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PI3K Signaling in B and T Lymphocytes: New Developments and Therapeutic Advances

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Synopsis

Activation of phosphoinositide 3-kinase (PI3K) is a shared response to engagement of diverse types of transmembrane receptors. Depending on the cell type and stimulus, PI3K activation can promote different fates including proliferation, survival, migration and differentiation. The diverse roles of PI3K signaling are well illustrated by studies of lymphocytes, the cells that mediate adaptive immunity. Genetic and pharmacological experiments have shown that PI3K activation regulates many steps in the development, activation and differentiation of both B and T cells. These findings have prompted the development of PI3K inhibitors for the treatment of autoimmunity and inflammatory diseases. However, PI3K activation has both positive and negative roles in immune system activation. Consequently, while PI3K suppression can attenuate immune responses it can also enhance inflammation, disrupt peripheral tolerance and promote autoimmunity. An exciting discovery is that a selective inhibitor of the p110δ catalytic isoform of PI3K, CAL-101, achieves impressive clinical efficacy in certain B cell malignancies. A model is emerging in which p110δ inhibition disrupts signals from the lymphoid microenvironment, leading to release of leukemia and lymphoma cells from their protective niche. These encouraging findings have given further momentum to PI3K drug development efforts in both cancer and immune diseases.

Introduction

The adaptive immune system is crucial for protection from recurring infections by various pathogens. T and B lymphocytes are the key cellular mediators of this system in which the enormous diversity and exquisite specificity of the antigen receptors enables T and B cells to recognize virtually any foreign molecule. Antigen recognition alone, however, is insufficient to initiate effective immune responses. A key feature of the adaptive immune system is that lymphocytes depend on signals from neighboring cells to perform specific functions in the context of various immune conditions. For example, B cells cannot properly complete their primary task of making antibodies without signals from CD4 T cells. Similarly, CD4 T cells can differentiate into a variety of effector subsets depending on costimulatory signals and the cytokine milieu generated from other immune cells of the microenvironment. To prevent autoimmunity, lymphocyte self-tolerance must be enforced and certain conditions should favor activation of regulatory cells that suppress rather than promote immune responses. Integration of diverse extracellular cues to achieve the required cellular response involves a complex network of intracellular signaling events. This concept also applies to tumors of

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lymphoid origin, whose survival typically depends on both cell-intrinsic and cell-extrinsic signals from the microenvironment.

The phosphoinositide 3-kinase (PI3K) signaling network plays a fundamental role in signal transduction in mammalian cells [1, 2]. Hence, it is not surprising that PI3K is activated by diverse stimuli in lymphocytes and is required for the maintenance of proper adaptive immunity and self-tolerance [3, 4]. In this review we summarize recent advances in the understanding of PI3K signaling in B and T cells. These advances have been spurred largely by two technological breakthroughs: refinements in gene-targeted mouse models and discovery of PI3K isoform-selective inhibitors. We emphasize two key concepts. First, PI3K activation should not be viewed as a simple on/off switch but rather as a "rheostat" whose output must be properly balanced for effective cellular responses. Both effector and regulatory cell populations engage PI3K signaling pathways, as do cells of the innate immune system. Therefore, decreased PI3K signaling can lead to immunosuppression or, in some contexts, to increased inflammation and improved pathogen clearance, and even to autoimmunity. The second concept is that despite this complexity, the p110δ catalytic isoform of class I PI3K has emerged as a central driver of lymphocyte clonal selection, differentiation and trafficking. We conclude by describing progress in the development of PI3K inhibitors for therapeutic uses. Indeed, development of highly specific PI3K inhibitors for clinical use is one of the major goals in the pharmaceutical industry today. Of particular interest is the surprising success of a selective p110δ inhibitor CAL-101 in human B cell malignancies, where CAL-101 seems to act mainly by perturbing the signals received from the tumor microenvironment.

Overview of PI3K

PI3Ks are a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphatidylinositol (PtdIns) and phosphoinositides (phosphorylated derivatives of PtdIns) [2]. Unlike yeast, whose genome encodes only one PI3K isoform (class III: Vps34) whose main role is in vesicle trafficking, the mammalian PI3Ks include eight enzymes with diverse roles in both vesicle trafficking and signal transduction. These enzymes are grouped into the categories known as class I, class II, and class III, based on substrate preference and structure. Only the class I PI3Ks have the ability to use PtdIns-4,5-bisphosphate (PtdIns-4,5-P_2) as a substrate to generate the important second messenger PtdIns-3,4,5-trisphosphate (PIP_3). Certain proteins containing a pleckstrin homology (PH) domain can specifically bind PIP_3 and be recruited to membranes where PI3K is active [5]. Hence, class I PI3K acts as a signaling hub at the plasma membrane to change the lipid composition in a way that links transmembrane receptors to the organization of multiprotein complexes, also known as signalosomes [6]. The composition of these signalosomes and the specific PH domain-containing PI3K effector proteins recruited to these assemblies varies according to the receptor that is engaged. In most cells, the serine/threonine kinase AKT (also known as protein kinase B, PKB) is a key PI3K effector and AKT phosphorylation is a common readout of PI3K activation [7]. Two amino acid residues in AKT are phosphorylated in a PI3K-dependent manner: T308 by phosphoinositide-dependent kinase-1 (PDK-1), and S473 by TORC2 (a complex of the target of rapamycin (TOR), rictor and other proteins).

PI3K and AKT coordinate many aspects of the response to antigen receptor engagement. In B and T cells, the formation of signalosomes that drive antigen receptor-dependent Ca^{2+} mobilization is partially dependent on PI3K [6, 8]. PIP_3 produced upon BCR or TCR clustering binds to PH domains in TEC family kinases to promote activation of phospholipase C-γ and subsequent hydrolysis of PtdIns-4,5-P_2. This results in release of soluble IP_3 to initiate Ca^{2+} mobilization, and accumulation of diacylglycerol in the membrane to activate protein kinase C isoforms and GTP exchange factors for RAS. A role

for PI3K upstream of RAS in lymphocytes is supported by the observation of decreased ERK phosphorylation in PI3K-deficient T and B cells stimulated through antigen receptors [9]. Activated AKT has many substrates with key roles in lymphocyte activation and trafficking. The Forkhead Box Subgroup O (FOXO) transcription factors are one key group of AKT substrates in lymphocytes [3]. AKT-mediated phosphorylation inhibits DNA binding by FOXO factors and promotes their nuclear exit and cytoplasmic sequestration and degradation. AKT also can phosphorylate Tuberous Sclerosis 2 (TSC2) and Proline-Rich AKT substrate of 40kDa (PRAS40) to increase the activity of TORC1 (TOR complex-1), a multifunctional signaling protein that coordinates cell growth and metabolism [10]. However, the dependence of TORC1 activation on PI3K and AKT varies in lymphocytes according to the cell subset and stimulus [11, 12].

Elevation of cellular PIP_3 is transient and is controlled by lipid phosphatases [13, 14]. Phosphatase and Tensin homolog (PTEN) is a phosphoinositide 3-phosphatase that converts PIP_3 back to PtdIns-4,5-P_2. In cells lacking PTEN, basal PIP_3 amounts are increased and receptor stimulation causes exaggerated PI3K signaling. SH2 domain-containing inositol phosphatases (SHIP1 and SHIP2) are 5-phosphatases that convert PIP_3 to PtdIns-3,4-bisphosphate (PtdIns-3,4-P_2). The latter lipid species can still recruit and activate PDK-1 and AKT but has other signaling functions that do not overlap with PIP_3. For example, the PH domains of TEC family kinases have high affinity for PIP_3 but not PtdIns-3,4-P_2, whereas the converse is true for the adaptor proteins Bam32, TAPP1 and TAPP2 [15].

Class I PI3Ks are further divided into two subgroups: class IA and class IB [2]. Class IA PI3Ks contain one of three distinct 110 kDa catalytic isoforms (p110α, p110β, or p110δ) that forms a heterodimer with one of the five regulatory (adaptor) isoforms (p85α, p55α, p50α, p85β, or p55γ). The overall structure of p110 subunits is very similar with the following domains: adaptor-binding (ABD), Ras-binding, C2, helical and lipid kinase (Figure 1). The class IA regulatory isoforms share a similar C-terminal half that contains two Src-homology-2 (SH2) domains that flank a coiled-coil domain (also called iSH2) that binds tightly to the p110 ABD to form the heterodimer. The N-terminal portions of p85α and p85β contain an additional Src-homology-3 (SH3) domain and a RhoGAP-homology region flanked by proline-rich motifs. Class IB PI3K is composed of a single catalytic isoform (p110γ) bound to one of two regulatory isoforms (p101 or p84). In general, class IA PI3Ks are activated by tyrosine kinase (TK)-based signals whereas class IB PI3Ks are activated by G protein-coupled receptors (GPCRs). However, these distinctions have been challenged by numerous studies linking p110β and p110δ to GPCRs and p110γ to TKs [2, 16]. Whereas p110α and p110β are expressed ubiquitously, p110δ and p110γ are predominantly expressed in the cells of the immune system compared to other cell types. This suggests that p110δ and p110γ may have evolved to serve specific roles in immunity, a hypothesis supported by many published studies and emphasized herein. The possibility that p110α and p110β isoforms might also be important in lymphocytes will also be discussed.

