferative capacity, due to their ability to persist longer without becoming exhausted (Sukumar, Kishton, & Restifo, 2017). As the T cells become more differentiated, their ability to perform effector functions increases, as does their reliance on aerobic glycolysis, rather than oxidative phosphorylation (Chang et al., 2013; Krauss, Brand, & Buttgereit, 2001; Rathmell, Vander Heiden, Harris, Frauwirth, & Thompson, 2000; Roos & Loos, 1973; G. J. W. van der Windt et al., 2013). The different metabolic pathways involved in T cell metabolism can be seen in figure 1.

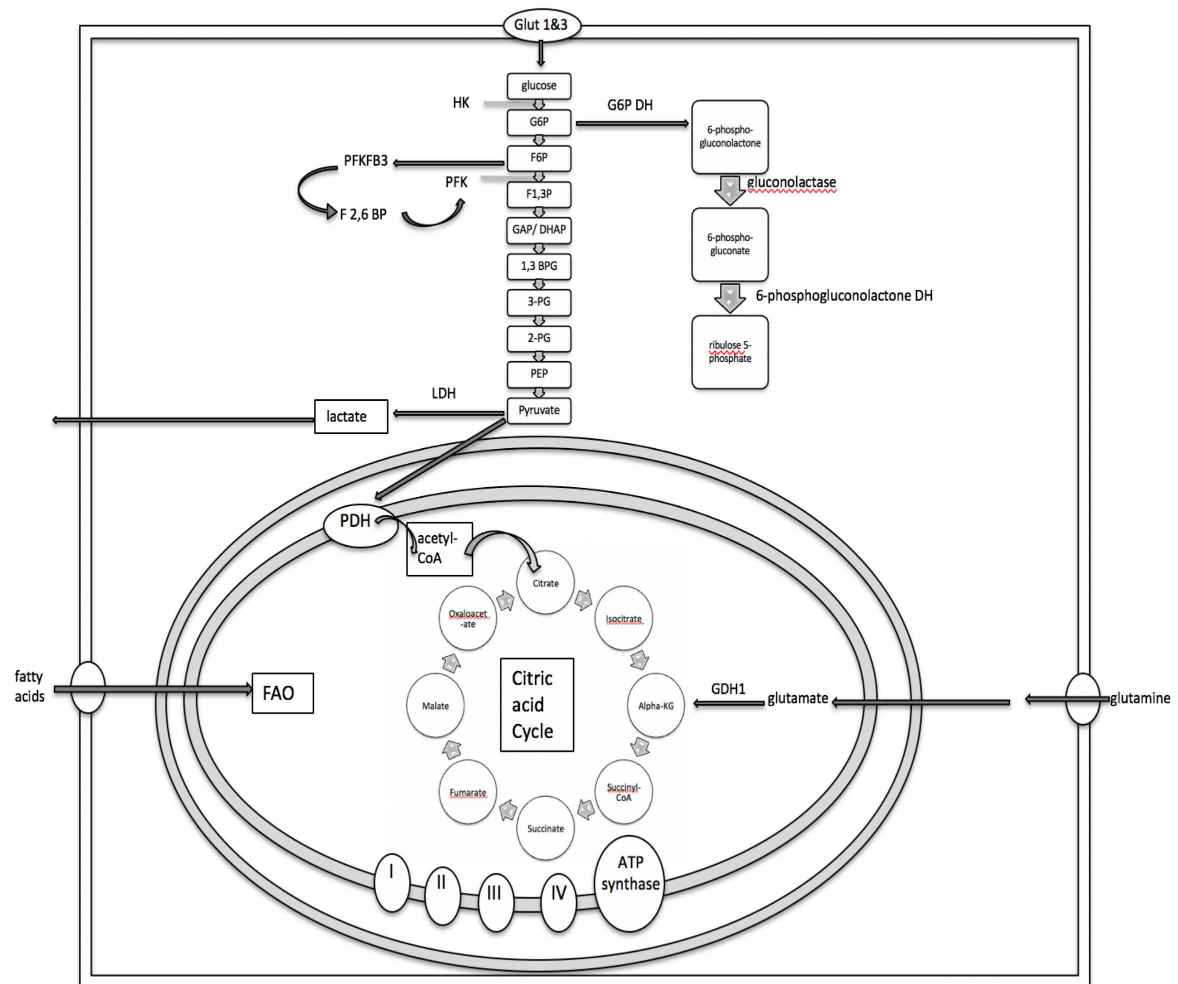


Figure 1: Metabolic pathways involved in T cell metabolism. Throughout the growth and differentiation of T cells, the metabolic pathways they predominantly use changes. Quiescent T cells predominantly utilize oxidative phosphorylation, whereas activated effector T cells actively fighting an infection rely more on glycolysis.

T cell Metabolism

The predominant use of aerobic glycolysis is seen in many cancer cells where it is known as the Warburg effect. Aerobic glycolysis produces lactic acid even when oxygen is present. Though ATP production is fast through this metabolic pathway, it is energetically inefficient because it only yields two ATP molecules per glucose, as compared to 30-36 molecules of ATP per glucose molecule generated through oxidative phosphorylation. Most of the molecules of pyruvate at the end of aerobic glycolysis are converted to lactate by lactate dehydrogenase, even when oxygen is present, and then excreted from the cell, rather than going on to mitochondrial metabolism (Salmond, 2018). The benefit of aerobic glycolysis in highly active cells, such as effector T cells, is that it also generates metabolic intermediates from glucose that can be utilized by the T cell for synthesis of lipids, proteins, carbohydrates, and amino acids required for cell division and synthesis of effector molecules needed to fight infections (Abbas, Lichtman, Pillai, & Preceded by: Abbas, n.d.; O'Sullivan & Pearce, 2015). Once the disease has been eliminated and the antigenic stimulus has been cleared most of the effector T cells die off and only a fraction of the memory T cells remain that serve as the basis for an anamnestic and secondary expansion of antigen specific T cell in case the antigen is encountered again. Memory T cells that remain have the ability to persist without requiring lots of metabolic fuel and energy, giving them a survival advantage. The memory T cells rely on oxidative phosphorylation, as well as fatty acid oxidation to persist in a quiescent state until the antigen is encountered again. Memory T cells have a larger mitochondrial mass, fused cristae, and a larger spare refractory capacity (SRC), all

of which favor oxidative metabolism (Gerritje J W van der Windt et al., 2012). This enhanced SRC is due to the build of energy from oxidative phosphorylation, as well as through the use of fatty acid oxidation, adding to the mitochondrial energy produced (Buck et al., 2016; Salmond, 2018). The mitochondrial capacity of a cell can be measured using the Seahorse assay, as shown in figure 2A. The seahorse assay involves successive addition of inhibitors of mitochondrial proteins and measurement of proton flux across the mitochondrial membrane. As inhibitors are added (figure 2B), the measured energy flux corresponds with specific metabolic functions involved in oxidative phosphorylation of the cells (figure 2C).

