THE DIFFERENTIAL ROLES OF PI3K P110 ISOFORMS IN REGULATING CD4 T CELL SUBSET POLARIZATION

By

Mason James Webb

Submitted to the Faculty of the Graduate School

of Augusta University in partial fulfillment

of the Requirements of the Degree of

Doctor of Philosophy

May

2017

THE DIFFERENTIAL ROLES OF PI3K P110 ISOFORMS IN REGULATING CD4 T CELL SUBSET POLARIZATION

This thesis/dissertation is submitted by Mason James Webb and has been examined and approved by an appointed committee of the faculty of the Graduate School of Augusta University.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis/dissertation has received final approval with reference to content, form and accuracy of presentation.

This thesis/dissertation is therefore in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____   _____

Date   Major Advisor

                                                   _____

                                                   Departmental Chairperson

                                                   _____

                                                   Dean, Graduate School

ACKNOWLEDGEMENTS

This work would not have been possible without the continual assistance and dedication of a village's worth of co-workers and advisors. Missing any one of these individuals, this thesis would not be on these pages.

First and foremost, I would like to acknowledge and thank my PI and mentor, Dr. Samir Khleif. When I was first searching for a research home in which to earn my graduate degree, I could not find any open positions in the cancer research field. I approached the then Director of the Cancer Center, Dr. Khleif, and asked him whether he knew of any researchers currently seeking PhD students. He graciously suggested I rotate in his own lab, and afterwards offered to accept me as a student. I am deeply grateful for the opportunity he provided me, as well as his constant mentorship in my professional and scientific development.

For my benchtop training, I would like to thank Dr. Shamim Ahmad. Dr. Ahmad has been invaluable in helping me plan and execute my experiments. We have worked together closely, and I have learned much from his example which will serve me well in my future research career.

My PhD committee members have been instrumental in guiding my professional development and in the editing of this thesis. They are mentioned in no particular order. Dr. Wendy Bollag, whose questions I appreciate as an opportunity to challenge myself and improve my work; Dr. Yukai He, who always has a moment for a quick question; Dr. Leszek Ignatowicz, who provided me with guidance on T cell development; Dr.

allowing me to use her laboratory's equipment on a regular basis. Also thanks to the Flow Cytometry Core, specifically Jeanene Pihkala, who sorted most of my cells and to the Cancer Flow Cytometry Core's Ningchun Xu and Aileen Lopez for their help and dedication in managing and maintaining the LSR II's, without which this thesis would be far less lengthy.

Lastly, I would like to thank my family and friends. Without their support and love, this work would never have started. Thank you, and all my love goes out to you.

ABSTRACT

MASON JAMES WEBB

The Differential Roles of PI3K p110 Isoforms in Regulating CD4 T Cell Subset
    Polarization

(Under the direction of SAMIR N KHLEIF)

Class IA phosphatidylinositol-4,5-bisphosphate 3-kinases, or PI3K's, are one of
the earliest bottlenecks for T cell receptor signaling transduction, without which
phosphorylated phosphatidylinositides cannot be generated and the T cell activation
cascade becomes impaired. Of the catalytic class IA PI3K subunits, there are three
isoforms designated as p110α, p110β, and p110δ. The Khleif laboratory has discovered
that these catalytic subunits display unique roles in T regulatory cells and non-polarized
activated CD4$^+$ T cells. This thesis aims to determine what differential control these
p110 isoforms have upon distinct polarized CD4$^+$ T cell subsets.

In our experiments, we have polarized naïve CD4$^+$ T cells to the Th0, Th1, Th2,
and Th17 subsets in the presence of pharmacologic inhibitors for PI3K p110α, β, and δ
and in p110δ kinase-dead D910A mouse models. Upon examination of cytokine
expression, transcription factor activation, and proliferative capacity, we discovered that
while p110δ signaling attenuation enhances the polarization of the Th2 and Th17 subsets
and impairs the Th1 subset at the transcription factor level, cytokine production is
drastically reduced. We determined that this inhibition of cytokine production is partially
due to control of pSTAT5 activation by p110δ signaling. Despite this reduction in
cytokine production with p110δ inhibition, restimulation experiments showed that cells

further Th2-skewed when polarized with this drug do produce Th2 cytokines when the p110δ inhibitory drug is removed. Further, p110α inhibition also additionally enhances Th2 and diminishes Th1 subsets, although to a lesser extent than p110δ signaling loss. We next examined the effect that the Th1 and Th2 polarizing cytokines IL-12 and IL-4 had on the activation of individual p110 isoforms and discovered that these cytokines modify PI3K signaling such that IL-12 strengthens isoform activation, while IL-4 attenuates it.

These results define an as-yet unknown and novel model for how PI3K isoforms modify CD4^{+} T cell fate. Further, as PI3K p110δ inhibitors have entered human cancer therapy trials, our data define in greater detail possible challenges that must be overcome for more effective therapeutic outcomes. This research may also be beneficial to therapies requiring the polarization of other specific CD4^{+} T cell subsets for disease clearance.

KEY WORDS: (PI3K, Isoforms, CD4, Th1, Th2, Th17, Cytokines, Plasticity, Polarization, STAT5)

Table of Contents

List of Tables

List of Figures

I. INTRODUCTION

A. How can we use novel PI3K-isoform targeting drugs to best direct immune response to selectively down-regulate pro-tumor suppressor cells without enhancing anti-tumor effector cell types?

I. Are the distinct T helper cell subsets Th1, Th2, and Th17 regulated by different PI3K isoforms during their polarization from naïve CD4$^+$ splenocytes?

The discovery of discrete differences between cells is the basis of therapeutic exploitation that underlies pharmacological treatment mechanisms. To this end, isoforms of various proteins are of particular interest as a way to target specific harmful or beneficial actors in the human system. The protein of interest in this case is phosphatidylinositol-3-kinase, or PI3K, an enzyme kinase present in a wide number of cellular systems [1]. While the family itself is quite broad and ubiquitously expressed, we focus on the class IA PI3K family, specifically the catalytic isoforms (Figure I). This is because these catalytic isoforms are the predominant signal transducers for downstream signaling cascades [2, 3], including the T cell receptor (TCR). The TCR, in combination with its co-signaling factors, such as CD28, utilize these tyrosine cascades for signaling initiation through the generation of Phosphatidylinositol (3,4,5)-trisphosphate, or PIP$_3$ [4, 5]. Through this class IA PI3K family a vast variety of cellular

1

signaling pathways are initiated [6, 7]. Without them, cellular growth, cell cycle progression, and cytokine production are significantly impaired [8-12]. As CD4^{+} T helper cells require some activation of their TCR complex to leave the naïve state and begin polarization towards effector or suppressor functionality, all T cells are dependent upon class IA PI3Ks [13].

Since T cells are highly dependent on these PI3Ks for TCR signal transduction, it follows that PI3K is a potential target for therapies that attempt to control T cell activation. As a potential site for therapeutic intervention, it is important to consider that class IA PI3Ks are split into three distinct catalytic isoforms, those being p110α, p110β, and p110δ [14]. Should these isoforms of PI3K play distinct roles in T cell activation, differentiation towards polarized T cell subtypes, or T cell functionality, their manipulation through selective pharmacologic inhibitors of PI3K could be a powerful tool to modulate immune responses. This idea has already moved past the realm of theory and into fact with some lymphomas and chronic lymphocytic leukemias (CLL), indicating the potential of this therapeutic model [15, 16].

Idelalisib, or CAL-101, has shown promise in both clinical and experimental cancer models [15, 16]. The Khleif laboratory has shown that CAL-101, as a

Figure I: Model of TCR Signaling through PI3K. Brief diagram of class IA PI3K isoform signaling in a CD4 T cell.

highly selective inhibitor for p110δ, strongly represses the induction of T regulatory cells (Tregs) [17]. We reported that the PI3Kδ isoform is sufficient for TCR downstream signaling, proliferation, and survival for either T conventional cells (Tconvs) or Tregs. In detail, Tregs solely depend on PI3Kδ to regulate these properties, while PI3Kα and PI3Kβ do not play any demonstrable role in these biologic processes. However, rather than observing the conservation of this schema in Tconvs, the two other isoforms, PI3Kα and PI3Kβ combined, provide a redundant pathway to PI3Kδ and can compensate for PI3Kδ in TCR signaling, proliferation and survival for Tconvs. This exclusivity of signaling redundancy in Tconvs provides a direct mechanism of selective PI3K inhibition therapy, such that we can use CAL-101 to diminish signaling, proliferation, and survival almost exclusively in Tregs, and thereby reduce the suppressive aspect of tumor escape. This is a refinement of our previous work, in which we showed that generalized inhibition of the PI3K/Akt pathway using wortmannin also selectively inhibited Tregs and led to enhanced therapeutic outcomes in several murine cancer models [18].

This previous research and related literature like it have begged the question: Are there other such redundancies in PI3K isoform requirements between polarized CD4^{+} T cell types? While PI3Kδ dominates the established literature as the most vital component of lymphocyte signaling, we have established a precedent that would be useful to further dissect. Our next insight occurred when we polarized naïve Tconvs into induced T regulatory cells (iTregs). We were attempting to determine whether iTregs and thymic Tregs (tTregs) were equally suppressed by the PI3Kδ inhibitor CAL-101. Upon flow analysis, we discovered that iTreg induction was attenuated in the presence of CAL-101 treatment, as we expected. What was unusual was that while FoxP3, a Treg specific

transcription factor, was decreased, RORγt was significantly elevated. This led us

through further experimentation to conclude that PI3Kδ inhibition greatly enhances Th17

induction while preventing iTreg maturation. This even occurs in the iTreg condition

without the addition of IL-6, a cytokine considered necessary for Th17 polarization [19].

This ability of CAL-101 inhibition to not only prevent iTreg induction, but to redirect

naïve CD4$^+$ T cell polarization to another subset altogether, led us to the following

hypothesis: Distinct CD4$^+$ T cell polarized subsets, those being Th1, Th2, and Th17,

require different PI3K isoforms for their polarization. Since iTreg end polarization in the

presence of CAL-101 is vastly inhibited to the benefit of the Th17 phenotype, it follows

that iTregs and Th17 cells have different PI3K requirements, or at least that iTregs

require more p110δ signaling than their Th17 counterpart. This difference is highly

exploitable within the context of pharmacologic therapy. It also follows that other

distinct CD4$^+$ T cell subsets, in this case Th1 and Th2, may display differences in the

utilization of PI3K isoforms during their polarization that can be likewise exploited.

To this end, I will create *in vitro* conditions skewing towards Th1, Th2, and Th17

polarization and examine their resulting phenotypes both with and without highly

selective p110α, p110β, and p110δ inhibitors. We will use these results to determine

which specific IA PI3K isoforms are required for specific CD4$^+$ T cell subset activation,

and thus create a 'roadmap' for which specific isoforms are critical or redundant in each

CD4$^+$ T cell subtype. This will provide a scientific blueprint for how best to control the

immune system through selective inhibition of PI3K isoforms, such that we can better

direct the immune response that leads to the desired therapeutic outcome.

II. Do the cytokines IL-12 and IL-4 effect differential change upon naïve CD4^{+} T cell PI3K isoform activation, and thereby modulate Th1 and Th2 polarization outcomes?

Cytokines are the predominant mechanism of control that the immune system uses to direct immune response [20, 21]. In many cases, the addition of a single cytokine within an *in vitro* culture is sufficient to induce a highly diverse set of T cell subtypes. As previously stated, our laboratory has defined differences in PI3K isoform utilization requirements between the iTreg and Th17 CD4^{+} T cell subtypes. All T cell subsets require activation of their TCR complex to move from their naïve to effector types, so the divergence of PI3K utilization is unlikely to result from the TCR complex alone. This implicates the cytokines, in this case IL-6 and TGF-β, as modulators of differential PI3K isoform signaling through direct or indirect effects. Based on this reasoning, we also expect the Th1 and Th2 T cell subtypes to display unique PI3K isoform utilization profiles through their polarizing cytokines IL-12 and IL-4.

In order to discover the exact importance of these cytokines, we must examine how the Th1, Th2, and Th17 polarizing cytokines affect individual PI3K isoforms. While literature does discuss the roles of specific cytokines for generating specific polarized CD4^{+} T cell subsets, there has been no detailed examination of whether these cytokines have differential effects on specific catalytic class IA PI3K isoforms. Our initial hypothesis was that TGF-β conservatively activated PI3Kδ, while IL-6 was an inhibitor of PI3Kδ, thus explaining why specific CAL-101 inhibition uniquely increased Th17 induction at the expense of iTregs. We have shown this to be correct in another paper [22]. As such, we expect Th1 and Th2 to show similar effects, in that IL-12 and IL-4 will

also modulate PI3Kδ signaling, as well as possibly other PI3K isoforms. We will approach this by differentially activating naïve CD4$^+$ T cells in the presence of these single polarizing cytokines and determining the activation of PI3K isoforms in each case via immunoprecipitation of phosphor-Tyrosine residue-containing proteins and then probing with antibodies specific to individual p110 isoforms.

While our previous work on pharmacological inhibition provides a working method to direct T cell polarization responses, directly breaking apart and mapping the roles of specific cytokines in PI3K isoform activation would provide us a mechanism to direct immune responses through PI3K manipulation. It would also provide a simple introductory mechanism to further examine PI3K isoforms within other T cell subsets. Pharmacological experiments are ultimately necessary for establishment of the soundness of potential therapies. However, discovering that polarizing cytokines are modifiers of PI3K isoform utilization signaling would make immunoprecipitation an excellent introductory experiment to determine likely redundancies and requirements for other T cell subtypes. Results can more specifically be tested with the selective PI3K isoform inhibitors as determined by the experimental results of immunoprecipitation, and ultimately fit into the 'roadmap' we will establish for the Th1, Th2, Th17, and iTreg subsets.

III. Can we recapitulate our results in a non-pharmacologic model system?

While our results are designed to clearly show differences in PI3K isoform utilization between the polarization of the CD4$^+$ T cell subsets, the lion's share of our experiments are based on pure pharmacological inhibition. While the isoform-specific

inhibitors we are using have been demonstrated to be highly selective to their indicated target, it is vital that we rule out off-target effects. Previous work has been performed with 110delta-PI3K-D910A transgenic mice, a PI3Kδ kinase-dead mutant [23], and as such we decided to use this method to verify our results. This provides a previously-verified mechanism to abrogate PI3Kδ isoform signaling transduction. We expect the knockout model to clearly align with our CAL-101 pharmacological inhibition data, demonstrating that our results are not artefactual or off-target effects. In the unlikely event that our results do not match, we will examine the off-target effects of our inhibitors to determine what truly is causing the differences between polarized subsets. Regardless of the results, the effects of specific cytokines on PI3K isoform activation will remain unchanged, and pharmacological inhibition still provides a mechanism to selectively target the polarization of specific CD4$^+$ T cell subtypes. The only likely change in the event of unexpected results when using the D910A model will be which specific signaling molecule or molecules are the targets of our pharmacologic inhibitors.

B. Review of Related Literature

I. What is PI3K and its role in cellular signaling?

Phosphatidylinositol-3-kinase, or PI3K, is the name of a family of enzyme kinases that are present throughout the human body. Discovered by Lewis Cantley and associates in 1985 [24], these molecules have been found to be important in many systems, spanning from insulin signaling [25] to cancer [18]. Despite this diversity of functionality, members of the PI3K family all share one defining trait; they are all signaling enzymes that phosphorylate phosphatidylinositol or polyphosphoinositides at the 3-position on the hydroxyl group of the inositol ring [26]. Mammalian PI3Ks are further sub-divided into distinct classes, those being class I, II, and III. While studies do exist on class II/III PI3Ks [25, 27], the focus of this thesis will be on class I PI3Ks. Class I PI3Ks as a whole are activated by tyrosine kinase receptors and G protein-coupled receptors [28]. They produce three phosphatidylinositols, each with sequentially increasing numbers of phosphates, these being phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P_2), and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P_3) [3]. Upon phosphorylation, these inositol rings create a docking position for intracellular proteins within the inner leaflet of the cell's plasma membrane, and thereby serve as initiator sites for a huge variety of intracellular signals. These signals span a gamut of possible functions, which include motility, metabolism, vesicular trafficking, differentiation, proliferation, and survival, amongst others. Class I PI3Ks can be still further sub-divided into class IA and IB PI3Ks [29]. These differences between subclasses are predominantly defined by what signals the PI3K heterodimer best propagates. Class IB PI3Ks are unique in that they are adapted to allow heterotrimeric G

proteins to transduce signal, while class IA PI3Ks transduce signals within tyrosine kinase cascades initiated by cell surface receptors [29]. A major aspect of T cell activation and signaling is through the T cell receptor, and as such class IA PI3Ks are the focus of this thesis.

Class IA PI3Ks are heterodimeric, composed of both a catalytic and a regulatory subunit [30]. The regulatory subunit is commonly called the p85 subunit, but exists in 5 splice variants p85α, p55α, p50α, p85β, and p55γ [31]. Befitting their name, the regulatory subunit controls the actions of the catalytic group by both stabilizing the catalytic unit and suppressing it in the absence of upstream initiator signals [32, 33]. The catalytic subunits allow the downstream transduction of signal and are composed of three variants called p110α, p110β, and p110δ. The p110α and p110β isoforms are expressed in all cells, with the exception of β in some types of lymphocytic cells [34]. The third variant, p110δ, is confined predominantly to leukocytes [35, 36]; however, it has been identified within neurons at moderate levels [37].

II. What are the mechanisms of signaling in class IA PI3Ks in lymphocytes?

While lymphocytes are unique in their expression of p110δ when compared to other cell types, gross functionality of class IA PI3Ks remains the same. They convert PI(3,4)P2 to PI(3,4,5)P3, which serves as a scaffold point for downstream intracellular signaling [7]. Despite this, differences in the roles of PI3K between lymphocytic types have been discovered.

Currently, the role of p110α isoform in lymphocytic signaling appears to be minimal. Broadly, p110α signaling is necessary for growth and metabolism [38]. This appears to be because p110α is selectively recruited by insulin-receptor substrate proteins, more so than p110β and p110δ. Because of p110α's importance in growth and metabolism, it is a frequently activated mutation in a variety of cancers [39], most commonly colorectal, brain, gastric, and liver [40]. Despite this, mutations of PIK3CA, the gene encoding p110α, do not appear to be particularly relevant within the context of lymphocytic cancers, with only about 1% of acute lymphocytic leukemias expressing mutated PIK3CA [41]. Our own previous research in the Khleif laboratory shows that the p110α isoform does have some importance outside of being a ubiquitous metabolic activator. We showed that p110α is a redundant T cell receptor signaling isoform for CD4^{+} conventional T cells [17], and that it is able to compensate for the inhibition or loss of other class IA PI3K catalytic isoforms, particularly p110δ. This redundancy can also be seen in B cell signaling. With complete knockout of p110δ, the development of B cells in the bone marrow is mildly attenuated [42, 43]. With the combined deletion of p110α and p110δ, however, pre-B cell receptor (BCR) signaling to promote the development of B cell progenitors is almost completely eliminated, leading to all but total depletion of B cells [34]. This mechanism is further parsed out in that either p110α or p110δ can activate tonic signaling from the BCR, while p110β cannot.

The p110β PI3K isoform seems more intermediate in its characteristics, in that it still displays metabolic signaling potential like p110α, as well as distinct importance in some lymphocytic pathways. Similarly to p110α, the β catalytic isoform appears to play roles in insulin signaling and cancer [44, 45], such that elimination of p110β can lead to

long-term insulin resistance and impaired glucose homeostasis. Ablation of p110β also seems to be protective against a few cancer types, notably some prostate and breast tumors in mice. This implicates it in having some importance related to cancer development. With regards to lymphocytes, p110β has been shown to play unique roles in macrophages, neutrophils, and in conventional T cells. While class IA PI3Ks are shown to be involved predominantly in tyrosine cascade signaling [29], p110β displays redundancy in macrophages with the class IB PI3K p110γ [46]. In macrophages and fibroblasts, p110β associates with G protein-coupled receptors (GPCRs) and activates downstream Akt. Further, p110β was downregulated in cells where p110γ expression was induced. Despite this GPCR functionality, PI3Kβ still seems warranted to remain in the class IA PI3Ks. While it seems to have some role in GPCR signaling, in the tyrosine-kinase specific Fc IgG receptors (FcγRs) of neutrophils, PI3Kβ has been demonstrated to be necessary for the activation and production of reactive oxygen species (ROS) [47]. Again, the idea of redundancy is brought up, in that neutrophilic FcγR activation can only be eliminated by dual targeting of PI3Kβ and δ. Likewise, our laboratory has seen similar redundancy in Tconvs. Notably, while the PI3Kδ isoform is the predominant driver of downstream signaling, proliferation, and survival in Tconvs, PI3Kβ is able to partially compensate for its loss in conjunction with PI3Kα [17].

The last class IA PI3K isoform, p110δ, largely and exclusively effects the immune system, with some expression in neuronal tissue [14]. As discussed, unlike PI3Kα and β, PI3Kδ is not ubiquitously expressed [13-16] and is seen in cells of hematopoietic origin and in the central nervous system (CNS). Further, while overexpression of PI3Kα and β through mutation of their encoding genes PIK3CA and

PIK3CB has been implicated in several cancers, PI3Kδ overexpression appears less prevalent in cancer development and pathogenesis, with only weak to moderate aberrant expression confined to some skin, pancreatic, and possibly urothelial cancers [48]. Mutations of the p110δ isoform's gene, PIK3CD, are instead the source of primary immunodeficiencies [14]. Two major subsets of these immunodeficiencies have been classified, initiated from either the loss or gain of p110δ isoform functionality. The loss of function in p110δ itself, rather than the regulatory unit p85, is extremely rare and only characterized in a single human patient [49]. In this case the disease was characterized by severe B cell lymphopenia and a depressed number of total T memory cells. The gain of function diseases are more common, with a prevalence sufficient to warrant a name for the specific immunodeficiency. The overall gain of function syndromes of PI3Kδ are either called APDS, for activated PI3Kδ syndrome, or PASLI, for p110δ-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency [14]. These syndromes appear largely associated with hyper IgM and aberrancy in B cell functionality [50-52]. Mouse models also provide additional insight into the p110δ isoform and its importance to lymphocytes. As demonstrated by ourselves and others, p110δ is vitally important for the signaling, proliferation, and functionality of Tregs [53]. It has also been implicated as a major player in CD4$^+$ T helper cell differentiation and expansion, although questions concerning redundancies and unique isoform utilization remain [23, 54]. Our own hypotheses do not contest PI3Kδ as the predominant driver of CD4$^+$ T cell functionality and signaling. Rather, because of the unique redundancies of PI3K isoforms in Tconvs not present in Tregs, and the discovery that PI3Kδ inhibition in

the iTreg condition favors Th17 polarization, we want to further detail the intricacies of isoform signaling and redundancies between other CD4^{+} T helper subsets.

III. T cell polarized subsets and cytokines

The initial discoveries that CD4^{+} T cells existed in distinct differentiated subsets gave us the Th1 and Th2 axis [55, 56]. The cells that were named Th1 cells were initially distinguished from their Th2 counterparts in their cytokine expression profile, namely Th1 cells made IFN-γ, lymphotoxin, TNF-α, and large quantities of IL-2 (Figure II). Th2 cells were discovered to produce TNF-α and IL-2 as well, but were lacking in IFN-γ and lymphotoxin and instead produced the interleukins 4, 5, 13, and, to some extent, 9 [57]. The next widely recognized T helper subset to come from naïve CD4^{+} T cell precursors was the Th17 subset, named due to its unique production of the IL-17 cytokine, not found in either the Th1 or Th2 subsets [58-60]. Around the same time it was also determined that naïve CD4^{+} T cells could be differentiated into a T regulatory cell type, designated induced Tregs (iTregs) and distinct from thymic origin Tregs (tTregs), which do not have a peripheral naïve T cell precursor [61-65]. There is discussion about the addition of other distinct CD4^{+} T cell subsets, but the Th1, Th2, Th17, and iTreg groups are the most widely accepted and broadly studied at this point.

As helper cells, Th1 cells do not directly kill pathogens—rather, they recruit and activate other immune effector cells in what is termed cell-mediated immunity (CMI). Macrophages are a major contributor to CMI, and B cells are activated to produce complement-fixing and opsonizing antibodies [66]. The primary role of Th1 helper cells
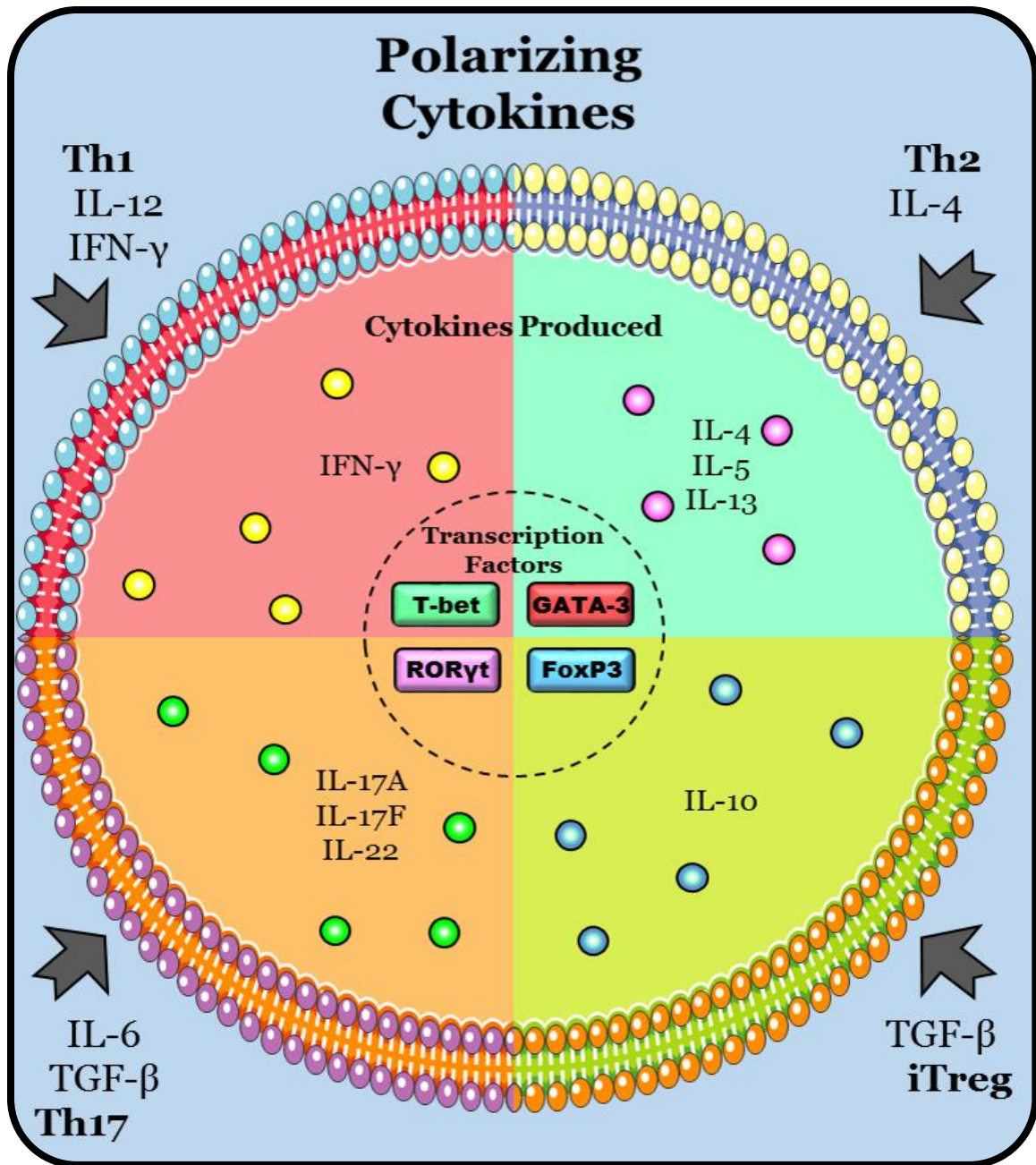
Figure II: Overview of Polarization. Model of polarizing cytokines, transcription factors, and produced cytokines for Th1, Th2, Th17, and iTreg T helper cell

in the immune system is to combat bacterial and viral infections [67]. Differentiation of the Th1 subset begins with the activation of the innate immune system, which causes the initial production of the cytokines IL-12 by dendritic cells (DCs) and macrophages, while initial IFN-γ expression is generated by natural killer (NK) cells. These polarizing cytokines induce the expression of the transcription factor T-bet, the prime driver of the Th1 cell type. These newly-developed Th1 cells in turn produce IL-12 and IFN-γ, creating a local cascade of differentiated Th1 cells.

T-bet, or Tbx21, is the master regulator of the Th1 CD4$^+$ T cell subset, as well as a vital transcription factor for a variety of other lymphocytic cell types [68]. This designation as a master regulator was proven through retroviral gene transduction of T-bet into polarized Th2 lineage cells, which afterwards began producing IFN-γ and downregulating their Th2 cytokines IL-4 and IL-5 [69]. Besides existing purely as an inducer towards the Th1 subset on the bipolar Th1/Th2 axis, retrovirally transfected T-bet was able to repress Th17 cytokines and transcription factors, as well as cause a much greater induction of IFN-γ [70]. Signaling begins, as discussed, with signal transduction from the IL-12 and IFN-γ receptors. IFN-γ signaling leads to the activation of STAT1 (Figure III) [71, 72]. This, combined with TCR-complex activation, allows the formation of a Tbx21 promoter complex and the production of the T-bet transcription factor. T-bet then is required for production of Th1's prime effector cytokine, IFN-γ [73].

Initiation of Th1 polarization begins with the production of IL-12 by the innate immune system. The IL-12 receptor expresses a heterodimeric receptor [74]. It shares this trait with other members of the IL-12 receptor family, which additionally include IL-
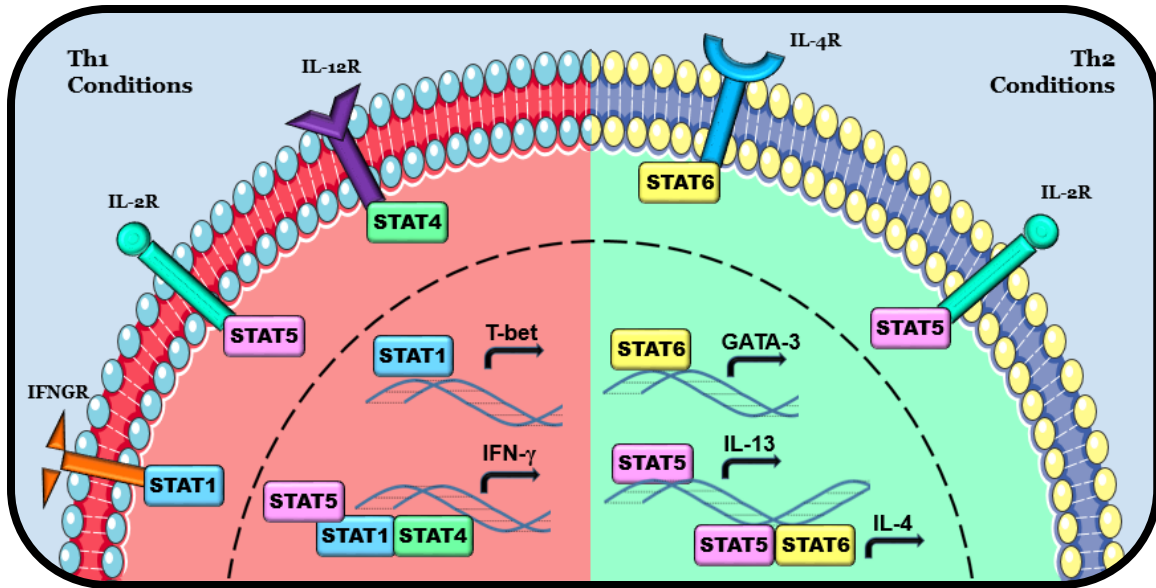
Figure III: STAT Signaling. Model of select STAT proteins involved in transcription factor and cytokine expression for Th1 and Th2 conditions.

23, -27, and -35. This heterodimer is formed from the distinct units IL-12Rβ1 and IL-12Rβ2, through which signaling is transduced [75]. These units exist as distinct and separate monomers, forming signaling dimers after cytokine engagement. As expected given that IL-12 signaling drives towards the Th1 subset, engagement of this receptor leads to a largely pro-inflammatory response. This cytokine-receptor engagement leads to the activation of the JAK/STAT pathway [76]. In the IL-12 receptor heterodimer, ligation of the IL-12 cytokine leads to the activation of Jak2 and Tyk2, both receptor-associated JAK kinases [77]. These kinases are vital for the recruitment and phosphorylation of STAT4, which forms a homodimer before translocation to the nucleus, driving Th1 differentiation [78-80]. This has also been shown in human cells in response to IFN-γ.

The cytokine IFN-γ also relies on the JAK/STAT signaling cascade [81]. Its receptor is formed of a heterodimer of interferon gamma receptor 1 and 2 (IFNGR1,

IFNGR2). While there is some debate on exact models, similarly to IL-12 JAK kinases are activated and lead to the phosphorylation of STAT1α [82, 83]. This forms a homodimer which translocates to the nucleus, leading to the activation of additional IFN-γ through binding to the IFN-γ activated sequence (GAS) promotor.

Unlike the Th1 subset, which mediates effector actions mainly through CMI, the Th2 group is the predominant driver of what is known as humoral immunity. Functionally, this means that the Th2 response is independent of phagocytic immune mediated response and co-requisite inflammation. Rather, they aid basophils, mast cells, and eosinophils through the promotion of the production of IgE antibodies. This response is targeted towards parasites rather than microorganisms targeted by the Th1 response. The IL-4 cytokine is the initial driver of Th2 differentiation from naïve CD4$^+$ T cells. The transcription factor expressed by activation of the IL-4 receptor is GATA-3, which commits the cell to the Th2 lineage and causes the production of the cytokines IL-4 and IL-5. As with Th1, this production of cytokines in the local tissue environment creates a positive feedback mechanism where additional naïve CD4$^+$ T cells in the specific area also are skewed towards the Th2 effector phenotype. The initial producer of the Th2 polarizing cytokine IL-4 is thought to be the basophil, as they produce IL-4, interact with Th2 cells, and, following infection, have been seen to be recruited to secondary lymphoid tissues.

Similarly to T-bet for the Th1 subset, GATA-3 is seen as the predominant and critical driver of Th2 subset polarization [84]. As previously discussed, the loss of T-bet leads to the inability of T cells to polarize towards the Th1 condition and instead skew towards the Th2 and Th17 subsets [85-88]. This conclusion that GATA-3 is required for

Th2 is based on the fact that deletion of GATA-3 prevents differentiation into the Th2 phenotype. Further, GATA-3 overexpression is able to switch Th1 polarized T cells to the Th2 phenotype [89, 90]. While the targets of GATA-3 are not exhaustively known, this transcription factor is vital for a variety of what are considered Th2-specific products. Just as T-bet binds to regions flanking the IFNG locus [91, 92], GATA-3 binds to regions specific to the production of interleukins 4, 5, and 13 [93-99]. Beyond this capability to direct different immune polarizing responses, however, these transcription factors also interact in such a way as to create a bipolar, diametric Th1/Th2 axis.

The concept of the Th1 and Th2 subsets acting in opposition comes from the mechanistic behavior of their transcription factors, T-bet and GATA-3. This extends beyond the concept of plasticity between $CD4^+$ T helper cell subtypes [100]. The plasticity model describes how differing circumstances, such as a change in the overall cytokine milieu in the cellular environment, can change the polarization subset of the T cell to better reflect the changing needs of the specific environment. Rather, the transcription factors T-bet and GATA-3 act antagonistically, suppressing the promotors that produce cytokines of the opposing lineage [84]. Specifically, T-bet not only drives forward Th1-favoring factors such as IFN-γ, but also binds to the IL-4 promotor and its downstream silencer [93, 101-103]. This opposition is not unilateral in that GATA-3 also downregulates Th1-specific promotors. GATA-3 does this by binding to the IFNG locus and then recruiting histone methyltransferase polycomb repressive complex-2 (PRC2) [104]. This repressor complex is a strong silencer, and a mark of non-active, silent chromatin. Beyond this evidence alone, it has been shown that T-bet and GATA-3 directly associate with each other when co-expressed [105]. This additionally leads to the

inability of GATA-3 to bind to the IL-5 promotor and severely curtails the Th2-specific cytokine IL-5. This oppositional nature of the Th1 and Th2 transcription factors is of vital importance to understanding our experiments relating to these subsets.