The mechanism of class IA PI3K activation has been clarified through structural studies and has been reviewed in detail elsewhere [17, 18]. Briefly, the regulatory subunit has different functions in the absence or presence of stimulation. In the basal state, the regulatory subunit stabilizes the catalytic subunit while suppressing its activity. This suppression is mediated by several inter-subunit contacts including helical(p110)/N-SH2(p85) and C2(p110)/iSH2(p85) (Figure 1). The C-SH2 domain of p85β can also form an inhibitory contact with the kinase domain of p110β. In response to tyrosine kinase-based signals, the SH2 domains become bound to phosphotyrosine (pTyr) residues on receptors and adaptor proteins (consensus motif pTyr-X-X-Met), releasing the inhibitory contacts and bringing the catalytic subunit to the membrane where its substrates reside. Additional interactions involving the N-terminal domains of p85 proteins might also contribute to PI3K recruitment and activation in

some contexts. Naturally occurring mutations in p85α and p85β have been identified in tumors of humans and mice [19–23]; in general these are gain-of-function mutations that disrupt the inhibitory interfaces and elevate PI3K enzyme activity while maintaining the stability of the catalytic subunit.

How do receptors on lymphocytes engage class I PI3K? Antigen receptors, costimulatory molecules, cytokine receptors, and chemokine receptors can all trigger an increase in PIP_3 and phosphorylation of AKT. Crosslinking of the B cell receptor (BCR) leads to tyrosine phosphorylation of the co-receptor CD19 and B-cell adaptor protein (BCAP), and each of these proteins recruits class IA PI3K through SH2 domain interactions [4, 24, 25] (Figure 2). T cell receptor (TCR) engagement also activates class IA PI3K but the molecular details remain to be fully established. Possible mechanisms include: binding of p85 proline-rich domains to SH3 domains of Src family kinases [26, 27]; interaction of p85 Rho-GAP domains with Rac-GTP downstream of the Vav exchange factors [28–31]; and association of SH2 domains in p85 or p50 with pTyr residues in proteins associated with TCR signalosomes [32, 33]. Two co-stimulatory molecules on T cells, CD28 and ICOS, recruit PI3K through pTyr-X-X-Met motifs [34]. In the case of CD28, recruitment of PI3K appears dispensable for most of the initial costimulatory signals but is essential for CD28 function in effector T cells [4]. The role of PI3K in ICOS signaling will be discussed below. Other costimulatory molecules and cytokine receptors on B and T cells recruit and activate class IA PI3K through diverse mechanisms [35, 36]. The class IB p110γ isoform is mainly activated by GPCR signals (i.e. chemokine receptors) in cells of the innate immune system such as neutrophils [37]. The βγ subunits of heterotrimeric G proteins directly bind to p110γ and mediate enzyme activation through p101 or p84 adaptor subunits (Figure 1). However, the situation is more complicated in lymphocytes in that chemokines activate p110γ in T cells but p110δ in B cells [38, 39]. The mechanism linking GPCRs to p110δ in B cells is not known. Furthermore, there is some evidence that TCR signaling activates p110γ, possibly via G-alpha proteins or the small GTPase RAS [40, 41]. All of the class I catalytic subunits including p110γ contain RAS-binding domains (Figure 1), and the interaction of p110γ with RAS is required for T cell development at the β-selection stage [41]. An interesting and largely unexplored question is whether different receptors generate distinct pools of PIP_3 during lymphocyte activation, with selective localization and downstream effectors. This concept was validated in a study of p110γ activation in mast cells, where p101-bound and p84-bound p110γ produced distinct pools of PIP_3 with separable function [42].

Tools to study PI3K signaling in lymphocytes

Following the molecular cloning of class I PI3K isoforms in the early 1990s, PI3K signaling in lymphocytes was studied mainly using established cell lines derived from lymphoid tumors. This approach was convenient for biochemical studies because cell lines have more cytoplasm and protein content than primary T and B cells. Cell line studies also avoided the problem that primary lymphocytes die rapidly in culture and cannot easily be metabolically labeled or transfected. However, tumor cells have severe drawbacks for signal transduction research as they usually have dysregulated PI3K signaling, and cell proliferation is typically uncoupled from physiological extracellular controls. Furthermore, the PI3K inhibitors used in early experiments (wortmannin and LY294002) are non-selective compounds that inhibit all PI3K isoforms as well as mTOR and other lipid and protein kinases in cells [43–45]. A more precise understanding of PI3K signaling required better tools.

The first major technical advance was the creation of genetically modified mouse strains lacking individual class I PI3K isoforms. Germline and/or tissue-specific knockouts of the genes encoding each class I catalytic subunit, and of the genes encoding p85α/p55α/p50α

and p85β, have been generated and characterized (Supplementary Table 1). Insights gained from these mouse strains have been summarized in many reviews (for example, [4, 35, 46]) and new findings are discussed herein. However, PI3K gene knockouts have significant limitations even when deletion is tissue-specific. One problem is that loss of one isoform of PI3K often leads to altered expression of non-targeted isoforms [47]. For example, deletion of the *Pik3r1* gene encoding p85α/p55α/p50α causes reduced expression of p110 proteins and increased expression of p85β. Deletion of individual p110 isoforms results in functional compensation of other catalytic isoforms. Hence, an important step forward was the generation of knock-in mice that express intact proteins with point mutations causing loss-of-function. This strategy was first used by Okkenhaug, Vanhaesebroeck and colleagues to generate $p110\delta^{D910A/D910A}$ mice with a point mutation inactivating p110δ kinase function [9], and has been used subsequently to generate kinase-inactive (KI) alleles of each of the p110 isoforms (Supplementary Table 1). This strategy prevents compensatory changes in expression or function and provides a more precise model of chemical kinase inhibition. However, the knock-in strategy is still limited to some degree by the fact that the mutation can affect lymphocyte development, resulting in an altered pool of mature T or B cells. In addition, p110αKI mice display embryonic lethality at a stage before lymphoid precursors can be isolated. Therefore, data from knockout and knockin mice are most conclusive when combined with studies of wild-type cells treated with selective PI3K inhibitors.

The discovery and validation of selective, ATP-competitive PI3K inhibitors has been driven by efforts from both industry and academia. Although the kinase domains of class I PI3K isoforms are highly conserved, X-ray crystal structures have shown that the ATP-binding pockets have distinct topologies and flexibilities that can allow selective binding of distinct chemical structures [18]. These properties have allowed the development of compounds with good selectivity for single class I PI3K isoforms. There are also several compounds targeting all class I isoforms with minimal off-target effects on other kinases (termed “pan-class I PI3K inhibitors”). Examples of isoform-selective and pan-class I PI3K inhibitors are shown in Table 1. Many of these can be purchased from commercial vendors, and most have optimized pharmacological properties to allow dosing of animals *in vivo*. Despite these powerful additions to the PI3K inhibitor toolkit, many investigators continue to test PI3K function using wortmannin or LY294002. The importance of choosing the right PI3K inhibitor at the optimal concentration (or *in vivo* dose) cannot be overstated.

In addition to PI3K gene knockouts and knock-ins, other genetically engineered mouse models have been produced to study PI3K signaling in lymphocytes. These include knockouts and knock-in mutations in PI3K effectors such as PDK-1 and AKT, and knockouts of the genes encoding PIP_3 phosphatases PTEN and SHIP1. These models have proven very useful for dissecting PI3K signaling pathways in lymphocytes, and some recent advances are described in this review. Mouse strains with mutations affecting TOR signaling, FOXO transcription factors and other PI3K-regulated signaling components have also yielded important advances that we will discuss. Another common approach has been to generate transgenic mice expressing membrane-targeted forms of AKT in lymphocytes. The rationale for this strategy is that it will reveal the functions of AKT, independent of other PI3K effectors. These mice exhibit dramatic phenotypes with altered lymphocyte development, lymphoproliferation and loss of immune homeostasis [48–51]. However, the phenotypes of AKT gain-of-function mutations should be interpreted with caution. In normal immune cells, AKT is recruited transiently to cell membranes and once activated it travels to different subcellular compartments to phosphorylate diverse substrates. The activation state of AKT is also controlled by a large array of phosphatases and other regulatory proteins. Therefore, constitutive anchoring in the membrane does not faithfully recapitulate the functions of active AKT. Nevertheless, T cell-specific deletion of PTEN or expression of a constitutively active PI3K also causes lymphoproliferation and

autoimmunity in mice [52, 53]. Together these approaches concur that artificially elevating PI3K signaling output in T cells causes a loss of immune homeostasis.

Fluorescent bioprobes have provided valuable insights into the kinetics and subcellular localization of PI3K lipid production in lymphocytes, and will continue to be useful tools [54]. However, these probes do not provide a comprehensive and quantitative view of different PI3K lipid species. Further advances in this direction are likely to emerge from the application of novel mass spectrometry technologies. The group of Stephens and Hawkins has reported a method for sensitive quantitation of PIP_3 without radioactive or heavy isotope labeling [55]. The method also allows the discrimination of PIP_3 species based on distinct fatty acyl chain composition. Combining this technique with genetic or pharmacological inhibition of specific PI3K isoforms might uncover important aspects of PI3K signaling specificity in lymphocytes.