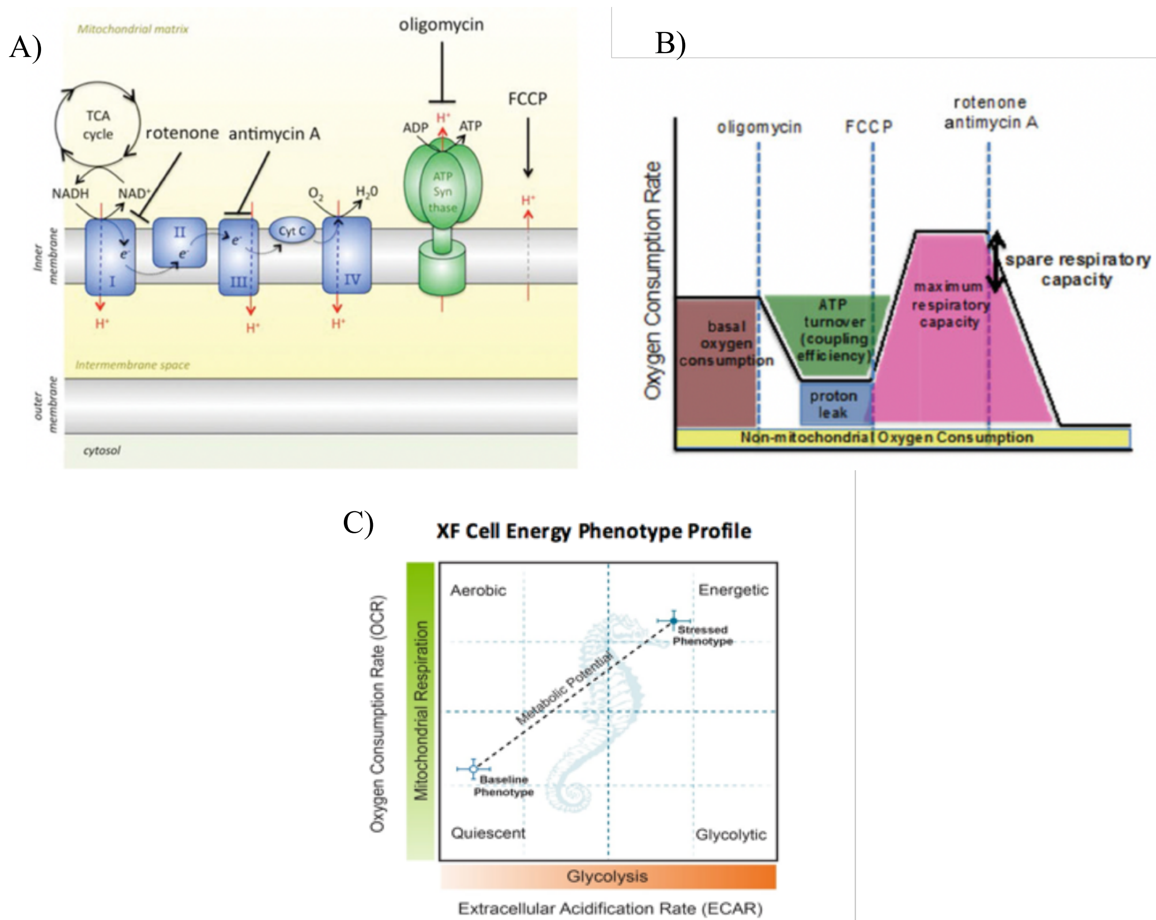


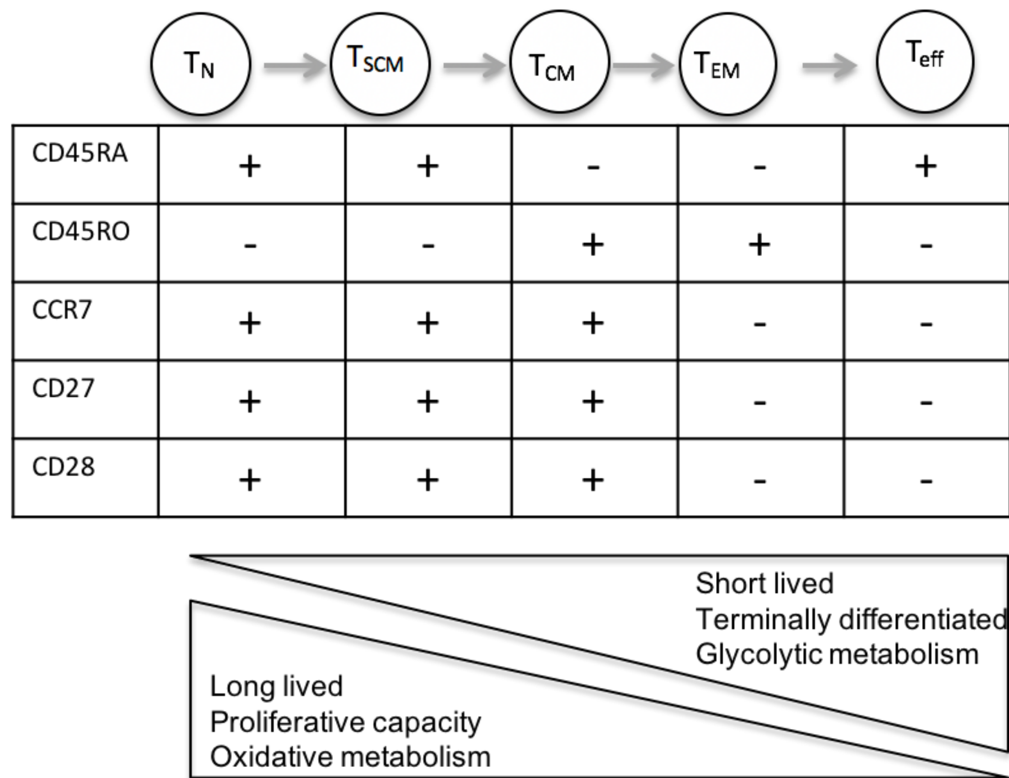
Figure 2: Schematic overview of Seahorse assay. A) Graphical overview of mitochondrial inhibitors including, oligomycin, an ATP synthase inhibitor, FCCP, an ATP synthase uncoupler, rotenone and antimycin A, are complex I and complex III inhibitors, respectively. B) Schematic view of mitochondrial stress test using the Seahorse extracellular flux analyzer. C) Graphic model for cell energy phenotype profile. (Gerritje J W Van Der Windt, Chang, & Pearce, n.d.)

In the event that an antigen is encountered again, memory T cells with a greater SRC will have a proliferative response that is faster and of greater magnitude. More rapid effector function is primarily facilitated because the genes that encode for cytokines and other effector molecules are on open chromatin, therefore leading to transcription of these cytokines at a faster rate. More rapid effector function is due to the greater TCR signaling and the enlarged SRC, leading to more efficient oxidative phosphorylation (DiSpirito & Shen, 2010; Farber, 2009; Klein Geltink et al., 2018; Salmond, 2018; G. J. W. van der Windt et al., 2013). This enables memory T cells to have an increased ability to use and promote oxidative phosphorylation , once reactivated, leading to not only a faster response, but also a larger one (Cho, Wang, Sugawa, Eisen, & Chen, 1999; Grayson, Harrington, Lanier, Wherry, & Ahmed, 2002; Veiga-Fernandes, Walter, Bourgeois, McLean, & Rocha, 2000). Overall, fast growth tends to lead to death and slow growth tends to lead to survival. Our overall hypothesis is that inhibition of the fast growth might result in net increase in T cells by limiting activation induced cell death.

Inhibitors for Metabolic Reprogramming

The use of inhibitors of metabolism to balance activation and senescence within the T cells is a novel approach to promote an optimal phenotype for persistence.

According to recent literature, certain T cell phenotypes, specifically the central memory T cells, are advantageous in the field of adoptive T cell therapy due to their increased survival and persistence in vivo. Central memory T cells are slightly more differentiated than the naïve T cells, but retain expression of CD27 and CD28, which are lost when T cells are fully differentiated, and the loss of these costimulatory receptors is characteristic of terminally differentiated T cells, including those that are considered senescent as seen in Figure 3 (Petersen et al., 2018).



	T_N	T_{SCM}	T_{CM}	T_{EM}	T_{eff}
CD45RA	+	+	-	-	+
CD45RO	-	-	+	+	-
CCR7	+	+	+	-	-
CD27	+	+	+	-	-
CD28	+	+	+	-	-

Figure 3: Differentiation of T cells. As T cells differentiate from naïve to terminal effector there is a difference seen in life span, predominant metabolism, and cell surface marker

Naïve and central memory T cells are also CCR7 positive, which is a lymphoid homing receptor. The presence of CCR7 helps coordinate the migration of naïve and memory T cells to secondary lymphoid organs where they may encounter peptide antigens on MHC molecules presented by dendritic cells. Effector T cells downregulate expression of CCR7 following activation in response to peptide antigen and co-stimulation and do not require interaction with a dendritic cell in a secondary lymphoid organ for antigen presentation. CD8+ effector T cells instead migrate to sites of inflammation based upon a different set of chemokine receptors and homing molecules to help fight the infection by killing virally infected cells that express virally encoded peptides expressed by MHC class I and are short-lived without replicative capacity, a state that is termed "senescent"(Finlay et al., 2012; Sinclair et al., 2013).The predominant use of glycolysis as an energy source has been associated with effector T cells, which have been shown to persist poorly ex vivo, as well as in vivo. Therefore, modulating metabolism by inhibiting or suppressing glycolysis via small molecule inhibitors could be advantageous to ex vivo production and expansion of T cells with the desired memory phenotype. A decrease in glycolytic flux could help promote the use of oxidative phosphorylation of T cells, therefore directing them towards the more persistent phenotype associated with memory T cells.

In this thesis research the effects on T cell expansion and phenotype of small molecule inhibitors of metabolism are investigated. As T cell phenotype is known to be heavily determined by metabolism, it may be hypothesized that by inhibiting or forcing a certain metabolism, will lead to T cells with the desirable memory phenotype. An

inhibitor of glycolysis was chosen because short-lived effector T cells are highly glycolytic and we reasoned that inhibiting glycolysis would promote cells to use oxidative phosphorylation, which is associated with long-lived memory T cells. The inhibitor of glycolysis that was used was an inhibitor of 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase 3 (PFKFB3), an enzyme which catalyzes the synthesis of fructose 2,6-bisphosphate (F2,6P2) (Tian). F2,6P2, is a potent allosteric activator of 6-phosphofructo-1-kinase (PFK-1), a rate-limiting enzyme in glycolysis. PFK-1 catalyzes the conversion of glucose-6-phsophate to fructose-6-phosphate (Tian). F2,6P2 binding to PFK-1 increases it's affinity for F6P, therefore allowing glycolysis to persist in the presence of ATP, which is inhibitor of PFK-1. In a highly active cells that produce a lot of ATP, this override by F2,6P2 allows glycolysis to continue even though there is tonic allosteric inhibition by ATP, allowing cells produce additional energy for effector functions. We hypothesized that inhibiting PFKFB3 would limit the cells ability to continue glycolytic metabolism, thus leading it to a more quiescent metabolic state.

As an alternative to directly inhibiting glycolysis, other compounds that were studied indirectly interfered with glycolysis through other mechanisms. Two inhibitors of phosphoinositide 3-kinase (PI3K) inhibit different subunits of PI3K were examined and their pathways can be seen in figure 4 below.

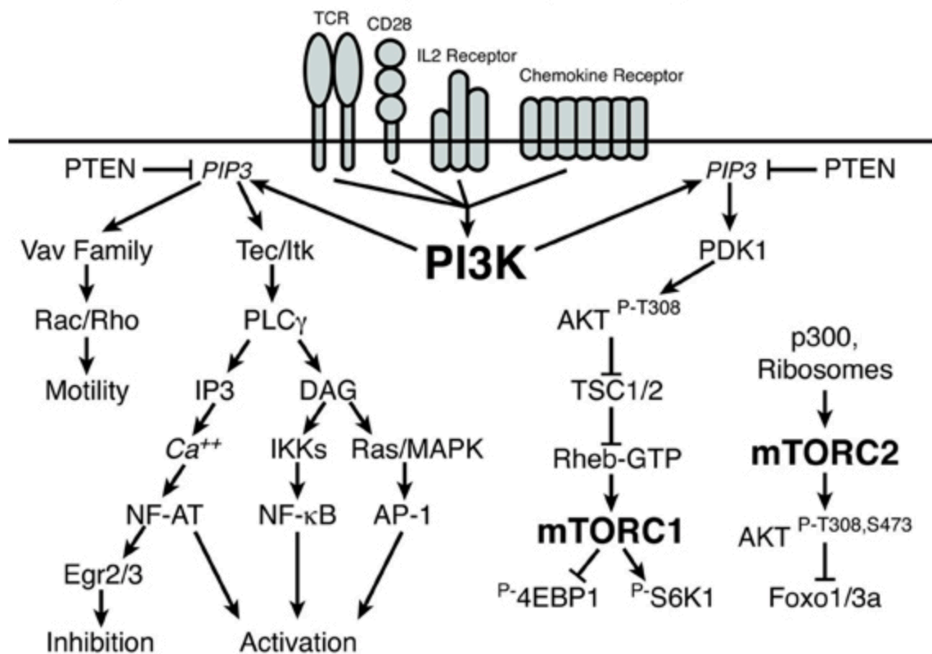


Figure 4: Pathway for PI3K leading to activation of mTOR1 and for ITK leading to activation of NFAT. Inhibition of PI3K will inhibit downstream proteins, such as AKT and mTOR1(Gamper & Powell, 2012)**.**