As IL-12 and IFN-γ are largely seen as Th1-specific cytokines, likewise the interleukins -4, -5, and -13 are predominantly seen as tied to the Th2 subset [67]. As with many cytokine receptors, these three cytokine-specific receptors activate specific JAK/STAT cascades which lead to their primary cellular responses [106]. The IL-4 and IL-13 receptors are closely related in that they share a common IL-4Rα unit. The IL-4 receptor differs in that the IL-4Rα unit complexes with a common cytokine receptor γ-chain, creating what is known as a type I receptor. This receptor only signals through interaction with IL-4. The IL-13 receptor is less conservative, binding not only IL-13 but also IL-4. The complex itself is of the IL-4Rα unit with IL-13Rα1, and this complex is known as the type II receptor. These two receptors also display unique patterns of expression, in that the type I receptor is predominantly seen on B cells, T cells, monocytes, and eosinophils, while the type II receptor is more common on activated B cells, monocytes, epithelial cells, and smooth muscle cells, with both types present on fibroblasts. IL-5 is not as closely related, and has its own distinct receptor units. These are the IL-5Rα chain complexed with a β-chain. This subunit leads to the production of granulocyte-macrophage colony-stimulating factor (GCSF) and IL-3 signaling.

While the major focus of our research is on how we can affect the Th1/Th2 axis by manipulating PI3K isoform signaling, we also decided to examine the Th17 group to gain greater understanding of the iTreg/Th17 axis that we discussed in a previous article [22]. This subset of CD4^{+} helper T cells is called the Th17 group, induced by the IL-6

cytokine. Matching their name, they secrete IL-17 and are defined by the predominance of the transcription factor RORγt [107, 108]. While IL-6 is required for polarization of the naïve precursors of Th17 cells, TGF-β and, it is suspected, IL-16 [109], are required for their development. These secondary cytokines are required for the Th17 cell to produce IL-21, which activates STAT3, another transcription factor essential for Th17 differentiation, in an autocrine fashion [110]. The predominant functionality of these cells is inducing epithelial and stromal cells to secrete CXCL8, among other cytokines, in order to recruit neutrophils to infected tissue [111]. As their effect is largely on epithelial and stromal cells, Th17 cells are seen in large numbers in skin tissue and gut mucosa.

The retinoic acid receptor-related orphan receptor-γ, or RORγt, is the master transcription factor for the Th17 phenotype. This is the same dynamic seen with T-bet towards the Th1 subset and GATA-3 towards Th2. Even the antagonism of action between the Th1 and Th2 transcription factors is seen in parallel with RORγt and the T regulatory cells' master transcription factor FoxP3 [71]. Again, besides merely driving forward and directing effector function at the IL-17A promoter, RORγt, in combination with Runx1, also inhibits FoxP3 expression [112]. Taking the Th1/Th2 axis into consideration, should the same mechanism for T-bet/GATA-3 hold, RORγt and FoxP3 would also bind to each other and lead to mutual inhibition of their respective polarizing functions. This was in fact shown to be the case in 2008 [113]. While the transcription factors between subsets are highly distinct, these bipolar, opposing axes of polarization provide an important mechanism for understanding how differentiation is ultimately completed, and amongst which competitors. This similarity of mechanisms also implies that some of the lessons learned in previous studies of how PI3K isoforms can

differentially drive cells towards different differentiation groups can be more broadly

applied, such as towards Th1 and Th2.

The specific Th17 polarizing cytokine is IL-6, which also displays a variety of

pleiotropic effects that extend beyond immune polarization to include vasculature, the

neuroendocrine system, and metabolism [114]. IL-6 also can be activated strongly

through a variety of pathways, specifically by tumor-necrosis factor and IL-1β signaling.

The cells that produce IL-6 are generally stromal and immune cells, and almost all cells

of these types have the capacity to produce IL-6. Beyond IL-6's pleiotropic role, it has

been implicated as a vital lymphocyte-stimulating factor. In both innate and adaptive

immune responses, impairment of IL-6 functionality worsens the response to infections

of viral, bacterial, and parasitic origin [115-122]. The actual structure of the receptor is

similar to other cytokine receptors and signaling mechanisms. It is composed of both an

α and β receptor subunit. The α subunit is known as IL-6R or CD126, and is the portion

that actually binds the IL-6 cytokine. The β subunit, also known as gp130 or CD130, is

the signal transducer [123-126]. 'Classical' signaling from the IL-6R works as

previously described cytokine receptor signaling does, with activation of the Jak/Tyk/Stat

phosphorylation cascade. Unique to this cytokine, however, is the concept of 'trans'

activation. This signaling mechanism involves the secretion of a soluble form of IL-6R,

which increases the bio-availability and half-life of IL-6 [127]. This signaling has a host

of effects in a large number of cell types. This includes roles in survival, proliferation of

Th1 and Th2 polarized subsets, lymphocyte recruitment, and immune responsiveness.

Specifically, though, we will be focusing on IL-6's importance in polarizing the Th17

subset and opposing iTreg polarization [128, 129], thus promoting an inflammatory rather than suppressive immune response.

In contrast, TGF-β polarizes naïve CD4$^+$ T cells towards the suppressive iTreg subset in the absence of IL-6, thereby enhancing FoxP3 expression [130]. Through this stimulation of the master suppressive transcription factor FoxP3, peripheral naïve CD4$^+$CD25$^-$ T cells become CD4$^+$CD25$^+$ induced T regulatory cells [61, 62]. Unlike the previously discussed cytokines, TGF-β's mechanism of action is not through Jak/Tyk/Stat cascades, but instead through distinct Smads binding to the FoxP3 promotor as well as some Erk/Jnk signaling transduction [131]. While this FoxP3 induction is vital for iTregs, TGF-β is also required for Th17 polarization, albeit in combination with IL-6. IL-6 is necessary for the elimination of iTreg polarization, but TGF-β also plays a distinct and important role in Th17 induction. It does this through inhibiting suppressor of cytokine signaling (SOCS)3, thereby enhancing STAT3 activation [132]. Thus, the Th17 phenotype requires both IL-6 and TGF-β in combination, while when polarizing iTregs TGF-β alone is sufficient.

As discussed, the interleukins generally act through tyrosine phosphorylation cascades that activate Jak/Tyk/Stat protein kinases. One specific Stat kinase, STAT5, is of specific interest in this thesis. This protein is activated by signal transduction from the IL-2 receptor [71, 133]. STAT5 is vital for the expression of a wide variety of cytokines across several polarized CD4$^+$ T helper subsets. Firstly, STAT5 has a role in IFN-γ signaling in that it has been shown to activate the GAS promotor region and leads to type I interferon-dependent gene transcription [134, 135]. Rather than being specific to Th1 though, it is also conserved in importance in the Th2 subset [136]. STAT5A and B bind

to multiple locations required for the regulation of Th2 cytokines, specifically the *Il4* and *Il13* gene loci, as well as the *Rad50* gene [137]. While not directly affecting cytokines, STAT5 also is important for the maintenance of expression of FoxP3 in Tregs [138]. These effects are not limited to these specific subgroups either. Although it is beyond the scope of this thesis, STAT5 has also been shown to be vital for Th9 differentiation [139]. As such, STAT5 is vital for a wide variety of T helper subsets and cytokines.

IV. PI3K Targeting in Cancer Therapies

Targeting of the PI3K/Akt pathway is being explored as an avenue of immune modulation to enhance beneficial cancer outcomes and clearance. Our own laboratory specializes in exploring this pathway. We have previously shown that Tregs and their suppressive functions are more strongly inhibited in the presence of pan PI3K/Akt inhibitors, such as wortmannin [18]. This led to increased tumor clearance in a variety of tumor models in mice, including TC-1 and B16 cancer lines. While this has led to increased survival in murine tumor models, we sought to explore more specific mechanisms that could be better exploited to lead to enhanced therapeutic outcomes. Other laboratories have demonstrated that targeting specific PI3K isoforms, specifically PI3Kδ, has a strong therapeutic effect in a variety of hematologic cancers. One of particular promise, CAL-101, or Idelalisib, was shown to have strong effects in murine models of multiple myeloma [140] and B cell malignancies [141]. Due to strong effects in murine models, this modality was taken to clinical trials.

Inhibition of PI3K isoforms moved into clinical trials with select hematological malignancies. In a CLL trial, there was a significant increase in overall survival with

Idelalisib and rituximab over rituximab alone, with a drastically improved rate of progression-free survival, 93%, versus the control group, 46%, at 24 weeks [16]. While the indolent lymphoma trial was uncontrolled, 90% of patients experienced tumor reductions, with 57% meeting the criteria for objective tumor response [15]. Due to the strong positive effects seen in these clinical trials, the PI3Kδ inhibitor Idelalisib (CAL-101) was approved by the FDA for patients with relapsed chronic lymphocytic leukemia in combination with rituximab, as well as patients with relapsed follicular B-cell non-Hodgkin lymphoma (FL) or relapsed small lymphocytic lymphoma (SLL), so long as they had received at least two previous therapies [142]. While these therapies have shown promise, recently the FDA has stopped some combination clinical trials due to high rates of adverse events, including deaths [143]. This not only shows the immense promise of this mechanism of immune modulation in cancer therapeutics, but the necessity of further understanding the mechanics of specific PI3K isoforms in order to minimize or prevent adverse side effects.

II.   MATERIALS AND METHODS

A. List of Required Materials

Inhibitory drugs for specific PI3K catalytic isoforms α, β, and δ and a pan-PI3K inhibitor were all purchased from Selleckchem. In order, they are A66, TGX-221, CAL-101 (Idelalisib, GS-1101), and GDC-0941 (Pictilisib).

All flow cytometry antibodies were purchased from BD and are as follows: APC Rat Anti-Mouse CD25 Clone PC61, PE Rat Anti-Mouse CD4 Clone H129.19, PerCP-Cy5.5 Rat Anti-Mouse CD4 Clone RM4-5, Alexa Fluor 488® Rat Anti-Mouse IFN-γ Clone XMG1.2, PE Rat Anti-Mouse IL-4 Clone 11B11, V450 Rat Anti-Mouse IL-17A Clone TC11-18H10, Alexa Fluor 488® Mouse anti-T-bet Clone O4-46, PE Mouse anti-GATA3 Clone L50-823, PE Mouse anti-Mouse RORγt Clone Q31-378, Alexa Fluor® 488 Rat anti-Mouse Foxp3 Clone MF23, Pacific Blue™ Mouse anti-Stat5 (pY694) Clone 47/Stat5(pY694).

The following antibodies and related products were purchased from CST: Phospho-Tyrosine Mouse mAb (P-Tyr-100) (Sepharose Bead Conjugate), PI3 Kinase p110α (C73F8) Rabbit mAb, PI3 Kinase p110β (C33D4) Rabbit mAb, PI3 Kinase p110δ (D1Q7R) Rabbit mAb, PI3 Kinase p85 (19H8) Rabbit mAb, Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Rabbit mAb, Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb,

Akt (pan) (C67E7) Rabbit mAb, β-Actin (8H10D10) Mouse mAb, Anti-rabbit IgG, HRP-linked Antibody, Anti-mouse IgG, HRP-linked Antibody.

All of the following polarizing cytokines and neutralizing antibodies were purchased from Peprotech: Recombinant human IL-2, Recombinant human IL-4, Recombinant human IL-6, Recombinant murine IL-12, Anti-murine IL-4 (polyclonal rabbit), Anti-murine IFN-γ (polyclonal rabbit), Anti-murine IL-12 (polyclonal goat).

Other cell culture antibodies used were obtained from the indicated sources: Purified NA/LE Hamster Anti-Mouse CD3e Clone 145-2C11 (BD), Purified NA/LE Hamster Anti-Mouse CD28 Clone 37.51 (BD), Recombinant Human TGF-beta 1 Protein (R&D Systems).

Media Supplies were obtained from the indicated companies: RPMI 1640 with HEPES and L-Glutamine (Lonza), Benchmark™ Fetal Bovine Serum (Gemini Bio-products), Penicillin/Streptomycin 5000 U/mL (Gibco), MEM Non-Essential Amino Acids Solution 100X (Gibco), 2-Mercaptoethanol (Gibco), Ethylenediamine Tetraacetate Acid (EDTA), 0.5M, pH 8.0 (Thermofisher Scientific).

Enrichment and CBA Kits were from Biolegend: MojoSort™ Mouse CD3 T Cell Isolation Kit, LEGENDplex™ Mouse Th Cytokine Panel (13-plex).

Fixation supplies were obtained from BD: Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit, Mouse Foxp3 Buffer Set, Phosflow™ Fix Buffer I, Phosflow™ Perm Buffer III.

Plasticware was purchased as indicated: Rapid-Flow™ Filter Units with 0.2 μm SCFA membrane, 75 mm diameter (Thermofisher), Sterile 48 Well Cell Culture Cluster Flat Bottom with Lid, Tissue Culture Treated (Costar), Sterile 96 Well Cell Culture

Cluster Flat Bottom with Lid, Tissue Culture Treated (Costar), Sterile 96 Well Cell

Culture Cluster Round Bottom with Lid, Tissue Culture Treated (Costar), Falcon™ 70

μm cell strainers (Thermofisher), Falcon™ Round-Bottom Polystyrene Tubes

(Thermofisher).

 Reagents for Cell Lysis were purchased from the indicated suppliers: RIPA Lysis

and Extraction Buffer (Thermofisher), Halt™ Protease Inhibitor Cocktail 100X

(Thermofisher), Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich).

 Western Blot Reagents were obtained as indicated: Precision Plus Protein™

Dual Color Standards (Bio Rad), iBlot® Gel Transfer Stacks, Nitrocellulose, Mini

(Invitrogen), NuPAGE® MOPS SDS Running Buffer 20x (Novex), NuPAGE® LDS

Sample Buffer 4x (Novex), Restore™ Western Blot Stripping Buffer (Thermofisher),

Tween® 20 (Thermofisher), Blotting-Grade Blocker, Non-fat Dry Milk (Bio Rad),

Bolt™ 4-12% Bis-Tris Plus Gels, 1.0mm X 10 Well (Invitrogen), SuperSignal® West

Femto Maximum Sensitivity Substrate (Thermofisher), SuperSignal® West Pico

Chemiluminescent Substrate (Thermofisher), Pierce™ Coomassie (Bradford) Protein

Assay Kit (Thermofisher).

 Miscellaneous reagents were obtained from the indicated companies:

LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nM excitation

(Thermofisher), CellTrace™ Violet Cell Proliferation Kit (Thermofisher), Leukocyte

Activation Cocktail, with BD Golgiplug™ (BD), Phorbol myristate acetate (PMA), 50

ng/μL (Sigma-Aldrich), Ionomycin, 750 ng/μL (Sigma-Aldrich).

B. Mice

C57BL/6 female 6-8-week-old mice were purchased from the Jackson Laboratory (Bar Harbor, ME). P110delta-PI3K-D910A (kinase-dead) PI3Kδ KO mice on C57BL/6 strain background were purchased from Charles River, Wilmington, MA and bred under pathogen-free conditions in the Augusta University animal facility. All procedures were carried out in accordance with approved institutional animal protocols.

C. Media

T cells are cultured in T cell media, made with the following materials in the stated ratios. Per our established laboratory protocols, 450 mL of RPMI-1640 with L-glutamine and 25 mM HEPES (Lonza) is mixed with 50 mL of FBS (Gemini Bio), 10 mL of 5,000 U/mL Pen-Strep (Gibco), 5 mL of MEM Non-Essential Amino Acids Solution 100x (Gibco), and 500 µL of 2-Mercaptoethanol (Gibco). This is then filtered via vacuum filtration in a tissue culture hood using 500 mL Rapid-Flow™ Filter Units with 0.2 µm SCFA membrane (Thermofisher).

FACS media is a preparation of sterile 1x PBS and filtered FBS (Gemini Bio) at the appropriate ratio to create 2.5% FBS in 1x PBS. For CD3 enrichment using MojoSort™ Mouse CD3 T Cell Isolation Kit, enrichment buffer is prepared. This enrichment buffer is 978 mL of 1x PBS, 20 mL FBS (Gemini Bio), and 2 mL of 0.5 M EDTA (Thermofisher), for a final concentration of 1 mM EDTA. This is then filtered in a tissue culture hood using 500 mL Rapid-Flow™ Filter Units with 0.2 µm SCFA membrane and left on the vacuum manifold for at minimum 1 hour to degas.

D. CD4^{+} T Cell Subset Polarization Protocols

Following the harvesting of spleens from mice, spleens were macerated using a syringe plunger in a sterile hood and filtered through Falcon™ 70 μm cell strainers (Fisher) with 1x PBS. Suspended cells were then enriched for CD3^{+} splenocytes via Mojosort™ Mouse CD3 T cell Isolation kit (Biolegend) using the protocol suggested by the company. This involves negative selection of the CD3^{+} fraction splenocytes, with an antibody cocktail targeting Biotin-Ly-6G/Ly-6C (Gr-1), CD45R/B220, CD49b, CD19, CD11b, CD24, and TER-119/Erythroid in enrichment buffer. After incubation at 4°C, Streptavidin nanobeads were added, the cells incubated again, and targeted cellular fraction-nanobead conjugates removed using a STEMCELL Technologies EasySep™ Magnet. Upon isolation, CD3^{+} splenocytes were counted, washed by centrifugation, resuspended in 2.5% FACS buffer, and stained with PE Rat Anti-Mouse CD25 and APC Rat Anti-Mouse CD4 antibodies (BD) for 30 minutes at 4°C. After staining, cells were washed with 2.5% FACS buffer and sorted for the CD4^{+}CD25^{-} population using a Beckman Coulter MoFlo XDP 7-color Cell Sorter.

Isolated CD4^{+}CD25^{-} cells are then polarized into specific T cell subsets via modified protocols obtained from the work of Reynolds and Flaherty [144]. For all conditions, 96- or 48- well flat bottom tissue culture treated plates (Costar Corning) were coated with either 100 or 200 μL, respectively, of 5 μg/mL purified NA/LE hamster anti-mouse CD3 (BD, clone 145-2C11) for between 2 and 3 hours in a cell culture incubator at 37°C. After such time, CD3 was removed from the wells via vacuum pipetting, and wells rinsed with 1x PBS. Either 100 or 300 μL, for 96- and 48- well plates, of 2x polarizing T cell media was then added to each well. To this either 0.2 or 0.3 million

CD4$^+$CD25$^-$ cells, respectively, were added in equivalent amounts of unpolarized T cell media. More specifically, in 96-well plates cells were concentrated at 2 million live cells per mL, such that when 100 μL of cells were added to the 100 μL of 2x polarization media, there were ultimately 0.2 million cells in 1x polarization media per well. In 48-well plates, cells were concentrated at 1 million live cells per mL, such that when 300 μL of cells were added to 300 μL of 2x polarization media, there were 0.3 million cells in 1x polarization media per well.

For Th0 conditions, final concentration of 2 ug/mL purified NA/LE hamster anti-mouse CD28 (BD, clone 37.51) and 40 U/mL recombinant human IL-2 (Peprotech) was used. For examination of non-activated T cells, cells were seeded on uncoated plates and only 40 U/mL IL-2 was added to the T cell media. The Th1 and Th2 conditions used the same final concentration of CD28 and IL-2. For the former, 10 ng/mL of recombinant murine IL-12 (Peprotech) was used for polarization, with 10 ug/mL of anti-murine IL-4 (Peprotech) to inhibit Th2 differentiation. For Th2 differentiation, we used 1000 U/mL of recombinant human IL-4 (Peprotech) and 10 ug/mL of both anti-murine IL-12 and anti-murine IFN-γ (Peprotech). For the Th17 condition, we used a higher concentration of IL-2, 100 U/mL—in this case for the control Th0 condition we also increased the amount of IL-2 to match. The concentration of CD28 remained the same at 2 ug/mL. For the addition of polarizing cytokines, we added 100 ng/mL of recombinant human IL-6 (Peprotech) and 3 ng/mL of recombinant human TGF-β (Peprotech). The PI3K inhibitors used in experiments listed under 'Reagents' were brought to twice the appropriate concentration through serial dilution in the prerequisite 2x polarizing media for the specific experiment, then an equal amount of media containing cells was added to

bring the final polarization and inhibitor concentration to 1x. Cells, unless stated

otherwise, were then allowed to grow for 72 hours in a cell culture incubator at 37°C.

Proliferation experiments followed the same protocol with staining using CellTrace™

Violet Cell Proliferation Kits (Thermofisher). Before cells were added to plates

containing 2x polarization media, our sorted cells were stained with dye using the

protocol provided.

Neutralizing antibodies were purchased from Peprotech in 100 μg vials. They

were purchased and stored in a lyophilized condition. When they were to be used, pure

deionized water was used to dilute them to the desired concentration. Recombinant

cytokines were also purchased from Peprotech, with the exception of TGF-β from R&D

Systems. Our stock concentrations were as follows: rhIL-2 was 200 U/μL, rhIL-4 was

500 U/μL, rhIL-6 was 1000 U/μL, rhIL-12 was 1000 U/μL, and recombinant human

TGF-β was 20 ng/μL. Reconstitution was done per manufacturer's specifications, with

resulting stock aliquoted and store in a -80° C freezer.

E. Cell Fixation and Staining

After 72 hours of incubation, cells were prepared for fixation. If intracellular cytokines were to be examined, Leukocyte Activation Cocktail with Golgiplug™ (BD) was added to the polarization media/cell mixture 4 to 6 hours before harvesting at a ratio of 2 uL per mL of media. Cells were transferred to either 96-well round bottom plates or round bottom Falcon™ Polystyrene tubes (Corning) and washed in 1x PBS twice before staining with Live/Dead® Fixable Near-IR Dead Cell Stain Kits, for 633 or 635 nm excitation (Thermofisher) using the manufacturer's protocol. If using 96-well round bottom plates, 150 μL of the Live/Dead® staining buffer was added per well, while if using Falcon™ tubes between 300-500 μL was used. After staining for 30 minutes in the dark, cells were then again washed in 1x PBS before addition of the appropriate fixation and permeabilization buffers.

For examination of intracellular cytokines, we used the BD Fixation/Permeabilization™ Solution Kit (BD) following the manufacturer's protocol. For the transcription factors RORγt and FoxP3 transcription factors, we utilized the Mouse FoxP3 buffer set (BD). T-bet and GATA-3 staining was also optimized for another fixation buffer set: Phosflow™ Fix Buffer I (BD) and Perm Buffer III (BD), again according to BD's protocol. Ultimately, after fixation and permeabilization all sets were washed twice in 2.5% FACS buffer, then stained with the appropriate flow cytometric antibodies, both intra- and extracellular, for 40 minutes in the dark. Cells were then washed twice in FACS media and analyzed via flow cytometry. The machines used for analysis were either a Becton Dickinson LSR II SORP 5-laser or a Becton Dickinson LSR II SORP 4-laser, depending on availability.

F. Cytometric Bead Array (CBA)

Supernatant from PI3K isoform inhibition during T cell polarization was collected for examination of cytokines. If not used immediately for CBA, supernatants were saved in 96-well round bottom plates at -80°C. The CBA assay used was the 13-plex LEGENDplex™ Mouse Th Cytokine Panel (Biolegend), and the assay was performed according to the manufacturer's protocol. Samples were run on either the Becton Dickinson LSR II SORP 5-laser or a Becton Dickinson LSR II SORP 4-laser analyzers, and saved data was analyzed using Biolegend LEGENDplex™ software, available at the company website.

G. Western Blot and Immunoprecipitation

For Western blot and immunoprecipitation, cells were polarized as above for 72 hours or in conditions specific to our experiments. Initially, 1x PBS was added to the wells and the plate was then centrifuged at 2000 RPM for 7 minutes. Supernatants were then collected, and 1x PBS was added for another wash at 2000 RPM for 7 minutes. Supernatants were again collected, and lysis buffer was added to individual wells. Lysis buffer was prepared from RIPA buffer (Thermofisher) at a ratio of 1 mL to 10 μL of Halt™ Protease Inhibitor Cocktail (Thermofisher) and 10 μL of Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). This was added to wells, with 60 μL generally added to each well in 48-well plates or 40 μL per well in 96-well plates. Plates were rocked using a mechanical shaker for between 30 to 90 minutes at 4°C, then solubilized cells were transferred into 1.5 mL microcentrifuge tubes. These tubes were centrifuged at 14,000 RPM for between 30 and 90 minutes at 4°C to clear cellular debris, and supernatant was transferred to fresh 1.5 mL tubes.

Lysate was quantified using a Bradford Assay. Commassie blue (Thermofisher) and BSA were added together to create a concentration of 50 μg/mL, and then serially diluted to create a concentration gradient of 50, 25, 12.5, 6.25, 3.125, and 0 μg/mL. For each lysate, 5 μL of lysate was added to 495 μL of Commassie blue. These samples were placed on a clear 96-well plate and analyzed at a wavelength of 570 nm by a SpectraMax M5 device. Resulting optical readings were then analyzed through Prism software using a 2nd order quadratic formula.

For Western blot analysis, equal amounts of protein for each lysate were diluted with 4x loading buffer (NuPAGE® LDS Sample Buffer 4x and 2-mercaptoethanol 4:1) to

create lysate in 1x loading buffer. The resulting lysates with loading buffer were then

heated at 95°C for 5-10 minutes, then loaded on Bolt™ 4-12% Bis-Tris Plus Gels.

Running buffer was diluted from 20x stock (Novex) in deionized water and used in the

apparatus. Dual-Color Protein Ladder (Bio Rad) was added to each gel to verify the size

of bands upon analysis. After separation, proteins were transferred from the gel to

nitrocellulose a nitrocellulose membrane using the manufacturer's protocol and the

iBlot® Gel Transfer Device. Nitrocellulose was blocked in 3% milk (Bio Rad) for 45-60

minutes, then washed in 1x PBST for 5 minutes. Nitrocellulose was then incubated on a

rocker at 4°C overnight, with the specific antibody probe of interest in 1x PBST using

manufacturer's recommendation for the antibody dilution. The next day the antibody

solution was removed, and then the blot was rocked quickly in 1x PBST for 10 minutes

for a total of 3 times at room temperature (RT). After washing, 1% milk (Bio Rad) in 1x

PBST with a 1:2500 ratio of either HRP-linked anti-rabbit or anti-mouse (depending on

the primary antibody probe) IgG was added and rocked slowly for 90 minutes at RT. At

the end of this incubation, the secondary solution was removed and blot was again

washed with 1x PBST for 10 minutes 3 times at RT. Western probes were visualized

using chemiluminescence (Thermofisher) and a Fugifilm LAS-3000 imager.

For immunoprecipitation, lysate was quantitated as above. After quantitation,

RIPA buffer was added to the highest concentration lysate in order to bring all to the

same concentration. For each sample, 1000 ug of total protein were added to 1.5 mL

tubes and 50 μL of anti-phospho-tyrosine mouse mAb (P-Tyr-100) sepharose bead

conjugate (CST) was added to each sample. Each sample was then incubated on a

rotator overnight at 4°C in the dark. The next day, samples were centrifuged at 2000

RPM at 4ºC for 5 minutes. Lysis buffer was prepared as above, then 500 µL was added to the sepharose bead pellet and gently mixed to wash before centrifugation again at 2000 RPM. This wash was performed 3 times. Next, 4x loading buffer, as prepared above with 2-mercaptoethanol reducing agent, was added to the bead pellet. This was mixed and boiled on a heat block at 95ºC for 10 minutes. Afterwards, each sample was centrifuged and the supernatant removed from the beads. The resulting eluted protein was loaded onto gels and the protein analyzed as above by Western blot.

III. RESULTS

A. The Inhibition of PI3Kδ Signaling Enhances Th17 Differentiation

Initial examination of the effects of inhibition of specific PI3K isoforms in controlling polarization of differential T cell subsets from naïve CD4^{+} T cells was seen in induced T regulatory cells (iTregs). As a major source of suppressive factors that have been shown to mediate immune suppression and cancer tolerance [145-149], iTregs are an ideal starting point for targeted therapeutics. From our previous work, we determined that iTregs were more susceptible to pan PI3K/Akt signaling inhibition than effector T conventional cells (Tconv) [18]. When further examined at the PI3K isoform level, we also discovered that iTregs are specifically dependent on the PI3Kδ isoform for their polarization and functionality [17]. However, iTreg polarization with PI3Kδ signaling abrogation did not result in complete apoptosis of the entire population. More specifically, it led to the loss of the master regulator transcription factor, FoxP3 [150], which would explain the decrease in suppressive functionality within treated iTregs. The question remained then, if these erstwhile iTregs were not dead or expressing FoxP3 as is their usual pattern, what had these cells become? Rather than simply remaining naïve and inactivated, we instead discovered that there was a significant increase in the Th17 master transcription factor, RORγt, at the expense of the single positive FoxP3, iTreg

phenotype [22]. This shift to the Th17 phenotype was present without the addition of IL-6, an important driver of the Th17 phenotype [19], so long as the PI3Kδ inhibitor CAL-101 was added to the iTreg polarizing media.

Because of this ability of a potent and selective PI3K isoform inhibitory drug to influence the polarization of naïve CD4$^+$ T cells to a completely different polarized subset, we set out to determine if the inhibition of PI3K isoforms during the polarization of other CD4$^+$ T helper subsets would shift the resulting cells to a different polarized subset. As this ability of PI3Kδ inhibition to shift iTregs to the Th17 phenotype condition occured exclusively in the iTreg condition, I started my examination by inhibiting PI3K isoforms under Th17 conditions. The Th17 condition in our experiments was identical to the iTreg condition, with the exception of the addition of the Th17 polarizing cytokine IL-6 [144]. We collected FACS-sorted naïve CD4$^+$CD25$^-$ splenocytes from 8 and 10 week old C57BL/6 mice and cultured them under Th0, Th17, or iTreg conditions for 72 hours, then stained and examined them for FoxP3 and RORγt transcription factors (Figure 1). As expected, the generalized activated Th0 cells expressed minimal levels of either FoxP3 or RORγt, while the iTreg cells strongly expressed FoxP3 with minimal expression of RORγt. The Th17 condition, which mirrored the iTreg condition except with the addition of IL-6, resulted in cells that showed a large number of single-positive FoxP3 transcription factor cells compared to the Th0 condition. However, the FoxP3$^+$ population was strongly reduced in comparison to the iTreg condition, while the RORγt$^+$ population was increased 10-fold. Whereas the Th17 polarizing condition did not completely eliminate iTreg induction, it did result in

1

Th0 · Th17 · iTreg

Th0: Q1 0.78, Q2 0.08, Q3 2.52, Q4 96.6

Th17: Q1 18.9, Q2 18.5, Q3 24.3, Q4 38.4

iTreg: Q1 2.51, Q2 2.27, Q3 62.0, Q4 33.2

RORγt
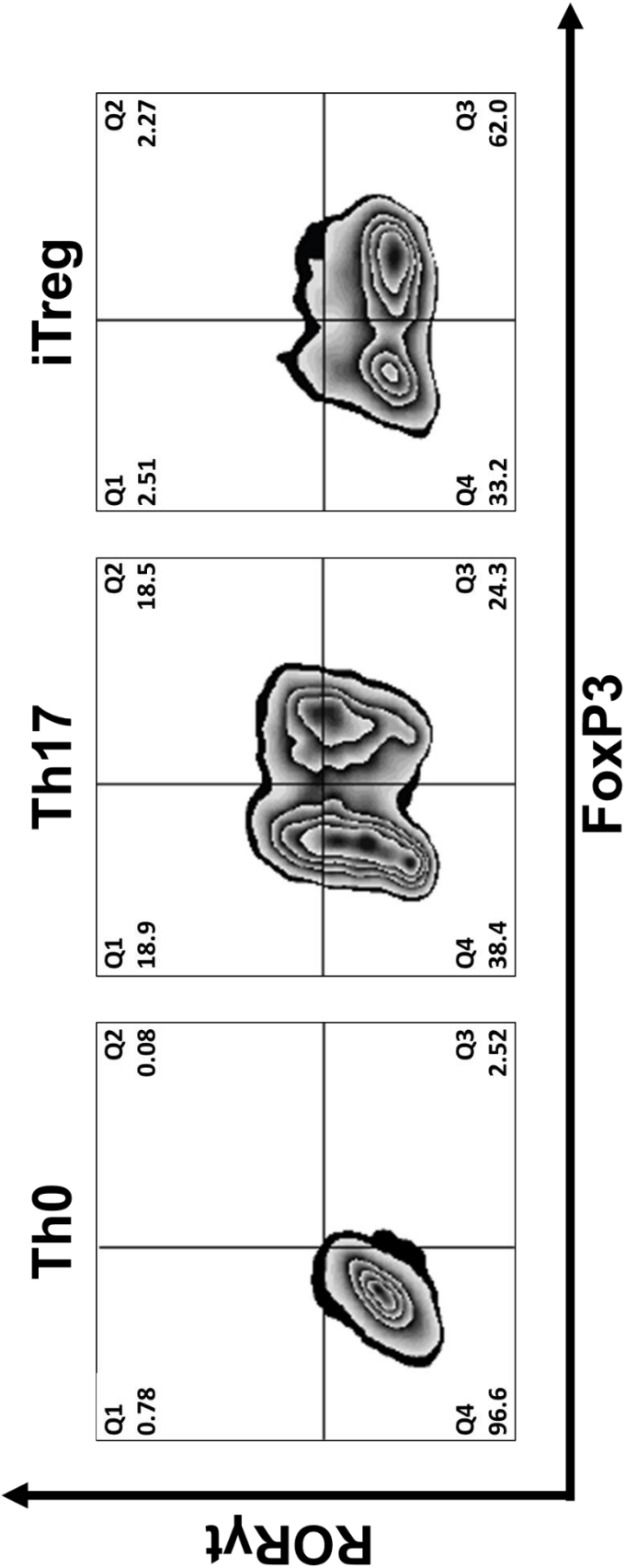
FoxP3

Figure 1: Examination of Th17 and iTreg Polarization using RORγt and FoxP3

Expression. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 coating and anti-CD28 and IL-2 in suspension. The cells cultured under iTreg and Th17 conditions were activated using the same method, with the addition of TGF-β for the iTreg condition and TGF-β and IL-6 together in the Th17 condition. After 72 hours, cells were harvested, washed, fixed, and stained for the FoxP3 and RORγt transcription factors. Representative matched set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated 3 times.

a large induction of Th17 cells and in turn we used that protocol as our basis for later experiments featuring Th17 polarization.