B lymphocytes

B cell development and tolerance

B cells develop in the bone marrow from lymphoid progenitors that undergo an ordered series of gene rearrangement steps punctuated by stringent checkpoints. The immunoglobulin (Ig) heavy chain rearrangement occurs at the pro-B cell stage and when successful, the heavy chain pairs with surrogate light chains and Igα-Igβ heterodimers to form the pre-BCR at the cell surface. Assembly of the pre-BCR triggers tonic (basal) signaling that is critical for repression of RAG (recombination-activating gene) expression to prevent further heavy chain rearrangement. At this stage cells proliferate and are termed large pre-B (or pre-B-I) cells. Ig light chain rearrangement proceeds at the small pre-B (pre-B-II) cell stage and this leads to surface expression of surface IgM containing μ heavy chains and either κ or λ light chains. Immature B cells expressing intact surface IgM are further screened for self-reactivity in the bone marrow and spleen before entering peripheral B cell pools.

The first evidence suggesting a role for PI3K in B cell development came from the original studies of p85α and p110δ knockout, and p110δKI models [9, 56–59]. The numbers of pre-B cells are reduced in mice lacking p85α or p110δ indicating a clear role for class IA PI3K in the pro-B/pre-B transition, though residual development suggested that other isoforms might also function in early B cell development. To address this possibility, Okkenhaug and colleagues analyzed mice with lymphocyte-specific (CD2-Cre) deletion of p110α or p110β [60]. No significant B cell deficits were observed. Crossing each conditional knockout strains with the p110δKI strain revealed that combined inactivation of p110α and p110δ results in a near complete absence of pre-B-II cells, whereas deletion of p110β does not alter the p110δKI phenotype. Using a fluorescence-based microscopy technique, it was demonstrated that sorted pro-B/pre-B-I cells lacking p110α/p110δ have elevated *Rag* gene expression. This defect in *Rag* repression correlates with enhanced frequency of rearranged heavy chain alleles in sorted pro/pre-B cells. Thus, both p110α and p110δ both seem to be important for allelic exclusion and developmental progression at the pre-BCR checkpoint whereas p110β is not involved. In addition, p110α and p110δ were found to have overlapping, required functions in IL-7-driven proliferation of pro-B cells *in vitro*. The class IB isoform p110γ is expressed in the B lineage but is not essential for B cell development or activation. However, combined inactivation of p110γ and p110δ does impair B cell development and reduce peripheral B cell numbers to a greater extent than p110δ inactivation alone [61]. Overlapping functions of p110γ and p110δ are also apparent in thymocyte development, as discussed below.

FOXO factors bind to the promoters of *Rag-1* and *Rag-2* genes and promote transcription [62]. Deletion of *Foxo1* in the B lineage using *Mb1*-Cre reduces expression of *Rag* genes, causing impaired heavy and light chain rearrangement and a reduction in pre-B cell numbers [63]. Together with the results of the p110α/δ deletion study this suggests a model in which tonic pre-BCR signaling activates PI3K and AKT, suppressing FOXO factors to extinguish *Rag* expression. In accord, Su and colleagues reported that deletion of Sin1, an essential component of TORC2, prevents phosphorylation of AKT on S473 and enhances Foxo1-dependent *Rag* expression in developing B cells [64]. This study also identified a selective role for the AKT2 isoform in Foxo1 phosphorylation and suppression of *Rag* expression in cultured pro-B cells. However, a required role for AKT2 in this process *in vivo* is challenged by a study of fetal liver chimeric mice reconstituted with AKT1/AKT2 double knockout cells [65]. In this system, combined deletion of both *AKT1* and *AKT2* actually resulted to an increase in bone marrow pre-B and immature B cells. One possibility is that the AKT3 isoform can compensate for loss of AKT1 and AKT2 in early B cell development *in vivo*; mRNA measurements suggest that AKT1 and AKT3 are expressed at higher levels than AKT2 at the pro-B and pre-B cell stages [65].

Kurosaki and colleagues reported that development of B cells from CD19/BCAP double knockout mice is blocked at the pre-BCR checkpoint stage [24]. Both CD19 and BCAP have multiple tyrosine residues that, when phosphorylated, represent a consensus motif for binding to p85 adaptor subunits. Reconstitution of wild-type but not tyrosine-mutated CD19 or BCAP into double knockout cells restores PI3K/AKT signaling and B cell development. These findings suggest that ligand-independent pre-BCR signaling triggers tyrosine phosphorylation of CD19 and BCAP, leading to recruitment of class IA heterodimers containing p85α and either p110α or p110δ. In order for light chain rearrangement to commence, PI3K/AKT signaling needs to be extinguished to allow reactivation of FOXO-dependent *Rag* expression. Another component of the pre-BCR signaling complex, the adaptor protein SLP-65 (also known as BLNK), seems to be required for attenuation of PI3K/AKT signaling [66].

There are several tolerance mechanisms that prevent the emergence of self-reactive B cells and the production of autoantibodies. In the bone marrow, immature B cells expressing surface IgM recognizing multivalent self-antigen undergo receptor editing, and eventually clonal deletion if self-reactivity persists. In the absence of self-antigen recognition, tonic signaling by the mature surface IgM on immature B cells activates PI3K to extinguish *Rag* expression, similar to the pre-BCR checkpoint [67]. Immature B cells recognizing monovalent self-antigens in the bone marrow or periphery enter a state known as anergy, in which they fail to respond even to strong stimulation. Anergic B cells have a short lifespan and are rapidly removed from the repertoire. Together, negative selection by clonal deletion and anergy induction produce a self-tolerant peripheral B cell repertoire. Rickert and colleagues have shown that both negative selection and anergy are regulated at the level of PIP_3 production [68]. Using a transgenic mouse model, it was observed that anergic B cells have significantly lower production of PIP_3 and activation of AKT upon BCR stimulation. Reduced PI3K activation correlates with diminished phosphorylation of CD19, and elevated expression of PTEN. Co-deletion of PTEN reverses the PI3K/AKT activation defects and prevents the induction of anergy. In a negative selection model using immature B cells derived from non-transgenic mice, BCR crosslinking causes a block in proliferation and differentiation that can be overcome by PTEN deletion [68]. An unanswered question is whether a specific PI3K isoform is involved in generating the PIP_3 pools responsible for preventing tolerance in PTEN-deficient B cells. Whether PI3K inhibition can induce tolerance in autoreactive B cells also remains to be determined, but is suggested by experiments described in the next section.

Peripheral B cells

At an early stage in B cell development, a lineage split occurs resulting in two subsets of peripheral B cells: the innate-like B-1 B cells that reside in body cavities, and B-2 B cells in blood and secondary lymphoid organs. The B-2 B cells are further divided into marginal zone (MZ) B cells that reside in the MZ of the spleen and do not circulate; and follicular (FO) B cells that recirculate through blood, lymph, and lymphoid tissues. In mouse strains lacking p85α or p110δ, or in chimeric mice with AKT1/AKT2-deficient B cells, B-1 and MZ B cells are nearly absent [9, 56, 58, 65, 69, 70]. This phenotype is similar to mice lacking CD19, a component of BCR signalosomes [71]. This suggests that a major of function of CD19 is to activate PI3K and AKT to allow commitment to the B-1 or MZ lineages at key stages of B cell development. The CD19/PI3K/AKT signal appears to act through inactivation of Foxo1, since *Foxo1* deletion in peripheral B cells expands the MZ B cell population and reverses the MZ deficiency in CD19 knockouts [72].

The absence of B-1 and MZ B cells in PI3K knockout strains made it difficult to interpret whether PI3K activity is important for the function of fully developed B-1 and MZ B cells. Gold and colleagues addressed this question by utilizing a p110δ-selective inhibitor IC87114 (Table 1) in immune assays using normal B-1 and MZ B cells [73]. Selective inhibition of p110δ in these cells completely inhibits AKT activation by Toll-like receptor (TLR) ligands and chemoattractants, suggesting that p110δ is the main isoform linking these extracellular signals to AKT. p110δ inhibition also suppresses chemotaxis, proliferation and antibody production by B-1 and MZ cells. Notably, treatment of mice with IC87114 disrupts the localization of B cells with a MZ surface phenotype ($IgM^{hi}IgD^{lo}$), suggesting that p110δ is required for chemotactic and adhesive signals that position MZ cells in the marginal zone. B-1 and MZ cells are thought to be the major source of natural antibodies to common microbial antigens and some self-antigens. Consistent with the reduced B-1 and MZ compartments in p110δKI mice, natural antibody production is reduced. Autoantibody production is also reduced in p110δKI mice and in mice treated with p110δ inhibitor. These findings suggest that selective p110δ inhibitors have potential for the treatment of autoimmune diseases driven by autoantibodies. Whether p110δ inhibition enforces B cell tolerance through anergy induction, or acts mainly by blocking proliferation and differentiation of self-reactive B cells remains to be established. Interestingly, p110δ inactivation suppresses the expansion of B-1 cells and MZ B cells in the absence of PTEN [74].

The main function of FO B cells is to produce antibodies in response to T cell-dependent (protein) antigens. It is well established that FO B cell function is highly dependent on class IA PI3K. B cells lacking p85α or p110δ, or expressing kinase-inactive p110δ show severely impaired signaling downstream of the BCR and a complete inability to proliferate following BCR crosslinking [9, 56–59, 70, 75]. These defects are not simply due to altered development because wild-type B cells treated with IC87114 display equivalent defects [76]. A primary role of PI3K following BCR crosslinking is to promote signalosome assembly leading to diacylglycerol production, PKCβ activation, and NFκB nuclear translocation to activate gene expression (Figure 2) [3, 8].