PI3Ks are involved in the generation of lipid second messengers and contain eight catalytic subunits of PI3K, with the P110 gamma and the P110 delta subunits being expressed at much higher levels in immune cells (Okkenhaug, 2013; Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010). The PI3K p110 subunits phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2), leading to the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 then functions as a second messenger that initiates a signaling cascade. The cascade starts with the phosphorylation of AKT, which is a serine/threonine protein kinase, leading to activation of downstream survival and differentiation signals such as mechanistic target of rapamycin 1 (mTOR) (Petersen et al., 2018; Vangapandu, Jain, & Gandhi, 2017). The activity of mTOR, which

is also a serine/threonine kinase, is regulated by amino acids and glucose levels. Upon T cell activation, there is an increase in uptake of amino acids and glucose leading to activation of mTOR1 and this activation is required to maintain the T cell effector functions (Finlay et al., 2012; Hukelmann et al., 2016; Rolf et al., 2013; Salmond, 2018). Activation of mTOR1 also promotes induction and maintenance of aerobic glycolysis. This leads to increased differentiation and effector functions, which are shown to be less efficacious for longer term persistence of adoptive T cell therapies (Salmond, 2018). By inhibiting mTOR through inhibition of PI3K, PI3K inhibitors may promote the beneficial memory T cell phenotype by inhibiting aerobic glycolysis and preventing terminal differentiation (Cantrell 12-14). One of the PI3K inhibitors studied, idelalisib, inhibits only the delta catalytic subunit, which has shown to block lymphocyte function (Okkenhaug, 2013). A second PI3K inhibitor studied was duvelisib, which has inhibitory activity on both the PI3K-delta and PI3K-gamma isoforms. The IC50 for the PI3K-delta subunit with idelalisib is 1.0 ± 0.2 μM, while the IC50 of PI3K-delta with duvelisib is 0.4 ± 0.1μM, therefore showing that duvelisib is a more potent inhibitor of PI3K-delta. Duvelisib, which is also known to work as an inhibitor of PI3K-gamma with an IC50 of 1.6 ± 0.2 μM, does demonstrate inhibition of both PI3K-delta and gamma, but much more selective for the delta subunit as demonstrated by the IC50s ((Vangapandu et al., 2017). The IC50 for PI3K-gamma with idelalisib is 9.4 ± 2.3 μM, which is too high for drug levels that can be achieved without untoward toxicity, therefore making it only a PI3K –delta inhibitor (Verastem document – private correspondence) By targeting either PI3K delta alone or both PI3K delta and gamma catalytic subunits that are predominantly

expressed on immune cells, it allows for partial blockade of terminal differentiation, while having little effect on the other cells of the body.

The fourth compound studied was ibrutinib, an irreversible inhibitor of Il-2 inducible T cell kinase (ITK) and Bruton's tyrosine kinase (BTK). Ibrutinib was used to modulate the metabolism of T cells through the inhibition of BTK and ITK. ITK, a member of the Tec family of tyrosine kinases that are important in TCR mediated signaling and is activated by PI3K (Li et al., 2015). When PI3K is activated ITK is transported from the cytoplasm to the plasma membrane. This leads to the activation of PLK-gamma, which then hydrolyzes PIP2 to IP3 and DAG. When IP3 binds it causes an influx of calcium leading to the downstream activation of transcription factors, such as nuclear factor of activated T cells (NFAT) (Zhong et al., 2015). NFAT then induces production of IL-2 in activated T cells (Macian, 2005). We hypothesized that inhibition of this pathway could be beneficial for enhanced T cell proliferation by limiting excess IL-2, which could lead to activation induced cell death. Therefore by inhibiting the NFAT pathway with ibrutinib, we may decrease activation-induced cell death (AICD) leading to increased live cell numbers (Long et al., 2017).

Experimental hypothesis

Our overall hypothesis was that the addition of these inhibitors would limit the terminal differentiation of T cells and retain them in a central memory phenotype with greater long-term replicative capacity and ex vivo and in vivo persistence.

SPECIFIC AIMS

A pressing issue seen with Adoptive T Cell Therapies is the in vivo persistence of the T cells once they are transferred to the patient. Studies have shown that once T cells are adoptively transferred to patients, their proliferation and persistence is minimal. Without the in vivo persistence of the T cells, they cannot proliferate and perform their function within the patient.

During ex vivo T cell expansion, having the optimal phenotype is essential for increased efficacy and persistence of cells in vivo. According to literature, T cells that are less differentiated and expressing both CD27 and CD28, have more proliferative capacity and enhanced functionality. These cells will also ideally rely more on oxidative phosphorylation rather than glycolysis, as they are shown to persist in vivo. Cells that rely more on glycolysis will become terminally differentiated and then become CD27 and CD28 negative, also known as senescent with no proliferative capacity. Therefore, the aim of this study is to prevent this senescence and push cells to rely more on oxidative phosphorylation, this study will use small molecule inhibitors that can help modulate metabolism in order to expand T cells that are more likely to survive once adoptively transferred to patients.

MATERIALS AND METHODS

Compounds

Idelalisib (CAL-101) was purchased from CombiBlocks (San Diego, CA) and stored as a 10mM stock solution in DMSO at -20 degrees Celsius. Duvelisib was purchased from Selleckchem and stored as a 20mM stock solution in DMSO at -80 degrees Celsius. Ibrutinib was purchased from Selleckchem (Houston, TX) and stored as a 50mM stock solution in DMSO in -20 degrees Celsius. The PFKFB3 inhibitor (GO-672) was produced by Gero Discovery, LLC (Moscow, Russia) and stored as a 20mM stock solution in DMSO at -20 degrees Celsius. Dilutions of all compounds were done in DMSO prior to addition to cell cultures, all with a final DMSO concentration of less than 0.1% in all wells.

Complete Media for Mouse and Human Apheresis Product Cultures

RPMI 1640 (Corning, Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM 2-mercaptoethanol, and 1mM sodium pyruvate.

Jurkat Cell Media

RPMI 1640 (Corning, Manassas, VA) supplemented with 10 % FBS and 1% penicillin/ streptomycin.

Fluorochrome Conjugated Antibodies

Cells were stained with the following antibodies. Anti-human CD3 phycoerythrin (PE)-CF594, CD4 allophycocyanin (APC)-Cy7, CD8 fluorescein isothiocyanate (FITC), CD27 PE, CD45RO Peridinin Chlorophyll Protein Complex (PerCP), and aqua live/dead fixation were purchased from BD biosciences (San Jose, CA). Anti-human CCR7 APC and CD45RA PE-Cy7 were purchased from Ebioscience (Santa Clara, CA). Anti-human CD28 Alexa Fluor (AF)-700 was purchased from Biolegend (San Diego, CA).

Western Blot Antibodies

The antibodies used for western blot were Bcl-2 (BD pharmigen, San Jose, CA), Bcl-Xl (Cell Signaling, Danvers, MA), Mcl-1 (Enzo, Farmingdale, NY)), Bim (Millipore, Burlington, MA), and Actin (Sigma, St. Louis, MO).

METHODS

Mouse T Cell Culture

T cells were isolated from spleen of a luciferase+ B6 mouse. Spleens were dissociated by macerating on a screen and then filtered through a cell strainer. Red blood cells were lysed with lysis buffer. Isolation of T cells was performed with a negative selection kit purchased from Miltenyi Biotec (Auburn, CA) was used to isolate T cells. Briefly, splenocytes were coated with a biotin antibody cocktail containing lineage markers exclusive of those on T cells and then coated with anti-biotin microbeads. The coated cells were then run through a magnetic column with eluate enriched for T cells. 100,000 purified T cells were cultured with RPMI –complete media supplemented with 50 U/mL of recombinant murine IL-2 in 96 – well plates pre-coated with 1ug/mL of anti-CD3 antibody (eBiosceince, Santa Clara, CA). After 24, 48, and 72 hours, cells were counted, stained for flow cytometry and assessed by bioluminescence. Bioluminescence was assessed by adding 150 μg/mL luciferin to each well with image collection on the IVIS spectrum. Assessment of the bioluminescence was conducted by creating a region of interest over each well and measuring the luminescence. Measurements were reported as photons per second per square centimeter using Living Image Software (PerkinElmer, Waltham, MA).

Human T Cell Culture

Frozen cells from a healthy apheresis product, containing 78% T cells, were thawed from liquid nitrogen and placed in a cell culture flask to rest overnight at 37

degrees Celsius 5% CO_2 humidified incubator in complete media supplemented with 30 U/mL recombinant human IL-2 (R&D systems, Minneapolis, MN). Cells were then cultured in a 24 well G-Rex plates, which have 2 cm^2 of gas permeable area, allowing for rapid expansion (Wilson Wolf, Saint Paul, MN). 1,000,000 cells were added to each well along with a 1:1 bead to cell ratio of anti-CD3/CD28 beads for activation and cultured in a 37 degrees Celsius incubator (Thermofisher-Dynabeads, Waltham, MA). Compounds were added at the initiation of the cultures and every four days thereafter along with new media supplemented with IL-2. On day 8, fresh anti-CD3/CD28 beads at a 1:1 bead to cell ratio were added for re-stimulation. Final DMSO concentration was kept below 0.1% in all wells.

Phenotypic Analysis

Phenotypic analysis was done prior to and during expansion cultures. Cells were first incubated with a Live/dead fixable Aqua Dead Cell Stain (Invitrogen), washed twice with PBS, and stained with the respective antibodies. Files were acquired in a. fst mode on a FACS Aria flow cytometer (BD) and data was analyzed using the FlowJo software.

Seahorse Assay

The day prior to the assay, Agilent Seahorse Sensor cartridge was coated in Agilent Seahorse XF Calibrant at 37 degrees Celsius in a non-CO_2 incubator. On the day of the assay, assay medium was prepared by supplementing the Agilent Seahorse XF Base Medium with 1mM pyruvate, 2mM glutamine, and 10mM glucose., warmed to 37 degrees Celsius and adjusted pH to 7.4 with 0.1 N NaOH.

Expanded T cells were harvested from G-Rex plates were plated at 200,000 cells per well in the pre-coated Seahorse plate, and incubated for one hour in assay medium. Stressor drugs were then pipetted directly into the delivery ports of the top sensor cartridge in the following order: 20µl of oligomycin, 22 µl of trifluormethoxy carbonylcyanide phenylhydrazone (FCCP), 24 µl of rotenone and antimycin A. Oligomycin is an inhibitor of complex V (ATP synthase), injection of this will indicate the amount of oxygen that is consumed during the production of mitochondrial ATP. FCCP is a protonophore that uncouples ATP synthesis from oxygen consumption. This will reveal the maximum respiratory capacity of the cell. Rotenone and antinomycin then completely inhibit the electron transport chain by inhibiting complex I and complex III, respectively. SRC, which is determined by comparing the ratio of maximal oxygen consumption rate (OCR), which is found once FCCP is added to basal OCR, which is found prior to any mitochondrial inhibitors are added (Gerritje J W van der Windt, Chang, & Pearce, 2016). All products for the Seahorse assay were purchased from Agilent (Santa Clara, CA).