In order to determine which specific PI3K isoform or isoforms was/were critical for the specific polarization phenotypes, we began polarizing Th17 subsets in the presence of selective PI3K isoform inhibitors. These inhibitors (Table 1) were A66, TGX-221, CAL-101 (Idelalisib, GS-1101), and GDC-0941 (Pictilisib). These preliminary experiments were performed at 100 and 500 nM concentrations. Mirroring the results seen in the skewing of iTregs [17], we saw that A66 and TGX-221 had little effect on further skewing Th17 cells to increase the number of RORγt single-positive cells (Figure 2-3). However, the inhibition of PI3Kδ through CAL-101 did increase the RORγt single-positive population (Fig. 4), similarly to the previous skewing of iTregs in the same inhibitory condition. While these results strongly supported PI3Kδ as the primary PI3K isoform affecting FoxP3 production and that its inhibition drove polarization towards the Th17 subset, the kinetics of the inhibitors obliged us to further examine this effect on a logarithmic dosing scale. Since CAL-101 has an IC_{50} of 2.5 nM, albeit in a cell-free system, our 100 and 500 nM CAL-101 samples were 40 and 200x, respectively, above our IC_{50}. The PI3Kβ inhibitor, TGX-221, has a cell-free IC_{50} of 5 nM. This is not drastically different from the cell free kinetics of CAL-101 on its primary target, and the 100 and 500 nM TGX-221 were 20 and 100x the cell-free IC_{50}. The concern lies primarily with A66, with a cell-free IC_{50} of 32 nM. This means that our treatment groups of 100 and 500 nM were only 3.125 and 15.625x. We thus could not rule out that PI3Kα was as or even more important in skewing towards the Th17 group unless we created experiments with doses comparable to their IC_{50}s. Repeat

Table 1

Cell Free Assay IC$_{50}$'s	p110α	p110β	p110δ
A66	**32 nM**	>12.5 μM	>1.25 μM
TGX-221	5 μM	**5 nM**	100 nM
CAL-101 (Idelalisib)	820 nM	565 nM	**2.5 nM**
GDC-0941 (Pictilisib)	**3 nM**	**33 nM**	**3 nM**

2 **3** **4**
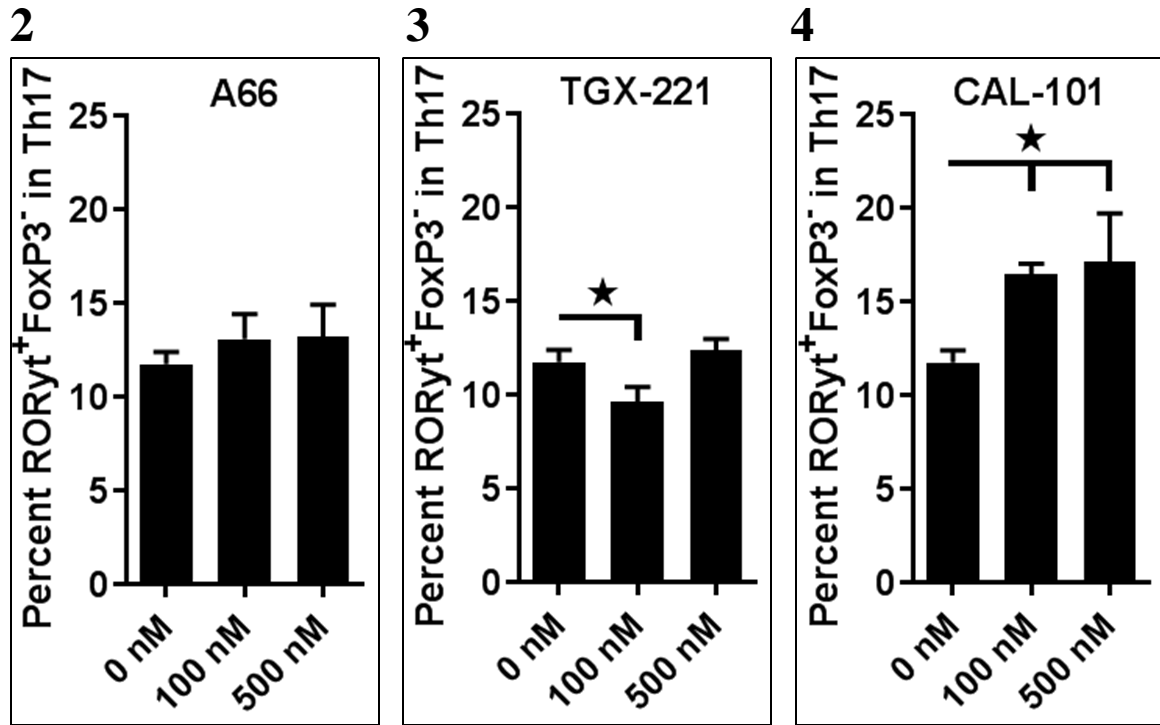
Figures 2-4: Effect of PI3K Isoform Inhibition on RORγt and FoxP3 in Th17

Polarization Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-

101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type

C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the

CD3$^+$ fraction through negative selection, then separated by FACS analysis for the

CD4$^+$CD25$^-$ naïve T cell population. The Th17 group was activated for 72 hours with

anti-CD3 and anti-CD28, IL-2, TGF-β, and IL-6 in suspension, with the addition of 100

and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were

harvested, washed, fixed, and stained for the FoxP3 and RORγt transcription factors.

Representative set, specific sample conditions performed in triplicate, n=5 mice per

experiment, repeated with matched conditions yielding similar results. Statistical

analysis was through a non-parametric one-way ANOVA with a Tukey *post hoc* test; * P

≤ 0.05

experiments were then created using doses matched to their IC_{50}s (Table 1). These results clearly showed that PI3Kδ inhibition was the primary driver towards the Th17 phenotype (Fig. 5). All other inhibitory groups failed to meet significance, while even 20 nM led to a significant increase in the RORγt single-positive population.

We next recapitulated the experiments with logarithmic dosing in order to determine whether the pharmacologic inhibitors were having an effect on cellular proliferation (Fig. 6). As our previous experiments were conducted on matched numbers of living cells, the effect of PI3Kδ inhibition was real—all naïve $CD4^+$ T cells were initially both $RORγt^-FoxP3^-$, so if the effect was merely due to toxicity the cells would have either been dead or double negative. Regardless, at the set of concentrations measured, there was no diminishment in proliferation observed in the A66 or TGX-221-treated groups (Fig. 6). There was, however, a reduction in the total proliferative population of the CAL-101-treated group. This reduction in proliferation was likely due to the decrease in the FoxP3 population, which still developed concomitantly in Th17 conditions. As we have previously shown, PI3Kδ inhibition has a strong effect on iTregs [22], and the decrease we observed in the $FoxP3^+$ population between the Th17 untreated group and the Th17 group in the presence of CAL-101 would explain this reduction of proliferation. Despite this reduction, the same number of cell generations were seen. Further, we showed an increase in the RORγt population and arrived at conclusions based on that result, rather than on a decrease in some marker that could be explained by toxicity.

We sought to verify our conclusion using a PI3Kδ kinase-dead mouse model, D910A. In our experimental conditions, splenocytes were harvested from age-matched

5
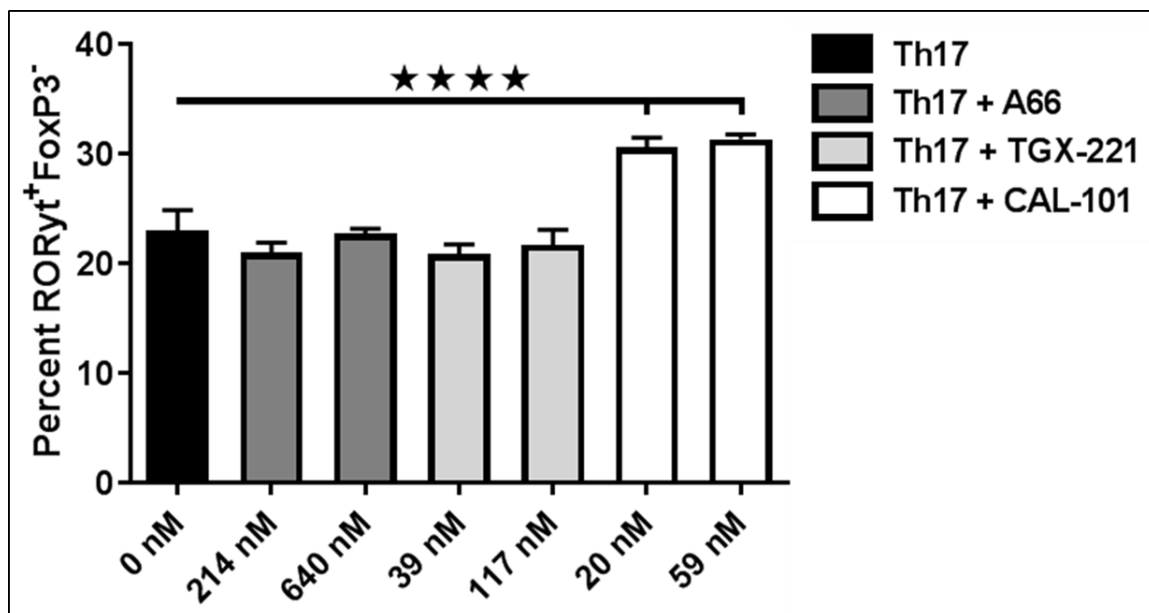
Figure 5: Effect of PI3K Isoform Inhibition on RORγt and FoxP3 in Th17 Polarization Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at Matched Cell-Free IC$_{50}$s. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th17 group was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, TGF-β, and IL-6 in suspension, with the addition of the listed concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the FoxP3 and RORγt transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Statistical

analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc*

test; **** P ≤ 0.0001

6

Th17 + GDC

VCT hi 23.4	VCT low 76.6	50 nM
VCT hi 37.3	VCT low 62.7	150 nM
VCT hi 64.0	VCT low 36.0	450 nM

Th17 + CAL

VCT hi 14.9	VCT low 85.1	11 nM
VCT hi 22.1	VCT low 77.9	33 nM
VCT hi 24.6	VCT low 75.4	100 nM

Th17 + TGX

VCT hi 11.4	VCT low 88.6	22.5 nM
VCT hi 11.8	VCT low 88.2	67.5nM
VCT hi 13.4	VCT low 86.6	202.5 nM

Th17 + A66

VCT hi 10.9	VCT low 89.1	45 nM
VCT hi 11.2	VCT low 88.8	135 nM
VCT hi 11.4	VCT low 88.6	405 nM

Th17

| VCT hi 11.5 | VCT low 88.5 |

Figure 6: VCT Proliferation Assay Under Th17 Polarization Conditions with A66 (α), TGX-221 (β), CAL-101 (δ), and GDC-0941 (pan) Treatment. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. Cells were stained with VCT, then activated in the Th17 condition for 72 hours with anti-CD3 and anti-CD28, IL-2, TGF-β, and IL-6 in suspension, with the addition of the listed concentrations of A66, TGX-221, CAL-101, and GDC-0941. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results.

wild-type and D910A mice and then sorted for naïve CD4$^+$CD25$^-$ T cells. These were then cultured for 72 hours in the Th17 condition before fixation and staining for RORγt and FoxP3. The resulting figure matched our previous pharmacological results, showing a significant reduction in the FoxP3$^+$ population and an increase in the RORγt$^+$FoxP3$^-$, or the Th17 population in the D910A mouse cells (Fig. 7). While this result is similar to what we have demonstrated previously in the iTreg condition [22], this experiment further showed that the Th17 population can be enhanced when PI3Kδ signaling is diminished, leading to more effective polarization of the Th17 subtype with less contamination from the iTreg group. Given the success of this modality, we next sought to apply our methods to other CD4$^+$ T helper subsets in order to determine if under other polarizing conditions, cells could have their subsets skewed with the addition of specific PI3K isoform inhibitors.

7
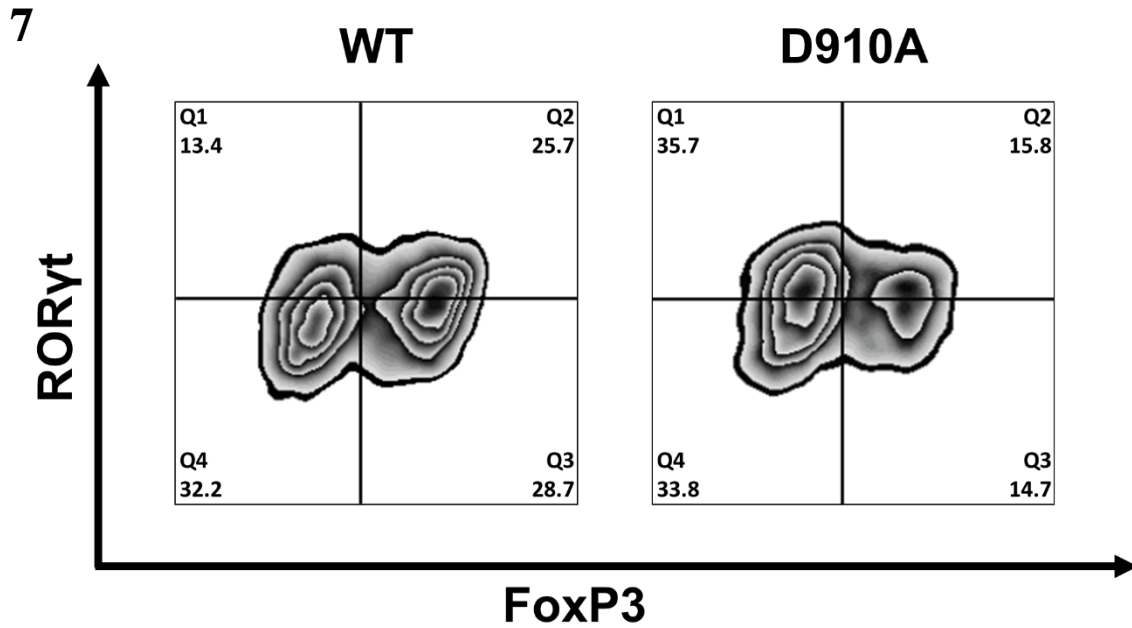
Figure 7: Examination of Th17 Polarization in Wild-type and D910A Mice by RORγt and FoxP3 Expression. Spleens were harvested from age-matched wild-type C57BL/6 mice and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th17 group was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, TGF-β, and IL-6 in suspension. After 72 hours, cells were harvested, washed, fixed, and stained for the FoxP3 and RORγt transcription factors. Representative set, specific sample conditions performed in triplicate, n=3 mice per experiment per strain, repeated with matched conditions yielding similar results.

B. Inhibitors of PI3K Delta and Alpha Decrease Th1 Subset Intracellular IFN-γ Production

Th1 cells are generated from naïve CD4^{+} T cells via TCR stimulation at the CD3/CD28 complex, IL2R activation, and cytokine stimulation of the IL-12 and IFN-γ receptors [67, 144]. We replicated the model of *in vitro* polarization we standardized in the Th17 experiments, incubating cells for 72 hours with specific cytokines, inhibiting certain cytokines with antibodies, and using PI3K isoform inhibitors (Table 1). Experimental results were initially examined for the highly Th1-specific intracellular cytokine IFN-γ after restimulation with BD Leukocyte Activation Cocktail with Golgiplug**™**. Th1 polarization was highly successful, with high levels of IFN-γ induction compared to the Th0 control (Figure 8) and minimal expression of Th2 or Th17 cytokines IL-4 or IL-17 (Supplement 1). As such, our Th1 polarized group matched the literature description, producing high quantities of IFN-γ cytokines [55, 69].

Previous literature has tied PI3Kδ to the production of CD4^{+} T helper cytokines, including IFN-γ in the Th1 subset [23]. While we do not dispute this, there is limited empirical information on the importance of the remaining p110 PI3K isoforms, PI3Kα and PI3Kβ, in CD4^{+} T cell polarization. Further, we wished to see whether there would be differences in polarization when utilizing pharmacological methods rather than a complete knockout of PI3Kδ. Instead of a conditional knockout of PI3Kδ in an adult animal, the D910A mice used have catalytically inactivated PI3Kδ from birth. Since the other p110 isoforms are closely related, and our own publications have implicated the p110 isoforms as having some degree of functional redundancy, we decided that further examination would be useful. As such, our next experiment focused on whether we

8
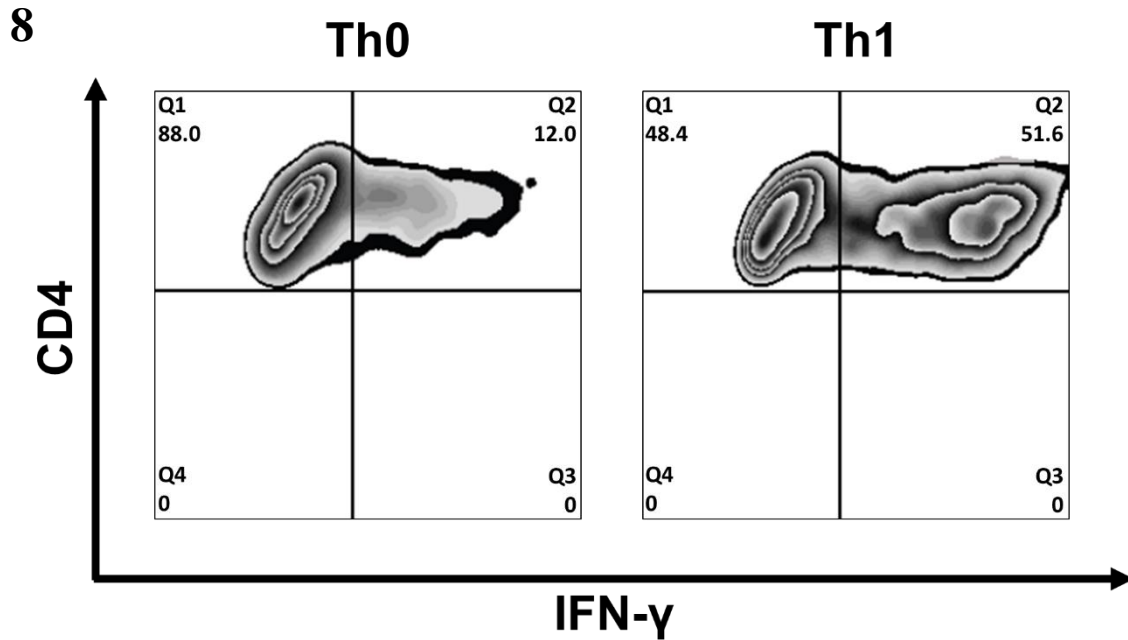
Figure 8: Examination of Th1 Polarization by IFN-γ Expression. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and both anti-CD28 and IL-2 in suspension. The Th1 condition was activated using the same method, with the addition of IL-12 and anti-IL-4. After 72 hours, cells were treated for between 4 to 6 hours with PMA/Ionomycin plus Golgiplug^{TM}, then harvested, washed, fixed, and stained for IFN-γ. Representative set, specific sample conditions performed in triplicate, n=3 mice per experiment, repeated greater than 3x with matched conditions yielding similar results.

could affect Th1 cytokines, in this case IFN-γ, using differential pharmacological

inhibitors of PI3K isoforms. We polarized for the Th1 condition in the presence and

absence of 100 and 500 nM of the alpha, beta, and delta inhibitors (Fig. 9-11). Amongst

this experimental set, we saw the greatest effect by far when cells were treated with the

PI3Kδ inhibitor CAL-101 (Fig. 11). However, we did see some reduction of IFN-γ

expression in the A66 and TGX-221 treatment groups at a 500 nM concentration (Fig. 9-

10). As previous literature implicates only PI3Kδ as playing a role in IFN-γ expression,

these results needed to be examined in further detail. The loss of IFN-γ in the A66 group

is highly interesting (Fig. 9), given that A66 has the highest IC_{50} and any changes have

the capacity to be more significant when the inhibitors are examined at doses of matched

effectiveness. The loss of expression in the TGX-221 group needs to be carefully

dissected (Fig. 10), as the IC_{50} of TGX-221, at 5 nM for inhibiting PI3Kβ, is already

close to that of the effect of CAL-101 on PI3Kδ, with an IC_{50} of 2.5 nM. Further, at a

concentration of 500 nM, the TGX-221 inhibitor was well over the point it should start to

affect the PI3Kδ isoform at 100 nM, meaning the loss of IFN-γ seen was almost certainly

due to inhibition of PI3Kδ. While these results strongly supported PI3Kδ as the primary

PI3K isoform affecting Th1 cytokine production, the kinetics of the inhibitors obliged us

to further examine this effect. Before moving onto the logarithmic dosing scale, as we

did in the Th17 set, we decided to use dual inhibitor treatment groups to determine the

truth of our initial hypothesis, which was that PI3Kδ and α both play a role in IFN-γ

expression while PI3Kβ did not.

The dual inhibitor dosing protocol used the same cells, times, and methods of

polarization, with the exception that the dual combinations of the PI3K inhibitors were

9   **10**   **11**
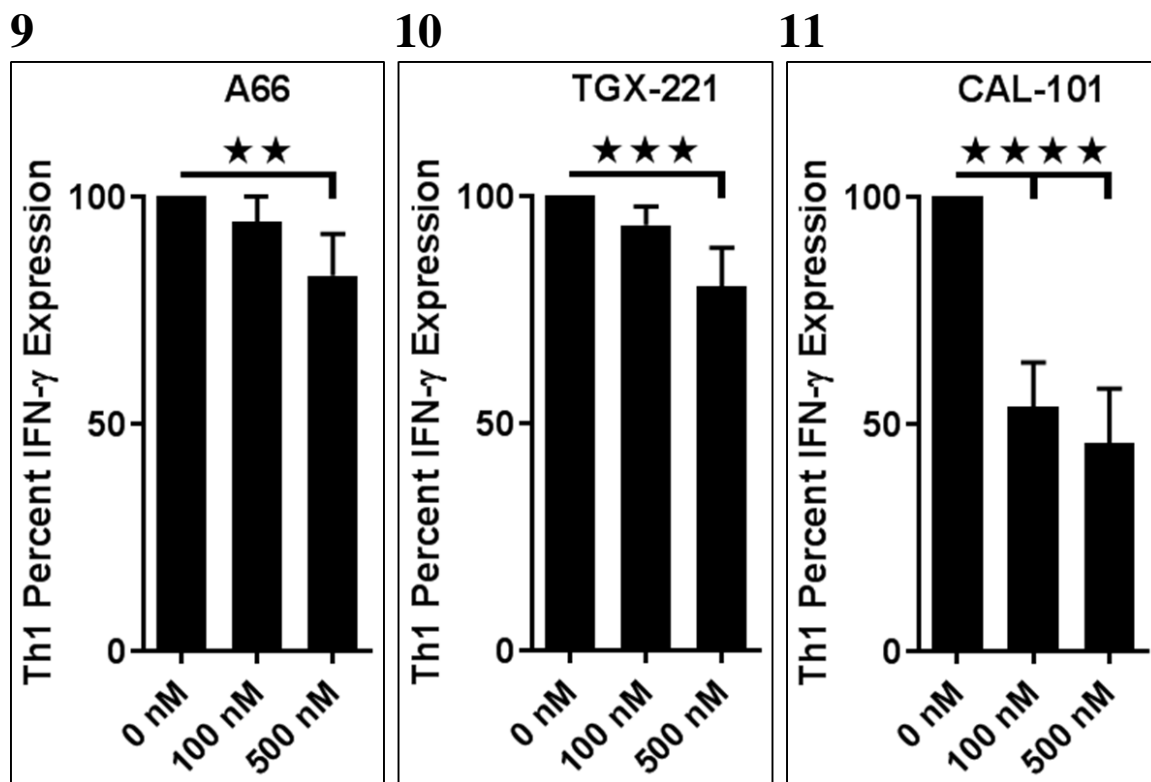
Figures 9-11: Effect of PI3K Isoform Inhibition on IFN-γ Expression in Th1 Polarization Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th1 group was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were treated for between 4 to 6 hours with PMA/Ionomycin plus Golgiplug™, then harvested, washed, fixed, and stained for IFN-γ. Combined normalized set of 2 experiments, specific sample conditions performed in triplicate, n=5 mice per

experiment, repeated 3x with matched conditions yielding similar results. Statistical

analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc*

test; ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

added at 100 and 500 nM for each drug. By using this experimental modality, we could

confirm the predominance of p110δ isoform signaling and whether its role is exclusive or

if p110α continued to demonstrate significance. After normalization of intracellular IFN-

γ to untreated Th1 condition-exposed cells, the cells exposed to the dual dose of 500 nM

CAL-101 and 500 nM of A66 showed a significant reduction of IFN-γ in comparison to

the 500 nM CAL-101 dose alone (Fig. 12), exactly as we expected given our hypothesis.

Further strengthening our initial conclusion that both the p110δ and p110α isoforms

specifically were vital for IFN-γ production, dual inhibition by CAL-101 and the β

inhibitor TGX-221 did not result in any significant difference when compared to the

CAL-101 alone treatment (Fig. 13). We next compared CAL-101 inhibition to the pan-

PI3K inhibitor GDC-0941 to see whether PI3Kδ isoform alone was responsible for IFN-γ

expression in the Th1 subset. GDC, with a cell-free p110δ inhibitory IC_{50} of 3 nM, was

comparable to CAL-101 PI3Kδ inhibitor, with its IC_{50} of 2.5 nM. When compared head-

to-head at 100 and 500 nM of CAL-101 and GDC-0941, we saw greater inhibition of

intracellular IFN-γ in the pan inhibition groups than with the CAL-101 alone (Fig. 14).

Given these results, we could fairly confidently state that, rather than depending purely

on PI3Kδ, IFN-γ expression in Th1 cells was also driven in part by PI3Kα. In order to

more fully determine which was more important, however, we needed to compare our

inhibitors using doses that are equivalent in effectiveness as determined by their cell-free

IC_{50}.

Due to the differences in IC_{50}s for the pharmacologic inhibitors used, we created

confirmatory experiments using overlapping doses that were comparable in their IC_{50}s.

12
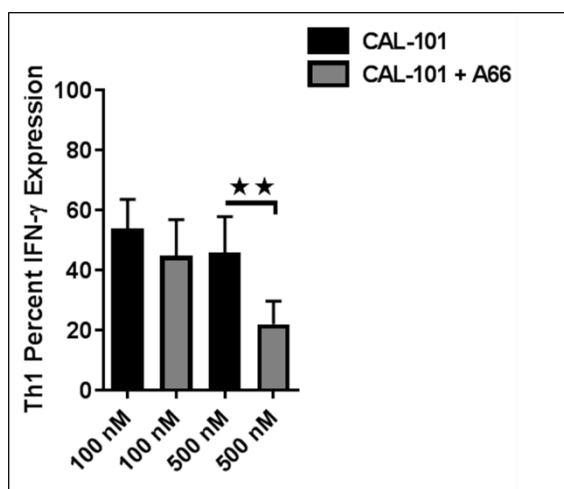
13
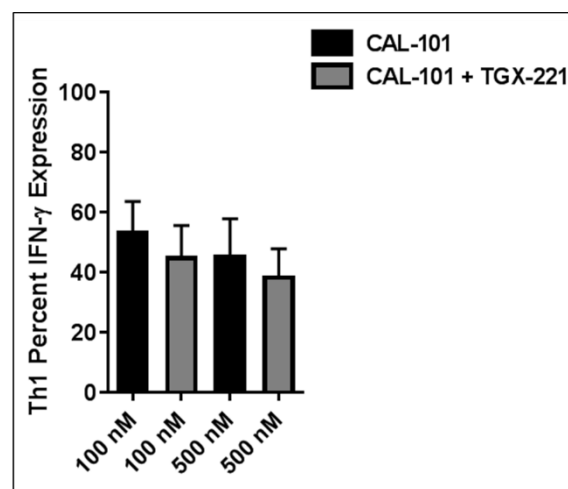
14
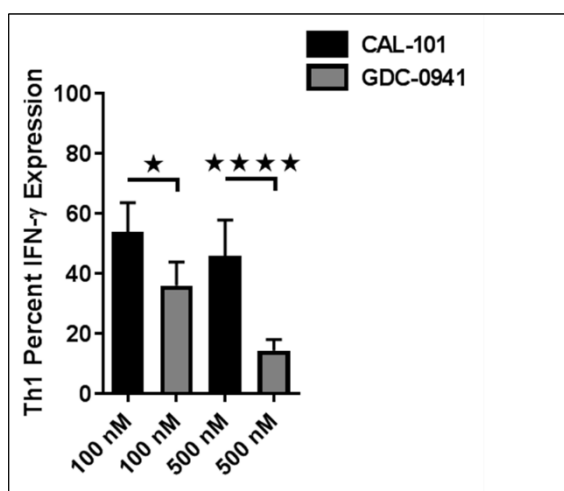
Figures 12-14: Effect of PI3K Isoform Inhibition on IFN-γ Expression in Th1 Polarization Conditions with CAL-101 (δ) Alone, CAL-101 (δ) and either A66 (α) or TGX-221 (β) Dual, and GDC-0941 (pan) inhibitors at 100 and 500 nM. Treatment groups are expressed as a percentage of IFN-γ expression in absence of inhibitor, which was standardized to 100% expression. Spleens were harvested from age-matched wild-

type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for

the $CD3^+$ fraction through negative selection, then separated by FACS analysis for the

$CD4^+CD25^-$ naïve T cell population. The Th1 group was activated for 72 hours with

anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension, with the addition of

100 and 500 nM concentrations of A66, TGX-221, CAL-101, and GDC-0941 as

indicated. After 72 hours, cells were treated for between 4 to 6 hours with

PMA/Ionomycin plus Golgi**TM**, then harvested, washed, fixed, and stained for IFN-γ.

Combined normalized set of 2 experiments, specific sample conditions performed in

triplicate, n=5 mice per experiment, repeated 3x with matched conditions yielding similar

results. Statistical analysis was performed using a non-parametric one-way ANOVA with

a Tukey *post hoc* test; * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$

15
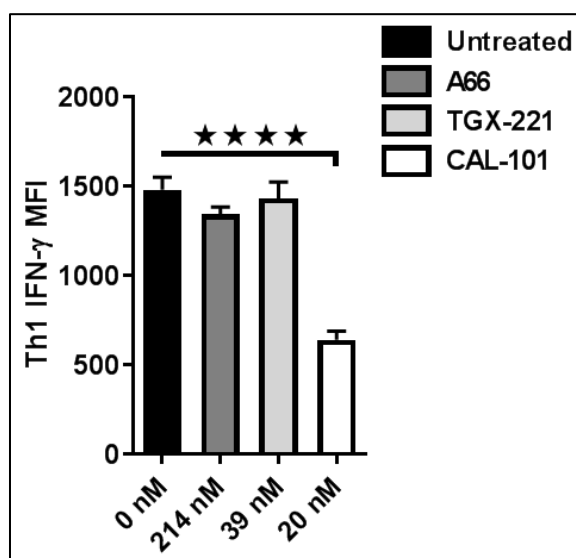
16
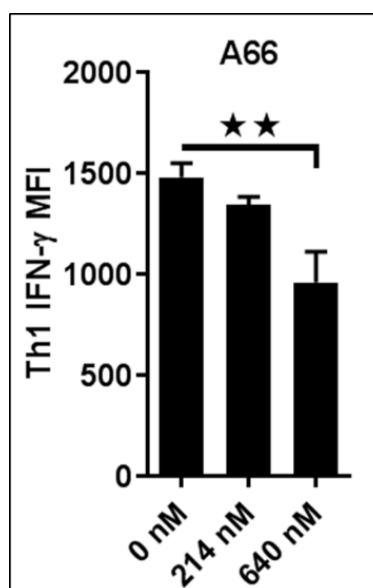
17
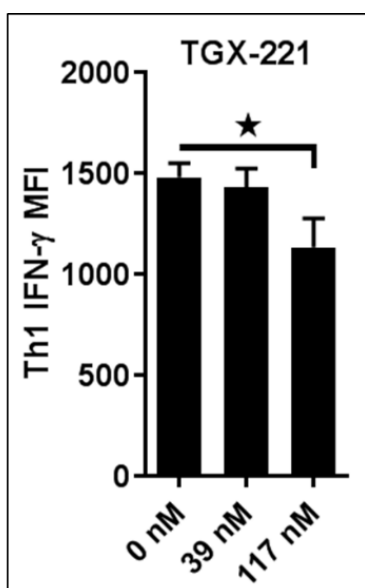
18
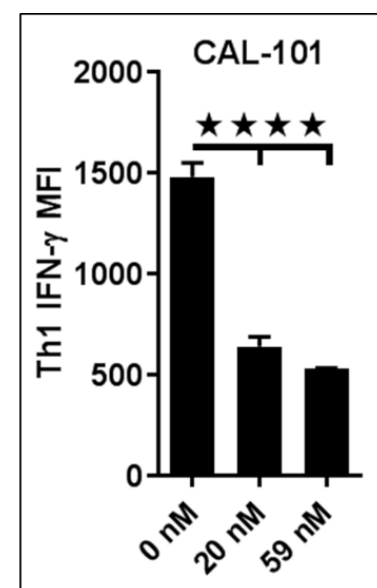
Figures 15-18: Effect of PI3K Isoform Inhibition on IFN-γ in Th1 Polarization Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at Matched Cell-Free IC$_{50}$s. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$

fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and both anti-CD28 and IL-2 in suspension. The Th1 condition was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension, with the addition of the listed concentrations of A66, TGX-221, and CAL-101. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Statistical analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$

To this goal we used a logarithmic scale, scaling by multiples of three across three doses per drug (Fig. 15-18). At matched, approximately 7x IC$_{50}$ equivalents, there was a clear and highly significant reduction of intracellular IFN-γ in the p110δ treatment group that was not seen in either the α or β treatment (Fig. 15). This mirrors the literature conclusion previously discussed [23], in which PI3Kδ is the predominant isoform for CD4$^+$ T helper cell cytokine signaling. However, we also examined higher treatment doses using this matched logarithmic scale. Significant inhibition of IFN-γ was achieved with the higher doses of both A66 and TGX-221, equivalent to 20 and 23x their cell free IC$_{50}$s (Fig. 16-17). While the same equivalent in CAL-101, 59 nM, at 23x its cell free IC$_{50}$, led to a much greater decrease in IFN-γ MFI (Fig. 18), these results indicated a possible importance of the other PI3K isoforms. In the A66 treatment group, the cell-free IC$_{50}$ for non PI3Kα isoforms was twice the highest dose of 640 nM that we used in the treatment group (Table 1). As such we can conclude with a high degree of certainty that p110α plays a secondary but non-insignificant role in Th1 cytokine production. For the TGX-221 treatment group, the only dosage that reached significance, 117 nM, was higher than the p110δ cell-free IC$_{50}$ of 100 nM. We thus could not definitively conclude on to the importance of PI3Kβ in this pathway; however, the results suggest that PI3Kβ is not as important as PI3Kδ.

Because the drugs used targeted individual PI3K isoforms, as well as other possible off-target proteins and pathways, one possible reason for the decreased IFN-γ expression with CAL-101 treatment was toxicity or some major roadblock to the cell cycle, which would ultimately lead to a relative decrease in cell number in of the treatment group as opposed to the control group. An important control we used was to

compare identical numbers of cells, but it is possible that these equivalent numbers of cells were anergic or otherwise unhealthy, thus creating the differences in intracellular IFN-γ observed. Unlike in the Th17 group, where an increase in RORγt expression in response to treatment was observed, in these experiments treatments decreased IFN-γ expression, and thus it was necessary to rule out anti-proliferative effects. Our hypothesis was that some downstream signaling pathway, dependent primarily on PI3Kδ followed by PI3Kα, was responsible for IFN-γ synthesis. Thus, we needed to eliminate the possibility of A66 and CAL-101 toxicity at the doses used for inhibition, and thereby reject the alternate hypothesis that decreased intracellular IFN-γ was primarily due to diminished cellular proliferation or impaired cell health. We examined this by staining sorted naïve $CD4^+$ T cells with VCT, as above, before polarizing them in the Th1 condition. At overlapping logarithmic concentrations, there was no significant inhibitory effect with A66 or TGX-221 (Fig. 19). While proliferation decreased slightly in the 100 nM CAL-101 group, no change was seen at the 34 nM dose, and a greater than 50% loss of IFN-γ was observed at doses as low as 20 nM CAL-101 (Fig. 18). We can thereby conclude that the reduction in IFN-γ seen is not due to toxic and/or anti-proliferative effects.

19
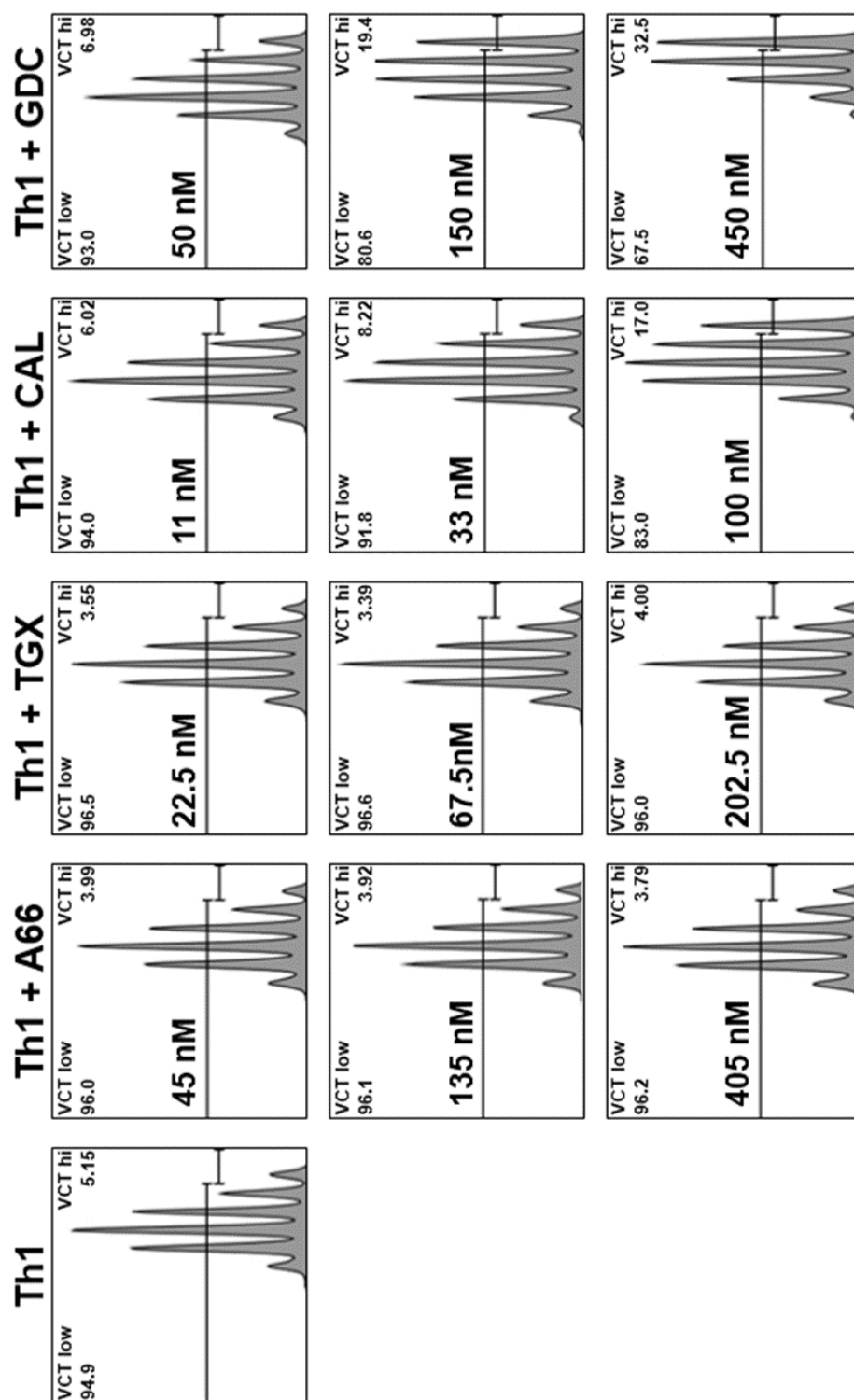
Figure 19: VCT Proliferation Assay for Th1 Polarization with A66 (α), TGX-221 (β), CAL-101 (δ), and GDC-0941 (pan) Treatment. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. Cells were stained with VCT, then activated in the Th1 condition for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension, with the addition of listed concentrations of A66, TGX-221, CAL-101, and GDC-0941. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results.