Class IA PI3K signals also contribute to antigen presentation by B cells [77], adhesion of T:B conjugates [78], and to differentiation of follicular helper T cells (Tfh cells, see below). This predicts that PI3K inhibition would prevent the differentiation and function of T cells capable of delivering helper signals to B cells. It is surprising, therefore, that T cell-dependent antibody responses, while reduced, are not abolished by genetic disruption of p110δ or p85α [9, 56, 59, 73] nor by IC87114 treatment of mice [73, 79]. It is important to note that PI3K is not required for that FO B cell proliferation *in vitro* driven by CD40 ligand (CD40L) and interleukin-4 (IL-4), a model for B cell response to T cell help [57, 80].

Proliferation of FO B cells driven by TLR ligands is also partially preserved when PI3K is inhibited [9, 56–59, 80, 81]. Therefore, the defects in BCR signaling might be overcome by PI3K-independent signals from T cells or TLR ligands. Further, PI3K inhibitors promote immunoglobulin (Ig) class switching under conditions where B cell proliferation is maintained (e.g. CD40L + IL-4, or LPS), whereas PTEN deletion suppresses class switching [79, 82, 83]. Ig class switching requires activation-induced cytidine deaminase (AID), whose gene transcription is controlled by Foxo1 [63]. All of these observations emphasize that PI3K is not a universal "on" switch for B cell responses or for T cell help (Figure 2). Ultimately, the balance of positive and negative effects of PI3K inhibition probably explains the retention of a reasonably robust T-dependent antibody response *in vivo*. Also of interest is the finding that p110δ inhibition or p85α deletion causes a selective elevation in basal and antigen-specific IgE production [79, 84]. Whether this phenomenon will affect the clinical success of p110δ inhibitors, particularly in inflammatory diseases, is a topic of interest.

FO B cell survival mainly depends on the cytokines B-cell activating factor (BAFF) and IL-4 as well as tonic (basal) signaling through the BCR. Cytokine-dependent survival is highly dependent on p110δ activity *in vitro* [76, 85]. However, FO B cell numbers are only modestly reduced in p110δKI mice suggesting that survival signals from cytokines and/or BCR tonic signaling *in vivo* are not absolutely p110δ-dependent. A study from Rajewsky's group showed that in the absence of the BCR, a constitutively active form of p110α is sufficient to maintain FO B cell survival in the periphery [86]. The converse experiment using a conditional p110α knockout approach showed that only in the absence of both p110α and p110δ were FO B cells completely depleted [60] (Figure 2). These results again show the importance of dissecting PI3K signaling through both gain-of-function and loss-of-function approaches. It is likely that expression of a constitutively active p110α isoform can elevate PIP_3 beyond the physiological level, bypassing the need for p110δ engagement by tonic signals from an intact BCR. Several interesting questions arise from this work. First, do p110α and p110δ produce distinct PIP_3 pools with different spatial location and effector molecules? Alternatively, each isoform might contribute quantitatively to shared signaling outputs. Is the absence of FO B cells in p110α/p110δ-double knockouts due only to impaired BCR tonic signaling or also to a block in survival signals from cytokines?

An intriguing issue raised by the p110α/p110δ-double knockout study is whether acute treatment with pan-class I PI3K inhibitors would cause a significant reduction in the peripheral B cell population, relative to selective inhibitors of individual PI3K isoforms. Given that pan-PI3K inhibitors are entering clinical trials for cancer therapy, it will be important to evaluate their impact on immune homeostasis compared to more selective compounds. One might predict that selective p110δ inhibitors would mainly affect the B-1 and MZ compartments without eliminating FO B cell numbers or function. Of particular interest is targeting p110α because activating mutations in the *PIK3CA* gene are very common in many solid tumors and are considered oncogenic drivers [87]. Development of a highly selective p110α inhibitor would perhaps have the potential to selectively act on *PIK3CA* mutant tumor cells while sparing B-1 and MZ B cells and maintaining normal FO B cell function in the periphery. Of course, the impact of different PI3K inhibitors on all immune subsets that regulate the tumor microenvironment is a subject of great significance. Later in this review we will address the issue of targeting PI3K isoforms in tumors of B cell origin.

T cells

Thymocytes

T cells develop in the thymus where CD4-CD8- double negative (DN) precursors arriving from the bone marrow undergo a differentiation program characterized by sequential VDJ

recombination of the TCRβ and TCRα chains. The DN precursor cells can further be divided into DN1-4 stages based on the differential expression of CD44 and CD25. Proper rearrangement of TCRβ is required at the DN3 stage to proceed to DN4 stage (β-selection) and subsequently to CD4+CD8+ double positive (DP) cells that now rearrange and express the TCRα chain. TCRαβ+ DP cells are subjected to positive and negative selection and CD4 or CD8 single positive (SP) cells migrate out of the thymus to populate the lymph nodes and spleen. It is now clear that PI3K activity plays a crucial role in thymocyte development especially during β-selection [88].

Genetic models have suggested roles for both class IA and IB PI3K in thymocyte development. Loss of p110δ, p85α or both p85α/β does not significantly reduce overall numbers of thymocytes. However, pre-TCR signaling is attenuated in the absence of p85α or p110δ [89, 90]. The role of class IB PI3K is more prominent. p110γ knockout mice have increased apoptosis of DP thymocytes and strikingly, p110δ/γ double knockout markedly reduces the number of thymocytes due to a defect at the DN3-DN4 transition stage [91, 92]. Turner and colleagues have addressed the molecular basis for this defect using genetic models and an *in vitro* DN3 differentiation system [89]. To a similar extent as the p110δ/γ double knockout mice, DP thymocyte numbers are reduced in mice lacking p110δ and the p101 regulatory subunit of class IB PI3K. Both p110δ and p101/p110γ are required at the DN3-DN4 stage but act downstream of different receptors. AKT activation through the pre-TCR is entirely dependent on p110δ whereas CXCR4-stimulated AKT activation is mainly dependent on p101/p110γ with a smaller role for p110δ. A functional role of CXCR4 in thymocyte development was confirmed using both genetic and pharmacological approaches. Using a knock-in approach in which p110γ is mutated to prevent its binding to RAS, the same group has shown that CXCR4 activates p110γ in a RAS-dependent manner during thymocyte β-selection [41]. These studies strongly support the concept that antigen receptors can cooperate with chemokine receptors to activate both class IA and IB PI3K isoforms to promote cellular responses. An important question is whether this concept also applies to activation of effector T cells, which express chemokine receptors with costimulatory function [93]. If so, it would suggest that dual p110δ/γ inhibitors would be more effective than isoform-selective compounds with respect to blocking effector T cell activation. An interesting detail to address is whether p110δ and p110γ create distinct pools of PIP_3 with different downstream effectors.

One process driven by preTCR-dependent PI3K activation is increased cellular metabolism driven by PDK-1 and AKT. A striking feature of thymocytes lacking PDK-1 is a severe defect in the ability to upregulate nutrient receptors CD71 (transferrin receptor) and CD98 (amino acid transporter subunit) at the DN3 stage. Conversely, PTEN-deficient thymocytes upregulate these receptors and progress to the DN4 stage in the absence of a pre-TCR signal [94]. Increased expression of nutrient transporters might be required for the rapid proliferative expansion at this stage. AKT is likely the key mediator downstream of PI3K and PDK-1, since nutrient receptor upregulation is maintained by a PDK1 mutant (L155E) that can activate AKT but not other PDK1 substrates [95]. AKT1/AKT2 double knockout DN3 cells show a defect in development and reduced glucose uptake [96].

CD4 T cells

Resting CD4 T cells recirculate among lymphoid tissues, searching for foreign antigenic peptides presented by antigen-presenting cells (APCs). Antigen recognition with costimulation from dendritic cells leads to T cell clonal expansion and differentiation into effector CD4 T cells. These are usually termed T helper (Th) cells as they provide help to other immune cells such as macrophages, B cells and CD8 T cells to orchestrate the overall immune response depending on the immune context. The functional variety of T helper cells is achieved by the differentiation of naïve uncommitted CD4 T cells to distinct subsets

including Th1, Th2, Th17, and T follicular helper (Tfh) cells (Figure 3). Distinct types of CD4 T cells with suppressive potential, termed regulatory T cells (Tregs), are also produced either during thymocyte selection (natural Tregs; nTreg) or upon stimulation under tolerogenic conditions (induced Tregs; iTreg). The differentiation of CD4 T cells to each subset is dictated by sensing of extracellular cues in the form of cytokines and cell contact-dependent signals from the microenvironment, with distinct outcomes depending on the immune context.

The proliferation of CD4 T cells *in vitro* driven by antibody-mediated clustering of TCR with CD28 is largely PI3K-independent [9, 57, 97–99]. It is likely that artificial TCR/CD28 clustering overrides certain physiological signaling requirements, as clonal expansion of CD4 T cells driven by antigen (or superantigen) and APCs is markedly suppressed by PI3K blockade, with a major role for p110δ [97–99] (Figure 3). Furthermore, numerous studies indicate that CD4 T cell differentiation is highly regulated by PI3K signaling and its downstream effectors, particularly the AKT/FOXO axis and TOR.