Western Blot

Cells were lysed in 25µl of 1% Triton lysis buffer for 15 minutes on ice, then centrifuged at 18000 x g for 10 minutes at 4 degrees Celsius in order to pellet insoluble material. Protein concentration in the lysate was determined using bicinochoninic acid assay (BCA) and then normalized. Approximately 10 µg of lysate were loaded onto a 4-20% acrylamide gradient gel and run at 100 V for 1.5 hours. Protein were wet transferred to nitrocellulose paper at 0.3 A for 2 hours. The membrane was then blocked in 5% non-fat milk in tris-buffered saline (TBS-T) for 1 hour and then incubated with primary

antibody in 5% BSA TBS-T overnight at 4 degrees Celsius. The membrane was then washed in TBS-T and incubated with secondary antibody conjugated to HRP in 5% milk TBS-T for 1 hour at room temperature. After washing again, enhanced chemiluminescence (ECL) was applied to the membrane for 1 minute prior to exposing the membrane to film for developing. The membrane was then stripped with 0.8% NaOH and blocked again in 5% milk TBS-T for 3 minutes prior to incubating with the next primary antibody.

Statistical analysis:

Statistical analyses were conducted using Prism version 8 (GraphPad, La Jolla, CA). Statistical significance amongst 3 or more groups was determined using 2-way analysis of variation (ANOVA) with post- tests that included Tukey or Dunnett. Significant differences between 2 groups were determined using a 2-tailed t test as a p value of <0.05.

RESULTS

Jurkat T cells cultured with 1nM of idelalisib showed a significant increase in viability

First we tested whether the metabolic inhibitors were generally toxic to cultured T cells. Jurkat T cells, which grow autonomous of mitogenic stimuli were cultured with graded concentrations of PFKFB3 inhibitor, ibrutinib, and idelalisib. 100,000 jurkat cells were plated in a 96-well plate and counted at 24 and 48 hours using Trypan blue. At 24 hours there was no significant difference in number of live cells between the control and treated groups (Figure 6A). At 48 hours there was a significant increase in the number of live cells comparing the control group and cultures containing with 1 nM of idelalisib (Figure 6B).

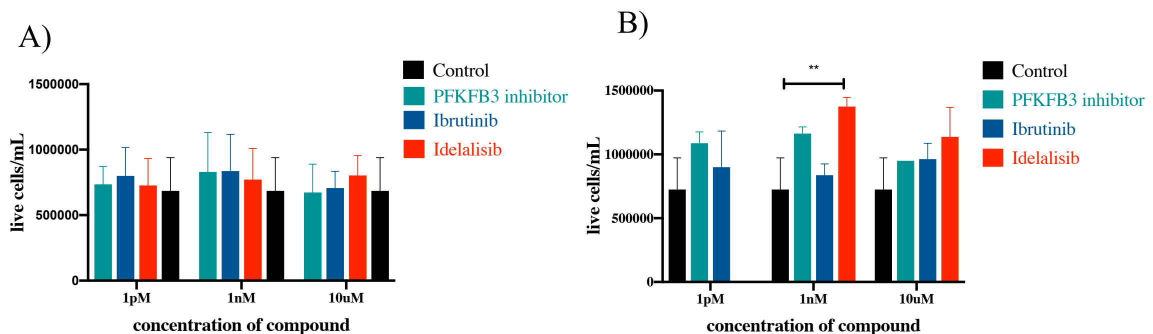
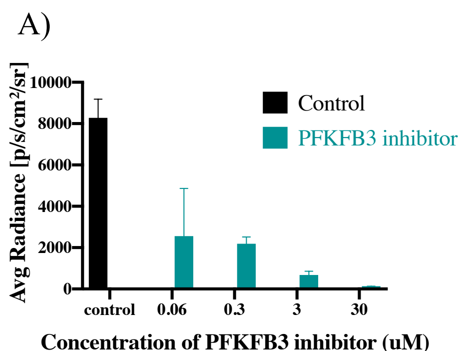


Figure 5: Viability of Jurkat cells at 24 and 48 hours. Jurkat T cells were cultured in complete media for 24 or 48 hours with different concentrations (0-10 μM) of PFKFB3 inhibitor/ibrutinib/idelalisib. The number of live cells under each culture condition was then quantified using trypan blue staining for 24 hour (A) and 48 hour (B) time points $^{**}P<0.002$

PFKFB3 inhibitor inhibits glycolytic metabolism and increases mouse T cell proliferation in vitro

In order to test whether PFKFB3 inhibitor inhibited the glycolytic metabolic pathway of T cells, T cells from B6 luciferase mice were cultured with 0 µM, 0.06 µM, 0.3 µM, 3 µM, or 3µM of a PFKFB3 inhibitor for 48 hours and then bioluminescence analyzed. Cells in the control group had an average radiance of 8.28 x 10^3 p/s/cm^2/sr ± 901.52. However, as the concentration of PFKFB3 inhibitor increased from 0.06 µM to 30 µM there was a drop in average radiance corresponding with the amount of inhibition (Figure 5A). To test whether decreased bioluminescence and metabolic activity was due to effects of drug-induced overall changes in T cell metabolism, T cells were cultured with 1 nM, 100 nM, and 1µM of PFKFB3 inhibitor for 24, 48, or 72 hours and then analyzed by flow cytometry. Cells treated with PFKFB3 inhibitor had an increased frequency of live cells as compared to control, which at 72 hours had 71% live cells, as compared with 1 nM with 86.1 %, 100nM with 91.2%, and 1µM with 73.9% (Figure 5B). Thus PFKFB3 inhibited T cell metabolism without killing cells.

A)



B)

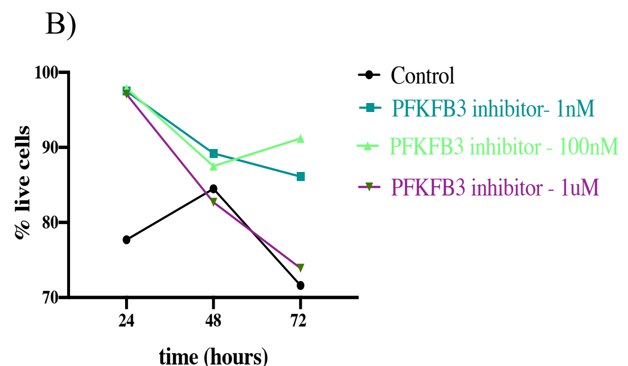


Figure 6: PFKFB3 inhibitor effect on T cell proliferation. T cells isolated from B6 luciferase + mice were cultured in a 96 well-plate with complete media and activated with anti-CD3. Quantification of metabolic inhibition was shown as change in (A) average bioluminescence radiance of T cells following exposure to 0 µM, 0.06 µM, 0.3 µM, 3 µM, or 30 µM of PFKFB3 inhibitor and (B) the frequency of live T cells at 24, 48, and 72 hours with 1nM, 100nM or 1µM of PFKFB3 inhibitor.

T cell viability increased with PFKFB3 inhibitor, ibrutinib, and idelalisib

In order to test the effect of small molecule inhibitors on T cell viability and fold expansion, T cells were cultured for 15 days with the PFKFB3 inhibitor, ibrutinib, and idelalisib at concentrations of 0, 10 nM 100 nM, and 1µM. Live and dead cells were then counted with ethidium bromide acridine orange. There was a significant difference in viability between every treated group and control with the most significant increase in the proportion of viable cells seen comparing control and those cultured with1µM of idelalisib (Figure 7A). The net fold expansion of T cells was significantly increased with ibrutinib at 10 nM and 1µM, PFKFB3 at 100 nM, and idelalisib at all concentrations (Figure 7B). Increased T cell expansion was seen at all doses with the greatest effect seen in cultures containing1µM idelalisib, in which a 52.8 ± 1.77-fold expansion was observed.

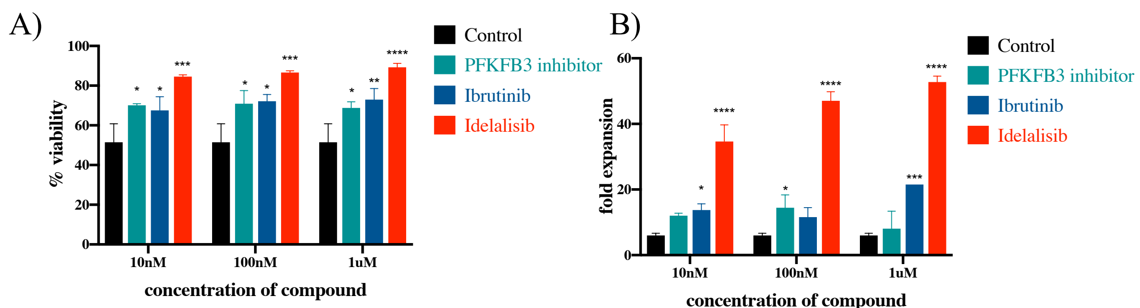


Figure 7: Viability and fold expansion of T cells cultured with PFKFB3 inhibitor, Ibrutinib, and Idelalisib. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then counted using ethidium bromide acridine orange. Viability (A) and mean fold expansion (B) are shown with significance compared to control cultures shown.. *$p<0.05$, **$p<0.002$, ***$p<0.0002$, ****$p<0.0001$.

Cells treated with idelalisib increased the ratio of CD8+: CD4+ T cells

To see if treatment with metabolic inhibitors effected the ratio of CD8+ to CD4+ T cells, cultures were analyzed by flow cytometry after 8 days and at 15 days of expansion. Following 8 days of expansion about the same percentage of CD3+ T cells and a slight increase in the frequency of CD8+ T cells was observed in the 1μM of idelalisib treated group with a corresponding decrease in the frequency of CD4+ T cells (Figure 8B). At day 15, the frequency of the CD8+ T cells had continued to largely decrease in the control cultures and those with the PFKFB3 inhibitor and ibrutinib, but remained high in the cultures containing 1μM of idelalisib (figure 8C).