C. Loss of PI3Kδ Signaling Decreases T-bet Transcription Factor Expression in Th1 Polarized Cells

As previously discussed, the transcription factor T-bet is both necessary and sufficient for Th1 polarization [68-70]. Our previous experiment demonstrated that selective pharmacological inhibition of PI3Kδ led to a marked decrease in the intracellular expression of the Th1 cytokine IFN-γ, whereas inhibition of PI3Kα reduced IFN-γ to a lesser degree. We hypothesized that this would translate into a decrease in T-bet expression of Th1 cells polarized in the presence of CAL-101, and possibly A66, as compared to a non-treated Th1 control. Preliminary experiments were conducted in non-specifically polarized activated splenocytes, also known as Th0 cells. These cells were activated for 72 hours exclusively with anti-CD3, anti-CD28, and recombinant human IL-2, with no polarizing cytokines added. When examined for T-bet and GATA-3, we saw that these Th0 cells expressed high levels of both T-bet and GATA-3 (Fig. 20-21). This made Th0 splenocytes ideal for examining T-bet and GATA-3 in the same experimental set. We conducted these experiments in the presence of the specific PI3K isoform inhibitors, and after three days fixed and stained the cells with fluorophore-conjugated antibodies targeting intracellular T-bet and GATA-3. While A66 and TGX-221 produced no significant decrease in T-bet expression (Fig. 22-23), a reduction in T-bet transcription factor was observed at 500 nM CAL-101 (Fig. 24). GATA-3 transcription factor expression was also reduced in both the TGX-221 and CAL-101 groups, with no effect seen with A66 (Fig. 25-27). What is important to consider in these results is the antagonistic relationship between the T-bet and GATA-3 transcription factors, which lead to the Th1 and Th2 phenotypes, respectively. As discussed in the introduction [84], T-bet
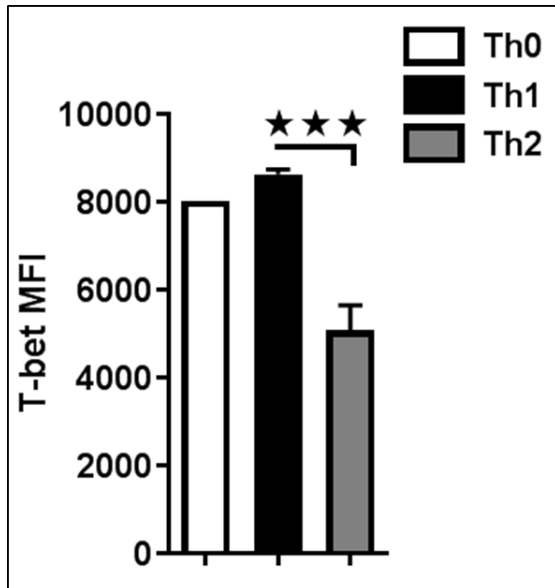
20 **21**
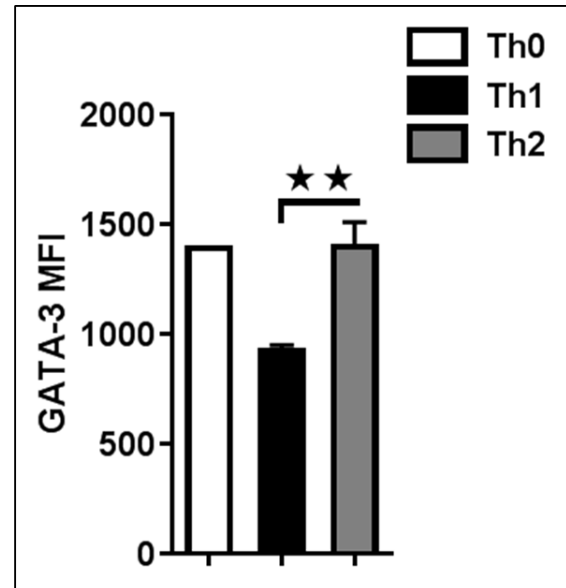
Figures 20-21: Examination of Th1 and Th2 Polarization by T-bet and GATA-3 Expression. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in suspension. The Th1 and Th2 conditions were activated using the same method, with the addition of IL-12 and anti-IL-4 for the Th1 condition and IL-4, anti-IL-12, and anti-IFN-γ for the Th2 condition. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative matched set, specific sample conditions performed in triplicate, n=3 mice per experiment, repeated greater than 3 times. Statistical analysis was performed using an unpaired two-tailed Student's *t* test (between Th1 and Th2 sets); ** P ≤ 0.01, *** P ≤ 0.001
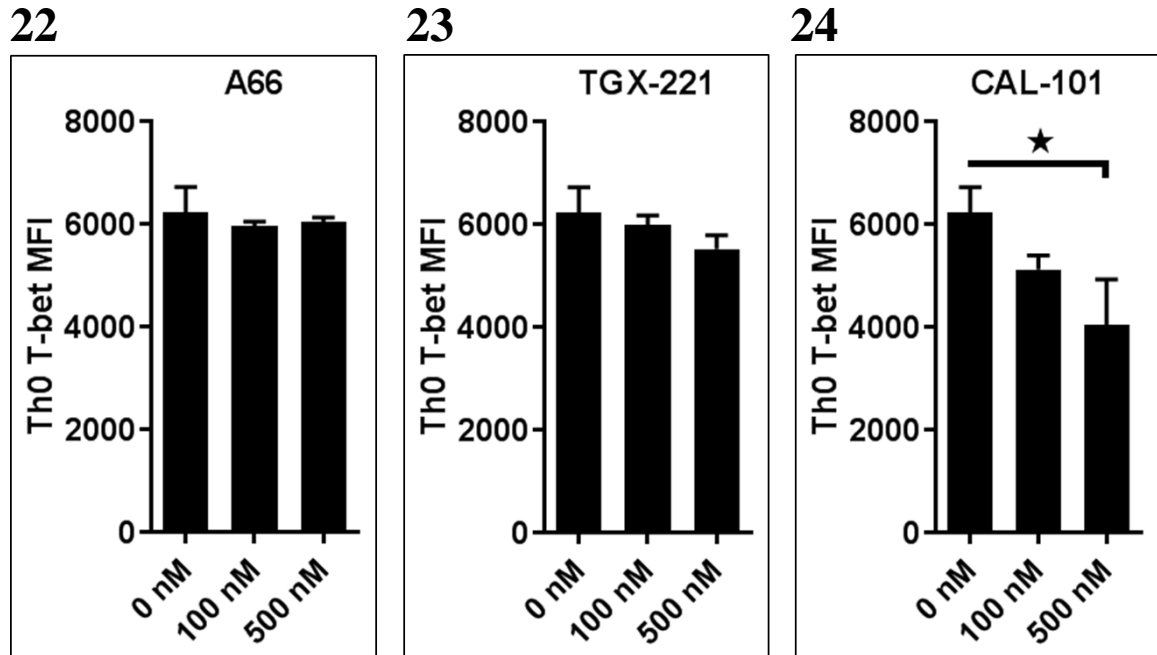
Figures 22-24: Effect of PI3K Isoform Inhibition on T-bet in Th0 Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment. Statistical analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; * $P \leq 0.05$

25   **26**   **27**

Figures 25-27: Effect ofPI3K Isoform Inhibition on GATA-3 in Th0 Conditions

with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500

nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the

ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through

negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell

population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and

IL-2 in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-

221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for

the T-bet and GATA-3 transcription factors. Representative set, specific sample

conditions performed in triplicate, n=5 mice per experiment. Statistical analysis was

done through non-parametric one-way ANOVA with a Tukey *post hoc* test; * $P \leq 0.05$,

** $P \leq 0.01$

not only directs Th1 polarization, but also prevents Th2 polarization. When T-bet is expressed, it sequesters the GATA-3 transcription factor away from its Th2 cytokine-driving sequences, which include the promotors for IL-4 and IL-13. This explained the minimal expression of IFN-γ in Th0 polarized cells compared to the Th1 group, despite almost identical expression of T-bet (Fig. 8). Due to this diametric transcription factor Th1/Th2 axis, we decided to see if the ratio of GATA-3 to T-bet expression in these groups changed in such a way as to favor either Th1 or Th2 polarization when Th0 cells were exposed to single inhibitors. Upon normalization to the Th0 control, we discovered that 500 nM CAL-101 inhibited T-bet more strongly than GATA-3, which we hypothesized was more favorable to the Th2 phenotype (Fig. 28). We decided to see if this trend continued for the Th1 subset.

We repeated Th1 polarization experiments with the Th0 group. As expected, the Th1 condition can be characterized by relatively high T-bet expression and low GATA-3 levels (Fig. 20-21). Regardless of inhibitor dose, GATA-3 remained low and did not change from its basal Th1 state (Fig. 29-31). This was likely due to the very low level of expression of GATA-3 in the Th1 subset, which is known to be characterized by minimal expression of GATA-3. Similarly to our results with Th0, we saw a significant reduction in T-bet signaling in the 100 and 500 nM CAL-101 treatment groups (Fig. 34). This same reduction was not observed in the A66 treated group at either concentration, while the TGX-221 groups showed significant decreases, albeit not to the same level as with CAL-101 (Fig. 32-33). Also, while not directly comparable, the inhibition of T-bet in the Th0 experiment was much greater than the inhibition observed in the Th1 group. One

28
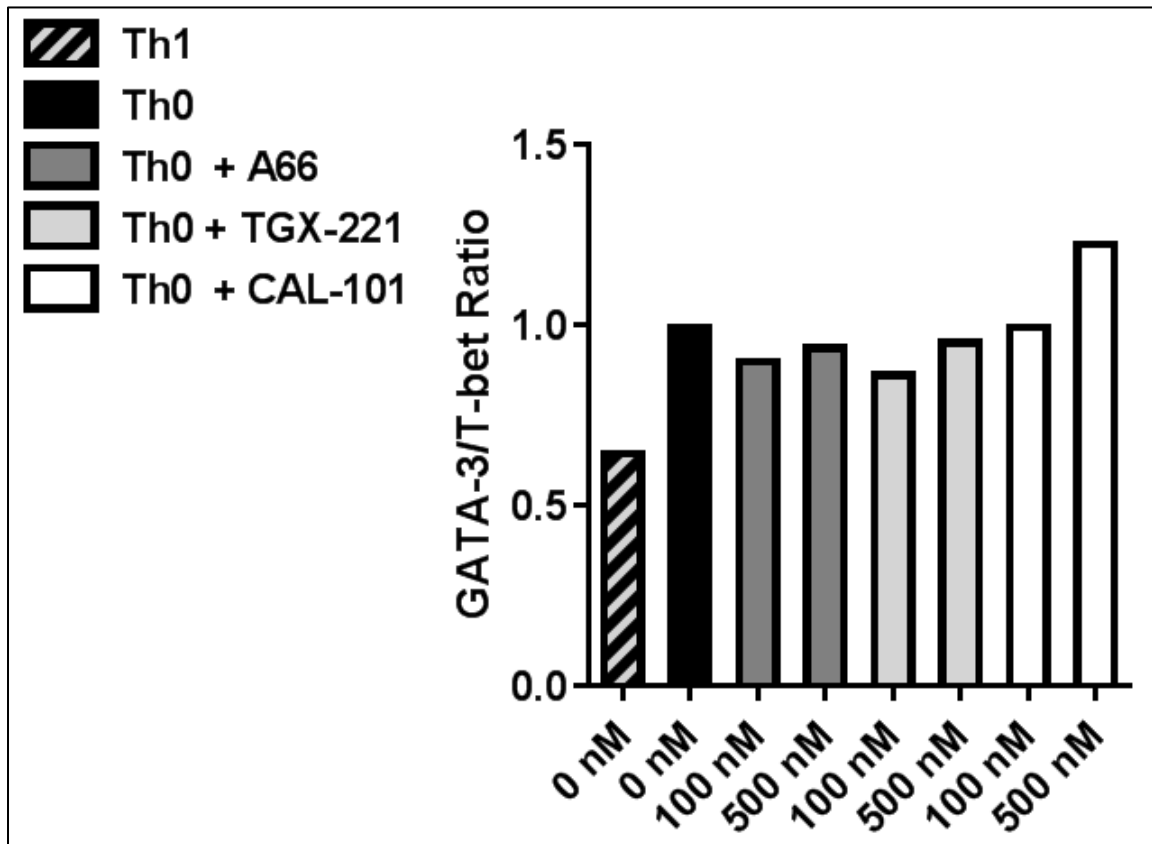
Figure 28: Effect of PI3K Isoform Inhibition on GATA-3/T-bet Ratio in Th0 Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. For determining the ratio, the MFIs of

GATA-3 and T-bet in the Th0 conditions were averaged from triplicates, then converted to a GATA-3/T-bet ratio that was normalized to 1. All other groups' ratios were calculated as compared to the normalized Th0. Representative set, performed in triplicate, n=5 mice per experiment.

29   **30**   **31**
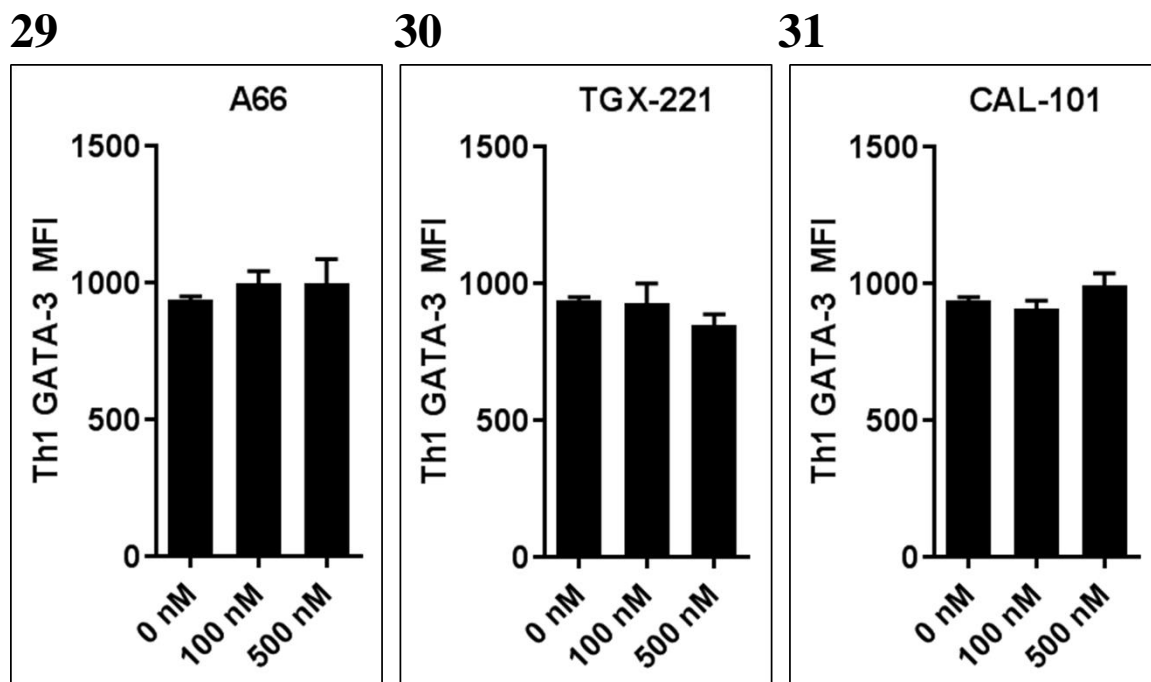
Figures 29-31: Effect of PI3K Isoform Inhibition on GATA-3 in Th1 Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th1 condition was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Statistical analysis was done through non-parametric one-way ANOVA with a Tukey *post hoc* test.

Figures 32-34: Effect of PI3K Isoform Inhibition on T-bet in Th1 Conditions with

Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM.

Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of

8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative

selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population.

The Th1 condition was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12,

and anti-IL-4 in suspension, with the addition of 100 and 500 nM concentrations of A66,

TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained

for the T-bet and GATA-3 transcription factors. Combined normalized set of 2

experiments, specific sample conditions performed in triplicate, n=5 mice per

experiment. Statistical analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; * P $\leq$ 0.05, *** P $\leq$ 0.001, **** P $\leq$ 0.0001

explanation of this is the addition of IL-12 in Th1 polarization, which was not present for Th0 polarization. As an inducer of Th1 and therefore T-bet, IL-12 may have been acting in opposition to the CAL-101 inhibitor, possibly even activating the PI3K pathway. The next conundrum was the loss of T-bet in the PI3Kβ inhibition group. We hypothesize this is a result of unwanted PI3Kδ inhibition due to the effect of the drug TGX-221 on PI3Kδ at higher doses. Despite this, T-bet did consistently remain decreased in the Th0 and Th1 subsets under CAL-101 treatment.

The D910A PI3Kδ kinase-dead model was then used to verify our hypothesis that the loss of T-bet was due to the loss of signal transduction through PI3Kδ. We polarized cells from both WT and D910A mice in the Th1 conditions for 72 hours, then compared their expression of T-bet and GATA-3. In both sets there was relatively high expression of T-bet and low GATA-3 (Supplement 2), matching Th1 phenotype characteristics. However, in the D910A set there was a marked reduction in the expression of the T-bet transcription factor when compared to the WT (Fig. 35). This matched our pharmacological inhibition results with CAL-101 treatment in the Th1 condition.

Given the strong reduction of T-bet transcription factor expression when PI3Kδ signaling was eliminated, we suspected that the Th1/Th2 axis was similar to the previously explored Th17/iTreg axis. In studies of the latter [22], we discovered that when treating naïve CD4$^+$ T cells with CAL-101 in the Th17/iTreg conditions, they were more strongly polarized towards the RORγt$^+$, Th17 condition than the untreated group. Given this strong inhibition of T-bet, with minimal change in GATA-3, we decided to examine PI3Kδ inhibition in the Th2 phenotype to see if treatment would increase the

35
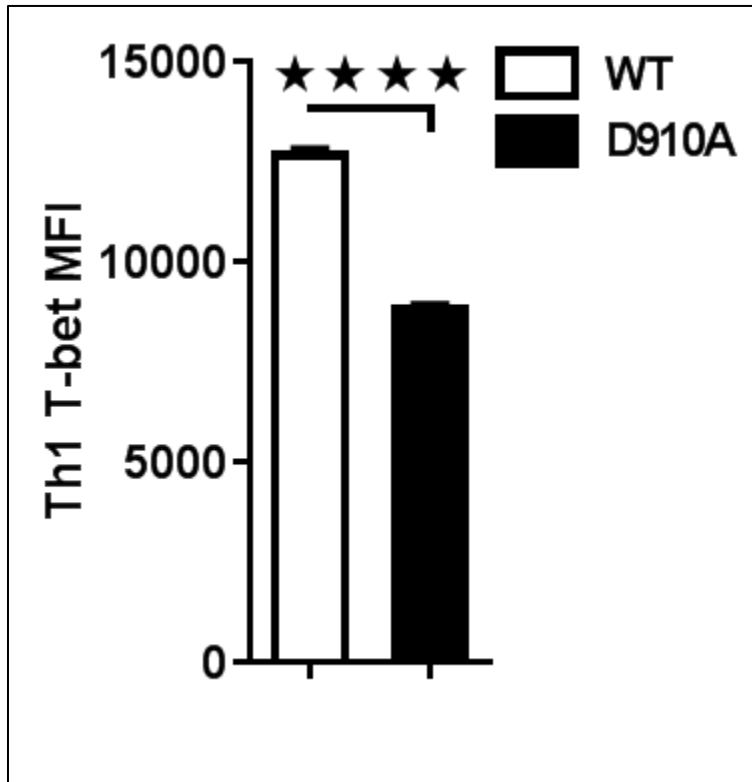
Figure 35: Examination of T-bet Expression in Th1 Polarization of Wild-type and D910A Mice. Spleens were harvested from age-matched wild-type C57BL/6 mice and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th1 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 in suspension. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=3 mice per experiment per strain, repeated with matched conditions yielding similar results. Statistical analysis was performed using an unpaired two-tailed Student's *t* test; **** P $\leq$ 0.0001

Th2 condition at 72 hours when compared to the untreated Th2 group, exactly as happened with the Th17 CAL-101 treated versus untreated groups (Fig. 1-5, 7).

D. Loss of PI3Kδ Signaling Increases Th2 Character in Th2 Polarized Cells

As CAL-101 appears to have the greatest effect on the T-bet transcription factor, we next examined the effect of our PI3K inhibitors on Th2 polarized splenocytes. We hypothesized that CAL-101 would further diminish T-bet while affecting GATA-3 to a lesser extent, ultimately leading to a stronger Th2 polarization phenotype. We polarized splenocytes as detailed in the methods section for 72 hours in the presence of single inhibitors at 100 and 500 nM concentrations, then washed and fixed the cells in preparation for staining with T-bet and GATA-3. We then confirmed the Th2 phenotype by comparison to the Th0 condition (Fig. 36). The Th2 phenotype can be further enhanced by longer polarization times [144], but as our experimental goal was to see whether PI3K isoform inhibition led to enhanced or redirected polarization, the additional time was not necessary. Despite the decreased expression of T-bet in the Th2 subset when compared to either the Th0 or the Th1 subsets (Fig 20-21), all inhibition groups, with the exception of 100 nM A66, significantly reduced the expression of T-bet (Fig. 37). Matching our previous results seen with Th0 and Th1 polarization, however, CAL-101 elicited the greatest reduction in T-bet expression. Likewise, however, GATA-3 expression was diminished with the highest TGX-221 dose, as well as with both doses of CAL-101 (Fig. 38-40). Regardless, the hypothesized changes of increased Th2 phenotype in the CAL-101 treatment versus control group were observed (Fig. 41-42).

In order to verify our hypothesis, we first replicated our results using matched cell-free IC_{50} concentrations with the same concentrations utilized for verification of IFN-γ inhibition in the Th1 experimental set. These results were much clearer, as we saw
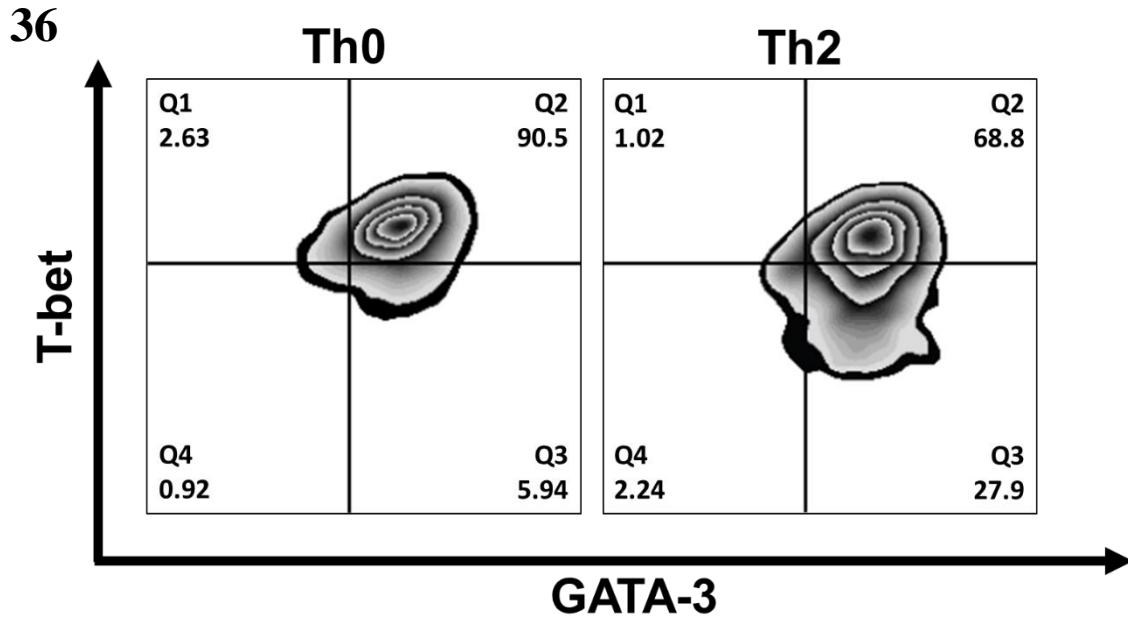
Figure 36: Examination of Th2 Polarization by T-bet and GATA-3 Expression.

Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of

8 and 10 weeks. Splenocytes were enriched for the CD3+ fraction through negative

selection, then separated by FACS analysis for the CD4+CD25- naïve T cell population.

The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in

suspension. The Th2 condition was induced using the same method, with the addition of

IL-4, anti-IL-12, and anti-IFN-γ in media suspension. After 72 hours, cells were

harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors.

Representative matched set, specific sample conditions performed in triplicate, n=3 mice

per experiment, repeated greater than 3 times.

37
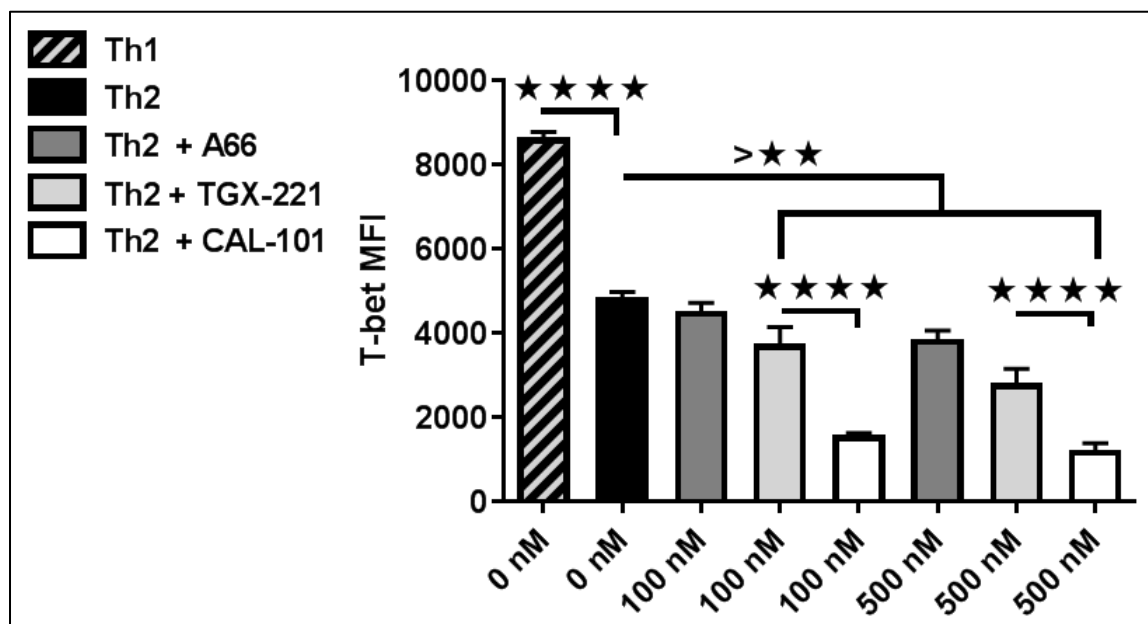
Figure 37: Effect if PI3K Isoform Inhibition on T-bet in Th2 Conditions with

Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM.

Spleens were harvested from age-matched wild-type C57BL6/ mice between the ages of

8 and 10 weeks. Splenocytes were enriched for the CD3+ fraction through negative

selection, then separated by FACS analysis for the CD4+CD25- naïve T cell population.

The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4,

anti-IL-12, and anti-IFN-γ in suspension, with the addition of 100 and 500 nM

concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested,

washed, fixed, and stained for the T-bet and GATA-3 transcription factors.

Representative set, specific sample conditions performed in triplicate, n=5 mice per

experiment, repeated with matched conditions yielding similar results. Statistical

analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; ** $P \leq 0.01$, **** $P \leq 0.0001$

Figures 38-40: Effect of PI3K Isoform Inhibition on GATA-3 in Th2 Conditions with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Statistical analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; * $P \leq 0.05$, ** $P \leq 0.01$

41

Figure 41: Effect of PI3K Isoform Inhibition on the GATA-3/T-bet Ratio in the Th2 Condition with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at 100 and 500 nM. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of 100 and 500 nM concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors.

For determining the ratio, the MFIs of GATA-3 and T-bet in the Th2 conditions were averaged from triplicate samples, then converted into a GATA-3/T-bet ratio that was normalized to 1. All other groups' ratios were calculated in comparison with the normalized Th2 control. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results.
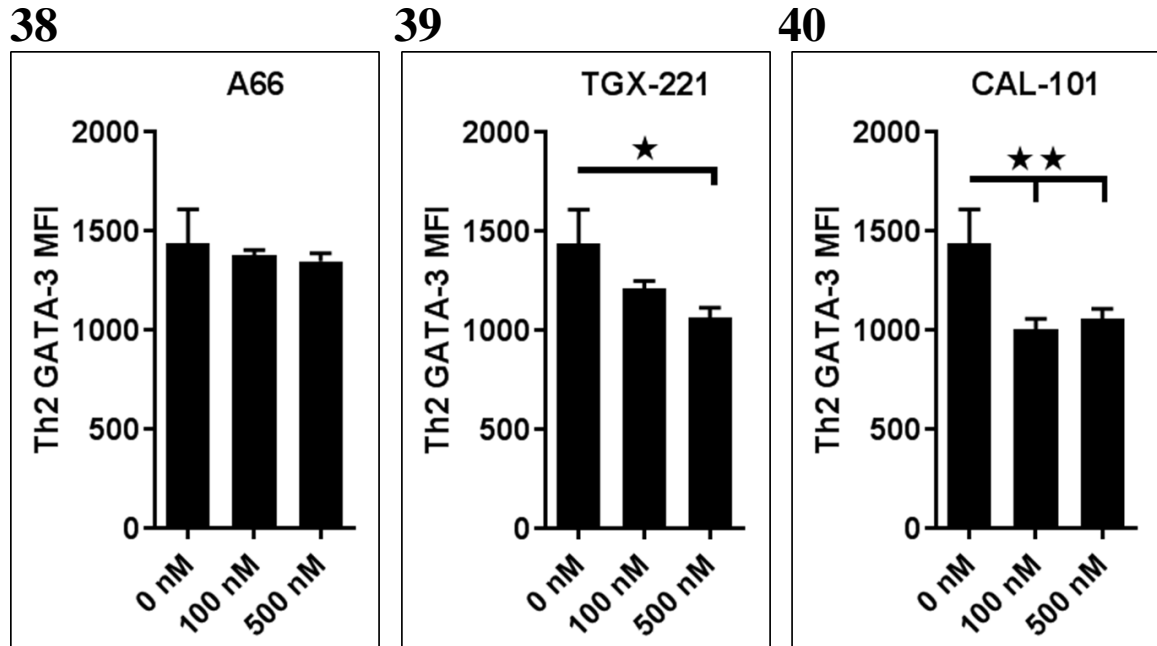
42
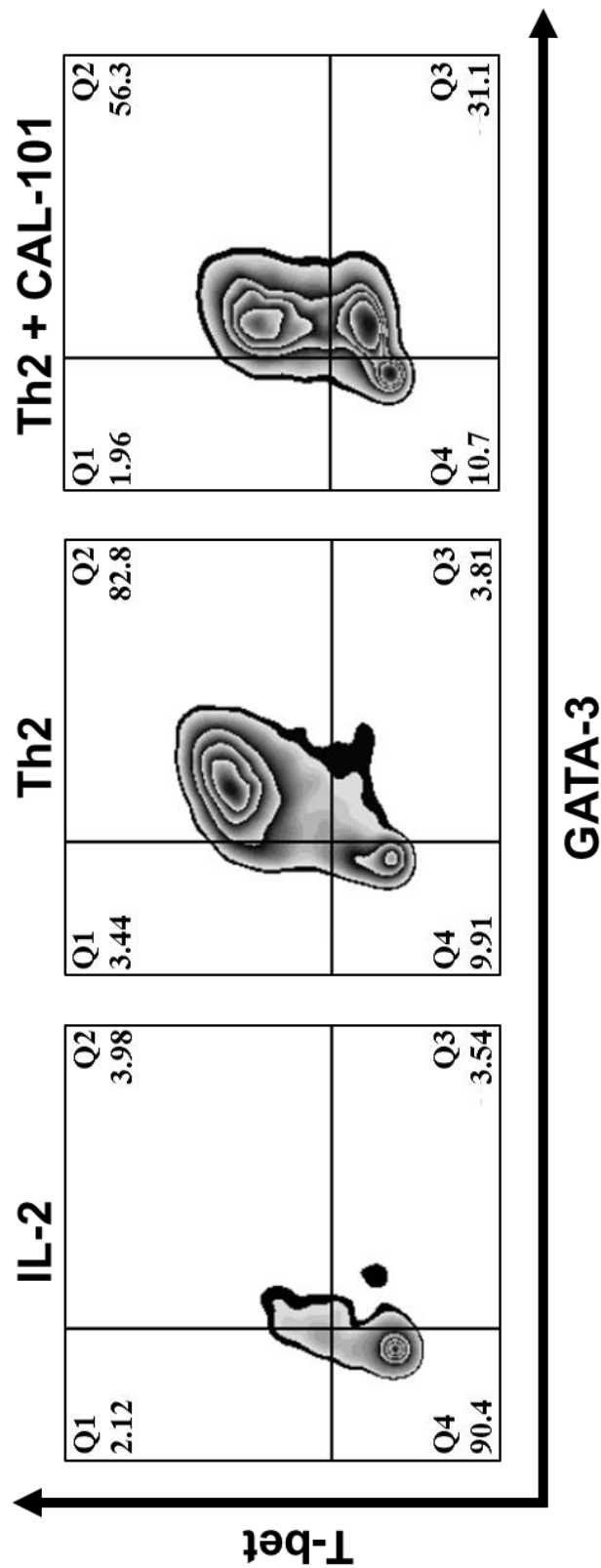
Figure 42: Comparison of CAL-101 (δ) treated and untreated Th2 Polarization by T-bet and GATA-3 Expression. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of 100 nM of CAL-101 in the treatment group. The control group was placed on non-CD3 coated plates with a media containing IL-2 alone. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative matched set, specific sample conditions performed in triplicate, n=3 mice per experiment, repeated greater than 3 times.