Th1 and Th2 cells

Th1 and Th2 cells were the first subsets of CD4 T cells to be discovered and categorized by the distinctive cytokines they secrete. The signature cytokine produced by Th1 cells is IFNγ, which provides essential help to macrophages for the destruction of vesicular pathogens. IFNγ and IL-2 produced by Th1 cells also help CD8 T cells differentiate into cytotoxic T lymphocytes (CTL) that are essential for cell-mediated immunity to viruses. Th2 cells secrete IL-4 and other cytokines to provide help to B cells for optimal antibody production to combat extracellular pathogens. These general distinctions are not absolute as Th1 cytokines can drive class switching to certain antibody isotypes, and Th2 cytokines can contribute to alternative macrophage polarization. T cells from p110δKI mice have a defect in both Th1 and Th2 differentiation and cytokine production, suggesting a main role of p110δ in this process [98]. p110δKI mice also are protected from Th2-mediated inflammation in a model of ovalbumin-dependent airway hypersensitivity [100]. In a more recent study the Okkenhaug group used IC87114 to revisit the role of p110δ in proliferation and differentiation of both murine and human CD4 T cells [101]. A noteworthy finding was that concentrations of IC87114 above 1 μM inhibit proliferation of p110δKI T cells lacking p110δ kinase activity. Therefore, off-target effects likely contribute to the cellular actions of IC87114 when used at > 1 μM. Nevertheless, lower concentrations (0.01–1 μM) of IC87114 do partially suppress proliferation and strongly inhibit Th1 and Th2 cytokine production from naïve and effector/memory T cells from both murine and human sources (Figure 3). *In vivo* treatment with IC87114 also suppresses Th1 cytokine production and reduces ear swelling in a contact hypersensitivity model.

Genetic or pharmacological inhibition of p110δ does not fully block proliferation of naïve CD4 T cells or differentiation of Th1 or Th2 subsets [98, 101]. Therefore, other class IA isoforms might contribute to PI3K signaling that drives T helper expansion and differentiation (Figure 3). Supporting this idea, total class IA PI3K activity measured by an *in vitro* kinase assay using immunoprecipitated p85 is reduced only 50% in p110δKI T cells suggesting that p110α and/or p110β activity might also be linked to signaling by the TCR and other receptors on T cells [98]. Immune assays using isoform selective inhibitors along with generation of T cell-specific p110α or p110β conditional knockout mice can help resolve the contribution of these isoforms in T cell activation.

PI3K influences Th1 and Th2 differentiation through complex mechanisms. One key signaling protein linking PI3K activation to Th differentiation is TOR (often referred to as mTOR, for mammalian (or mechanistic) target of rapamycin). Originally identified in searches for rapamycin targets in yeast and mammalian cells, this serine/threonine kinase is

now known to have multiple functions and only some of these are rapamycin-sensitive [102, 103]. TOR is encoded by a single gene in mammals (*MTOR*) but is the catalytic subunit of two distinct multi-protein assemblies known as TOR complex-1 (TORC1) and TOR complex-2 (TORC2). TORC1 contains the protein raptor and is nutrient-sensitive; TORC2 contains a distinct protein rictor and is nutrient-insensitive. The activation of both TORC1 and TORC2 is at least partially PI3K-dependent [103–105]. TORC1 phosphorylates many substrates, but the best studied are two protein families that control mRNA translation: the ribosomal S6 kinases (S6Ks) and the eIF4E-binding proteins (4EBPs). TORC2 phosphorylates the hydrophobic motif of AKT and other AGC family kinases including serum and glucocorticoid-inducible kinase (SGK) and certain protein kinase C (PKC) isoforms. In mouse T cells, conditional knockout of *Mtor* (the gene was previously known as *Frap1*) blunts clonal expansion and completely blocks differentiation into Th1, Th2 or Th17 subsets [106]. In contrast, the induction of FoxP3 expression, the hallmark transcription factor driving a Treg program, is increased in TOR-deficient T cells [106]. This observation fits with abundant literature that rapamycin promotes Treg formation [107, 108].

Both TORC1 and TORC2 contribute to Th differentiation (Figure 3). Deletion of the small GTPase Rheb1 to block TORC1 activation prevents Th1 and Th17 differentiation, but not Th2 differentiation [109]. Deletion of rictor, an essential component of TORC2, impairs Th2 but not Th17 differentiation [109, 110]. Boothby and colleagues reported that rictor deletion also impairs Th1 differentiation, but this was not observed in the study of Powell and colleagues [109, 110]. Inhibition of both TORC1 and TORC2 is required for efficient induction of Tregs. Rheb1-deficient or rictor-deficient T cells show only a small increase in Treg generation [109]. Low concentrations of rapamycin, which inhibit TORC1 but not TORC2, achieve weak Treg induction unless TORC2 is genetically inactivated by rictor knockout [109].

The mechanisms that modulate Th differentiation downstream of TORC1 and TORC2 are beginning to be defined. Powell's group provided evidence that TORC1 and TORC2 control differentiation programs through distinct effects on signal transducer and activator of transcription (STAT) phosphorylation and suppressor of cytokine signaling (SOCS) protein expression [109]. The signaling components linking TOR complexes to the STAT pathway are not yet assigned. Boothby and colleagues showed that a constitutively active PKCθ restored Th2 differentiation in rictor-deficient T cells [110]. This suggests that TORC2-dependent phosphorylation of PKCθ is required for signals leading to the Th2 program.

There is also evidence that AKT activity promotes Th1 differentiation. An early study indicated that constitutively active AKT could restore Th1 cytokine production in CD28-deficient T cells [111], and a rictor knockout study reported that active AKT could restore Th1 differentiation in TORC2-deficient T cells [110]. Activated AKT can associate with the CARMA-1/MALT1/BCL10 complex in T cells and promote NFκB activation [112–114]. A selective inhibitor of AKT1 and AKT2 (AKTi-1/2) suppresses a subset of NFκB-dependent genes, particularly the Th1 cytokine TNFα [115]. PI3K might also promote NFκB activation through PDK-1, which associates with PKCθ following TCR stimulation to promote the interaction of the CARMA-1/MALT1/BCL10 and IKK complexes [116, 117] (Figure 3).

The ability of AKT to phosphorylate and inactivate the FOXO family of transcription factors might also regulate Th1 and Th2 differentiation. Mice with FOXO-deficient T cells develop inflammatory disorders with elevated Th1 and Th2 cytokines [118–120]. However, FOXO factors activate the *FoxP3* promoter and FOXO inactivation causes a dramatic impairment in Treg numbers and function [118, 120, 121]. Conversely, AKT inhibition potentiates Treg

induction [122]. Thus, modulation of AKT activity in T cells can indirectly restrain the differentiation of T helper subsets through increased Treg-mediated suppression.

A related concept is that during immune responses *in vivo*, PI3K inhibition can affect T cells indirectly through altered function of accessory cells. PI3K/TOR signaling in dendritic cells and macrophages suppresses production of inflammatory cytokines [107, 123, 124]. Hence, PI3K inhibition augments the production of inflammatory mediators such as IL-12 that can promote Th1 differentiation. This likely explains an observed Th1 bias in p85α-deficient mice [125] and could contribute to elevated Th1 cytokine production in an allergy model using p110δKI mice [100].

Th17 cells

The Th17 subset of CD4 T cells, discovered in 2003, is developmentally and functionally distinct from Th1 or Th2 cells. These cells produce a different set of cytokines (IL-17A, IL-17F, IL-21 and IL-22) and are specialized to protect against microbial pathogens at epithelial surfaces. Th17 cells gained major interest based on their primary role in autoimmune diseases such as colitis and experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. The role of PI3K in Th17 differentiation has been explored recently. However, data from three different groups show apparently conflicting results. SHIP KO T cells, which presumably have elevated PIP_3 levels, fail to differentiate into Th17 cells both *in vitro* and *in vivo*. [126]. T cells expressing a myristoylated AKT transgene (myr-AKT) likewise cannot differentiate into Th17 cells *in vitro* [127]. The simplest interpretation of these results is that PI3K signaling suppresses Th17 differentiation. However, PI3K inhibition also blocks Th17 differentiation. p110δKI T cells cannot differentiate into Th17 cells *in vitro* and p110δKI mice are less susceptible to EAE due to a Th17 defect *in vivo* [128] (Figure 3). It is possible to reconcile these observations by considering that PI3K lipid production and hydrolysis is not a simple on/off switch; each of these genetic models has qualitatively distinct effects on the PI3K signaling network. SHIP dephosphorylates the 5-position of PIP_3 to produce PtdIns(3,4)P_2. Since both PIP_3 and PtdIns(3,4)P_2 can promote PDK-1- mediated AKT phosphorylation, loss of SHIP should not necessarily affect AKT activation. Indeed, activation of AKT through TCR engagement in SHIP KO T cells is unchanged [129]. It is possible that loss of SHIP prevents the recruitment of effector proteins that specifically bind to PtdIns(3,4)P_2, and that these proteins transmit signals to induce Th17 differentiation. This model is consistent with the data from p110δKI T cells, which should also have reduced PtdIns(3,4)P_2 levels. Both p110δKI and SHIP knockout T cells also have reduced Th2 differentiation [98, 129]. The observation that myristoylated AKT blocks Th17 differentiation could result from the atypical localization or activation kinetics of this AKT variant. The increased Treg differentiation observed in this Myr-AKT strain [127] is also at odds with other approaches to modulate PI3K/AKT activity (see below). An important future question to address is whether PI3K isoforms other than p110δ have a distinct role in Th17 differentiation. It will also be interesting to identify PtdIns(3,4)P_2 effectors that function in T cells; PtdIns(3,4)P_2-selective binding proteins Bam32, TAPP1 and TAPP2 have important roles in B cells [78, 130]. In addition, it is worth considering whether SHIP and potential PtdIns(3,4)P_2 effectors function mainly in signaling from the TCR or from cytokine receptors that influence Th17 differentiation.