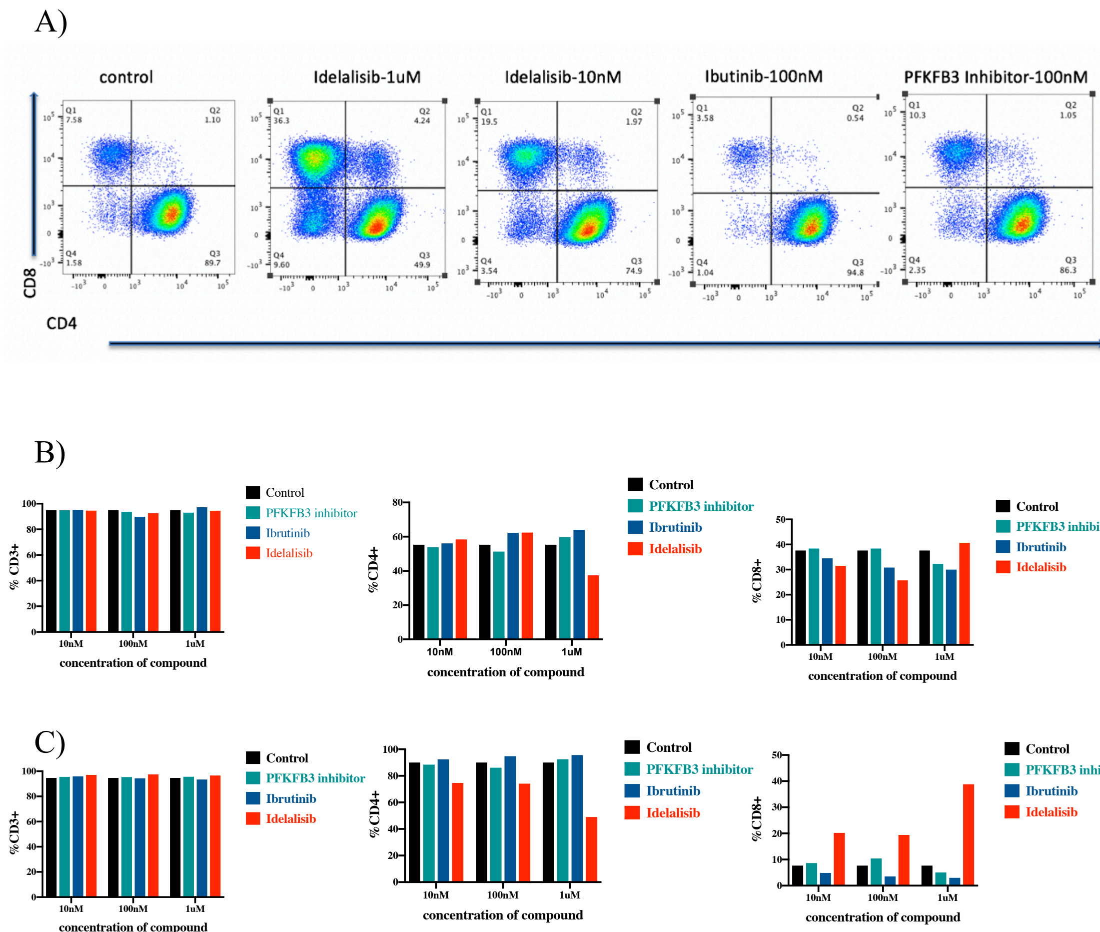
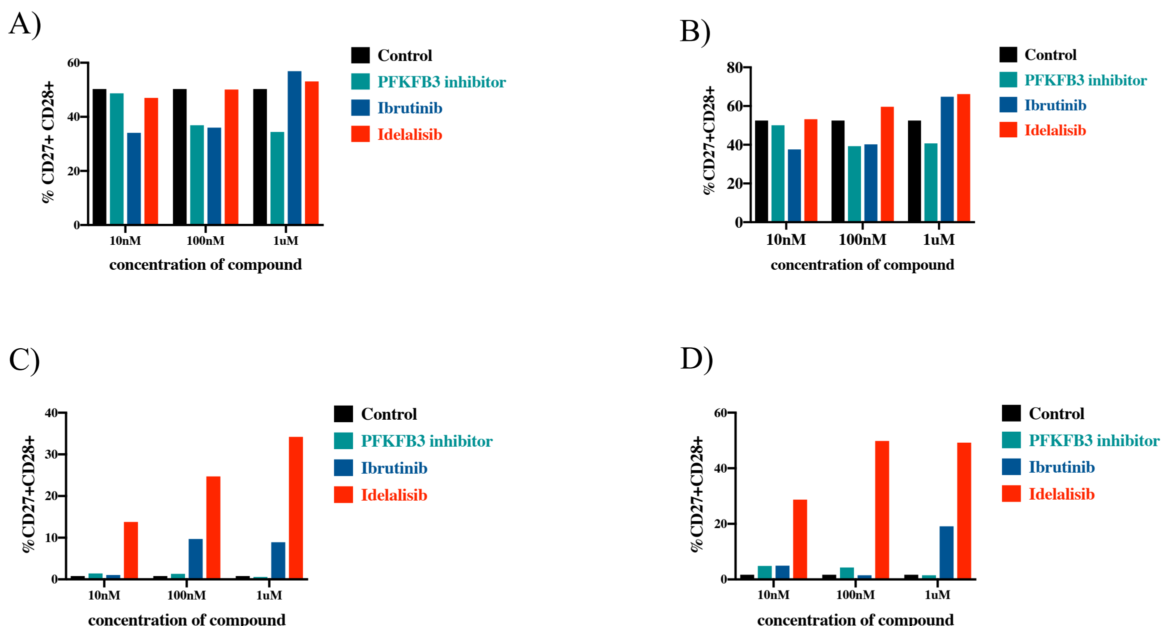


Figure 8: Inhibition of PI3K delta subunit leads to increased frequency of CD8+ T cells. Human mononuclear cells were cultured for 8 or 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS. A representative dot plot shows the ratio of CD8+ to CD4+ T cells with the different compounds (A). The frequency of CD3+, CD4+, and CD8+ (from left to right) T cells after 8 days of expansion (B) and the frequency of CD3+ CD4+, and CD8+ (from left to right) T cells after 15 days of expansion (C) are shown.

T cells cultured with ibrutinib and idelalisib showed an increase in frequency of CD27+CD28+ T cells and a decrease in frequency of CD27-CD28- cells

Based on the fact that expression of the costimulatory receptors CD27 and CD28 are lost during maturation of naïve and central memory T cells to effector T cells, and that the presence of T cells lacking both CD27 and CD28 expression is associated with senescence we next asked if the addition of metabolic inhibitors could decrease the frequency of CD27-CD28- senescent T cells. At 8 days of expansion, there was a slight increase in CD27+CD28+ (double positives) and a larger decrease CD27-CD28- (double negative seen with 1uM of ibrutinib and idelalisib as compared to control (Figure 9A, B, E, F). At 15 days there was almost a complete loss of the double positives in all groups except for the idelalisib treated groups and the 1µM of ibrutinib treated group C and D). In regards to the double negative cells, cultures containing 1µM of idelalisib showed the largest decrease in the frequencies of these senescent T cells, (Figure 9G and H). and the effect of idelalisib decreased senescent T cells was dose-dependent as seen in the flow plots. (Figure 9I).



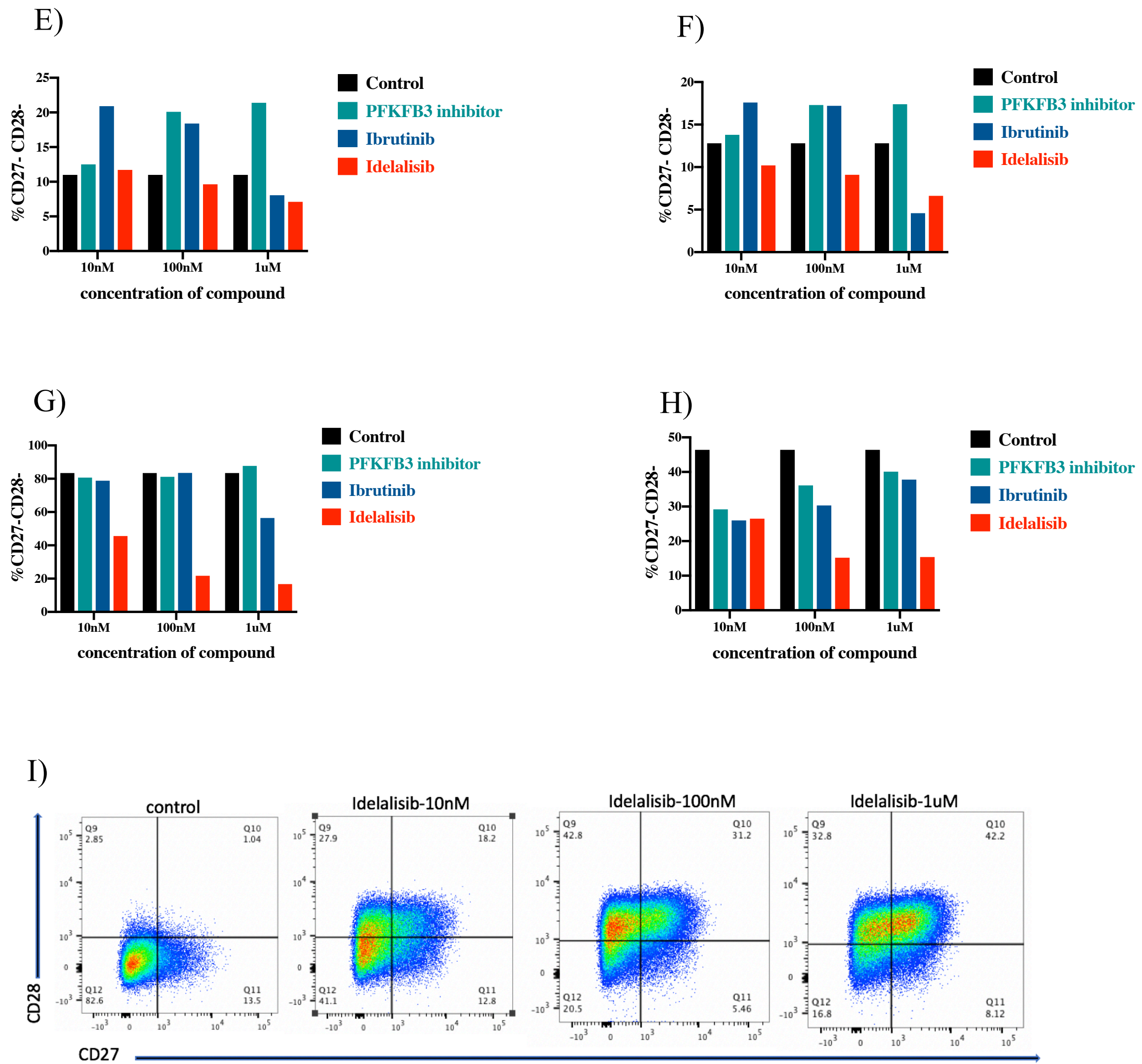
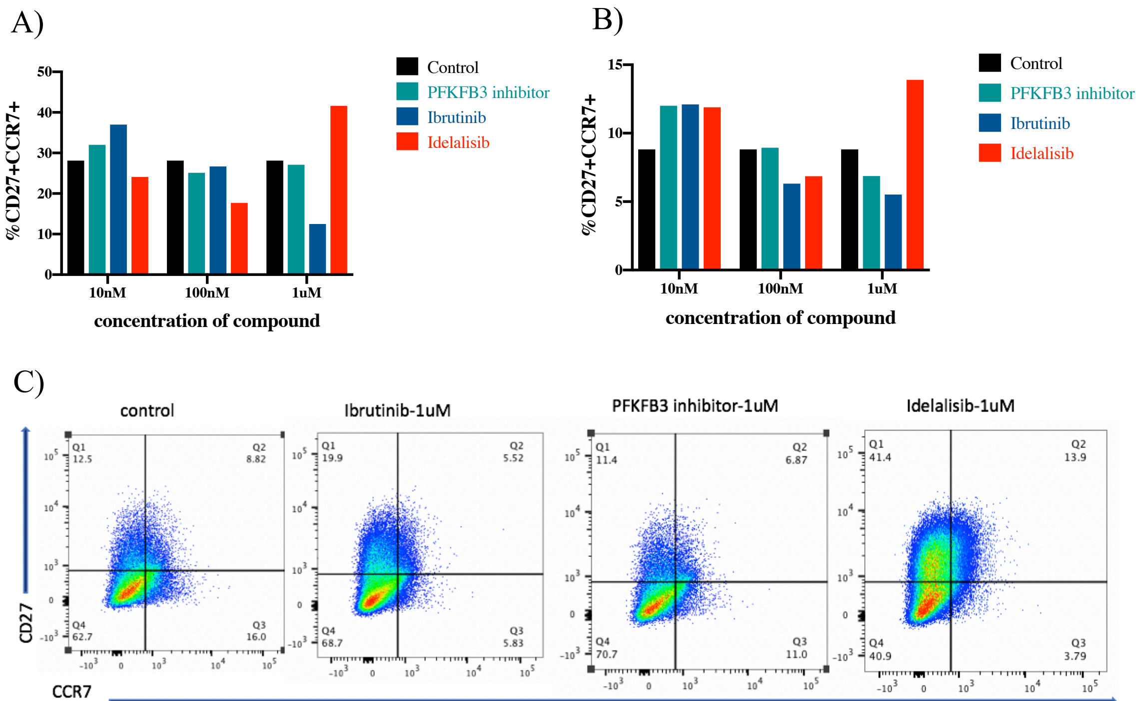