43
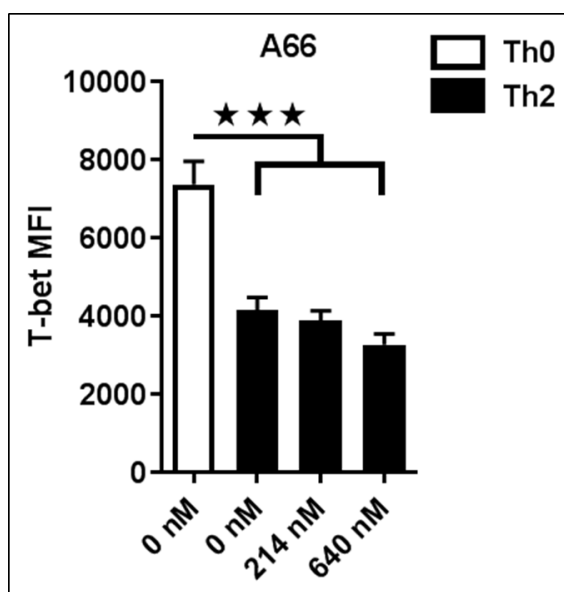
44
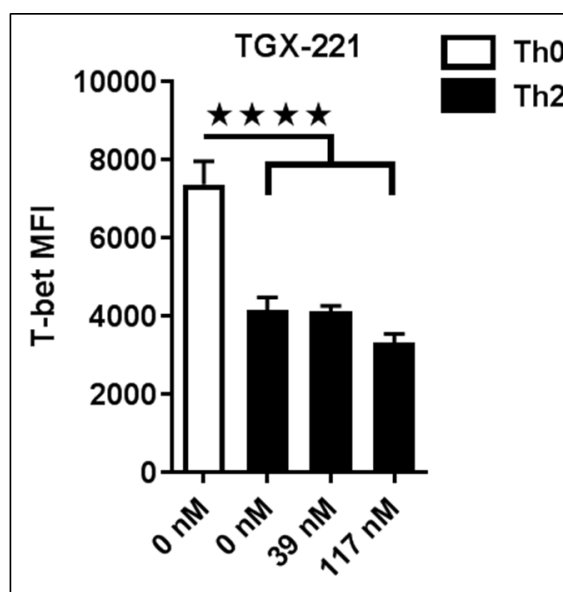
45

Figures 43-45: Effect of PI3K Isoform Inhibition on T-bet in the Th2 Polarization Condition with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at Matched Cell-Free IC$_{50}$s. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$

fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$

naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and

both anti-CD28 and IL-2 in suspension. The Th2 groups were activated for 72 hours with

anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the

addition of the listed concentrations of A66, TGX-221, and CAL-101. Representative

set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated

with matched conditions yielding similar results. Statistical analysis was performed

using a non-parametric one-way ANOVA with a Tukey *post hoc* test; ***** P ≤ 0.001,**

**** P ≤ 0.0001
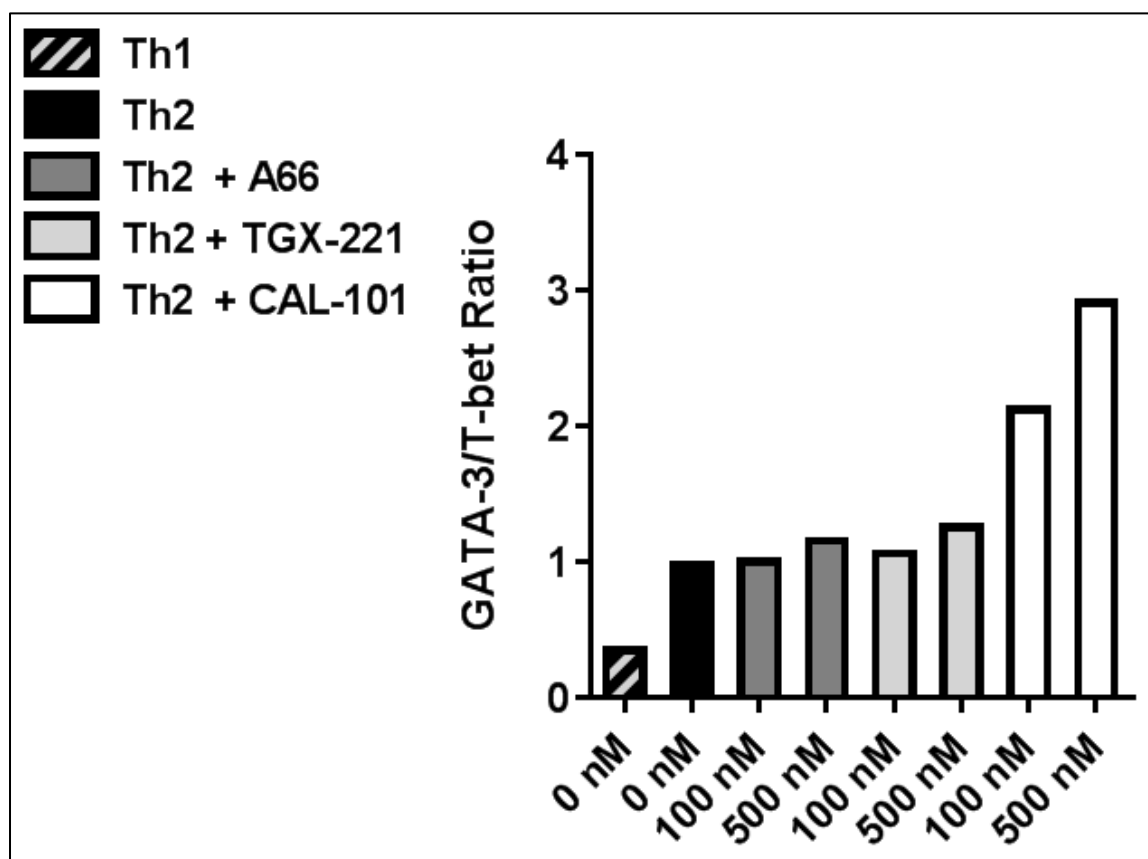
no significant inhibition of T-bet in either the A66 or TGX-221 treatment sets (Fig. 43-44), while even at a 20 nM dose of CAL-101 we observed greater than a 50% decrease in T-bet expression (Fig. 45). Upon examination of GATA-3 expression, we saw a moderate reduction of expression across all treatment groups (Fig. 46-48). Despite this, overall Th2 character was enhanced as the reduction in T-bet expression was greater than the reduction in GATA-3 (Fig. 49). This matches our earlier results with 100 and 500 nM concentrations of specific inhibitors; however, we decided to examine proliferation of the Th2 cells with VCT in order to see if there were any confounding effects due to drug toxicity, as we did with the Th1 and Th17 sets. From our previous results (Fig. 6, 19), we expected neither the A66 nor TGX-221 sets to have any significant change in the proliferative population, while CAL-101 would show a slight drop of about 10% at the higher concentrations. Quite interestingly, none of the PI3K inhibitors had a significant effect at any of the utilized concentrations of A66, TGX-221, or CAL-101 (Fig. 50). In fact, even the pan-PI3K inhibitor, GDC-0941, at the highest inhibitory concentration induced only a minimal drop in proliferation, while in the Th17 and Th1 conditions large decreases were observed (Fig. 6, 19). This not only proves that the drugs were not affecting this Th2-skewing through some toxic or anti-proliferative effect, but possibly that Th2 polarized cells did not have as great a requirement for PI3K signaling or activation and proliferation as did the Th1 and Th17 subsets. This has been partially explored in previous literature [151], in that diminished levels of TCR signaling bias towards a Th2 response while increasing antigen activation in otherwise identical conditions leads to a stronger Th1 response. Final verification of how PI3Kδ signaling elimination drives Th2 polarized cells to a stronger Th2 response was performed using
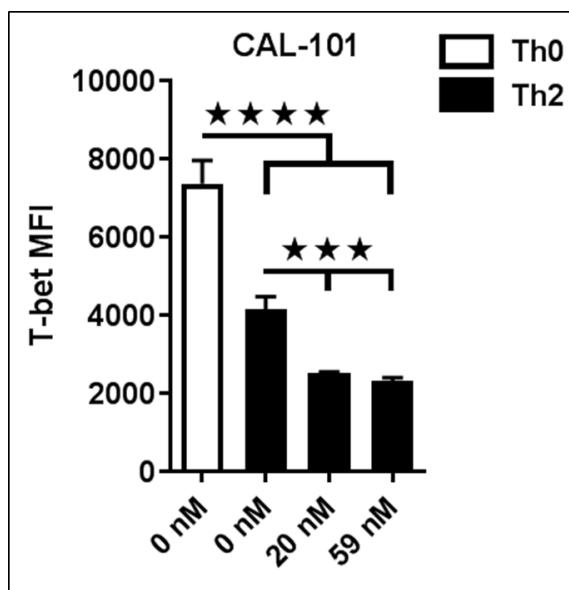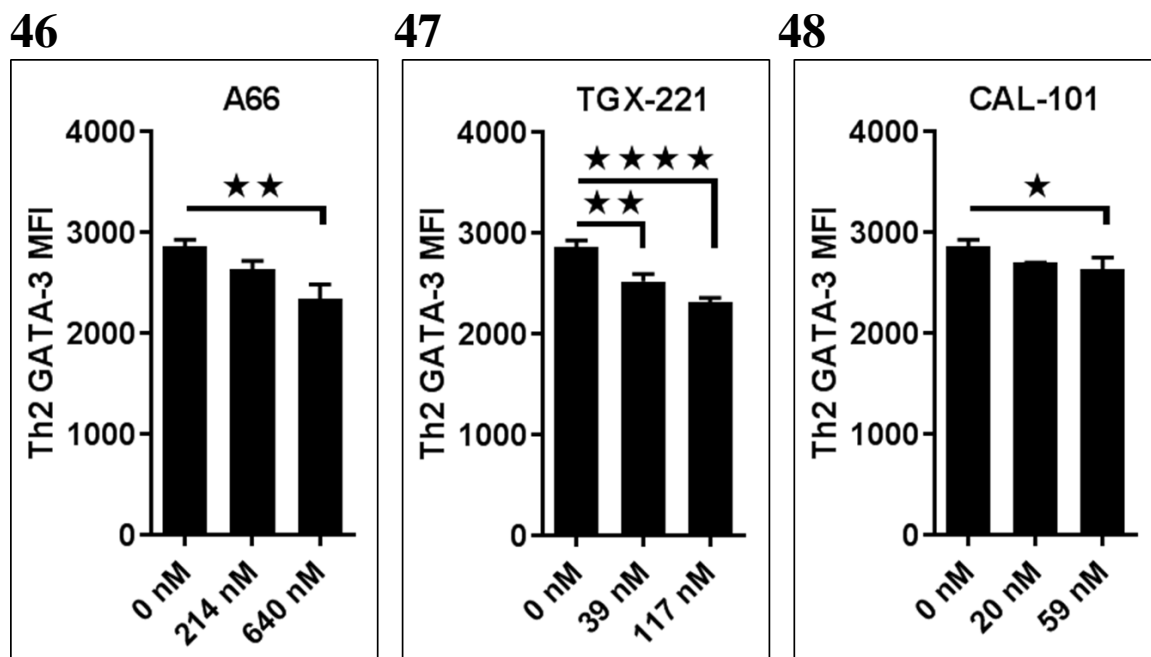
Figures 46-48: Effect of PI3K Isoform Inhibition on GATA-3 in the Th2 Polarization Condition with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at Matched Cell-Free IC$_{50}$s. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of the listed concentrations of A66, TGX-221, and CAL-101. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Statistical analysis was performed using a non-parametric one-way ANOVA with a Tukey *post hoc* test; * P ≤ 0.05, ** P ≤ 0.01, **** P ≤ 0.0001

49

Figure 49: Effect of PI3K Isoform Inhibition on the GATA-3/T-bet Ratio in Th2 Condition with Individual Inhibitors A66 (α), TGX-221 (β), and CAL-101 (δ) at Matched Cell-Free IC$_{50}$s. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, using the listed concentrations of A66, TGX-221, and CAL-101. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. For determining the ratio, the MFIs of GATA-3 and T-bet in the Th2 conditions were

averaged from triplicate samples, then converted into a GATA-3/T-bet ratio that was normalized to 1. All other groups' ratios were calculated as compared to the normalized Th2 control. Representative set, n=5 mice per experiment, repeated with matched conditions yielding similar results.

50

Figure 50: VCT Proliferation Assay for Th2 Polarization with A66 (α), TGX-221 (β), CAL-101 (δ), and GDC-0941 (pan) Treatment. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population. Cells were stained with VCT, then activated in Th2 conditions for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, using the listed concentrations of A66, TGX-221, and CAL-101. Representative set, n=5 mice per experiment, repeated with matched conditions yielding similar results.

D910A mice.

Knockout verification experiments were performed with age-matched WT and D910A mice, harvested and sorted as previously described, then cultured for 72 hours in Th2 conditions. In the Th1 condition, we previously showed that T-bet was markedly reduced with D910A cells when compared to WT Th1 polarized cells (Fig. 35). We thus expected this reduction in T-bet to be conserved, despite the changed polarizing conditions. The WT Th2 condition showed the expected transcription factor profile of T-bet and GATA-3 after 72 hours, with a population of approximately 30% GATA-3^{hi}T-bet^{lo} (Fig. 36, 42, 51). According to our hypothesis, we predicted an increase in the GATA-3^{hi}T-bet^{lo} population in the D910A model, as we saw with CAL-101 treatment. As expected, the GATA-3^{hi}T-bet^{lo} quadrant increased to 55%, an increase of almost twice the WT control (Fig. 51). Because of this, we concluded that the elimination of PI3Kδ signaling in the Th2 polarizing condition shifted polarization towards a stronger Th2 outcome. We then sought to examine whether PI3Kα or β inhibition had an effect to further skew towards the Th2 condition in the D910A mouse model. While neither A66 nor TGX-221 alone had a marked effect on T-bet expression, we thought that since Yamane et al. [151] showed that decreased TCR signaling drove stronger Th2 responses, maybe additional PI3K isoform inhibition in the already Th2-skewed D910A cells would further enhance this effect.

Wild-type and D910A naïve CD4^{+}CD25^{-} splenocytes were isolated and cultured for 72 hours, with groups of 1 μM A66 and 250 nM TGX-221. Again, D910A cells polarized in the Th2 condition showed an enhanced Th2 transcription factor phenotype (Fig. 52). However, when the untreated D910A group was compared to the set treated

51
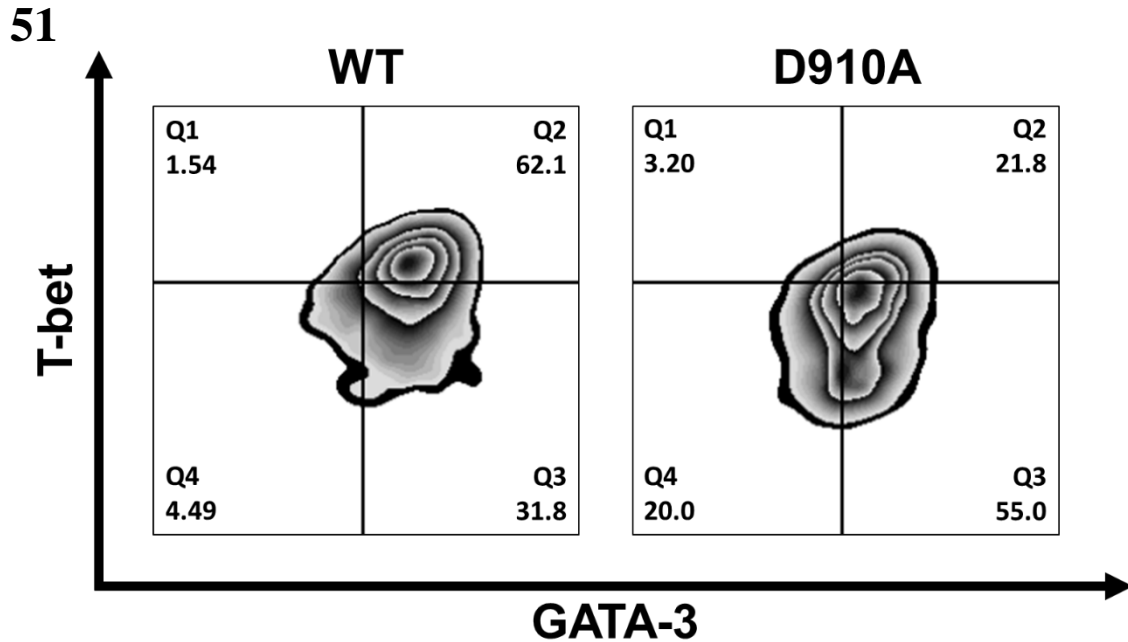
Figure 51: Examination of T-bet and GATA-3 Expression in Th2 Polarization of Wild-type and D910A Mice. Spleens were harvested from age-matched wild-type C57BL/6 mice and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment per strain, repeated with matched conditions yielding similar results.

52

Figure 52: Effect of A66 (α) Treatment on Th2 Polarization in D910A Mouse CD4⁺ Naïve T Cells. Spleens were harvested from age-matched D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3⁺ fraction through negative selection, then separated by FACS analysis for the CD4⁺CD25⁻ naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of 1 μM of A66 to the treatment group. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment.

with A66, we saw a significant increase in the GATA-3^{hi}T-bet^{lo} population (Fig. 52).

Rather than also being replicated in the TGX-221 treated group, we saw no significant

change in the GATA-3^{hi}T-bet^{lo} population from the D910A control set (Fig. 53). This

indicates that, while PI3Kδ abrogation was able to strongly shift towards the Th2 subset,

PI3Kα inhibition by A66 further skewed the phenotype while PI3Kβ inhibition through

TGX-221 did not. While TGX-221 does show some cross inhibition, it acts toward the

PI3Kδ isoform. As the D910A mouse has a catalytically inactivated PI3Kδ isoform, the

high concentration of TGX-221 could not be having an effect on the PI3Kδ isoform.

Further, the 250 nM concentration was nowhere near its ability to affect PI3Kα, with an

IC$_{50}$ of 5 μM (Table 1). Thus, it is unlikely that TGX-221 is acting through non-PI3Kβ

inhibiting effects.

Based on our results, we created a mechanistic model that could potentially

explain our results through the lens of the established literature (Fig. 54). We explore our

reasoning more in the discussion. However, the polarization of CD4 T cells is not solely

dependent on activation by PI3K through TCR transduction. We touch on this in our

previously published work, where we demonstrate that one mechanism utilized for

differential signaling between the Th17 and iTreg polarized subsets is control directly

through specific polarizing cytokines [22]. Utilizing immunoprecipation methods, we

were able to determine that TGF-β, the iTreg polarizing cytokine, has a strong pro-PI3Kδ

signaling effect. This was contrasted by the Th17 polarizing cytokine IL-6, which

inhibited PI3Kδ signaling. Given the similarity in the Th17/iTreg axis with Th1/Th2, we

hypothesized that the Th1/Th2 specific cytokines mirror the Th17/iTreg ones in their

ability to modify PI3K isoform signaling. More specifically, we expected the Th1

cytokine IL-12 to activate PI3Kδ, as TGF-β activated PI3Kδ, while the Th2 cytokine IL-4 would reduce PI3Kδ signaling, as IL-6 did. In addition, as PI3Kα inhibition was able to skew cells further towards a Th2 phenotype and decrease IFN-γ in the Th1 condition, we also hypothesized that IL-12 and IL-4 would affect PI3Kα signal transduction as well.
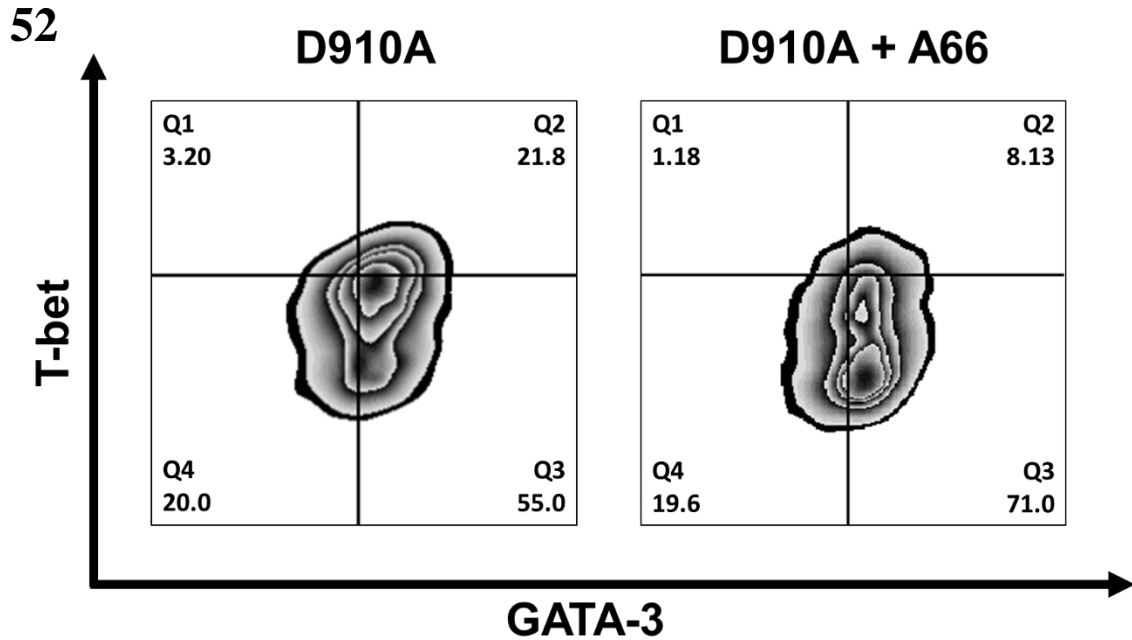
53
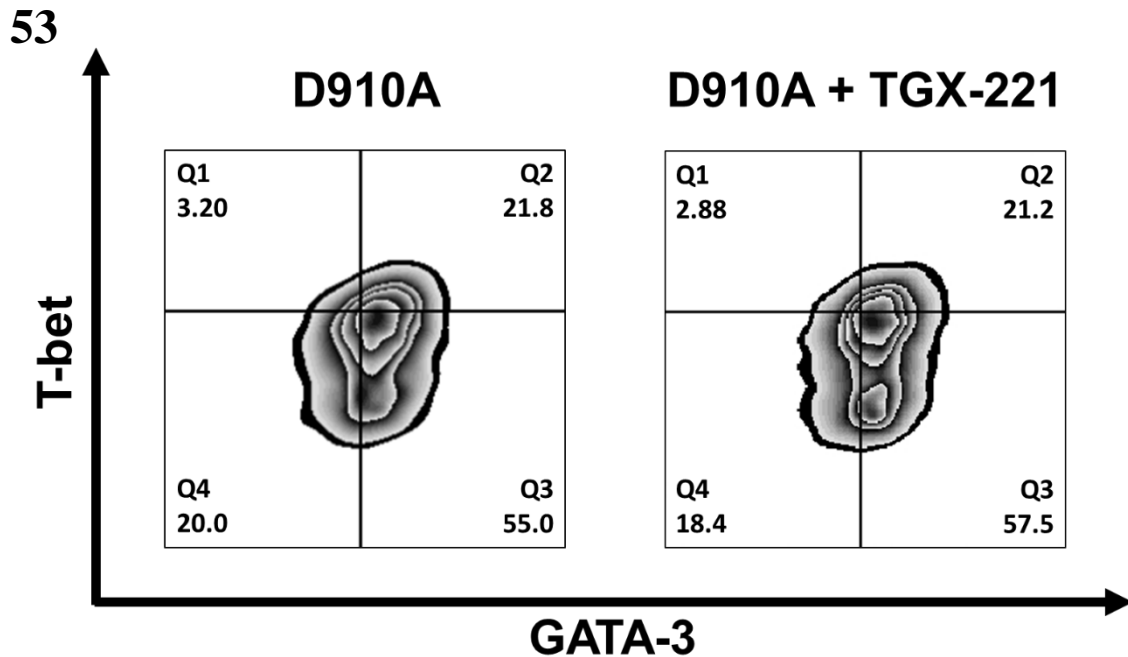
Figure 53: Effect of TGX-221 (β) Treatment on Th2 Polarization in D910A Mouse CD4+ Naïve T Cells. Spleens were harvested from age-matched D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3+ fraction through negative selection, then separated by FACS analysis for the CD4+CD25- naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension, with the addition of 250 μM of TGX-221 to the treatment group. After 72 hours, cells were harvested, washed, fixed, and stained for the T-bet and GATA-3 transcription factors. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment.

54
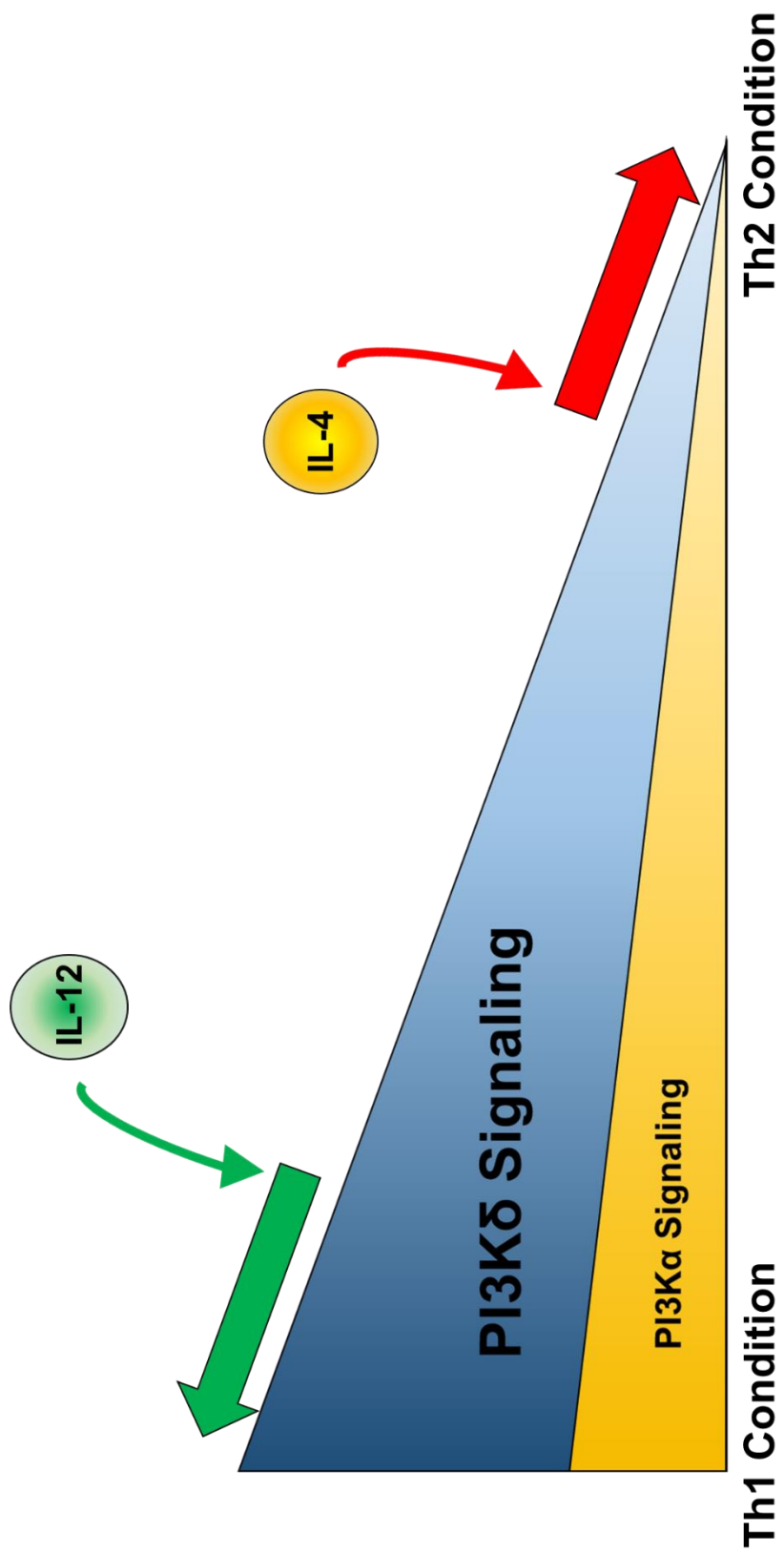
Figure 54: Model of PI3K Isoform Control of Th1/Th2 Polarization, with Signaling Modification from the Th1 and Th2 Cytokines IL-12 and IL-4. Signaling through the TCR in T helper cells is most strongly dependent on the PI3Kδ isoform, with lesser dependence on the PI3Kα isoform. High levels of PI3K αδ signaling bias towards a Th1 response, while activation with low levels of αδ signaling preferentially drives Th2 polarization. In addition to their JAK/STAT signaling, IL-12 and IL-4 influence PI3K αδ signaling to bias towards the activation level preferred by their respective polarization subtypes, specifically, IL-12 increases PI3K αδ signaling, while IL-4 inhibits it.

E. IL-12 and IL-4 Modify PI3K Isoform Signaling

As discussed in the introduction, PI3K is vital for downstream signaling and a host of effector functions in CD4$^+$ T cells, including polarization [3, 54]. Our own experiments have shown that by inhibiting PI3Kδ, in general activated cells were skewed more strongly towards the Th2 and Th17 subtypes and the Th1 and iTreg groups were inhibited. But how does this occur mechanistically? We hypothesized that this may occur because of the strength of antigenic activation, with strong TCR transduction biasing towards Th1/iTreg while low promotes Th2/Th17. But is this mechanism purely based on TCR affinity? If so, that would mean that CD4$^+$ T helper polarization is largely random, since antigen specificity is randomly generated [67]. This also ignores the huge role that cytokines play in polarizing T helper subsets. Given the role that PI3K isoforms have been shown to play in promoting specific polarized cell types, it follows that cytokines, the ultimate supervisors of T helper subset polarization, may affect specific PI3K isoforms. In our previous work, we discovered that TGF-β activated PI3Kδ, while IL-6 inhibited it [22]. This indicates that, besides their specific polarizing pathways, these cytokines have some effect on PI3K activation, which may in turn lead to differential polarization outcomes.

Initial experiments focused on the Th1 subset, which, if it mirrors the Th17/iTreg axis, should have increased PI3K isoform signaling. As of writing this thesis, there were no verified monoclonal antibodies available which were highly specific to individual phosphorylated PI3K isoforms. However, there were highly specific PI3Kα, β, and δ p110 catalytic subunit antibodies. As such, for these immunoprecipitation experiments, lysates were precipitated with sepharose beads conjugated to an anti-phospho-tyrosine

residue specific antibody to pull down proteins with phosphorylated tyrosine. This

immunoprecipitate was then split into three equivalent samples and probed for PI3Kα, β,

and δ p110. Upon examination of the individual blots, we saw a huge increase in

phosphorylated PI3Kα and δ, with minimal change in β (Fig. 53). Within the non-

immunoprecipitated lysates there was also an 18% increase in total p85, the regulatory

subunit of PI3K that we use as a measure of total PI3K protein. This confirms what we

expected, in that there is increased PI3K signaling in the Th1 subset compared to the Th0

group. However, this experiment does not allow a conclusion on the effect of IL-12, as

the Th1 subset has two variables that are different from the Th0 group, with the addition

of both IL-12 and anti-IL-4, which blocks IL-4 signaling. In order to generate a

conclusion on specific effects of individual cytokines, we need to compare groups that

are different by only a single variable.

To compare groups with an individual variable, we removed the neutralizing

antibodies from our 72 hour polarization sets. All cells were cultured in what we have

labeled the Th0 condition, with the single variable addition of either 1000 U of IL-4 or 20

ng of IL-12. After lysis, these samples were immunoprecipitated with anti-phospho-

tyrosine conjugated sepharose beads and run on blots probed for PI3Kα, β, and δ p110.

Consistent with our hypothesis, we show that IL-4 induces a decrease in PI3K signaling

across all class IA PI3K isoforms, while IL-12 elicits activation of PI3Kα and δ (Fig. 54-

57). The mechanism of action is almost certainly indirect, but this result does show that

individual polarizing cytokines have some role in modifying PI3K isoform signaling.

Thus, we propose a model where IL-12 and IL-4, besides their polarizing JAK/STAT

signaling, also modify PI3K isoforms to better match the level of antigenic TCR

transduction preferred by their polarized T helper cell, with IL-12/Th1 preferring 'high'

TCR transduction while IL-4/Th2 prefers 'low' TCR signaling (Fig. 54) [151]. While

our results as yet favor this system, we have only demonstrated skewing towards Th2

subset polarization through GATA-3^{hi}T-bet^{lo} transcription factor profile, rather than

functionality. As cytokine expression is another important indicator of T helper

polarization, we next moved to demonstrate that Th2 polarized cells skewed by PI3Kδ

signaling inhibition expressed Th2 cytokines, which include IL-4, IL-5, and IL-13 [67].

55
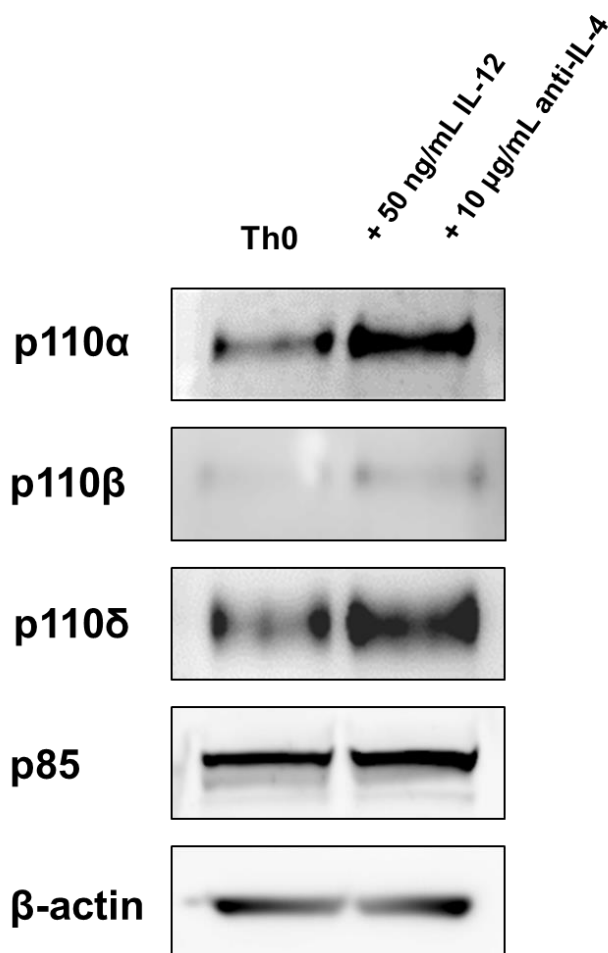
Figure 55: Immunoprecipitation of phospho-Tyrosine Residue-Containing Proteins from Th0 and Th1 Polarized T Cells. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3+ fraction through negative selection, then separated by FACS analysis for the CD4+CD25- naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in suspension. The Th1 polarized cells were activated using the same method, with the addition of IL-12 and anti-IL-4. After 72 hours, cells

were washed and lysed in RIPA buffer, quantitified, and immunoprecipitated for

phospho-tyrosine residue-containing proteins. Each sample was then split into three

equivalent portions and analyzed via Western blot, before being probed for PI3K α, β,

and δ protein. p85 and β-actin were used as loading controls on remaining, non-

immunoprecipitated lysate. Representative set, n=10 mice per experiment.

56
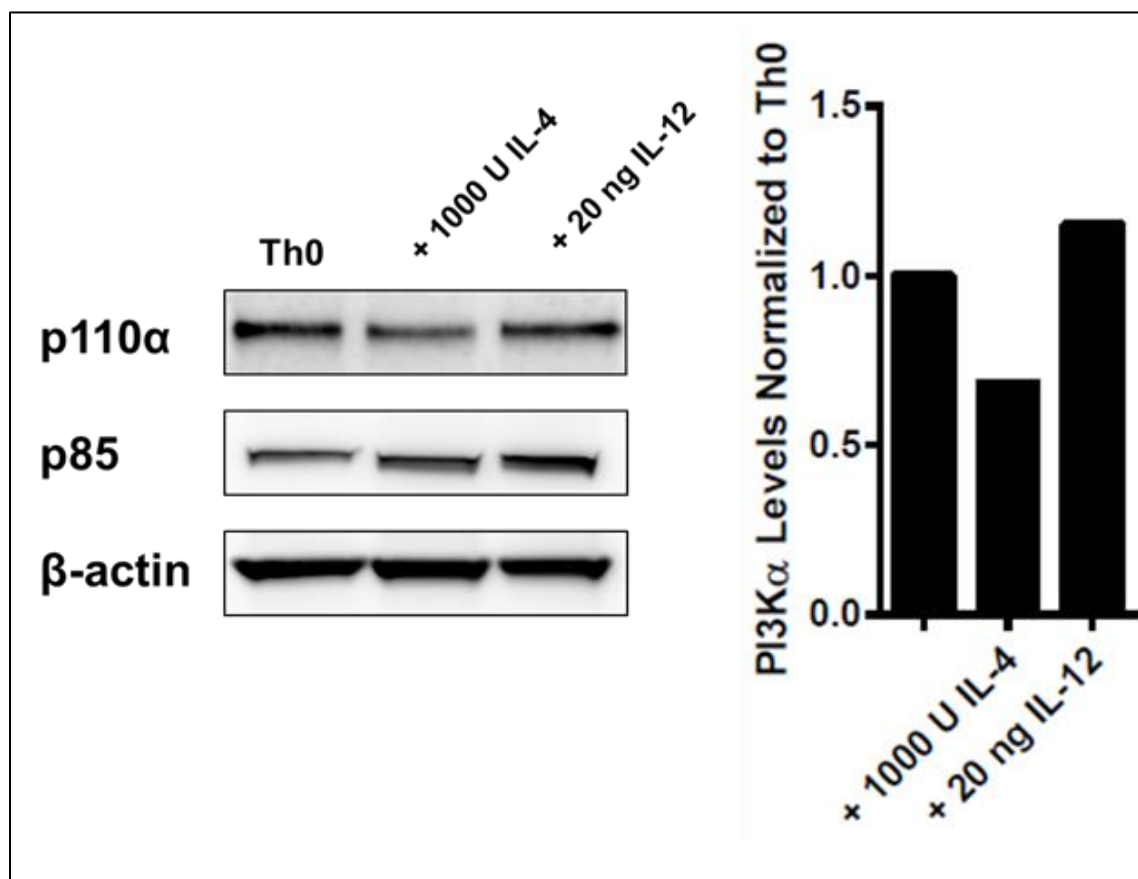
Figure 56: Immunoprecipitation of phospho-Tyrosine Residue-Containing Proteins

from Th0 and Single Cytokine Addition Th0 Sets Probed with p110α Antibody.

Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of

8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through negative

selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population.

The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in

suspension. The additional conditions were activated using the same method, with the

addition of 1000 U of IL-4 for one set and 20 ng of IL-12 for the other. After 72 hours,

cells were washed and lysed in RIPA buffer, quantified, and immunoprecipitated for

phospho-tyrosine residue-containing proteins. Each sample was then split into three equivalent portions and analyzed via Western blot for PI3K α protein. p85 and β-actin probes were used as loading controls on remaining, non-immunoprecipitated lysate. Representative set, n=10 mice per experiment, repeated with matched conditions yielding similar results, normalized to β-actin.

57

Figure 57: Immunoprecipitation of phospho-Tyrosine Residue-Containing Proteins from Th0 and Single Cytokine Addition Th0 Sets Probed with p110β Antibody.

Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and IL-2 in suspension. The additional conditions were activated using the same method, with the addition of 1000 U of IL-4 for one set and 20 ng of IL-12 for the other. After 72 hours, cells were washed and lysed in RIPA buffer, quantified, and immunoprecipitated for

phospho-tyrosine residue-containing proteins. Each sample was then split into three

equivalent portions and analyzed via Western blot for PI3K β protein. p85 and β-actin

probes were used as loading controls on remaining, non-immunoprecipitated lysate.

Representative set, n=10 mice per experiment, repeated with matched conditions yielding

similar results, normalized to β-actin.

58

59

Figures 58-59: Immunoprecipitation of phospho-Tyrosine Residue-Containing

Proteins from Th0 and Single Cytokine Addition Th0 Sets Probed with p110δ

Antibody. Spleens were harvested from age-matched wild-type C57BL/6 mice between

the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3^{+} fraction through

negative selection, then separated by FACS analysis for the CD4^{+}CD25^{-} naïve T cell

population. The Th0 group was activated for 72 hours with anti-CD3 and anti-CD28 and

IL-2 in suspension. The additional conditions were activated using the same method,

with the addition of 1000 U of IL-4 for one set and 20 ng of IL-12 for the other. After 72 hours, cells were washed and lysed in RIPA buffer, quantified, and immunoprecipitated for phospho-tyrosine residue-containing proteins. Each sample was then split into three equivalent portions and analyzed via Western blot for PI3K δ protein. p85 and β-actin probes were used as loading controls on remaining, non-immunoprecipitated lysate. Representative set, n=10 mice per experiment, repeated with matched conditions yielding similar results, normalized to β-actin.
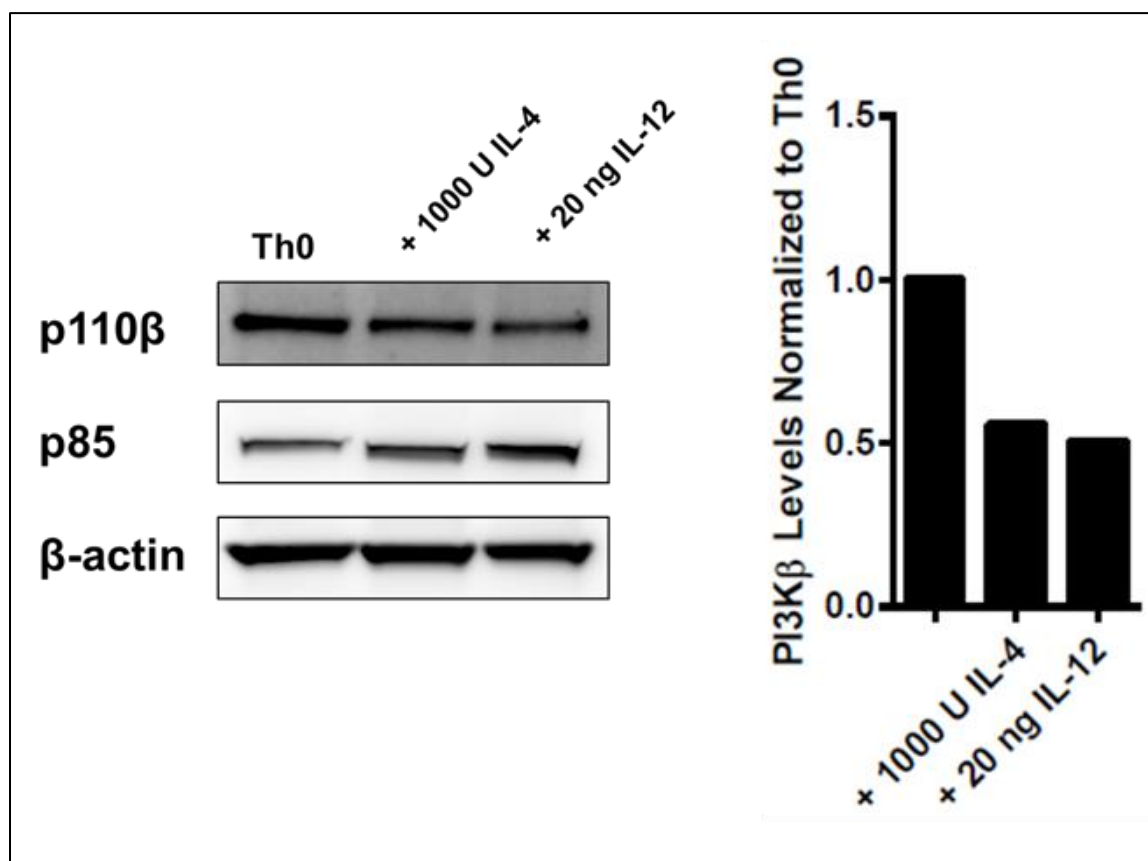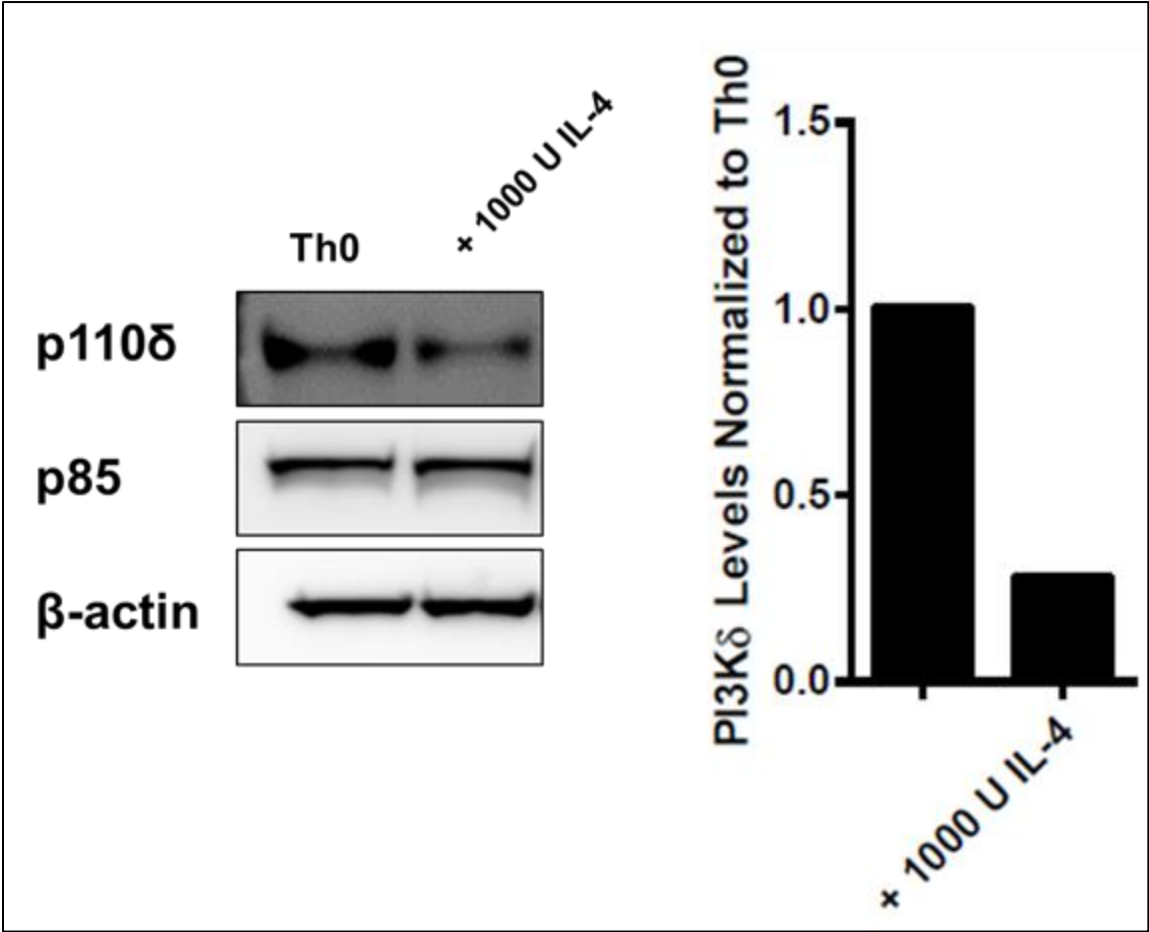
F. PI3Kδ is Required for Cytokine Production through pSTAT5 Signaling

While cells skewed towards the Th2 condition display a strong Th2 transcription factor phenotype (Fig. 42), a crucial component of what defines a Th2 cell is the production of a specific cytokine profile. As we proposed that PI3Kδ inhibition promoted a Th2 condition to a greater extent than would otherwise occur in non-treated conditions, we sought to further confirm this through the examination of cytokines produced in the CAL-101 treatment group compared to the non-treated Th2 condition. Because of the broad number of Th2 cytokines, we decided to examine the supernatant using cytometric bead arrays (CBA). This method provides a broad spectrum of cytokine data, which allowed us to more accurately determine the polarized T helper subset. Also, as the supernatant from our experimental groups were used, we were able to verify our polarization results in terms of transcription factors and match these to the cytokine profile. This strengthened our overall results, as flow analysis examining all required transcription factors and cytokines is limited by the number of different dyes and is not possible in a single panel. As such, we analyzed the supernatants of our previous experiments in which we polarized towards CD4$^+$ T helper subsets in the presence and absence of PI3K isoform inhibitors. Given that PI3Kδ inhibition skews more strongly towards the Th2 phenotype, we hypothesized that these cells would express higher levels of IL-4, IL-5, and IL-13 than Th2 polarized splenocytes without CAL-101 treatment. Instead, when the supernatant was examined, we saw drastically decreased expression of all Th2 cytokines in the treatment groups (Fig. 60-62). This loss of cytokine production was consistent and dose dependent. We then recapitulated this experimental set up using our D910A model to confirm that these results were not due to off-target effects of our

60

61
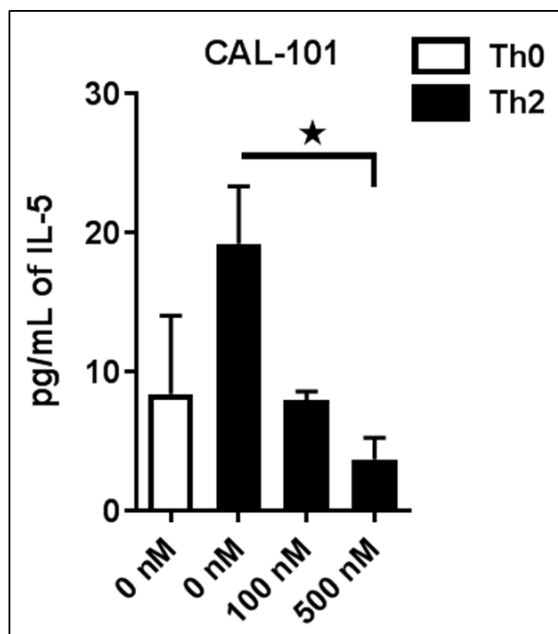
62
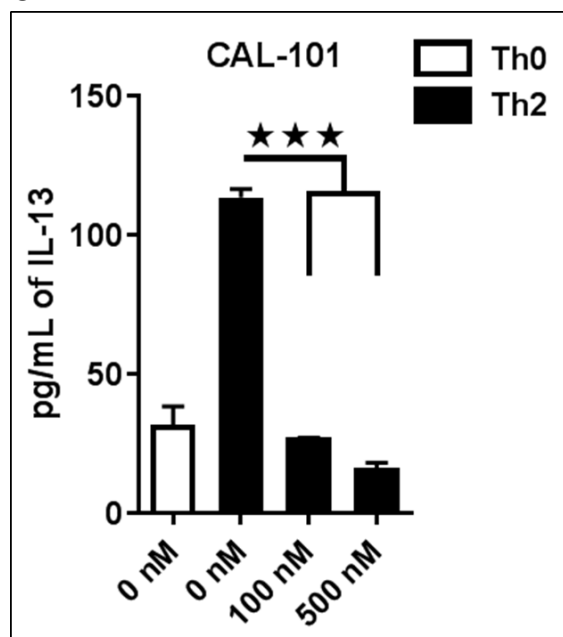
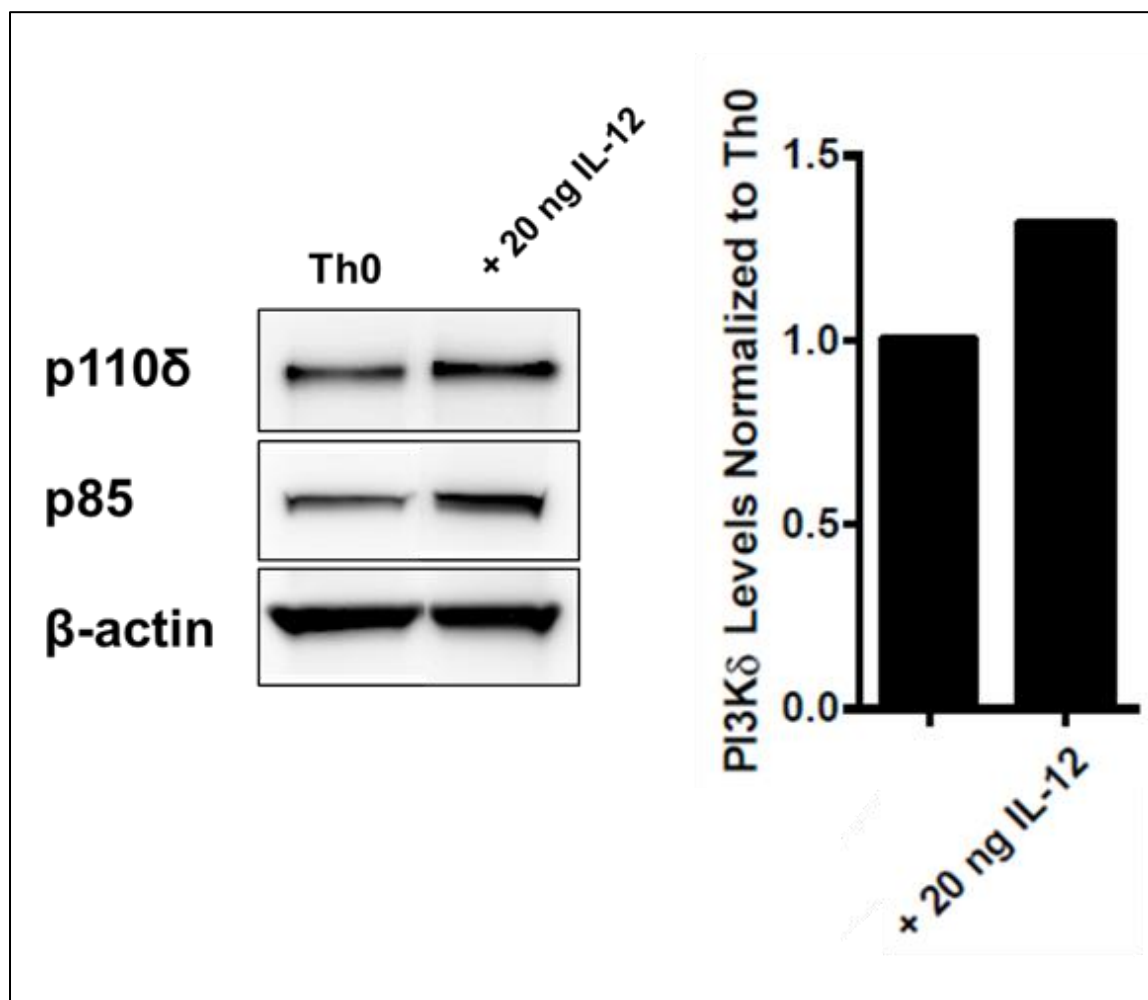**Figures 60-62: Effect of PI3Kδ Inhibition by CAL-101 on Th2 Cytokine Production at 100 and 500 nM.** Spleens were harvested from age-matched wild-type C57BL/6 mice
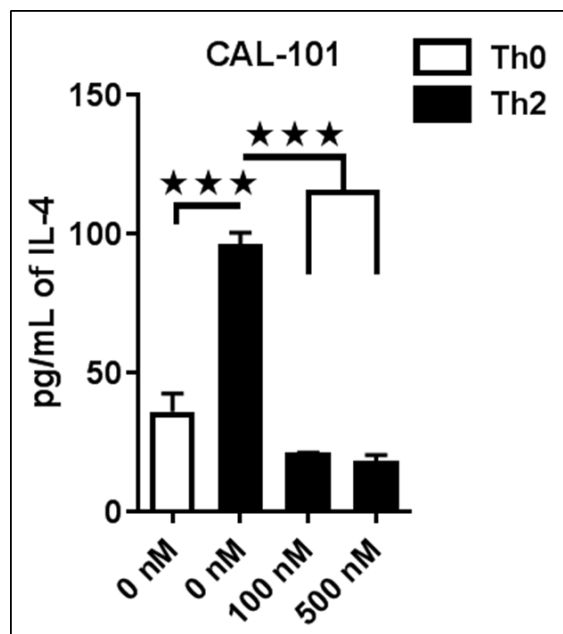
between the ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction

through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T

cell population. The Th0 group was activated for 72 hours with anti-CD3 and both anti-

CD28 and IL-2 in suspension. The Th2 groups were activated for 72 hours with anti-CD3

coating and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in media suspension, with

the addition of 100 and 500 nM concentrations of CAL-101. After 72 hours, supernatant

media was removed and analyzed by CBA. CBA is specific for examination of murine

cytokines, while recombinant human cytokines are used for polarization. Representative

set, specific sample conditions performed in duplicate, n=5 mice per experiment.

Statistical analysis was performed using a non-parametric one-way ANOVA with a

Tukey *post hoc* test; * $P \leq 0.05$, *** $P \leq 0.001$

inhibitors.

We again used the CBA assay on supernatants collected from matched WT and D910A verification experiments performed previously (Fig. 51). In this model, we also saw an overall reduction of most cytokines, with some notable exceptions (Fig. 63). Across almost all cytokines examined, a measurable, consistent drop in the Th0, Th1, Th2, and Th17 conditions was observed with D910A cells when compared to the WT. The only exception was IL-5, which is increased in the D910A groups across all conditions (Fig. 64). This brought us to the conclusion that while PI3Kδ loss increased the GATA-3/T-bet and RORγt/FoxP3 ratios in the Th2 and Th17 conditions, respectively, it decreased the production of most cytokines. We thereby hypothesized that PI3Kδ signaling was also necessary for the production of most cytokines through some other factor(s) than the GATA-3, T-bet, RORγt, and FoxP3 transcription factors. There are a host of various nuclear-translocating factors that are necessary for the production of cytokines [71], but one that is known to be required in both Th1 and Th2 cytokine production, as well as several others, is STAT5 [133-139]. In order to determine if STAT5 signaling was affected by PI3Kδ signaling loss, we stained for activated pSTAT5 in the WT and D910A model, hypothesizing that pSTAT5 would be uniquely decreased in D910A cells across all polarizing conditions when compared to the WT.

Upon stimulation of WT and D910A splenocytes for 72 hours in the Th0, Th1, and Th2 conditions, we collected the supernatant for confirmation of previous cytokine trends and then fixed and stained the polarized cells using anti-Stat5(pY694). Upon normalization to the WT control, we measured a consistent drop of activated STAT5 in

63

pg/mL	IFN-γ	IL-5	TNF-a	IL-2	IL-4	IL-9	IL-17A	IL-17F	IL-21	IL-22	IL-13
WT Th0	7126	22.36	268.38	14530	174.5	<15.6	25.46	2.04	<2.6	2.42	55.26
WT Th0	7786	23.76	263.32	15602	191.86	<15.6	18.9	2.52	<2.6	2.74	63.04
WT Th1	>42240	20	442.72	11910	7.66	62.44	18	3.72	<2.6	45.36	55.18
WT Th1	>42240	26.98	427.14	11680	9.56	61.68	21.28	4.7	18.08	47.7	54.88
WT Th2	20.58	78.16	415	15310	445	<15.6	36.8	1.92	<2.6	2.74	274.06
WT Th2	27.32	50.98	472.52	15208	343.24	<15.6	37.1	1.92	<2.6	3.98	289.42
WT Th17	1563.72	3.92	180.22	662.04	131.58	531.96	742.06	188	161.02	2.42	54.92
WT Th17	1756.1	5.86	177.12	620.98	125.42	546.92	590.28	165.58	122.72	2.42	49.64
WT IL-2	<3.36	<1.94	<1.96	<2.18	2.3	<15.6	1.22	1.28	<2.6	1.36	<2.1
WT IL-2	<3.36	<1.94	<1.96	<2.18	2.14	<15.6	1.56	1.38	<2.6	1.08	<2.1
KO Th0	2804	109.82	148.48	11202	32.92	<15.6	35.02	2.16	<2.6	5.7	46.78
KO Th0	2720	84.78	143.44	10538	35.08	<15.6	23.66	1.8	<2.6	5.9	38.84
KO Th1	16786	96.08	178.56	7154	4.08	<15.6	38.28	3.04	2.66	4.74	50.02
KO Th1	16462	121.02	172.3	6950	4.82	<15.6	19.78	2.04	<2.6	8.76	53.52
KO Th2	32.02	188.22	213.46	15064	33.92	<15.6	62.16	3.16	<2.6	8.14	86.72
KO Th2	33.22	203.76	207.82	14286	35.56	20.68	42.12	3.3	<2.6	5.7	94.38
KO Th17	1170.24	9.02	37.76	276.32	18.16	55.28	99.02	90.02	16.22	2.58	12.36
KO Th17	1165.28	13.56	36.46	280.28	19.06	48.32	117.74	71.2	12.66	2.74	17.28
KO IL-2	4.16	2.06	2.28	<2.18	3.34	<15.6	1.74	1.8	<2.6	1.8	2.44
KO IL-2	25.02	5.24	5.56	4.94	7.28	28.46	4.96	4.7	2.66	4.16	8.44

10^5

10^4

10^3

10^2

10^1

0

Figure 63: Changes in Th0, Th1, Th2, and Th17 Cytokine Production Between WT and D910A Mouse Models. Spleens were harvested from age-matched wild-type C57BL/6 and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and both anti-CD28 and IL-2 in suspension. The Th1, Th2, and Th17 groups were activated using the same method, with the addition of IL-12 and anti-IL-4 for the Th1 condition, IL-4, anti-IL-12, and anti-IFN-γ for the Th2 condition, and both TGF-β and IL-6 in the Th17 condition. After 72 hours, supernatants were removed and analyzed by CBA. Representative set, specific sample conditions performed in duplicate, n=5 mice per experiment per strain, repeated with matched conditions yielding similar results. Units in pg/mL.

64
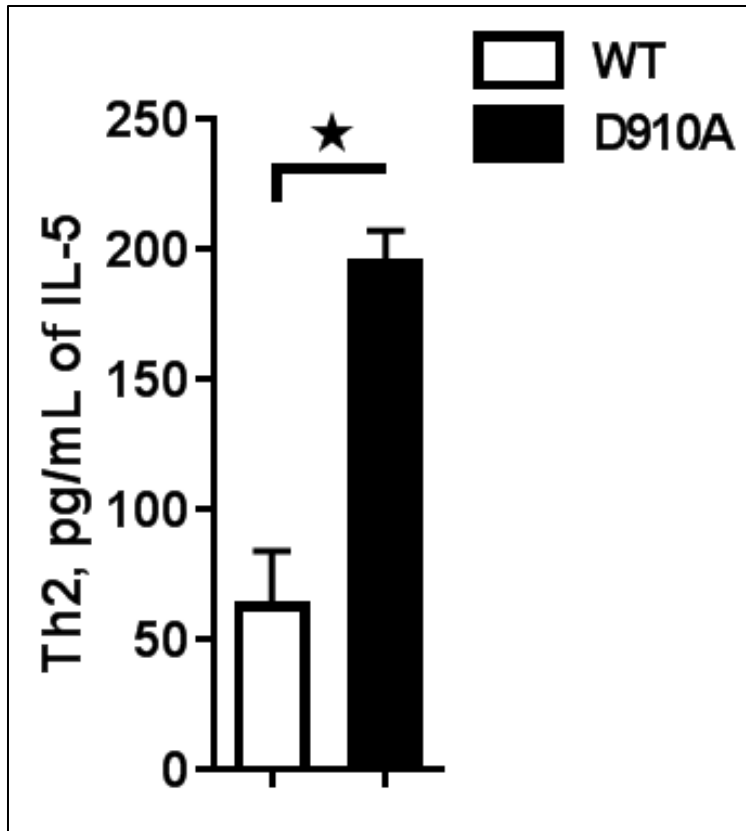
Figure 64: D910A Th2 Polarized Cells Showed Marked Increases in IL-5 Cytokine Production Compared to Wild-type. Spleens were harvested from age-matched wild-type C57BL/6 and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th2 group was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in suspension. After 72 hours, supernatants were removed and analyzed by CBA. Representative set, specific sample conditions performed in duplicate, n=5 mice per experiment per strain,

repeated with matched conditions yielding similar results. Statistical analysis was performed using an unpaired two-tailed Student's *t* test; * P $\leq$ 0.05

all experimental D910A polarized conditions when compared to their respective WT

controls (Fig. 65-67). Since STAT5 has been implicated as an important requirement for

the expression of IFN-γ, IL-4, IL-13, and other cytokines, we concluded that PI3Kδ

inhibition, while increasing Th2 and Th17 polarization, also inhibited cytokine signaling.

Despite this decrease, we have confidence that the Th2 cells skewed by the inhibition of

PI3Kδ signaling are still classical Th2 cells. In addition to the increase in the GATA-

3/T-bet ratio, we also saw a consistent increase in the Th2 cytokine, IL-5 (Fig. 63-64).

As a Th2-specific cytokine [66], this increase in the D910A model compared to the WT

indicated a shift to a stronger Th2 phenotype. However, this is just a subset of the

cytokine-producing Th2 milieu. In order to more fully verify the increased Th2 nature of

our PI3Kδ signaling abrogated models, we designed an experiment to determine if the

Th2-skewed cells would be cytokinetically functional after removal of PI3Kδ inhibition

mediated by CAL-101.

65
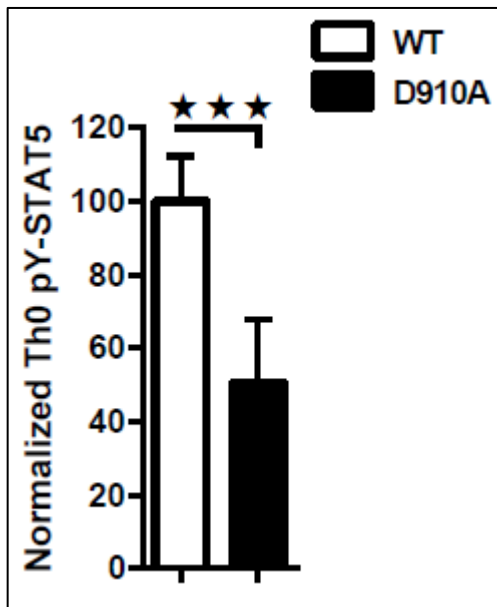
66
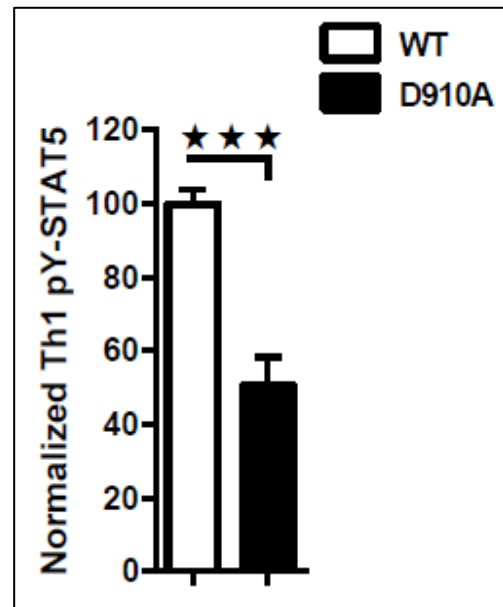
67
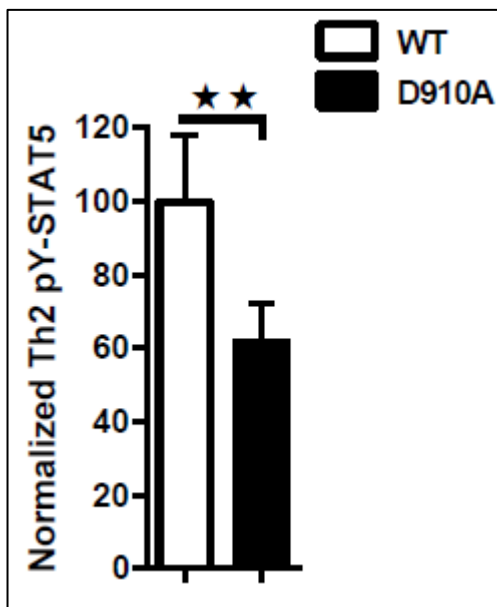
Figures 65-67: D910A Th0, Th1, and Th2 Polarized Subsets Showed Strongly Inhibited pSTAT5⁺ Populations when Compared to Wild-type. Spleens were

harvested from age-matched wild-type C57BL/6 and D910A mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3and both anti-CD28 and IL-2 in suspension. The Th1 and Th2 groups were activated using the same method, with the addition of IL-12 and anti-IL-4 for the Th1 condition and IL-4, anti-IL-12, and anti-IFN-γ for the Th2 condition. After 72 hours, cells were harvested, washed, fixed, and stained for pSTAT5. WT control was converted to 100%. Pooled from 2 experiments, in each experiment sample conditions were performed in triplicate, n=5 mice per experiment. Statistical analysis was performed using an unpaired two-tailed Student's *t* test; ** P ≤ 0.01, *** P ≤ 0.001

G. Th2 Cells Further Polarized by PI3Kδ Inhibition Produce High Levels of IL-5, IL-13, and IL-2 upon Restimulation

While D910A cells polarized in the Th2 condition exhibited a stronger Th2 transcription factor phenotype and increased IL5 expression compared to the wild-type cells, other Th2 cytokines such as IL-4 and IL-13 were still drastically reduced. We have determined that this cytokine reduction was likely due in part to PI3Kδ loss leading to global reductions of STAT5 activation, which is known to be important for the expression of a variety of cytokines, including those of the Th1 and Th2 subsets. In order to bypass this secondary effect on STAT5, we designed an experiment where the Th2 and PI3Kδ-inhibited Th2 subsets were cultured for 72 hours, leading to the previous increased Th2 character in the treated versus the non-treated group (Fig. 42). These cells were then harvested and washed multiple times to remove any remaining CAL-101, then 'rested' in T cell media with IL-2 alone for 48 hours before stimulation for 4 to 6 hours with PMA and ionomycin. The overall goal of this experiment was to create the more fully Th2-skewed subset in the treatment group through CAL-101 inhibition, but to then to remove the PI3Kδ signaling blockade after polarization to allow STAT5 activation. This paradigm would then result in the control Th2 and the more strongly Th2-skewed treatment groups, yet we would no longer have the PI3Kδ signaling blockade that previously led to diminished cytokine production. Thus, we hypothesized that upon PMA/ionomycin stimulation, there would be increased Th2 cytokines produced in the more strongly skewed Th2 CAL-101 treatment subset than in the Th2 control. Before restimulation, both groups still retained their GATA-3/T-bet profiles, with the stronger Th2 character present in the CAL-101 treatment group than in the control (Supplement

3). Cytokine production after PMA/ionomycin treatment was compared as a fold increase to the basal cytokine level, which was obtained from the sample supernatant directly before the addition of PMA/ionomycin. Within these experiments, we still saw a large reduction in IL-4 production in the CAL-101-treated Th2 group after restimulation when compared to the control (Fig. 68). However, the fold expression of IL-5 was equivalent (Fig. 69), and, in addition, we saw an increase in the fold expression of IL-13 in the treatment group when compared to the control (Fig. 70). Lastly, there was a dramatic shift in IL-2 expression, such that the CAL-101-treated Th2 group produced vastly more IL-2 than the Th2 control, both in fold and absolute expression (Fig. 71).

Thus, while IL-4 expression in the treatment group was low compared to the control, we saw equivalent IL-5 expression between the groups and large increases in IL-13 and IL-2. Further, we learned that this was not an intermediate Th1/Th2 cell, as IFN-γ expression was negligible (Supplement 4). We can thereby state with confidence that additional skewing towards the high GATA-3/low T-bet phenotype through PI3Kδ inhibition led to Th2 helper subset polarization. Further, we propose a cellular signaling modality for Th1/Th2 subset polarization, which we explore fully in the discussion (Fig. 72).

68
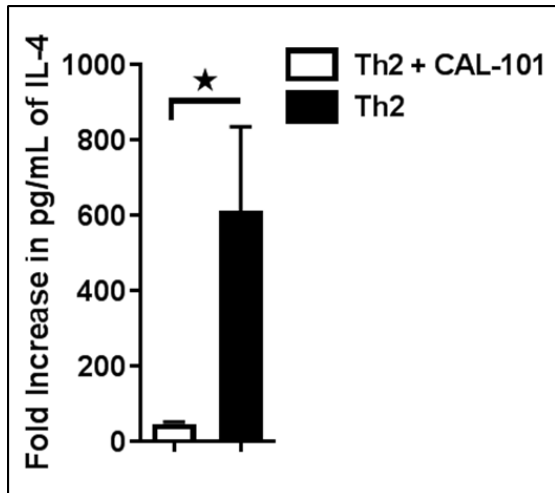
69
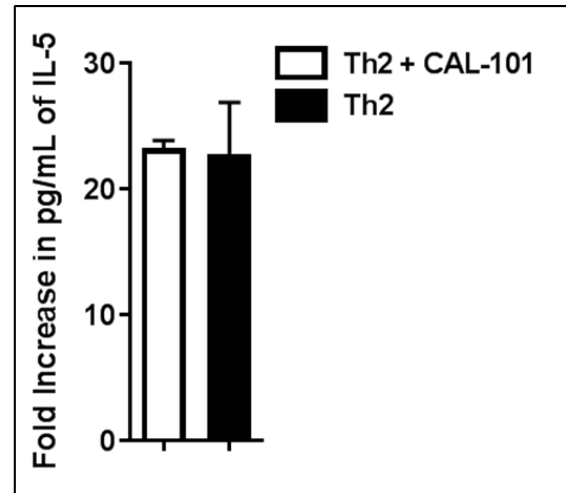
70
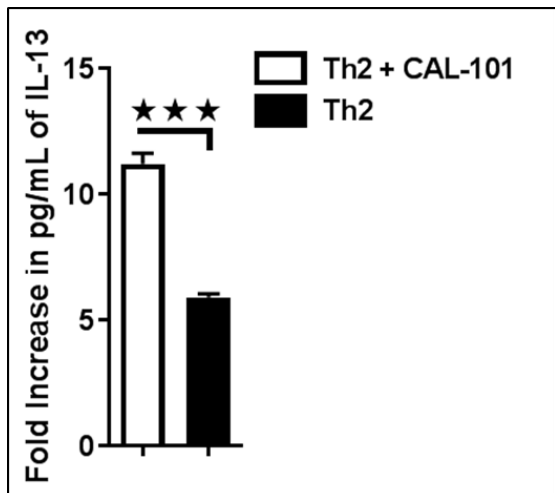
71
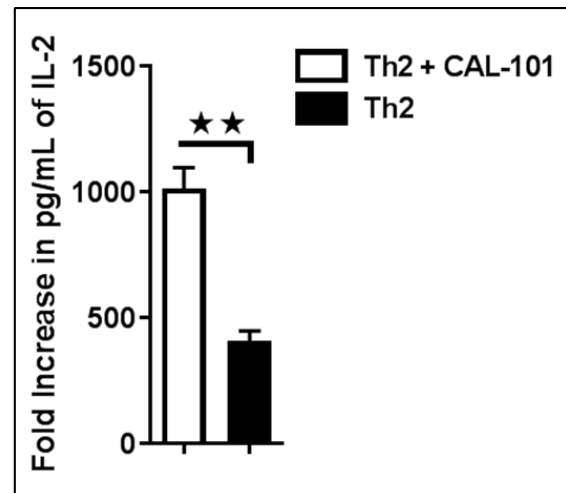
Figures 68-71: Splenocytes Skewed by CAL-101 (δ) Treatment towards Higher Th2 Character Express Th2 Cytokines Upon Restimulation. Spleens were harvested from age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3^{+} fraction through negative selection, then separated by FACS analysis for the CD4^{+}CD25^{-} naïve T cell population. The Th2 groups were activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IL-12, and anti-IFN-γ in

suspension, with the addition of 100 nM of CAL-101 in the treatment group. After 72

hours, cells were harvested and washed three times in 1x PBS to remove as much residual

CAL-101 as possible. Cells were counted and re-plated in matched numbers with T cell

media containing IL-2 alone and 'rested' for 48 hours. After this time period, a portion of

supernatant was collected as a basal control before cells were restimulated with

PMA/ionomycin for 4 to 6 hours, and then the supernatant was collected and analyzed by

CBA. Cytokine expression is measured as a fold increase from basal cytokine levels

before PMA/ionomycin addition. Representative set, specific sample conditions

performed in triplicate, n=5 mice per experiment, repeated with matched conditions

yielding similar results.