A positive role for PI3K/AKT signaling in Th17 is supported by studies of T cells lacking TORC1 function. Mice with T cell-specific deletion of *Mtor* or *Rheb1* have a drastic reduction in Th17 differentiation and are protected from EAE [106, 109] (Figure 3). Further supporting a role for TORC1, rapamycin treatment also suppresses Th17 differentiation and ameliorates EAE [131, 132]. Other work suggests that TORC1 regulates the balance of Th17/Treg differentiation through a central regulator of cellular metabolism, hypoxia-

inducible factor-1α (HIF-1α) [133, 134]. HIF-1α expression is increased by TORC1 in a manner independent of hypoxia, and contributes to increased glycolysis during Th17 differentiation. The idea that TORC1 controls metabolic programs that determine Th differentiation is supported by other recent work [135–137].

Human memory Th17 cells can be defined by surface expression of the chemokine receptor CCR6. Culturing these cells in the presence of cytokines that signal through the γ_C (gamma-common) chain (e.g. IL-2, IL-7, IL-15) induces production of IL-17, IL-22 and other cytokines. Under these conditions, pharmacological inhibition of PI3K or AKT suppresses IL17/IL-22 production by human $CCR6^+$ cells [138]. This indicates that PI3K activation is important not only for establishment of Th17 effector cells but also for their function (Figure 3). Further mechanistic experiments suggest that PI3K/AKT signaling promotes Th17 cytokine production through suppression of FOXO and KLF transcription factors [138]. The ability of PI3K/AKT signaling to suppress FOXO and KLF-dependent gene expression is also responsible for changes in homing and trafficking that accompany activation of both CD4 and CD8 T cells in mice [139].

Tfh cells

The T follicular helper (Tfh) cell subset is specialized to provide help to B cells during germinal center (GC) reactions. Tfh cells are defined by the expression of the chemokine receptor CXCR5 and their specific requirement for the transcription factor B cell lymphoma-6 (BCL6) and the inducible costimulatory (ICOS) transmembrane receptor. Following antigen encounter, B cells that initiate the GC reaction undergo somatic hypermutation and isotype switching to increase antibody affinity and diversify effector functions. By expressing CXCR5, primed T cells can migrate into the B cell follicle along the CXCL13 gradient and first receive signals from B cells in the form of antigen-mediated TCR signaling and costimulatory signals to initiate the Tfh program. Many costimulatory signals provided by activated B cells are crucial for Tfh signaling. Of these, PI3K signaling downstream of ICOS appears to play a nonredundant role in regulating the abundance and effector functions of Tfh cells [140].

Interruption of ICOS-ICOS ligand (ICOSL) interaction or ICOS deficiency (ICOS−/− mice) leads to impaired GC responses [140]. To address the question of whether PI3K signaling downstream of ICOS is the main mediator for this defect, an ICOS knock-in transgenic mouse (ICOS-YF) was generated that harbors a mutation in the crucial tyrosine Y^{181} that is required for p85 recruitment [141]. The ability of ICOS to strongly enhance TCR-mediated AKT phosphorylation is blocked in ICOS-YF T cells. Class switched Ig levels as well as the magnitude of the GC response are severely impaired in these mice. This phenotype is mainly due to a defect in the generation of Tfh cells and the effects are very similar in ICOS−/− mice. This suggests that class IA PI3K is a central player in ICOS signaling leading to Tfh differentiation (Figure 3). FOXO inhibition might play an important role in this pathway as *Foxo1*/*Foxo3*-deletion in T cells promotes Tfh generation [118].

A subsequent study from Turner and colleagues provided evidence that p110δ is the primary catalytic isoform that couples ICOS engagement to Tfh generation [142] (Figure 3). Conditional deletion of *Pik3cd* (encoding p110δ) in mature T cells (using *CD4*-Cre) or in primed T cells (using *OX40*-Cre) prevents the expression of Tfh-selective cytokines IL-4 and IL-21. T cell-specific deletion of p110δ reduces Tfh numbers with an accompanying decrease in GC B cells and high affinity antibody production. Importantly, amplifying PIP_3 through PTEN deletion in activated T cells has the converse effect of increasing Tfh cells, GC B cells and antibody affinity. A surprising finding from this study was that B cell-specific ablation of p110δ using *CD19*-Cre does not impair the GC response or antibody production, other than an increase in IgE production. Hence, the decreased T-dependent

antibody response in the original "whole body" p110δKI mice can be attributed to defects in T cells rather than in B cells. Although p110δ controls many facets of B cell development and T cell-independent antibody production, this experiment suggests that the B cell intrinsic role of p110δ in T-dependent responses is mainly restricted to suppression of IgE isotype switching during the germinal center response.

A number of observations add complexity to the simple model that p110δ in T cells controls Tfh differentiation and antibody responses. Acute inhibition of p110δ using IC87114 in mice reduces GC B cell numbers, but does not reduce antigen-specific IgG1 and markedly increases the IgE response [73, 79]. This indicates that extrafollicular B cell differentiation and class switching can still proceed when the germinal center response is suppressed. It is also possible that PI3K isoforms other than p110δ might contribute to ICOS signaling. Using synthetic ICOS peptides and ICOS immunoprecipitations, a significant amount of p110α bound to ICOS can be detected after stimulation of the CD4 T cell line D10 and primary CD4 T cell blasts [143]. siRNA-mediated knockdown or pharmacological inhibition of p110α partially reduces ICOS-dependent AKT phosphorylation in D10 cells. The application of newer inhibitors with greater selectivity and improved pharmacological properties should further resolve the roles of PI3K isoforms in ICOS function *in vitro* and *in vivo*. With the entry of isoform-selective inhibitors and pan-class I PI3K inhibitors in clinical trials, we will also begin to learn how different PI3K target profiles affect antibody isotypes in humans.

Tregs

The discussion thus far makes it clear that PI3K activation plays a generally positive role in signaling to generate different effector CD4 T cell subsets (Figure 3). The major catalytic isoform responsible in most contexts seems to be p110δ although other isoforms contribute. This function for PI3K in CD4 T cell expansion and differentiation is not surprising given the central role of PI3K in the growth factor responses of many other cell types. In this respect, it was initially perplexing that reduced PI3K function in immune cells could lead to autoimmunity in mice. p110δKI mice develop colitis [9] and r1δT/r2n mice (a strain with T cell-specific deletion of *Pik3r1* encoding p85α, and germline deletion of *Pik3r2* encoding p85β) develop an exocrinopathy that resembles primary Sjögren's Syndrome [144]. It now seems likely that both of these phenotypes ban be attributed to reduced numbers and/or function of Tregs ([144, 145] and unpublished data). Accumulating data suggest that PI3K activity is intimately linked to both development and function of Tregs.

Tregs are essential for peripheral tolerance induction. Both nTregs and iTregs can suppress proliferation of potentially autoreactive T cells, through direct contact with effector T cells and dendritic cells as well as through secreted factors. Thymic development of nTregs seems to be constrained by PI3K activity as p110δKI mice have increased thymic Treg numbers [145]. Recent studies suggest a critical role for FOXO transcription factors in linking PI3K/AKT signaling to the thymic development of nTregs. Although a single allele of *Foxo1* or *Foxo3* is enough to maintain nTreg development, combined deletion of both genes leads to a severe defect in FoxP3+ nTregs in the thymus [118, 120]. FOXO factors directly activate FoxP3 expression through direct binding to FOXO consensus sequences in the *Foxp3* promoter [120, 121]. Thus, when PI3K activity is reduced FOXO factors can relocate the nucleus to turn on a Treg transcriptional program that includes FoxP3 expression (Figure 3). On the other hand, elevated PI3K/AKT signaling does not necessarily suppress nTreg development as thymic nTreg development is unaltered in a mouse model where PTEN is deleted specifically in T cells (CD4-Cre) [146]. Conflicting results have been obtained using activated AKT transgenes in two different models of nTreg development in thymocytes [127, 147].

PI3K/AKT signaling has complex roles in mature Tregs. Despite the marked decrease in thymic nTregs in the T cell-specific *Foxo1*/*Foxo3* double knockouts, peripheral (spleen and lymph node) Tregs are only modestly reduced [118, 120]. This suggests that PI3K/AKT signaling promotes, and FOXO factors suppress, homeostatic maintenance of peripheral Tregs. In accord, peripheral Treg numbers are decreased in both p110δKI and r1δT/r2n mice [144, 145]. Adding further complexity, PI3K/AKT signaling is attenuated in mature Tregs. Compared to conventional CD4 T cells, isolated Tregs show blunted activation of AKT in response to TCR engagement or IL-2 [126, 146, 148]. This phenotype correlates with elevated PTEN expression in Tregs, and PTEN-deletion promotes IL-2-dependent proliferation of Tregs [120]. It is conceivable that a low level of PI3K/AKT signaling is required to prevent FOXO-dependent cell cycle arrest and apoptosis, but that higher levels could suppress the Treg phenotype.