Figure 9: Idelalisib decreases the frequency of CD27 and CD28 double negative T cells and increases the frequency of CD27+CD28+ cells during ex vivo T cell expansion. Human mononuclear cells were cultured for 8 or 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS. Frequency of CD4+CD27+CD28+ (double positive) on day 8 (A), frequency of CD8+ double positive on day 8 (B), frequency of CD4+ double positive on day 15 (C), and frequency of CD8+ double positive on day 15 (D) are shown. In terms of the CD27- CD28- (double negative) cells, frequency of CD4+ double negative on day 8 (E), frequency of CD8+ double negative on day 8 (F), frequency of CD4+ double negative on day 15 (G), and frequency of CD8+ double negative on day 15 (H) are shown. A representative dot plot showing a dose-dependent increase in double positive T cells treated with idelalisib (I).

Cells cultured with 1µM of idelalisib, 10nM of ibrutinib, and 10nM of PFKFB3 inhibitor increased the frequency of naïve and central memory T cells

Less differentiated T cell subsets, such as naïve and central memory T cells, are known to be longer lasting, allowing for these cells to survive and persist. Therefore, we looked to see if use of PFKFB3 inhibitor, ibrutinib, or idelalisib increased these T cell subsets. To test this, cultured for 15 days and then analyzed by flow cytometry. At 15 days, cells treated with 1uM of idelalisib had the highest frequency of naïve T cells with a frequency of 41.6% followed by those treated with 10 nM of ibrutinib that had a frequency of 37.0% (Figure 10A and C). For the central memory T cell population 1µM of idelalisib showed the largest increase (Figure 10B and C).



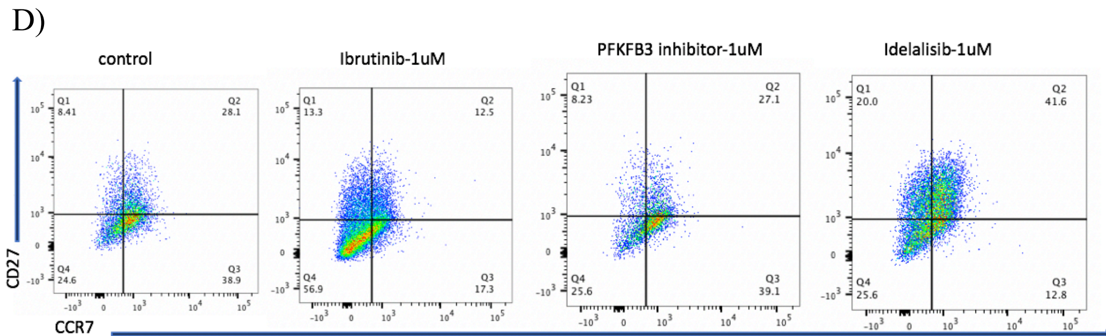


Figure 10: Increase in naïve and central memory T cell subsets with ibrutinib and idelalisib. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS. Frequency of naïve T cells after 15 days of expansion (A), frequency of central memory T cells after 15 days of expansion (B), a representative dot plot showing increased naïve T cells when treated with ibrutinib and idelalisib (C), and a representative dot plot showing increased central memory T cells with idelalisib (D) are shown.

T cells cultured with idelalisib and duvelisib show increased number of T cells

Since cultures with idelalisib had the greatest increase in T cell expansion we next tested whether duvelisib, a more potent PI3K inhibitor with activity in the inhibition of both the gamma and delta subunits, could increase T cell expansion. T cells from human mononuclear cells were cultured for 15 days and then counted with ethidium bromide acridine orange. The results show that there is a similar effect of both compounds on augmenting T cell expansion, although duvelisib was more potent with a maximal effect seen at 300nM concentration versus the 1 μM for idelalisib. Within the idelalisib group there was a significant difference between control and 100 nM and 1μM treated groups

(Figure 11A). In the duvelisib group there was a significant difference between the control and the 10 nM, 100 nM, 300 nM, and 1μM treated groups (Figure 11B).

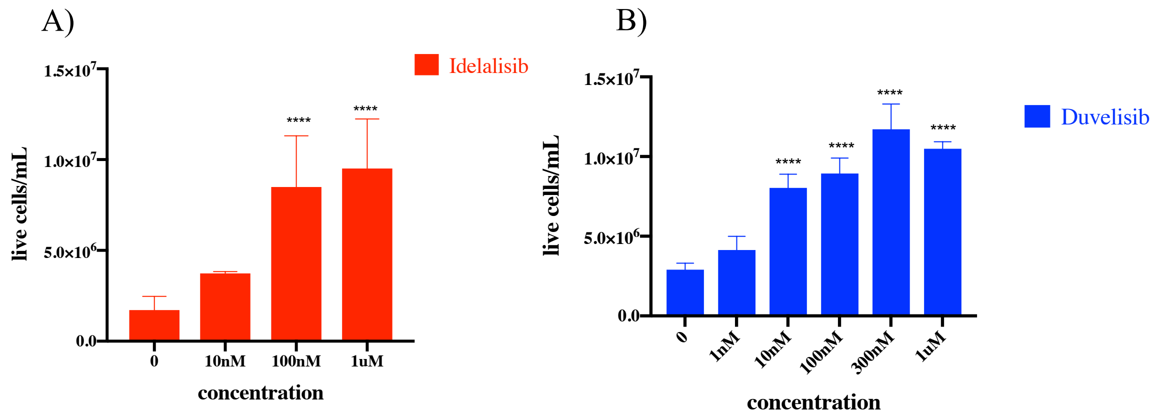
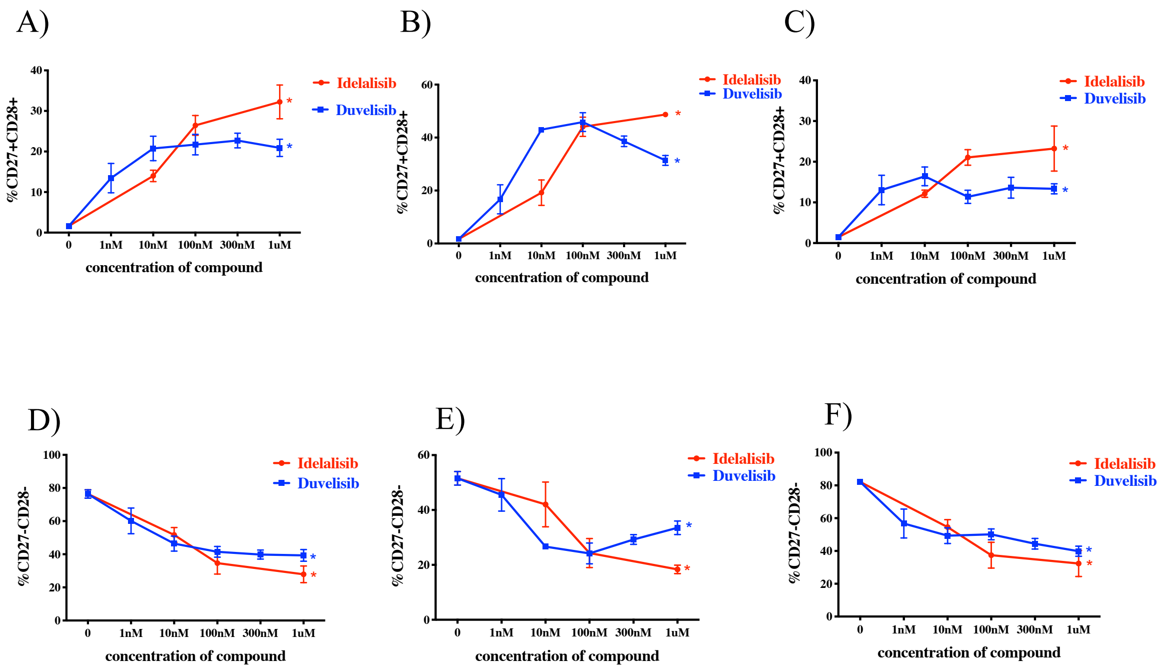


Figure 11: T cells cultured with idelalisib and duvelisib increased T cell expansion. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS. Number of live T cells cultured with idelalisib (A) and number of live T cells cultured with duvelisib (B) are shown. *$p<0.05$, **$p<0.002$, ***$p<0.0002$, ****$p<0.0001$.