72
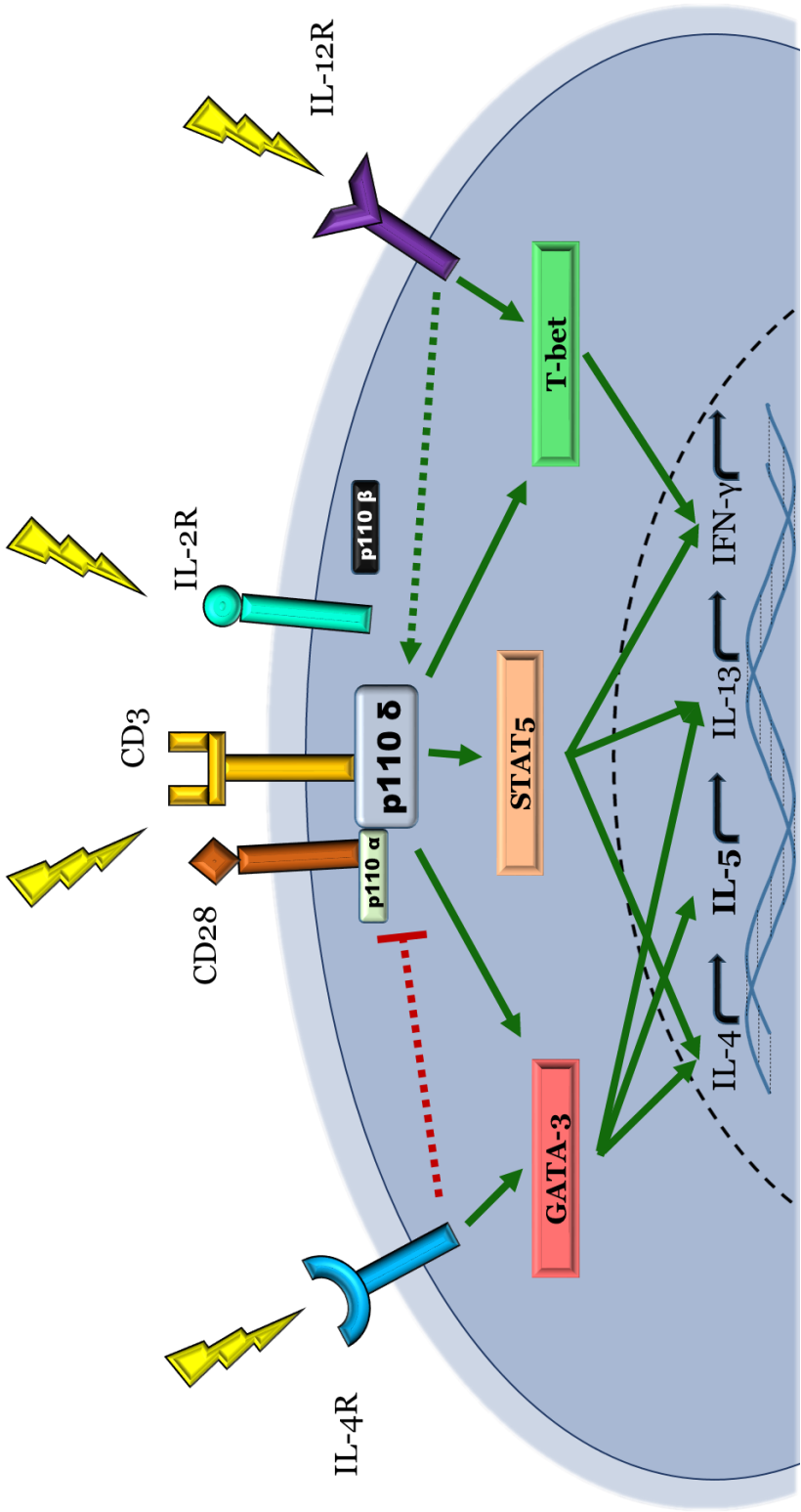
Figure 72: Signaling Model of Experimental Results. TCR transduction predominantly uses the PI3K p110δ isoform, followed by p110α, with minimal or negligible involvement of p110β to this specific pathway. Through p110δ, pSTAT5 is activated, which is a necessary signal for the production of IL-4, IL-13, and IFN-γ. STAT5 activation is not necessary for IL-5 production. Signaling through the IL-4R not only activates GATA-3 through JAK/STAT signaling, but crosstalks with the TCR/PI3K pathway to directly or indirectly diminish PI3K p110α, β, and δ activation. Conversely, IL-12R not only activates T-bet through JAK/STAT signaling, but crosstalks with the TCR/PI3K pathway to directly or indirectly activate PI3K p110α and δ, while p110β activity is decreased.

IV. DISCUSSION

A. Diminished PI3K Isoform Signaling Enhances Th2 and Th17 Polarization

The transcription factors T-bet, GATA-3, RORγt, and FoxP3 are necessary and sufficient to induce the CD4^{+} T helper subsets Th1, Th2, Th17, and iTregs, respectively [68, 88, 89, 106, 112]. From previous work [22], we have determined that iTreg polarization and functionality are strongly inhibited when PI3Kδ signaling was inhibited or eliminated. This was measured by a large decrease in FoxP3 transcription factor and IL-10 levels with CAL-101 inhibition and in the p110delta-PI3K-D910A model when compared to matched wild-type cells. This thesis also expands upon the literature pertaining to the Th1 subtype, which implicates the p110δ PI3K isoform as vital for IFN-γ production [23]. While PI3Kδ remains uncontested as the most vital TCR signal transducer leading to IFN-γ production in the Th1 subset, we posit that PI3Kα also plays a lesser but significant role, given the effects of the A66 inhibitor (Fig. 9, 12, 16). Further, mirroring the effect of PI3Kδ isoform inhibition on FoxP3 in the iTreg condition, we saw a significant reduction in T-bet transcription factor under both CAL-101 inhibition and in cells from D910A mice polarized in the Th1 condition (Fig. 34-35). By these measures, prevention of PI3Kδ signaling in Th1 and iTreg initial activation conditions reduces their respective polarization. Given the large divergence in the effect of these distinct polarized CD4^{+} T helper subsets, it would be reasonable to hypothesize

that the PI3Kδ isoform is vital for the differentiation of all T helper subsets, and that its inhibition reduces these immune populations across the board. After all, the PI3Kδ isoform is uniquely expressed within immune cells, with exceptions in some neuronal tissue [14]. As such, before examining the Th2 and Th17 populations, the initial hypothesis was that inhibition of PI3Kδ signaling would lead to a reduction of Th2 and Th17 polarization and functionality, as seen with Th1 and iTregs.

Unexpectedly, the inhibition/loss of PI3Kδ seemed to enhance Th2 and Th17 polarization. While cytokines for the Th2 and Th17 conditions were reduced when PI3Kδ signaling was inhibited (Fig. 63), polarization towards Th2 and Th17 transcription factor conditions were enhanced relative to their respective controls (Fig. 7, 51).   How was it that inhibition of PI3Kδ, a kinase vital for cytokine production, increased the Th2 and Th17 subsets, while massively reducing their Th1 and iTreg counterparts? Do the other class IA PI3K isoforms play any role in this, or is PI3Kδ the 'master' PI3K isoform? Clearly, since the complete catalytic elimination of PI3Kδ still leads to Th2 and Th17 production, PI3Kδ is not the sole TCR-transducing kinase of importance in T helper cells. Inhibiting the PI3Kα isoform using A66 in the PI3Kδ knockout model *further* enhanced Th2 polarization (Fig. 52), consistent with a 2005 paper by Yamane et al. [151]. Using pigeon cytochrome *c* peptide (pPCC), they were able to show that there was a strong bias towards a Th1 response with greater levels of pPCC stimulation, whereas by sequentially decreasing the amount of pPCC there was an increasing Th2 response. In short, the level of TCR signal transduction induced by antigen determined T cell polarization outcome along a diametric, bipolar axis. This opposition of the Th1 and Th2 polarization subsets is seen at the transcription factor level, with the antagonism of

T-bet and GATA-3 [84]. We thereby hypothesize that, by inhibiting PI3K isoforms through drugs or genetic knockout, we are affecting this preferential Th1/Th2 response through altering the level of TCR signal transduction, and that this model is applicable to Th17/iTreg polarization as well. In essence, we are using PI3K isoform inhibition to manipulate the signaling of a specific mix of these three gateway kinases with which the lymphocyte must work to propagate signaling downstream. As Th2 has a clear preference for lesser TCR signaling and Th1 requires greater TCR transduction, it follows that by decreasing, but not completely eliminating, signaling through the immediate downstream TCR signaling PI3K isoforms, we favor the Th2 phenotype and disfavor the Th1.

We propose a model works as follows (Fig. 73). The Th1 polarization condition requires high levels of TCR signaling for it to become a fully mature, differentiated Th1 helper cell. The cascade of kinase signaling from the immune synapse must utilize PI3K isoforms to propagate signaling downstream. As indicated specifically by the loss of T-bet in cells from the p110delta-PI3K-D910A model, the Th1 phenotype is strongly dependent on PI3Kδ isoform signaling. When PI3Kδ is absent, activating signal transduction must take place through the only remaining class IA PI3K isoforms, either PI3Kα or β. As we demonstrated that PI3Kα inhibition was able to reduce IFN-γ signaling in the Th1 subset as well as further reduce T-bet in D910A Th2 polarized cells, we hypothesize that PI3Kα is also involved in CD4^{+} T helper cell TCR transduction, although to a lesser extent than PI3Kδ. This is followed by PI3Kβ, which seems to have either minimal or at present indeterminate effects. We base this on the fact that TGX-221

73
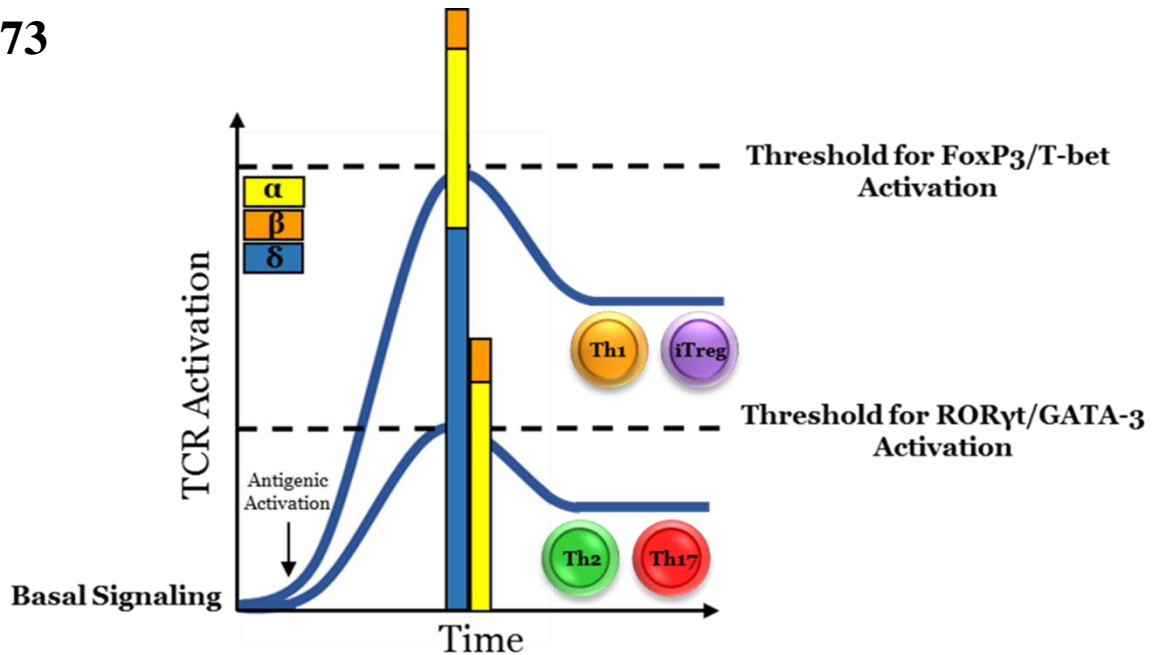
Figure 73: Threshold Model, Th1 and iTreg. We propose that the T-bet and FoxP3 transcription factors require a higher level of TCR signaling in order to be expressed. The PI3Kδ isoform is mostly, if not entirely, sufficient to transduce sufficient signal to overcome this high-level antigenic activation threshold. Upon elimination of PI3Kδ isoform in the p110delta-PI3K-D910A model, both T-bet and FoxP3 have reduced expression, although neither is totally eliminated. The PI3Kα isoform also is able to partially compensate for the loss of PI3Kδ signaling, explaining the decreased but still present T-bet and FoxP3 expression. Combined PI3Kδ and PI3Kα inhibition strongly reduces T-bet signaling, as PI3Kβ appears to have a minimal role in antigenic TCR activation propagation.

74
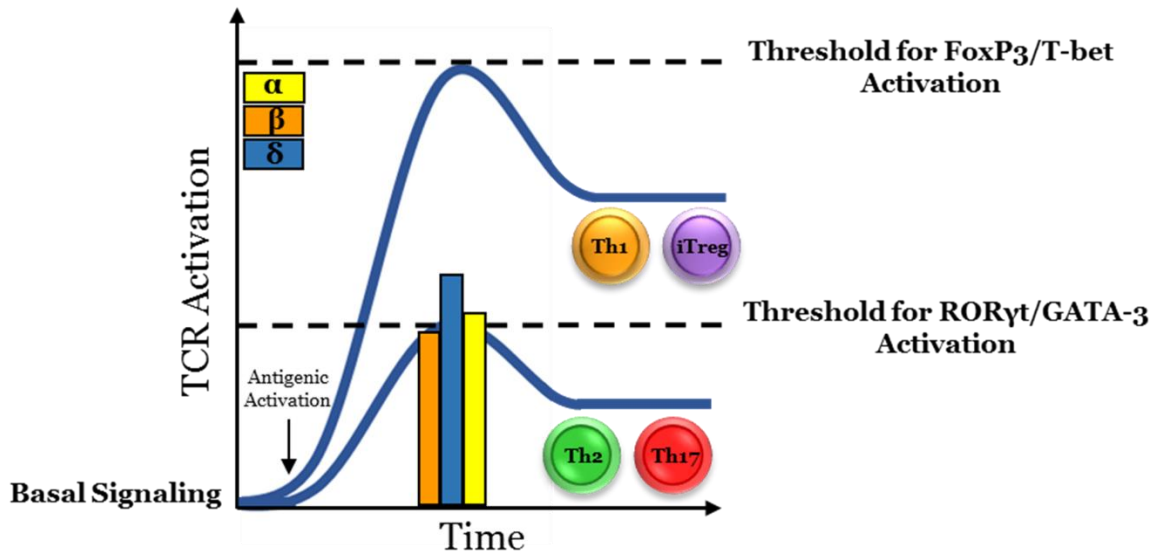
Figure 74: Threshold Model, Th2 and Th17. We propose that the RORγt and GATA-3 transcription factors require a lower level of TCR signaling in order to be expressed, relative to FoxP3 and T-bet. Both the PI3Kδ and PI3Kα isoforms are entirely sufficient to transduce signal to overcome this relatively low bar of antigenic activation, with some evidence that PI3Kβ may be able to as well. Upon elimination of the PI3Kδ isoform, T-bet and FoxP3 have reduced expression and there are increased RORγt$^+$FoxP3$^-$ and T-bet$^-$ GATA-3$^+$ populations. Inhibition of PI3Kα isoform in the D910A model lacking functional PI3Kδ via A66 further increased RORγt$^+$FoxP3$^-$ and T-bet$^-$GATA-3$^+$ populations, meaning either that the remaining PI3Kβ or some remaining signaling through PI3Kα was able to overcome the low antigenic threshold for Th2/Th17 polarization.

75
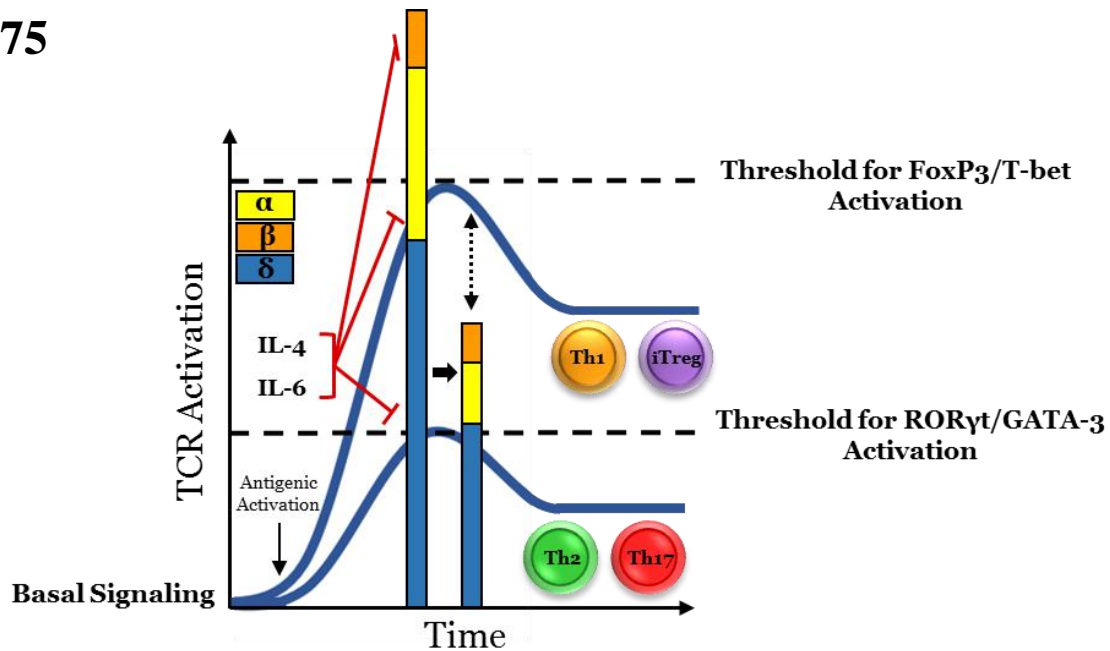
Figure 75: Stochastic Model, Th2 and Th17 Cytokines. Within the threshold model, we propose that specific polarizing cytokines crosstalk with the TCR/PI3K pathway to modify its signaling and thereby better match the level of antigenic activation preferred by the polarization subset. As such, since the antigenic threshold for the Th17 and Th2 transcription factors, RORγt and GATA-3, is low compared to their iTreg and Th1 counterparts, the IL-6 and IL-4 cytokines diminish signal transduction of all three class IA PI3K isoforms. This modification makes it less likely that the threshold for antigenic activation necessary for FoxP3 and T-bet expression will be reached, and therefore favors the expression of RORγt and GATA-3, leading to higher Th17 and Th2 polarization.

76
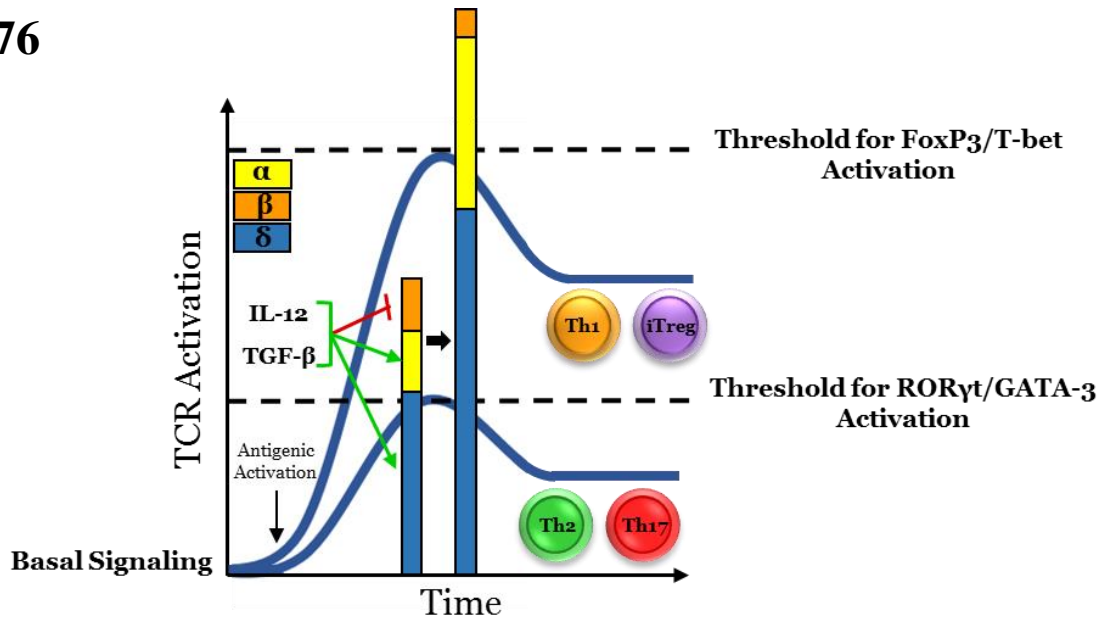
Figure 76: Stochastic Model, Th1 and iTreg Cytokines. We continue exploring this hypothesis of cytokines modifying the TCR/PI3K antigenic cascade with the Th1 and iTreg cytokines, IL-12 and TGF-β. As the antigenic threshold for the iTreg and Th1 transcription factors, FoxP3 and T-bet, are relatively high in our model, the TGF-β and IL-12 cytokines increase signal transduction through PI3Kα and δ isoforms. Interestingly, we see a reduction in PI3Kβ signaling, rather than the increase we expected. However, overall we have proposed that PI3Kβ has a minimal role in antigenic activation, as inhibition of PI3Kβ through TGX-221 had a minimal effect at concentrations specific to PI3Kβ alone. This modification of signaling by PI3Kα and δ makes it more likely that the threshold for antigenic activation necessary for FoxP3 and T-bet expression will be reached, leading to higher iTreg and Th17 polarization.

treatment was unable to increase Th2 character in Th2-polarized D910A cells or decrease IFN-γ in wild-type under Th1 conditions. Given that PI3Kβ has been implicated as a compensator for the class IB PI3K p110γ [46], it is possible that β is not as important for TCR transduction. This is comparable to B cells, as they require the elimination of both PI3Kα and PI3Kδ for complete prevention of B cell development, while PI3Kβ has no determine role [34]. As indicated by the figure, PI3Kδ signaling is mostly sufficient for Th1 polarization, as neither the inhibition of PI3Kα or PI3Kβ were able to strongly inhibit IFN-γ levels in Th1.

In contrast, Th2 has a lower threshold of TCR activation for appropriate differentiation. Cells polarized in the Th2 condition are more resistant to CAL-101- and GDC-0941-mediated reduction in proliferation than matched cells under Th1 conditions, strengthening this idea that Th2 differentiation is tied to lower TCR signaling transduction (Fig. 50). It is even possible that any of the three class IA PI3K isoforms is sufficient to allow Th2 polarization. When naïve CD4$^+$ cells from D910A mice were polarized in Th2 conditions in the presence of A66, the GATA-3^{hi}T-bet^{lo} population was further increased compared to the D910A cells without treatment (Fig. 51-53). This means that either the remaining uninhibited PI3Kα or PI3Kβ isoforms provided sufficient signaling to reach the lower 'threshold' for Th2 polarization (Fig. 74). We know that some TCR signal transduction must occur, as T cell activation is only possible with some level of signaling through the immune synapse [67]. These changes in combined PI3K isoform signaling are the crux of our model. All things being equal, high antigenic activation through the TCR activates PI3K isoforms, of which PI3Kδ is the strongest propagator of signaling, followed by PI3Kα, with minimal or no enzymatic activity

required from PI3Kβ. This allows attainment of a high Th1 'threshold,' which is required for Th1 polarization and T-bet expression. On the other hand, low levels of antigenic activation do not to reach this threshold of activation energy for the Th1 and, we predict, iTreg phenotypes. Instead, there is a lower threshold for GATA-3 and RORγt expression, preferentially biasing towards the Th2 and Th17 responses in less strongly activated naïve CD4$^+$ T cells. Our manipulation of PI3K isoforms by pharmacological inhibition or genetic knockout in effect creates a lower TCR activation response by reducing the amount of signal that is transduced downstream, thereby skewing towards the lower activation requirements of the Th2 and Th17 polarization subsets.

Physiologically, this model makes sense. The Th1 subset is important in antiviral and antibacterial responses [67]. Viral and bacterial infections can be fatal without strong, killing immune responses, as aided through the Th1 helper subset. However, pathogen clearance through the Th1 response can also be damaging to nearby tissue, as seen with autoimmune responses mediated by Th1 [152]. Cell-mediated immunity, while necessary for clearance or control of dangerous pathogens such as tuberculosis [153], causes recruitment of innate, phagocytic immune cells, such as macrophages, which produce toxic reactive oxygen species. Therefore, Th1 polarization is a dangerous option. It is critical for infectious response, but damaging to surrounding tissue and, if uncontrolled, can lead to autoimmunity. As such, the immune system requires high 'certainty' before polarization towards a Th1 helper. Should antigen requirements for a Th1 response be too low, low-level tonic TCR interactions or bystander activation from other immune cells could cause unwarranted strong responses against innocuous antigens, such as with Crohn's disease in gut epithelia [88]. The Th2 response, already

diametrically opposed to the Th1 response by the antagonism of their respective transcription factors [84], would make sense to be less regulated in its requirements for this 'certainty'.

The Th2 response is a non-phagocytic, humoral response, generally thought to clear parasitic infections. The Th2 response has also been suggested to be a 'healing' immune response against the destruction mediated by parasites [154]. Unwarranted Th2 responses, rather than causing potentially fatal autoimmunity through CMI due to Th1 polarization, is more strongly tied to allergies through the activation of basophils and eosinophils. While allergic responses can reach deadly levels, the Th2 end of the Th1/Th2 polarization axis seems more conservative and 'safer' than a Th1 response when naïve T cells are presented with ambiguous activation. This system can be seen in action with helminth infections [67]. Helminths, while damaging to the body, are less dangerous than a Th1 response to the helminth infection. While a Th2 response facilitates parasite evacuation by driving worms to the gut lumen for easier removal through feces elimination, the Th1 response decreases turnover of epithelial cells and can create chronic, debilitating disease. Given the dangers of an unwarranted Th1 response, it makes sense that there is a higher threshold of activation must be reached through the immune synapse that before the naïve $CD4^+$ cell is 'certain' and polarizes. As Th2 exists in a bipolar axis with the Th1 response, low level but non-basal TCR activation would be biased towards the low risk, humoral and healing response, so as to avoid dangerous responses to innocuous antigens or pathogens such as parasites, where the Th1 response is ineffectual and/or harmful.

This same reasoning can be applied to the effect observed between the Th17 and iTreg responses, replacing our Th1/Th2 axis with iTreg/Th17, respectively. However, this idea does directly contradict another model, in which it has been proposed that weak TCR interactions bias towards an iTreg fate while the opposite drives towards Th17 [155]. However, as with Th1 and Th2, reduction of TCR signal transduction by elimination of the highly important signaling kinase PI3Kδ results in skewing towards a Th17 response. This is in direct contrast with what would be expected with the weak TCR transduction model in iTregs versus high in Th17, where chemically or genetically reducing downstream TCR signaling should lead to greater numbers of iTregs.

While the TCR signaling strength model is a vital component of our hypothesis, we do not propose that naïve $CD4^+$ T helper cell polarization is solely or even mainly dependent on the strength of signaling through the immune synapse. Any discussion of T cell polarization cannot neglect the role of directing cytokines. As discussed previously, these directing cytokines are vital for activation of the JAK/STAT cascades which ultimately lead to the expression of the polarized subsets' respective transcription factors. We do not contest the critical importance of these cytokines in directing naïve cells to their mature T helper cell types. What we instead propose is that these cytokines, in addition to their JAK/STAT signaling, also crosstalk with the TCR/PI3K pathway in order to 'skew' towards the preferred activation potentials of their specific polarized subtype. Thus, IL-12 and TGF-β enhance PI3K isoform signaling and IL-4 and IL-6 inhibit it, as in our model we propose that Th1/iTregs require higher signaling thresholds than their Th2/Th17 counterparts.

B. Polarizing Cytokines Modify PI3K Isoform Signaling to Match the PI3K Signaling Potential of Their Respective CD4^{+} T Helper Subsets

According to our proposed model, the level of signaling through the immune synapse can bias towards specific CD4^{+} T helper polarization responses. As cytokines have been strongly tied to the polarization fate of naïve T cells [20, 21], we must reconcile this to our proposed mechanism. One explanation is a conservative model, where TCR signaling and cytokine-driven JAK/STAT signaling are entirely divorced from each other. In this model, cytokine signaling determines the possible polarization fate of the naïve T cell, setting a threshold for that specific polarization outcome. If the cytokine milieu favors Th1 or iTreg subtypes, the antigenic threshold for maturation of that naïve T cell is relatively high and strong signaling must take place for polarization. Conversely, should the microenvironment favor Th2 and Th17 signaling, the level of antigenic stimulation required is relatively low. This model depends almost entirely upon the cytokines themselves, and does not adequately explain how in matched environments, given differential antigen stimulation, distinct T cell polarization outcomes are observed [151]. As such, we propose a stochastic model.

In this model, signaling from polarizing cytokines interacts with the TCR cascade. In our previous paper [22], we determined that TGF-β increased PI3Kδ signaling compared to a control, while the addition of IL-6 to that mixture strongly inhibited PI3Kδ signaling. In our threshold model, we indicate that strong levels of TCR signal transduction favor the iTreg phenotype, while weak signaling favors Th17. The specific polarizing cytokines for these two conditions, TGF-β and IL6, modify PI3Kδ signaling to match what we hypothesize to be the preferential activation energies of their respective

polarization conditions. In this thesis, we expanded this modality to Th1/Th2 cytokines across all PI3K isoforms, and have shown the same overarching mechanism: IL-12, the Th1 polarizing cytokine, overwhelmingly enhances PI3Kα and PI3Kδ signaling, while IL-4, the Th2 polarizing cytokine, diminishes all signaling through class IA PI3K isoforms (Fig. 56-59). This exactly aligns with our TCR signaling strength model, where Th1 is favored by enhanced TCR transduction while diminished signaling favors the Th2 subset. Physiologically, the milieu present at an immunological hotspot can contain a variety of different polarizing cytokines. This could provide a potentially ambiguous and ineffective activation outcome. While strength of antigen binding plays a role in enhancing one response or another, ultimately the predominant cytokines present are involved as well. Rather than solely signaling through their JAK/STAT pathways, these cytokines crosstalk with PI3K isoforms to enhance their respective conditions (Fig. 75, 76). For example, should CD4$^+$ T cells be strongly but inadvertently activated towards a Th1 response, high IL-4 receptor signaling would diminish PI3K isoform signaling transduction, correcting polarization to favor a Th2 response. Conversely, should weak antigen activation of T cells be present where the innate immune response is strongly activated and thus the cells are producing high levels of IL-12 through DCs and macrophages [67], this IL-12 would enhance TCR signal transduction to the PI3Kα and δ isoforms to bias towards a Th1 response rather than an ambiguous mixed or unhelpful Th2 response.

The importance of this gradient, non-deterministic system can be modeled with generically activated CD4$^+$ T helper cells. These Th0 cells are activated solely through anti-CD3, anti-CD28, and IL-2, the signals for activation and proliferation, without any

specific polarizing cytokines. These cells seem like an intermediate CD4$^+$ T cell subtype, displaying transcription factors and cytokines from both the Th1 and Th2 subsets: They express high levels of both GATA-3 and T-bet (Fig. 20-21), and express moderate to low levels of both IFN-γ and IL-4. Despite having these Th1 and Th2 characteristics, they are functionally neither, due to the antagonistic interaction of their regulating transcription factors. Just as the addition of polarizing cytokines would drive to one outcome or another, modifying the level of TCR signal transduction, such as with our pharmacological inhibitors, also influences the outcome response. Generalized activation leads to the production of sufficient cytokines to allow a Th1 or Th2 response—what truly determines fate is the combination of antigenicity of TCR activation with the predominant skewing cytokine(s), with the cytokines modifying the level of TCR signaling transduction and antigenicity providing a 'baseline.' This provides the basis for a complex, stochastic system where CD4$^+$ T cell polarization is guided by multiple factors, with the central crux of control tied to the intensity of TCR signaling transduction, which is in turn modified by cytokine receptor signals.

V.   SUMMARY

Th17 Polarization is Enhanced at the Expense of iTreg Induction

when PI3Kδ Signaling is Ablated

We have demonstrated previously for iTregs that when PI3Kδ is inhibited pharmacologically or within the D910A transgenic model [22], the Th17 condition is enhanced significantly (Fig. 1-7).

Th1 Functionality is Dependent on PI3Kδ and α Signaling, with Minimal Importance of the PI3Kβ Isoform

While IFN-γ has previously been tied to PI3Kδ signaling [23], the roles of the other class IA PI3K isoforms were not fully explored due to the lack of a viable complete knockout animal. Using specific PI3K isoform inhibitors, we show that while PI3Kδ has the strongest connection to IFN-γ expression, PI3Kα inhibition also leads to a significant decrease in IFN-γ expression, while PI3Kβ shows a minimal effect (Fig. 8-19).

Th2 Polarization is Enhanced when PI3Kδ Signaling is Ablated at the Expense of Th1 Polarization

Using both chemical inhibitors and the p110delta-PI3K-D910A model, we demonstrate that T-bet expression is strongly tied to PI3Kδ isoform signaling, with effects also seen to a lesser extent with PI3Kα inhibition by A66 and no changes observed with PI3Kβ inhibition by TGX-221. While there are changes in GATA-3

148

expression with both pharmacological inhibition and the D910A model, these changes ultimately lead to an enhanced Th2 character and GATA-3^{+}T-bet[-] population (Fig. 20-53).

The IL-12 and IL-4 Polarizing Cytokines Modify PI3K Isoform Signaling to Match Their Respective T Helper Subset's Preferred Level of TCR Signal Transduction

We examined the effects of specific Th1 and Th2 cytokines, IL-12 and IL-4, upon individual PI3K isoforms in single variable, controlled immunoprecipitation experiments. From this we discovered that IL-12 upregulates the phospho-tyrosine levels (i.e. activation) of the p110 PI3K isoforms α and δ, with a decrease in β. Conversely, IL-4 decreases the phospho-tyrosine levels of all class IA PI3K isoforms (Fig. 55-59).