PI3K activity is also required for the function of peripheral Tregs. Purified Tregs from p110δKI mice have reduced suppressive activity and severely impaired secretion of IL-10, an anti-inflammatory cytokine [145] (Figure 3). Interestingly, this defect in Treg function provides protection of mice from *Leishmania major* infection [149]. Despite having impaired Th1 responses, p110δKI mice are able to contain the parasite and this phenotype is reversed by adoptive transfer of wild-type Tregs. This opens up the possibility of developing p110δ-selective inhibitors for *Leishmania* treatment.

An extensive focus in Treg biology has been the study of *ex vivo* expansion of naïve conventional CD4 T cells to iTregs. This process is relevant to peripheral tolerance induction and has therapeutic implications with respect to adoptive transfer of Tregs. There is strong evidence that limiting PI3K/AKT/TOR activity during T cell priming promotes the conversion of conventional CD4 T cells to iTregs. Merkenschlager and colleagues showed that withdrawal of TCR stimulus after 18 hours triggers iTreg conversion, and used small molecule inhibitors to probe the signaling pathways responsible for this effect [122]. Inhibition of PI3K and/or TOR, or AKT inhibition, led to greatly increased FoxP3+ iTregs in this system. Although the p110δ inhibitor IC87114 modestly increased iTregs, the effect of a p110α inhibitor with some activity against p110γ and p110δ (PIK-90; see Table 1) was more profound. This result further supports involvement of diverse p110 isoforms in different immune contexts. Other work supports a role for PI3K/AKT signaling to oppose iTreg conversion through suppression of FOXO activity (Figure 3). In CD4 T cells lacking the ubiquitin ligase Cbl-b, phosphorylation of AKT and FOXO factors are elevated and TGFβ-induced iTreg conversion is greatly reduced [121]. This phenotype can be rescued by overexpressing Foxo1 or Foxo3. Conversely, iTreg induction is abolished in *Foxo1*/*Foxo3* double knockout T cells or in cells expressing constitutively active AKT [79, 120, 147]. Together these data indicate that similar to thymic nTregs, FOXO-driven FoxP3 expression is also important for iTreg generation in peripheral T cells.

As discussed above, TOR activity also opposes iTreg induction. It is interesting that inhibition of both TORC1 and TORC2 are required for conversion of conventional CD4 T cells to Tregs [132, 138]. It makes sense that TORC2 inhibition should augment FOXO-dependent Treg programming through AKT suppression (Figure 3). What is the role of TORC1? We have obtained evidence that S6K activity downstream of TORC1 opposes iTreg differentiation. Genetic or pharmacological suppression of S6K increases iTreg induction *in vitro* and increases Treg numbers *in vivo* [135]. A search of the patent literature reveals S6K inhibition as a strategy for ex vivo expansion of Tregs (US 2009/0142318). TORC1 also opposes the iTreg fate through HIF-1α, which binds to FoxP3 and promotes its degradation [133, 134]. Whether PI3K inhibitors promote iTreg conversion primarily through the AKT/FOXO axis or also through TORC1 inhibition remains to be determined.

CD8 T cells

Upon antigen encounter, resting CD8 T cells differentiate into effector CTLs with a fundamental role in anti-viral and anti-tumor responses. Cantrell and colleagues have reported key roles for PI3K/ATK/TOR signaling in CD8 T cells, with interesting distinctions from PI3K function in CD4 T cells [150]. Whereas PI3K inhibition suppresses antigen-driven clonal expansion of CD4 T cells, PI3K is not required for CD8 T cell proliferation or associated metabolic changes such as increased glucose uptake. Clonal expansion of antigen-primed CD8 T cells cultured in the presence of IL-2 are not affected by the p110δ inhibitor IC87114, a specific AKT inhibitor, nor in CD8 T cells from p110δKI mice. Proliferation and glucose uptake are impaired in PDK-1 null cells, implying that AGC kinases regulated by PDK1 can compensate for AKT in this context. However, PI3K/AKT signaling is required in activated CD8 T cells to initiate the transcriptional program for CTL effector function. Expression of cytotoxic effector molecules including granzymes and perforin, and production of the cytokine IFNγ correlate with AKT activity and FOXO inactivation. AKT activation also coordinates changes in expression of homing and trafficking receptors to allow effector CTLs to leave lymph nodes and traffic to sites of infection. Specifically, AKT inhibition or genetic interference with PDK-1-mediated AKT activation prevents the downregulation of FOXO target genes encoding proteins important for naïve CD8 T cell trafficking: L-selectin (CD62L), KLF2, CC motif chemokine receptor 7 (CCR7), and sphingosine-1 phosphate receptor 1 (S1P1). An earlier study from this group reported that rapamycin treatment of antigen-primed CD8 T cells also prevents the downregulation of L-selectin (CD62L), KLF2, CCR7 and S1P1 [151]. Whether rapamycin acts in this system through inhibition of TORC2/AKT or TORC1 signaling remains to be resolved [139].

The role of PI3K signaling in cytotoxicity of mature CTLs has not been studied in detail. However, there is evidence that the p110γ isoform is required for CTL chemotaxis and trafficking to sites of infection [152]. Similarly, the p110γ isoform has a significant role in trafficking and survival of effector CD4 cells [153]. This raises the possibility that drugs in development that target p110γ, either alone or with other class I isoforms, will suppress effector T cell trafficking and compromise host defense.

Although natural killer (NK) cells are traditionally classified as part of the innate immune system, they are lymphocytes and display some features of adaptive immune cells [154]. Both p110δ and p110γ appear to play important roles in NK cell maturation and function, including cytokine production and cytotoxicity. The functions of p110δ and p110γ in NK cells and the discrepancies among experimental systems were reviewed recently by Kerr and Collucci [155].

CAL-101 and the potential of PI3K inhibitors in lymphoid malignancies

Starting from the initial description of wortmannin as a PI3K inhibitor that suppresses neutrophil responses [156, 157], there has been interest in developing PI3K inhibitors for inflammatory and autoimmune diseases. Most efforts have focused on inhibitors of p110δ and/or p110γ based on the leukocyte-restricted expression of these isoforms and the immune phenotypes of gene-targeted mice [158]. The p110γ isoform has received particular attention for inflammatory diseases, as p110γ plays an integral role in chemotaxis and respiratory burst of neutrophils during inflammation [159], and amplifies mast cell degranulation and allergic responses [160]. p110γ has a limited role in T cells and is largely dispensable in B cells. p110δ has been considered a more promising target for suppression of adaptive immune responses, based on the fundamental role of this isoform in both B and T cells [46]. The discovery of compounds with high selectivity for p110δ has proceeded more rapidly than for selective p110γ inhibitors, and many pharmaceutical companies have

selective p110δ inhibitors in their development pipelines [161] (current clinical candidates are listed in Table 1). These have potential application for allergy and autoimmune diseases, as discussed in the Future Directions section.

In parallel to studies of PI3K inhibitors in inflammation, there has been a tremendous effort to develop pan-class I PI3K inhibitors, isoform-selective PI3K inhibitors, dual PI3K/TOR inhibitors and selective TOR inhibitors for cancer [161–167]. Notably, the most exciting success in the clinic so far has been the efficacy of the p110δ inhibitor CAL-101 in a subset of cancers of B cell origin. Although the results of CAL-101 clinical trials have not been published in peer-reviewed journals, some of the data have been presented at professional meetings and conferences [168, 169]. Remarkably, treatment with CAL-101 as a single agent provided durable remissions to a significant percentage of patients with chronic lymphocytic leukemia (CLL), indolent non-Hodgkin's lymphoma (iNHL) or mantle cell lymphoma (MCL). Most of these patients had relapsed from multiple other treatment regimens, yet responded to the p110δ inhibitor. CAL-101 also showed impressive efficacy when combined with standard-of-care agents in CLL and iNHL (rituximab and bendamustine).

CAL-101 was discovered by Calistoga and is a chemical derivative of IC87114. CAL-101 inhibits p110δ *in vitro* at a concentration 40- to 300-fold lower than other class I PI3K isoforms [170, 171]. The compound also shows great selectivity when profiled against other protein and lipid kinases. In cell-based assays, CAL-101 inhibits p110δ-dependent responses a low nanomolar concentration. At concentrations above 1 μM, CAL-101 significantly inhibits cellular responses mediated by p110β and p110γ. These concentration ranges are important to remember when considering the mechanism of action of CAL-101 in cellular model systems as well as in patients.

The actions of CAL-101 have been studied in cell lines and primary patient samples representing diverse B cell malignancies including CLL, diffuse large B cell lymphoma (DLBCL) and multiple myeloma (MM) [170–173]. In general, CAL-101 has weak cytotoxic activity on isolated cancer cells at submicromolar concentrations, and variable cytotoxicity in the range of 1–10 μM. This suggests that p110δ is not required for intrinsic or serum-dependent survival signaling in culture. However, lower concentrations of CAL-101 (0.1–0.5 μM) do effectively suppress the survival signals provided by stromal cells and extracellular factors including TNFα, BAFF, fibronectin and soluble CD40L. Importantly, submicromolar concentrations of CAL-101 effectively suppress chemotaxis of CLL cells to CXCL12 and CXCL13 [173], chemokines that are commonly present in B cell follicles of lymph nodes. This suggests that in malignant B cells, similar to normal B cells, chemokine receptors signal via the class IA isoform p110δ. In addition, p110δ may serve important functions in stromal cells as CAL-101 suppresses the secretion of cytokines by monocyte-derived "nurse-like" cells in co-culture with CLL cells [173]. Together these findings indicate that CAL-101 disrupts the microenvironment and might block access of tumor cells to niches that provide extrinsic survival signals (Figure 4).