Both idelalisib and duvelisib increased the frequency of CD27+CD28+ T cells and decreased the frequency of CD27-CD28- T cells

Analysis of CD27 and CD28 expression showed that both PI3K inhibitors decreased the frequency of CD27-CD28- (double negative) T cells to a similar degree, as compared to control (Figure 12D). The frequency of CD27-CD28- T cells with 1uM of idelalisib was 28 ± 9% and 300 nM of duvelisib showed a frequency of 40 ± 6%, whereas control cultures had a frequency of 74 ± 2% CD27-CD28- T cells. There was a corresponding increase in the frequency of CD3+ T cells that displayed CD27+CD28+

(double positive) phenotype with cultures containing either PI3K inhibitor with the maximal effect seen at 100nM duvelisib (23 ± 3%) and 1 μM idelalisib (32 ± 7). The expression of CD27 and CD28 on the CD4+ and CD8+ populations showed the same pattern as seen in the CD3+ population as a whole (Figure 12 B, C, E, F). Notably, the viability of the double positive T cells in cultures treated with idelalisib and duvelisib was greater compared to control (Figure 12G).



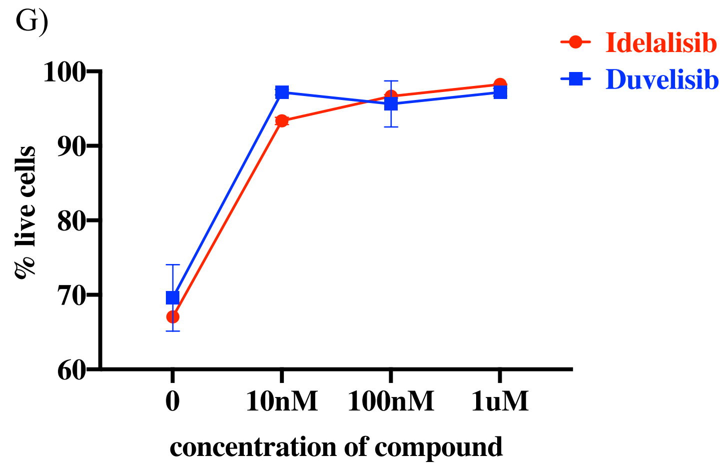


Figure 12: Frequency of double positive T cells increases with idelalisib and duvelisib and frequency of double negative T cells decreases. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS. Frequency of CD3+ double positive (A), CD8+ double positive (B), and CD4+ double positive (C) T cells are shown. Frequency of CD3+ double negative (D), CD8+ double negative, CD4+ double negative (F) and live CD3+ double positive T cells (G) are also shown. *p<0.05

Frequency of naïve and central memory T cells increases while frequency of terminal effect T cells decreases when cultured with idelalisib and duvelisib

To help determine the metabolic phenotype of a cell, T cells extent of differentiation was looked at after being cultured for 15 days with or without idelalisib or duvelisib. There was no significant difference between the compounds for naïve (CD45RA+ CD27+CCR7+), central memory (CD45RO+CD27+CCR7+), and terminal effector (CD45RA+CD27-CCR7-) T cells. However, a significant difference was seen between all treated groups when compared to control for all three T cell phenotypes. For

naïve T cells, peak values were seen in the 1μM idelalisib treated group, 30.21 ± 2.05% and the 300nM of duvelisib, 29.56 ± 3.55% (Figure 13A). For the central memory T cells the highest frequency was seen with 1uM of idelalisib, 25.65 ± 1.78 %, but there was no significant difference between this concentration and peak concentration of duvelisib, which had a value slightly lower at 19.83 ± 3.59% (Figure 13C). The terminal effector T cells showed a decrease in frequency with increasing doses of idelalisib and duvelisib (Figure 13B).

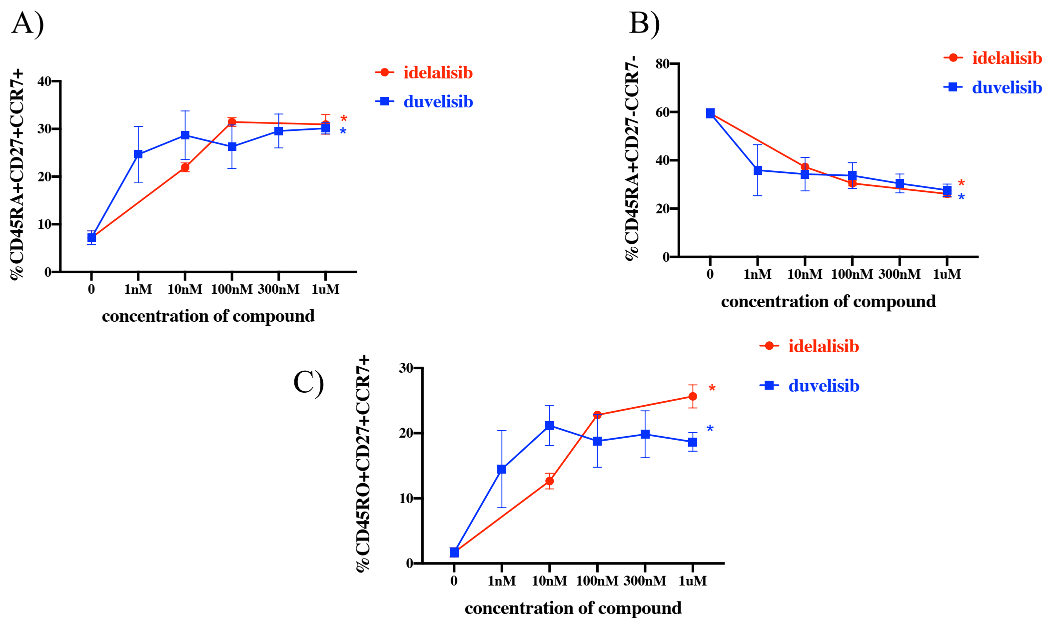


Figure 13: T cells cultured with idelalisib and duvelisib show an increased frequency of naïve and central memory T cells and a decrease in terminal effector T cells. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30 U/mL). The expanded cells were then phenotyped using FACS, which showed frequency of CD3+ naïve T cells (A), frequency of CD3+ central memory T cells (B), and frequency of terminal effector T cells (C). *$p<0.05$.

Cells cultured with duvelisib show increased spare respiratory capacity and other metabolic advantages

To examine the metabolic capacities of T cells cultured with idelalisib or duvelisib, T cell cultured for 15 days were assessed for their metabolic profile using the Seahorse assay. Both PI3K inhibitors led to higher oxygen consumption rate (OCR) at baseline and under stressed conditions, with duvelisib showing the greatest effect overall (Figure 14A). In terms of extracellular acidification rate (ECAR), which represents the rate of glycolysis in the cell, all groups had similar levels at baseline except the unstimulated group, which was much lower. Under stressed conditions, cultures of T cells with 300 nM of duvelisib had the highest ECAR, with the 1μM of idelalisib treated group, as well as the other duvelisib concentrations slightly lower (Figure 14B). The spare respiratory capacity (SRC) showed that the duvelisib treated groups had the highest SRC compared to all other groups (Figure 14C). The cells energetic phenotype shows the difference in the cells baseline energy phenotype, compared to the stressed energy phenotype. These results show that cells treated with the PI3k inhibitors have a more aerobic phenotype compared to that of the controls (Figure 14D). The stressed phenotypes of groups treated with 1μM and 300 nM of duvelisib showed that cells were able to utilize both oxidative phosphorylation and glycolysis for energy. The 100 nM of idelalisib treated group and the stimulated control group both show a stressed phenotype that is more glycolytic (Figure 14D).

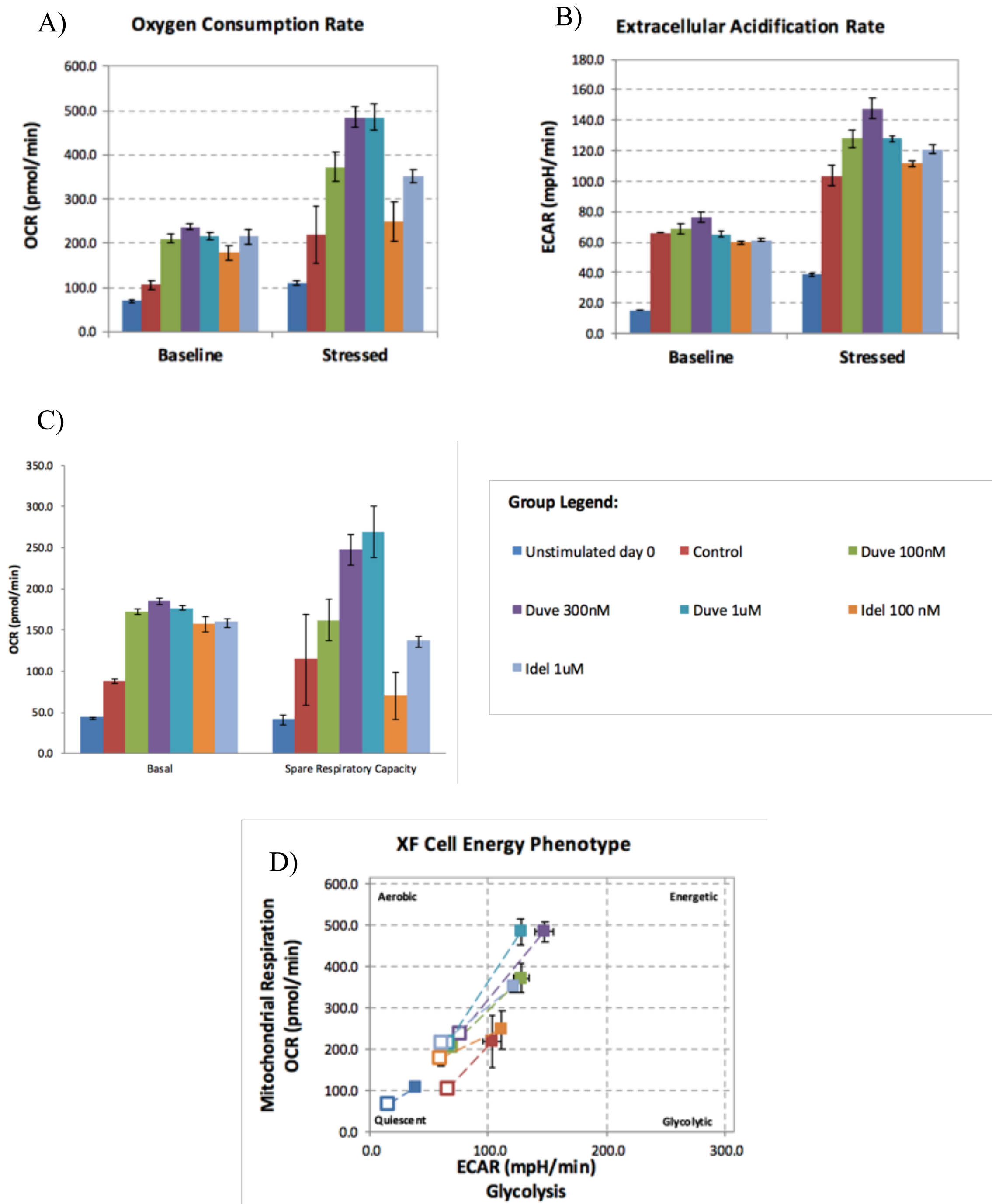


Figure 14: T cells treated with 1µM of idelalisib and all concentrations of duvelisib show metabolic advantages over control groups. Human mononuclear cells were cultured for 15 days in a G-Rex plate with complete media supplemented with IL-2 (30