Ablation of PI3Kδ Signaling Decreases Cytokine Production by Preventing the Activation of STAT5

While reduction of PI3Kδ signaling enhances the polarization of the Th2 and Th17 subsets, it leads to a sharp decrease in the production of many polarization subset-specific cytokines. We determined that activation of STAT5, a vital mediator of the expression of Th1 and Th2 cytokines, is inhibited within p110delta-PI3K-D910A mice compared to the WT controls (Fig. 60-67). Furthermore, the expression of the IL-5 cytokine appears independent of this PI3Kδ-STAT5 pathway, and is actually uniquely increased in the knockout condition (Fig. 63).

Enhanced GATA-3^{+}T-bet[-] Cells Polarized in the Th2 Condition with CAL-101 are Functionally Th2

Since cytokine production was strongly inhibited with PI3Kδ signaling reduction, due presumably to inhibited STAT5 activaiton, after polarization we removed CAL-101 and restimulated cells to determine if the CAL-101 treatment-skewed Th2 population expressed Th2 functionality. After 4 to 6 hours of PMA/ionomycin stimulation, the CAL-101 skewed Th2 population expressed predominantly Th2 cytokines, marked expression of IL-5 and IL-13. There was lesser, but still present, IL-4 expression than in the Th2 untreated control and a massive increase of IL-2 expression in the treated Th2 set (Fig. 68-71).

Proposed Model of Th1/Th2 Signaling Through TCR Signaling Strength and Modification of Said Signaling Through Cytokine Receptor Crosstalk

Lastly, we propose a stochastic mechanism where intensity of signaling through the immunological synapse and downstream to the PI3K isoforms is modified by crosstalk from the activated polarizing cytokine receptors. This interaction guides the final polarization fate of the activated T helper cell, with antigenicity setting a baseline and cytokines competing to modify PI3K isoform signaling toward a lower (Th2/Th17) or higher (Th1/iTreg) activation potential (Fig. 54, 72-76).

VI.   REFERENCES

1. Vanhaesebroeck, B., et al., *Synthesis and function of 3-phosphorylated inositol lipids.* Annu Rev Biochem, 2001. **70**: p. 535-602.
2. Franke, T.F., et al., *Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate.* Science, 1997. **275**(5300): p. 665-8.
3. Okkenhaug, K., *Signaling by the phosphoinositide 3-kinase family in immune cells.* Annu Rev Immunol, 2013. **31**: p. 675-704.
4. Ward, S.G., et al., *Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation.* Eur J Immunol, 1993. **23**(10): p. 2572-7.
5. Manning, B.D. and L.C. Cantley, *AKT/PKB signaling: navigating downstream.* Cell, 2007. **129**(7): p. 1261-74.
6. Vanhaesebroeck, B. and D.R. Alessi, *The PI3K-PDK1 connection: more than just a road to PKB.* Biochem J, 2000. **346 Pt 3**: p. 561-76.
7. Fruman, D.A. and L.C. Cantley, *Phosphoinositide 3-kinase in immunological systems.* Semin Immunol, 2002. **14**(1): p. 7-18.
8. Kane, L.P., et al., *Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines.* Nat Immunol, 2001. **2**(1): p. 37-44.
9. Rathmell, J.C., et al., *IL-7 enhances the survival and maintains the size of naive T cells.* J Immunol, 2001. **167**(12): p. 6869-76.
10. Frauwirth, K.A., et al., *The CD28 signaling pathway regulates glucose metabolism.* Immunity, 2002. **16**(6): p. 769-77.
11. Burgering, B.M. and G.J. Kops, *Cell cycle and death control: long live Forkheads.* Trends Biochem Sci, 2002. **27**(7): p. 352-60.
12. Brennan, P., et al., *Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F.* Immunity, 1997. **7**(5): p. 679-89.
13. Alberts B, J.A., Lewis J, et al., *Molecular Biology of the Cell. 4th edition.* Helper T Cells and Lymphocyte Activation, ed. N.Y.G. Science. 2002.
14. Lucas, C.L., et al., *PI3Kdelta and primary immunodeficiencies.* Nat Rev Immunol, 2016. **16**(11): p. 702-714.
15. Gopal, A.K., et al., *PI3Kdelta inhibition by idelalisib in patients with relapsed indolent lymphoma.* N Engl J Med, 2014. **370**(11): p. 1008-18.
16. Furman, R.R., et al., *Idelalisib and rituximab in relapsed chronic lymphocytic leukemia.* N Engl J Med, 2014. **370**(11): p. 997-1007.
17. Ahmad, S., et al., *Differential PI3Kdelta signaling in CD4+ T cell subsets enables selective targeting of T regulatory cells to enhance cancer immunotherapy.* Cancer Res, 2017.
18. Abu-Eid, R., et al., *Selective inhibition of regulatory T cells by targeting the PI3K-Akt pathway.* Cancer Immunol Res, 2014. **2**(11): p. 1080-9.

19.   Kimura, A. and T. Kishimoto, *IL-6: regulator of Treg/Th17 balance.* Eur J Immunol, 2010. **40**(7): p. 1830-5.

20.   Van der Meide, P.H. and H. Schellekens, *Cytokines and the immune response.* Biotherapy, 1996. **8**(3-4): p. 243-9.

21.   Swain, S.L., K.K. McKinstry, and T.M. Strutt, *Expanding roles for CD4(+) T cells in immunity to viruses.* Nat Rev Immunol, 2012. **12**(2): p. 136-48.

22.   Ahmad, S., et al., *IL-6 modulates TGF-b-Smad signaling for balancing Th17 and regulatory T-cells through inhibition of PI3Kδ.* Manuscript in press.

23.   Okkenhaug, K., et al., *The p110delta isoform of phosphoinositide 3-kinase controls clonal expansion and differentiation of Th cells.* J Immunol, 2006. **177**(8): p. 5122-8.

24.   Whitman, M., et al., *Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation.* Nature, 1985. **315**(6016): p. 239-42.

25.   Alliouachene, S., et al., *Inactivation of the Class II PI3K-C2beta Potentiates Insulin Signaling and Sensitivity.* Cell Rep, 2015. **13**(9): p. 1881-94.

26.   Vanhaesebroeck, B., M.A. Whitehead, and R. Pineiro, *Molecules in medicine mini-review: isoforms of PI3K in biology and disease.* J Mol Med (Berl), 2016. **94**(1): p. 5-11.

27.   Corvera, S. and M.P. Czech, *Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction.* Trends Cell Biol, 1998. **8**(11): p. 442-6.

28.   Leevers, S.J., B. Vanhaesebroeck, and M.D. Waterfield, *Signalling through phosphoinositide 3-kinases: the lipids take centre stage.* Curr Opin Cell Biol, 1999. **11**(2): p. 219-25.

29.   Andrews, S., L.R. Stephens, and P.T. Hawkins, *PI3K class IB pathway.* Sci STKE, 2007. **2007**(407): p. cm2.

30.   Carpenter, C.L., et al., *Purification and characterization of phosphoinositide 3-kinase from rat liver.* J Biol Chem, 1990. **265**(32): p. 19704-11.

31.   Shepherd, P.R., et al., *Differential regulation of phosphoinositide 3-kinase adapter subunit variants by insulin in human skeletal muscle.* J Biol Chem, 1997. **272**(30): p. 19000-7.

32.   Yu, J., et al., *Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit.* Mol Cell Biol, 1998. **18**(3): p. 1379-87.

33.   Luo, J., et al., *The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex.* J Cell Biol, 2005. **170**(3): p. 455-64.

34.   Ramadani, F., et al., *The PI3K isoforms p110alpha and p110delta are essential for pre-B cell receptor signaling and B cell development.* Sci Signal, 2010. **3**(134): p. ra60.

35.   Chantry, D., et al., *p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes.* J Biol Chem, 1997. **272**(31): p. 19236-41.

36.   Vanhaesebroeck, B., et al., *P110delta, a novel phosphoinositide 3-kinase in leukocytes.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4330-5.

37.   Eickholt, B.J., et al., *Control of axonal growth and regeneration of sensory neurons by the p110delta PI 3-kinase.* PLoS One, 2007. **2**(9): p. e869.

38.   Foukas, L.C., et al., *Critical role for the p110alpha phosphoinositide-3-OH kinase in growth and metabolic regulation.* Nature, 2006. **441**(7091): p. 366-70.

39.   Samuels, Y., et al., *High frequency of mutations of the PIK3CA gene in human cancers.* Science, 2004. **304**(5670): p. 554.

40.   Karakas, B., K.E. Bachman, and B.H. Park, *Mutation of the PIK3CA oncogene in human cancers.* Br J Cancer, 2006. **94**(4): p. 455-9.

41. Lee, J.W., et al., *PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas.* Oncogene, 2005. **24**(8): p. 1477-80.
42. Clayton, E., et al., *A crucial role for the p110delta subunit of phosphatidylinositol 3-kinase in B cell development and activation.* J Exp Med, 2002. **196**(6): p. 753-63.
43. Okkenhaug, K., et al., *Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice.* Science, 2002. **297**(5583): p. 1031-4.
44. Ciraolo, E., et al., *Phosphoinositide 3-kinase p110beta activity: key role in metabolism and mammary gland cancer but not development.* Sci Signal, 2008. **1**(36): p. ra3.
45. Jia, S., et al., *Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis.* Nature, 2008. **454**(7205): p. 776-9.
46. Guillermet-Guibert, J., et al., *The p110beta isoform of phosphoinositide 3-kinase signals downstream of G protein-coupled receptors and is functionally redundant with p110gamma.* Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8292-7.
47. Kulkarni, S., et al., *PI3Kbeta plays a critical role in neutrophil activation by immune complexes.* Sci Signal, 2011. **4**(168): p. ra23.
48. Uhlen, M., et al., *Proteomics. Tissue-based map of the human proteome.* Science, 2015. **347**(6220): p. 1260419.
49. Zhang, K.J., Husami, A., Marsh, R. & Jordan, M. B., *Identification of a phosphoinositide 3‐kinase (PI‐3K) p110δ (PIK3CD) deficient individual.* Journal of Clinical Immunology, 2013. **33**(3): p. 673–674.
50. Crank, M.C., et al., *Mutations in PIK3CD can cause hyper IgM syndrome (HIGM) associated with increased cancer susceptibility.* J Clin Immunol, 2014. **34**(3): p. 272-6.
51. Angulo, I., et al., *Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage.* Science, 2013. **342**(6160): p. 866-71.
52. Deau, M.C., et al., *A human immunodeficiency caused by mutations in the PIK3R1 gene.* J Clin Invest, 2014. **124**(9): p. 3923-8.
53. Ali, K., et al., *Inactivation of PI(3)K p110delta breaks regulatory T-cell-mediated immune tolerance to cancer.* Nature, 2014. **510**(7505): p. 407-11.
54. Soond, D.R., et al., *PI3K p110delta regulates T-cell cytokine production during primary and secondary immune responses in mice and humans.* Blood, 2010. **115**(11): p. 2203-13.
55. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
56. Killar, L., et al., *Cloned, Ia-restricted T cells that do not produce interleukin 4(IL 4)/B cell stimulatory factor 1(BSF-1) fail to help antigen-specific B cells.* J Immunol, 1987. **138**(6): p. 1674-9.
57. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
58. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
59. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-32.
60. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells.* Immunity, 2006. **24**(2): p. 179-89.
61. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

62.   Fu, S., et al., *TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors.* Am J Transplant, 2004. **4**(10): p. 1614-27.

63.   Fantini, M.C., et al., *Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7.* J Immunol, 2004. **172**(9): p. 5149-53.

64.   DiPaolo, R.J., et al., *Autoantigen-specific TGFbeta-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells.* J Immunol, 2007. **179**(7): p. 4685-93.

65.   Zheng, S.G., et al., *Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10.* J Immunol, 2004. **172**(9): p. 5213-21.

66.   Romagnani, S., *T-cell subsets (Th1 versus Th2).* Ann Allergy Asthma Immunol, 2000. **85**(1): p. 9-18; quiz 18, 21.

67.   Parham, P., and Charles Janeway., *The Immune System.* 2015, New York, NY: Garland Science, Taylor & Francis Group.

68.   Lazarevic, V., L.H. Glimcher, and G.M. Lord, *T-bet: a bridge between innate and adaptive immunity.* Nat Rev Immunol, 2013. **13**(11): p. 777-89.

69.   Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.

70.   Lazarevic, V., et al., *T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat.* Nat Immunol, 2011. **12**(1): p. 96-104.

71.   Hermann-Kleiter, N. and G. Baier, *NFAT pulls the strings during CD4+ T helper cell effector functions.* Blood, 2010. **115**(15): p. 2989-97.

72.   Marsters, S.A., et al., *Interferon gamma signals via a high-affinity multisubunit receptor complex that contains two types of polypeptide chain.* Proc Natl Acad Sci U S A, 1995. **92**(12): p. 5401-5.

73.   Avni, O., et al., *T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes.* Nat Immunol, 2002. **3**(7): p. 643-51.

74.   Vignali, D.A. and V.K. Kuchroo, *IL-12 family cytokines: immunological playmakers.* Nat Immunol, 2012. **13**(8): p. 722-8.

75.   Presky, D.H., et al., *A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits.* Proc Natl Acad Sci U S A, 1996. **93**(24): p. 14002-7.

76.   Wurster, A.L., T. Tanaka, and M.J. Grusby, *The biology of Stat4 and Stat6.* Oncogene, 2000. **19**(21): p. 2577-84.

77.   Sinigaglia, F., et al., *Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function.* Immunol Rev, 1999. **170**: p. 65-72.

78.   Bacon, C.M., et al., *Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes.* Proc Natl Acad Sci U S A, 1995. **92**(16): p. 7307-11.

79.   Cho, S.S., et al., *Activation of STAT4 by IL-12 and IFN-alpha: evidence for the involvement of ligand-induced tyrosine and serine phosphorylation.* J Immunol, 1996. **157**(11): p. 4781-9.

80.   Jacobson, N.G., et al., *Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4.* J Exp Med, 1995. **181**(5): p. 1755-62.

81.   Johnson, H., et al., *IFN Signaling: How a Non-Canonical Model Led to the Development of IFN Mimetics.* Frontiers in Immunology, 2013. **4**(202).

82. Chen, X., et al., *Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA.* Cell, 1998. **93**(5): p. 827-39.

83. Platanias, L.C., *Mechanisms of type-I- and type-II-interferon-mediated signalling.* Nat Rev Immunol, 2005. **5**(5): p. 375-86.

84. Kanhere, A., et al., *T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements.* Nat Commun, 2012. **3**: p. 1268.

85. Bettelli, E., et al., *Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis.* J Exp Med, 2004. **200**(1): p. 79-87.

86. Finotto, S., et al., *Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet.* Science, 2002. **295**(5553): p. 336-8.

87. Matsui, M., et al., *T-bet is required for protection against vaccinia virus infection.* J Virol, 2005. **79**(20): p. 12798-806.

88. Neurath, M.F., et al., *The transcription factor T-bet regulates mucosal T cell activation in experimental colitis and Crohn's disease.* J Exp Med, 2002. **195**(9): p. 1129-43.

89. Lee, H.J., et al., *GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells.* J Exp Med, 2000. **192**(1): p. 105-15.

90. Zhang, D.H., et al., *Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene.* J Biol Chem, 1997. **272**(34): p. 21597-603.

91. Hadjur, S., et al., *Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus.* Nature, 2009. **460**(7253): p. 410-3.

92. Sekimata, M., et al., *CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-gamma locus.* Immunity, 2009. **31**(4): p. 551-64.

93. Jenner, R.G., et al., *The transcription factors T-bet and GATA-3 control alternative pathways of T-cell differentiation through a shared set of target genes.* Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17876-81.

94. Lee, G.R., P.E. Fields, and R.A. Flavell, *Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level.* Immunity, 2001. **14**(4): p. 447-59.

95. Kishikawa, H., et al., *The cell type-specific expression of the murine IL-13 gene is regulated by GATA-3.* J Immunol, 2001. **167**(8): p. 4414-20.

96. Spilianakis, C.G. and R.A. Flavell, *Long-range intrachromosomal interactions in the T helper type 2 cytokine locus.* Nat Immunol, 2004. **5**(10): p. 1017-27.

97. Tykocinski, L.O., et al., *A critical control element for interleukin-4 memory expression in T helper lymphocytes.* J Biol Chem, 2005. **280**(31): p. 28177-85.

98. Yamashita, M., et al., *Identification of a conserved GATA3 response element upstream proximal from the interleukin-13 gene locus.* J Biol Chem, 2002. **277**(44): p. 42399-408.

99. Zhang, D.H., L. Yang, and A. Ray, *Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3.* J Immunol, 1998. **161**(8): p. 3817-21.

100. DuPage, M. and J.A. Bluestone, *Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease.* Nat Rev Immunol, 2016. **16**(3): p. 149-63.

101. Ansel, K.M., et al., *Deletion of a conserved Il4 silencer impairs T helper type 1-mediated immunity.* Nat Immunol, 2004. **5**(12): p. 1251-9.

102. Djuretic, I.M., et al., *Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells.* Nat Immunol, 2007. **8**(2): p. 145-53.

103. Naoe, Y., et al., *Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer.* J Exp Med, 2007. **204**(8): p. 1749-55.

104. Chang, S. and T.M. Aune, *Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells.* Nat Immunol, 2007. **8**(7): p. 723-31.

105. Hwang, E.S., et al., *T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3.* Science, 2005. **307**(5708): p. 430-3.

106. Gandhi, N.A., et al., *Targeting key proximal drivers of type 2 inflammation in disease.* Nat Rev Drug Discov, 2016. **15**(1): p. 35-50.

107. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.

108. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.

109. Nischwitz, S., et al., *Interferon beta-1a reduces increased interleukin-16 levels in multiple sclerosis patients.* Acta Neurol Scand, 2014. **130**(1): p. 46-52.

110. Wei, L., et al., *IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner.* J Biol Chem, 2007. **282**(48): p. 34605-10.

111. Pelletier, M., et al., *Evidence for a cross-talk between human neutrophils and Th17 cells.* Blood, 2010. **115**(2): p. 335-43.

112. Zhang, F., G. Meng, and W. Strober, *Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells.* Nat Immunol, 2008. **9**(11): p. 1297-306.

113. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.

114. Hunter, C.A. and S.A. Jones, *IL-6 as a keystone cytokine in health and disease.* Nat Immunol, 2015. **16**(5): p. 448-57.

115. Dienz, O., et al., *Essential role of IL-6 in protection against H1N1 influenza virus by promoting neutrophil survival in the lung.* Mucosal Immunol, 2012. **5**(3): p. 258-66.

116. Garbers, C., et al., *Inhibition of classic signaling is a novel function of soluble glycoprotein 130 (sgp130), which is controlled by the ratio of interleukin 6 and soluble interleukin 6 receptor.* J Biol Chem, 2011. **286**(50): p. 42959-70.

117. Hoge, J., et al., *IL-6 controls the innate immune response against Listeria monocytogenes via classical IL-6 signaling.* J Immunol, 2013. **190**(2): p. 703-11.

118. Kopf, M., et al., *Impaired immune and acute-phase responses in interleukin-6-deficient mice.* Nature, 1994. **368**(6469): p. 339-42.

119. Longhi, M.P., et al., *Interleukin-6 is crucial for recall of influenza-specific memory CD4 T cells.* PLoS Pathog, 2008. **4**(2): p. e1000006.

120. Neveu, W.A., et al., *IL-6 is required for airway mucus production induced by inhaled fungal allergens.* J Immunol, 2009. **183**(3): p. 1732-8.

121. Smith, K.A. and R.M. Maizels, *IL-6 controls susceptibility to helminth infection by impeding Th2 responsiveness and altering the Treg phenotype in vivo.* Eur J Immunol, 2014. **44**(1): p. 150-61.

122. van der Poll, T., et al., *Interleukin-6 gene-deficient mice show impaired defense against pneumococcal pneumonia.* J Infect Dis, 1997. **176**(2): p. 439-44.

123. Hibi, M., et al., *Molecular cloning and expression of an IL-6 signal transducer, gp130.* Cell, 1990. **63**(6): p. 1149-57.

124. Taga, T., et al., *Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130.* Cell, 1989. **58**(3): p. 573-81.

125. Taga, T., et al., *Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression.* J Exp Med, 1987. **166**(4): p. 967-81.

126. Yamasaki, K., et al., *Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor.* Science, 1988. **241**(4867): p. 825-8.

127. Peters, M., et al., *The function of the soluble interleukin 6 (IL-6) receptor in vivo: sensitization of human soluble IL-6 receptor transgenic mice towards IL-6 and prolongation of the plasma half-life of IL-6.* J Exp Med, 1996. **183**(4): p. 1399-406.

128. Korn, T., et al., *IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells.* Proc Natl Acad Sci U S A, 2008. **105**(47): p. 18460-5.

129. Pasare, C. and R. Medzhitov, *Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells.* Science, 2003. **299**(5609): p. 1033-6.

130. Huehn, J., J.K. Polansky, and A. Hamann, *Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage?* Nat Rev Immunol, 2009. **9**(2): p. 83-9.

131. Zhang, H., et al., *Subsets of regulatory T cells and their roles in allergy.* J Transl Med, 2014. **12**: p. 125.

132. Qin, H., et al., *TGF-beta promotes Th17 cell development through inhibition of SOCS3.* J Immunol, 2009. **183**(1): p. 97-105.

133. Mahmud, S.A., L.S. Manlove, and M.A. Farrar, *Interleukin-2 and STAT5 in regulatory T cell development and function.* JAKSTAT, 2013. **2**(1): p. e23154.

134. Uddin, S., et al., *Role of Stat5 in type I interferon-signaling and transcriptional regulation.* Biochem Biophys Res Commun, 2003. **308**(2): p. 325-30.

135. Tanabe, Y., et al., *Cutting edge: role of STAT1, STAT3, and STAT5 in IFN-alpha beta responses in T lymphocytes.* J Immunol, 2005. **174**(2): p. 609-13.

136. Li, P., et al., *Complex interactions of transcription factors in mediating cytokine biology in T cells.* Immunol Rev, 2014. **261**(1): p. 141-56.

137. Liao, W., et al., *Priming for T helper type 2 differentiation by interleukin 2-mediated induction of interleukin 4 receptor alpha-chain expression.* Nat Immunol, 2008. **9**(11): p. 1288-96.

138. Burchill, M.A., et al., *IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells.* J Immunol, 2007. **178**(1): p. 280-90.

139. Liao, W., et al., *Opposing actions of IL-2 and IL-21 on Th9 differentiation correlate with their differential regulation of BCL6 expression.* Proc Natl Acad Sci U S A, 2014. **111**(9): p. 3508-13.

140. Ikeda, H., et al., *PI3K/p110{delta} is a novel therapeutic target in multiple myeloma.* Blood, 2010. **116**(9): p. 1460-8.

141. Lannutti, B.J., et al., *CAL-101, a p110delta selective phosphatidylinositol-3-kinase inhibitor for the treatment of B-cell malignancies, inhibits PI3K signaling and cellular viability.* Blood, 2011. **117**(2): p. 591-4.

142. Administration, U.S.F.a.D. *Idelalisib.* 2015 09/15/2015 3/9/2017]; Available from: https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm406410.htm.

143. Administration, U.S.F.a.D. *FDA Alerts Healthcare Professionals About Clinical Trials with Zydelig (idelalisib) in Combination with other Cancer Medicines.* 2016 3/9/2017]; Available from: https://www.fda.gov/Drugs/DrugSafety/ucm490618.htm.

144. Flaherty, S. and J.M. Reynolds, *Mouse Naive CD4+ T Cell Isolation and In vitro Differentiation into T Cell Subsets.* J Vis Exp, 2015(98).

145. Antony, P.A., et al., *CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells.* J Immunol, 2005. **174**(5): p. 2591-601.

146. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.

147. Fu, J., et al., *Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients.* Gastroenterology, 2007. **132**(7): p. 2328-39.
148. Heimberger, A.B., et al., *Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas.* Clin Cancer Res, 2008. **14**(16): p. 5166-72.
149. Zhou, S., et al., *Depletion of CD4+ CD25+ regulatory T cells promotes CCL21-mediated antitumor immunity.* PLoS One, 2013. **8**(9): p. e73952.
150. Sakaguchi, S., *Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.* Annu Rev Immunol, 2004. **22**: p. 531-62.
151. Yamane, H., J. Zhu, and W.E. Paul, *Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment.* J Exp Med, 2005. **202**(6): p. 793-804.
152. Dardalhon, V., et al., *Role of Th1 and Th17 cells in organ-specific autoimmunity.* J Autoimmun, 2008. **31**(3): p. 252-6.
153. Wangoo, A., et al., *Contribution of Th1 and Th2 cells to protection and pathology in experimental models of granulomatous lung disease.* J Immunol, 2001. **166**(5): p. 3432-9.
154. Allen, J.E. and T.A. Wynn, *Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens.* PLoS Pathog, 2011. **7**(5): p. e1002003.
155. Yamane, H. and W.E. Paul, *Cytokines of the gamma(c) family control CD4+ T cell Differentiation and function.* Nat Immunol, 2012. **13**(11): p. 1037-44.

APPENDIX A: ADDITIONAL FIGURES

S1
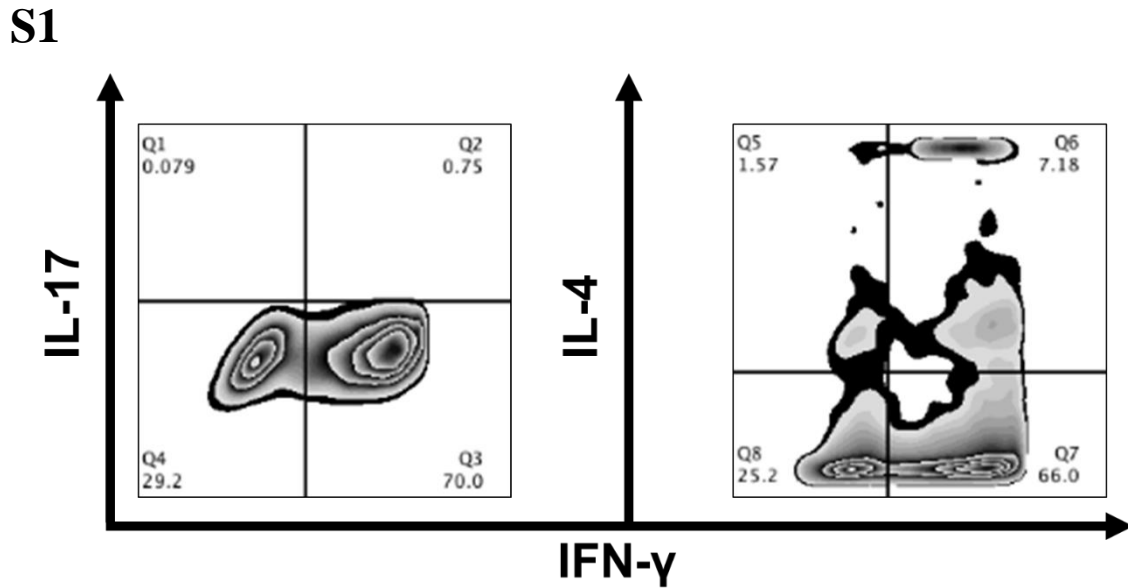
Supplemental Figure 1: Intracellular IL-17 and IL-4 Expression in Th1 Polarized WT naïve CD4$^+$ T cells. Spleens were harvested from female, age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th1 group was activated for 72 hours with anti-CD3 coating and anti-CD28, IL-2, IL-12, and anti-IL-4. After 72 hours, cells were treated for between 4 to 6 hours with PMA/ionomycin plus Golgiplug^{TM}, then harvested,

washed, fixed, and stained for IFN-γ, IL-4, and IL-17. Representative set, specific sample conditions performed in triplicate, n=3 mice per experiment, repeated with matched conditions yielding similar results.

S2
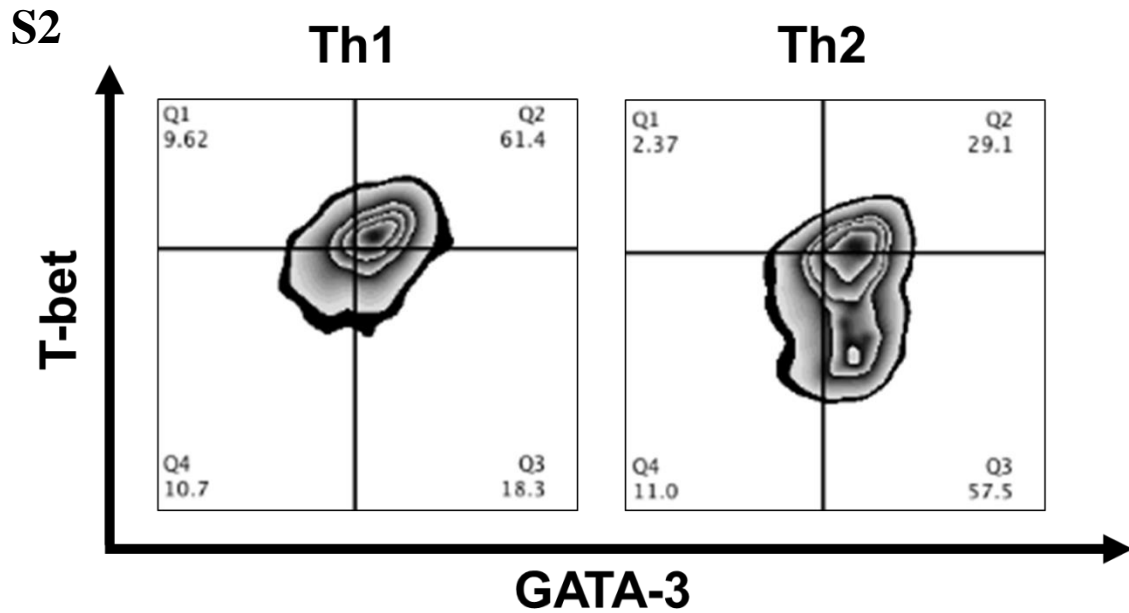
Supplemental Figure 2: T-bet and GATA-3 Expression in Th1 and Th2 Polarized

Cells of p110delta-PI3K-D910A Mouse Splenocytes. Spleens were harvested from

female, age-matched p110delta-PI3K-D910A mice between the ages of 8 and 10 weeks.

Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then

separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th1 group

was activated for 72 hours with anti-CD3 and anti-CD28, IL-2, IL-12, and anti-IL-4 and

the Th2 with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IFN-γ, and anti-IL-12. After 72

hours, cells were harvested, washed, fixed, and stained for T-bet and GATA-3

Representative set, specific sample conditions performed in triplicate, n=5 mice per

experiment, repeated with matched conditions yielding similar results.
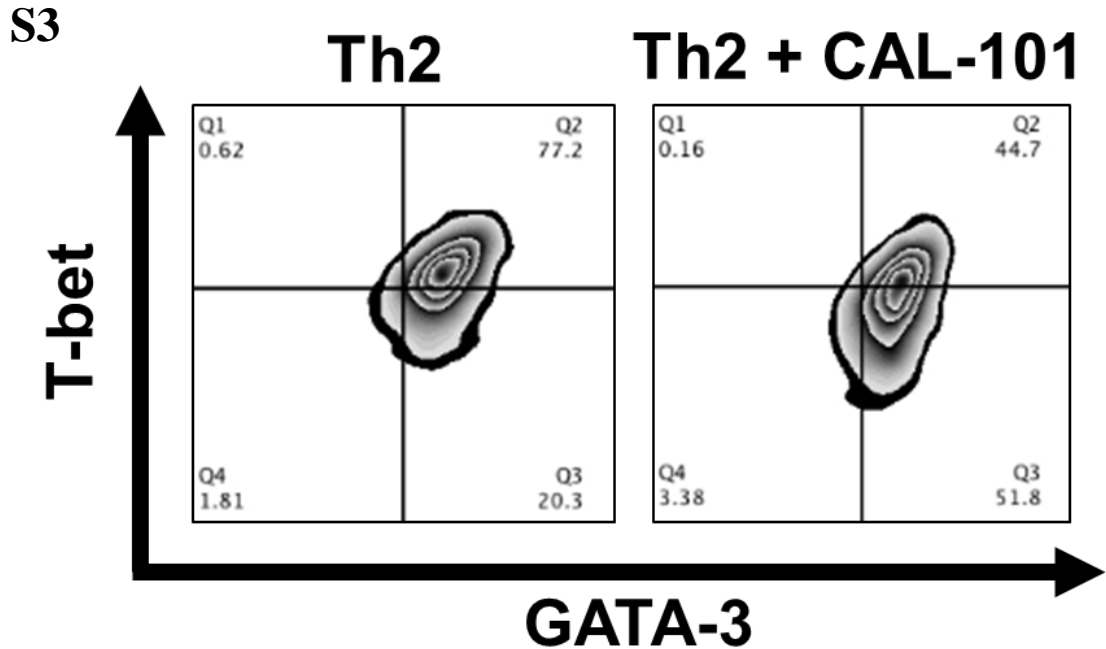
Supplemental Figure 3: T-bet and GATA-3 Expression of Polarized Th2 and Th2 CAL-101-treated Cells After a 48 Hour 'Rest' with IL-2. Spleens were harvested from female, age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the $CD3^+$ fraction through negative selection, then separated by FACS analysis for the $CD4^+CD25^-$ naïve T cell population. The Th2 groups were stimulated with anti-CD3 and anti-CD28, IL-2, IL-4, anti-IFN-γ, and anti-IL-12, with or without the addition of 100 nM of CAL-101 (δ). After 72 hours, cells were washed and re-plated in IL-2 alone-containing T cell media for 48 hours, then harvested, washed, fixed, and stained for T-bet and GATA-3 Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results.

S4

pg/mL

	IFN-γ	IL5	TNF-α	IL2	IL6	IL4	IL10	IL9	IL13
Basal Th0	413.04	5.34	13.84	2	1.3	<0.58	<8.30	<8.79	51.86
Basal Th0	338.82	4.94	12.78	2	<0.61	<0.58	<8.30	<8.79	47.7
Basal Th0	431.1	5.6	13.32	2.28	1.3	<0.58	<8.30	<8.79	51.12
Basal Th2	139.54	29.52	10.66	2.2	3.62	1.18	<8.30	137.84	229.68
Basal Th2	154.86	28.04	10.48	2.58	3.42	1.6	<8.30	129.76	225.54
Basal Th2	138.16	27.28	9.08	2.2	3.04	<0.58	<8.30	140.34	214.56
Basal Th2 + CAL	47.82	6	11.88	6.76	1.78	1.76	<8.30	51.36	54.88
Basal Th2 + CAL	45.5	4.8	10.48	6.64	1.78	1.46	<8.30	47.06	53.2
Basal Th2 + CAL	28.96	3.28	8.72	5.16	2.1	<0.58	<8.30	39.3	47.7
Restim Th0	1770.4	181.68	1483.68	5780	5.78	388.2	371.12	<8.79	679.22
Restim Th0	2098	177.8	1867.98	6712	7.66	415.82	503.96	21.64	790
Restim Th0	2878	144.8	2208	8138	4.04	546.56	337.92	<8.79	583.72
Restim Th2	228.32	546.6	1062	1014.06	23.5	729.62	933.24	268.66	1291.72
Restim Th2	209.86	608.28	1003.98	887.7	18.58	618.62	806	224.42	1337.2
Restim Th2	272.34	772.94	1905.38	2662	19.4	1202.2	720.06	303.8	2430
Restim Th2 + CAL	101.26	105.78	969.9	5746	3.42	71.92	133.02	118.38	555.18
Restim Th2 + CAL	131.24	111.72	1120.64	6296	4.04	83.48	128.42	117.72	596.8
Restim Th2 + CAL	127.1	109.08	979.34	6760	3.84	68.48	136.44	101.02	591.08

Supplemental Figure 4: Cytokine Production of Polarized Th2 and Th2 CAL-101-treated Cells After a 48 Hour 'Rest' with IL-2. Spleens were harvested from female, age-matched wild-type C57BL/6 mice between the ages of 8 and 10 weeks. Splenocytes were enriched for the CD3$^+$ fraction through negative selection, then separated by FACS analysis for the CD4$^+$CD25$^-$ naïve T cell population. The Th0 group was activated for 72 hours with anti-CD3 and both anti-CD28 and IL-2 in suspension. The Th1 and Th2 groups were activated using the same method, with the addition of IL-12 and anti-IL-4 for the Th1 condition and IL-4, anti-IL-12, and anti-IFN-γ for the Th2 condition. After 72 hours, cells were washed and re-plated in IL-2 alone-containing T cell media for 48 hours, then basal supernatants were collected. Cells were restimulated for 4 to 6 hours with PMA and ionomycin, then supernatants were collected. Both supernatants were analyzed by CBA. Representative set, specific sample conditions performed in triplicate, n=5 mice per experiment, repeated with matched conditions yielding similar results. Units in pg/mL.