U/mL). The expanded cells were than analyzed using Seahorse device for their oxygen consumption rate (OCR) at baseline and under stressed conditions (A), extracellular acidification rate (ECAR) at baseline and under stressed conditions (B), spare respiratory capacity (SRC) (C), and the cells energy phenotype profile (D), which are shown. (van der Windt, Chang, & Pearce, 2016)

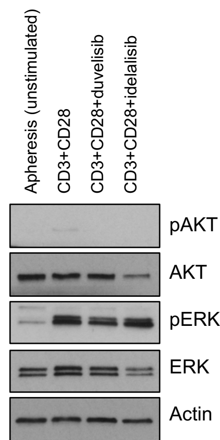
Inhibition of pAKT and increased Bcl-2 levels seen with western blot in the idelalisib and duvelisib treated groups

Examining anti-apoptotic pathways given the increased expansion and viability with PI3K inhibitors in order to look more in depth at the effects of the PI3K inhibitors on protein levels, western blots were performed on T cell lysates form cultures treated with 1 μM of idelalisib and 300 nM of duvelisib for 24 hours or 15 days. At 24 hours there is a slight presence of pAKT after being exposed for 20 minutes and there is a complete absence of a band for all other groups. Total AKT was similar across all groups, although slightly less for the idelalisib treated group. Phosph-ERK levels were much lower in the unstimulated group as compared to the activated control group, as well as the activated and treated groups (Figure 15A).

The CD3+CD28+ stimulated cells have decreased levels of Bcl-2 after 15 days of expansion, which is reversed by PI3K inhibition by idelalisib or duvelisib. In contrast, there was no difference between the groups in Bcl-2 and Bim expression (Figure 15B).

A)

24 hours after cells were stimulated and given compounds



B)

15 days of T cell expansion

- Compounds were last given at day 12
- Stimulation was at day 8

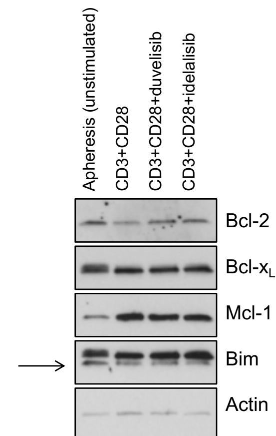


Figure 15: T cells cultured with idelalisib and duvelisib show loss of pAKT band and increased Bcl-2 levels. Human mononuclear cells were cultured, for 24 hours in a 6-well plate or for 15 days in a G-Rex plate, with complete media supplemented with IL-2 (30 U/mL). The expanded cells were than analyzed. Western blot of T cells cultured for 24 hours with or without idelalisib and duvelisib (A) and T cells cultured for 15 days with or without idelalisib or duvelisib (B).

DISCUSSION

Use of idelalisib and duvelisib in cultures lead to optimal T cell phenotype

Our overall hypothesis was that small molecule inhibitors of metabolism could limit terminal differentiation of T cells during ex vivo expansion yielding a cell population with increased potency when used for adoptive cell therapy. This is a relevant question for CART manufacturing, as only 30%-40% of patients with lymphoma achieve durable long-term remissions with current CART products (Neelapu et al., 2017; Schuster et al., 2019). Prior work in the Waller lab has shown that use of idelalisib increases proliferation of murine and human T cells leading to the question of whether other inhibitors of metabolism could have a similar effect on the proliferative capacity of T cells, and what the underlying mechanism of action was for the favorable effect of metabolic inhibitors on T cell expansion (Petersen et al., 2018). We compared the effects of a PKFB3 inhibitor, ibrutinib (BTK/ ITK inhibitor), idelalisib (PI3K ■ inhibitor), and duvelisib (PI3K ■ and ● inhibitor). Results of our experiments show that use of idelalisib and duvelisib had the greatest effect on augmenting T cell proliferation and also maintained higher frequencies of viable CD27/ CD28 double-positive T cells following T cell expansion as compared to control cultures lacking added inhibitors. The naïve and central memory T cell phenotypes have been shown to be critical for in vivo persistence and anti-cancer activity for adoptive T cell therapy (Sukumar et al., 2013). Notably, the effect of the Pi3K inhibitors also decreased the CD27-CD28- double negative T cell, a cell population that we and others have previously shown inhibits T cell expansion via

Fs-mediated fratricide (Petersen et al., 2018). Finally, the addition of PI3K inhibitors favorable altered the ratio of CD4:CD8 T cells during expansion and increased the frequency of CD8 single positive T cells as well as the CD4- CD8- double negative T cells. Since the CD8+ T cells have the majority of cytotoxic functions in adoptive T cell therapy, increasing the frequencies of CD8+ T cells is predicted to lead to better responses following adoptive transfer of expanded T cells. These results demonstrate that simple addition of metabolic inhibitors to T cell cultures during anti-CD3/CD28 driven expansion led to increased frequencies of phenotypes of effector cell populations that are predicted to result in advantageous clinical results when used clinically.

Doses used were based on IC50s for idelalisib and duvelisib

The doses of idelalisib and duvelisib used for these studies were chosen based on their IC50s. The IC50s for the different subunits of duvelisib were, 0.4 ± 0.1µM for the delta subunit and 1.6 ± 0.2µM for the gamma subunits. For idelalisib, the IC50 of the delta subunit is 1.0 ± 0.2µM and for the gamma subunit it is 9.4 ± 2.3µM (Verastem document – private communication). The idelalisib IC50 for the gamma subunit is too high to be clinically relevant at pharmacologically tolerable blood levels, which is why idelalisib is considered strictly a delta subunit inhibitor.

Metabolic phenotype

Our studies reveal a surprising effect of the PI3K inhibitors on the metabolic reserve capacity of the expanded T cells. The increase in baseline OCR seen in the Seahorse assay in the duvelisib treated groups is an attractive result for adoptive T cell therapy clinical applications for a number of reasons. First, because less differentiated naïve and memory T cells, with greater capacity for in vivo expansion and persistence, rely more on oxidative phosphorylation. Therefore, treatment with duvelisib pushes T cells to a phenotype that is more reliant on oxidative phosphorylation, which should allow these cells to persist longer in vivo following adoptive transfer. A second reason is that this result is favorable is because the increased OCR leads to an increased ratio of maximal OCR to basal OCR, which is known as the SRC. The SRC is the extramitochondrial capacity that is available for the cell to produce energy. Increased SRC is associated with increased ability to provide energy under conditions of stress and therefore an increased SRC is said to increase the fitness of the T cell (Gerritje J W van der Windt et al., 2016). A large SRC allows for a more rapid response in the case that T cells encounter their cognate antigen again, as well as a metabolic advantage, as they can rely more on oxidative phosphorylation, which relies on the mitochondrial capacity which is enriched in these cells (Gerritje J W van der Windt et al., 2016).

Duvelisib treatment of T cells in vitro also led to other metabolic differences that may underlie its enhanced ability to expand viable naïve and memory T cell subsets. The metabolic profile of the duvelisib-culture T cells has been described as an energetic phenotype, meaning that T cells able to utilize both oxidative phosphorylation and

glycolysis for its energy needs. This allows the T cell to access lots of energy for expansion and cytolytic functions, therefore leading to a faster and stronger immune response. The energetic phenotype is best seen by looking at the stressed OCR and ECAR levels. The groups treated with duvelisib, showed the highest levels of both under stressed conditions, further showing the energetic efficiency of duvelisib-expanded T cells.

The Seahorse assay also demonstrated marked differences between activated and unactivated T cells in control cultures lacking added metabolic inhibitors. The unstimulated group showed very little glycolytic flux, consistent with lack of prior engagement of the TCR. Once the T cells are activated with anti-CD3/ CD28 beads, glycolytic flux increases in T cells from the control culture as these cells increase their metabolism. The Seahorse assay demonstrates the increased energy production of activated T cells in the control cultures, and the even larger production of energy when activated T cells are treated with PI3K inhibitors.

Protein levels further explain the effects of idelalisib and duvelisib on T cells

Finally, we studied whether the addition of metabolic inhibitors changes the relative levels of pro- and anti-apoptotic proteins that regulate cell fate decisions between survival and apoptosis. Loss of pAKT in T cells treated with PI3K inhibitors, idelalisib and duvelisib, confirms on-target activity of the drugs, as a critical role of PI3K is to phosphorylate AKT. Levels of total AKT were slightly diminished by idelalisib (but not duvelisib) treatment, from which we infer that idelalisib may lead to a negative feedback

mechanism that not only leads to decreased phosphorylation of AKT, but also downregulates the presence of AKT itself. Further studies are needed to better define how AKT is regulated in the presence of PI3K inhibitors.

Following expansion for 15 days, T cells from cultures with added idelalisib and duvelisib showed increased Bcl-2 levels. The increased levels of an anti-apoptotic protein help explain the increased viability of the cultured cells in spite of continued exposure to strongly-activating signals through CD3 and CD28. Future studies will further examine the role of Bcl-2 protein family involvement in the enhanced survival and expansion of PI3K inhibitor-treated T cells by testing the effects of ventoclax, an inhibitor of Bcl-2, and navitoclax an inhibitor of Bcl-X_L, on T cell expansion.

Ongoing research and future directions

Additional planned experiments will be conducted to increase the statistical power of the preliminary results seen from the T cell expansions with the PFKFB3 inhibitor, ibrutinib, idelalisib, and duvelisib. Special attention will be given to the ratio of CD8+ to CD4+ T cells in the idelalisib and duvelisib treated groups as large variances observed in current data preclude clear conclusions as to the magnitude of the effect of these drugs on preferentially enhancing expansion of CD8+ T cells and CD4-CD8- T cells.

Planned experiments will involve sorting cells for naïve, central memory, and terminal effector phenotypes, to verify and further elucidate the metabolic differences within these less differentiated to more differentiated phenotypes using the Seahorse assay of T cell subsets cultured with and without the metabolic inhibitors currently used.

RNA analysis will also be done on sorted cell subsets to determine how gene expression pathways are altered at the level of transcription when T cells are cultured with the inhibitors.

Finally, to ensure reproducibility and generalizability of these findings, the same experiments will be performed on apheresis products from other volunteer donors, as well as fresh peripheral blood samples. These future results will then be analyzed and compared to the results seen as described in this thesis research which relied on mononuclear cells from a single volunteer who underwent apheresis.

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