This model is strongly supported by clinical data. In a phase I trial of CAL-101 in CLL (Clinical Trials identifier NCT00710528), a large majority of patients (> 80%) across all dose levels showed significant shrinkage of lymph nodes but this was accompanied by an elevation of circulating lymphocytes. This is consistent with release of CLL cells from lymphoid tissue and/or failure of circulating cells to home to lymph nodes. Patients treated with CAL-101 showed markedly reduced amounts of chemokines CCL2, CCL3 and CXCL13 [173], in accord with the *in vitro* experiments mentioned above. Importantly, the peripheral lymphocytosis resolved over time suggesting that tumor cells died out when prevented from accessing lymph nodes (Figure 4). It remains unclear whether direct

cytotoxic effects of CAL-101 contribute to efficacy in CLL patients. The peak plasma levels of CAL-101 are greater than 2 μM suggesting that partial blockade of p110β and/or p110γ together with complete p110δ inhibition might cause direct tumor cell killing. Nevertheless, the lymphocytosis indicates that many tumor cells do not die immediately. In patients treated with combination of CAL-101 and either Rituximab or Bendamustine, lymphocytosis was not observed. This suggests that release from the protective niche by CAL-101 sensitizes leukemia/lymphoma cells to the companion agent.

Calistoga is now part of the company Gilead, which has plans to develop CAL-101 (now GS-1101) rapidly for oncology applications. When representatives of these companies have presented clinical data on CAL-101, the excitement among audience members has been palpable. The emerging success of CAL-101 validates more than a decade of intense PI3K drug discovery efforts across academia and industry. The CAL-101 results also raise many questions, some of which are discussed here (also see [161]).

One question is whether inhibition of microenvironmental signals is also the basis for CAL-101 efficacy in iNHL and MCL. When clinical data become available it will be interesting to learn whether CAL-101 causes transient lymphocytosis and reduced plasma chemokine levels in these patient populations. A related issue is whether CAL-101 fails to block microenvironmental signals in malignancies where the drug has not shown efficacy, such as DLBCL, AML and MM. A more general point is that CAL-101 studies have underscored the importance of modeling cancer cell interactions with the microenvironment even at early stages of development of PI3K-targeted therapies. It is likely that the efficacy of PI3K inhibitors in cancer patients does not correlate linearly with the IC_{50} for cytotoxic effects *in vitro*. In other words, the potential of a certain inhibitor can be either underestimated or overestimated by studies of isolated tumor cell lines.

Another question is whether greater efficacy can be achieved using less selective PI3K inhibitors. Some tumors arising from mature B cells depend on chronic BCR signaling [174]; if these signals require both p110α and p110δ then a dual inhibitor of these two isoforms might provide a potent cytotoxic effect. Perhaps pan-PI3K inhibitors would have even broader activity in leukemia and lymphoma subtypes that have been resistant to CAL-101 in early clinical studies. The potentially greater efficacy might be counterbalanced by reduced tolerability and general immunosuppression. It is even possible that broader PI3K inhibition will promote tumor survival through increased production of Tregs.

Conclusions and Future Directions

Thus far, a p110δ inhibitor in B cell malignancies is the biggest success story in the world of PI3K drug development. Where does this leave the community of inflammation and autoimmunity investigators? In some ways the barriers are greater for development of drugs in this arena. Most of the target diseases are not terminal, so there is a higher standard for safety. There are usually other treatment options with reasonable efficacy. Furthermore, animal models of inflammation and autoimmunity are generally of limited predictive value for efficacy in humans. Nevertheless, there remains a medical need and large potential market for improved anti-inflammatory and immunosuppressive drugs. Also, PI3K inhibitors seem to cause more manageable side effects in humans than initially feared. Considering these issues, it makes sense to move PI3K inhibitors forward quickly, establishing safe doses first in healthy volunteers and then testing in patient populations. This is where the promise of PI3K inhibitors will truly be tested: in human beings.

If one accepts this premise, then the question is which isoform(s) to target. Both p110δ and p110γ are preferentially expressed in leukocytes and are central mediators of immune responses. Mice lacking either protein are healthy, suggesting that compounds selective for

one of these isoforms will be well tolerated. Yet given the overlap in function between these isoforms, and possible roles of p110α and/or p110β, it might not be possible to shut down pathological immune responses or cancer cells by inhibiting just one isoform. Compounds that inhibit both p110δ and p110γ (e.g. IPI-145, Table 1) might offer better control of autoimmunity and inflammation, but what will be the effect on host defense? Again, the best choice might be to test different target profiles in various disease states to establish which profile provides the best therapeutic window. It is important to keep in mind that compounds with "partial" selectivity might provide the best balance between efficacy and tolerability. For example, a p110δ inhibitor that partially blocks p110γ at the effective dose might suppress effector T cell function without interfering with the myriad functions of p110γ in innate immunity. An equally important consideration is that each compound has its own pharmacological profile. Two experimental agents with similar isoform selectivity might have distinct efficacy in humans based on their pharmacokinetics. Relating such findings back to the biology of PI3K in different immune cells will be a challenge.

One research question that deserves more detailed investigation is the role of different PI3K isoforms in memory lymphocytes. Unlike laboratory mice maintained in pathogen-free conditions, which have mostly naïve T and B cells, human beings are constantly exposed to microbes and the majority of peripheral lymphocytes are memory or effector cells. A typical autoimmune disease patient will have an even greater fraction of such cells. There has been some progress in studying PI3K signaling in human memory lymphocytes. As noted above, memory Th17 cells stimulated with cytokines produce IL-17 through a PI3K-dependent pathway [138], and p110δ inhibition reduces proliferation and cytokine production by effector/memory T cells from humans with inflammatory diseases [101]. Similarly, human lupus patients have increased PI3K activity in peripheral lymphocytes, with a direct correlation between p110δ activity and the fraction of activated/memory T cells [175]. We need to learn more about how different PI3K inhibitors affect the function and trafficking of pathogenic lymphocytes in different immune disease states. At the same time it will be important to consider whether PI3K inhibitors that effectively suppress autoimmunity also interfere with immunological memory to pathogens. Although the road ahead is certain to be filled with challenges, the fundamental premise that PI3K inhibition will be useful for human disease therapy seems more and more solid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4EBP	eIF4E-binding protein
ABD	Adaptor-binding domain
AID	Activation-induced cytidine deaminase
AKT/PKB	Protein kinase B
APC	Antigen-presenting cell
BAFF	B-cell activating factor

BCAP	B-cell adaptor protein
BCL6	B cell lymphoma-6
BCR	B cell receptor
CCR7	CC motif chemokine receptor 7
CD40L	CD40 ligand
CD62L	L-selectin
CD71	Transferrin receptor
CD98	Amino acid transporter subunit
CLL	Chronic lymphocytic leukemia
CTL	Cytotoxic T lymphocyte
DLBCL	Diffuse large B cell lymphoma
EAE	Experimental autoimmune encephalomyelitis
eIF4E	Eukaryotic translation initiation factor 4E
FO	Follicular
FOXO	Forkhead Box Subgroup O
GC	Germinal center
GPCR	G protein-coupled receptor
HIF-1α	Hypoxia-inducible factor-1 alpha
ICOS	Inducible costimulatory
IFNγ	Interferon-gamma
IKK	IκB kinase
IL-4	Interleukin-4
iNHL	Indolent non-Hodgkin's lymphoma
iTreg	Induced Treg
KLF	Kruppel-like factor
LPS	Lipopolysaccharide
MCL	Mantle cell lymphoma
MM	Multiple myeloma
mTOR	Mammalian/mechanistic target of rapamycin
Myr-AKT	Myristoylated AKT
MZ	Marginal zone
NFκB	Nuclear factor kappa B
NK cell	Natural killer cell
nTreg	Natural Treg
PDK-1	Phosphoinositide-dependent kinase-1
PI3K	Phosphoinositide 3-kinase

PIP_3	PtdIns-3,4,5-trisphosphate
PKC	Protein kinase C
PRAS40	Proline-Rich AKT substrate of 40kDa
PtdIns	Phosphatidylinositol
PTEN	Phosphatase and Tensin homolog
pTyr	Phosphotyrosine
RAG	Recombination-activating gene
Raptor	Regulatory-associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
S1P1	Sphingosine-1 phosphate receptor 1
S6K	Ribosomal S6 kinase
SGK	Serum and glucocorticoid-inducible kinase
SH2	Src-homology-2 domain
SH3	Src-homology-3 domain
SHIP	SH2 domain-containing inositol phosphatase
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
Tfh	Follicular helper T cell
Th	T helper cell
TK	Tyrosine kinase
TNFa	Tumor necrosis factor alpha
TOR	Target of rapamycin
TORC1	TOR complex 1
TORC2	TOR complex 2
Treg	Regulatory T cell
TSC2	Tuberous Sclerosis 2

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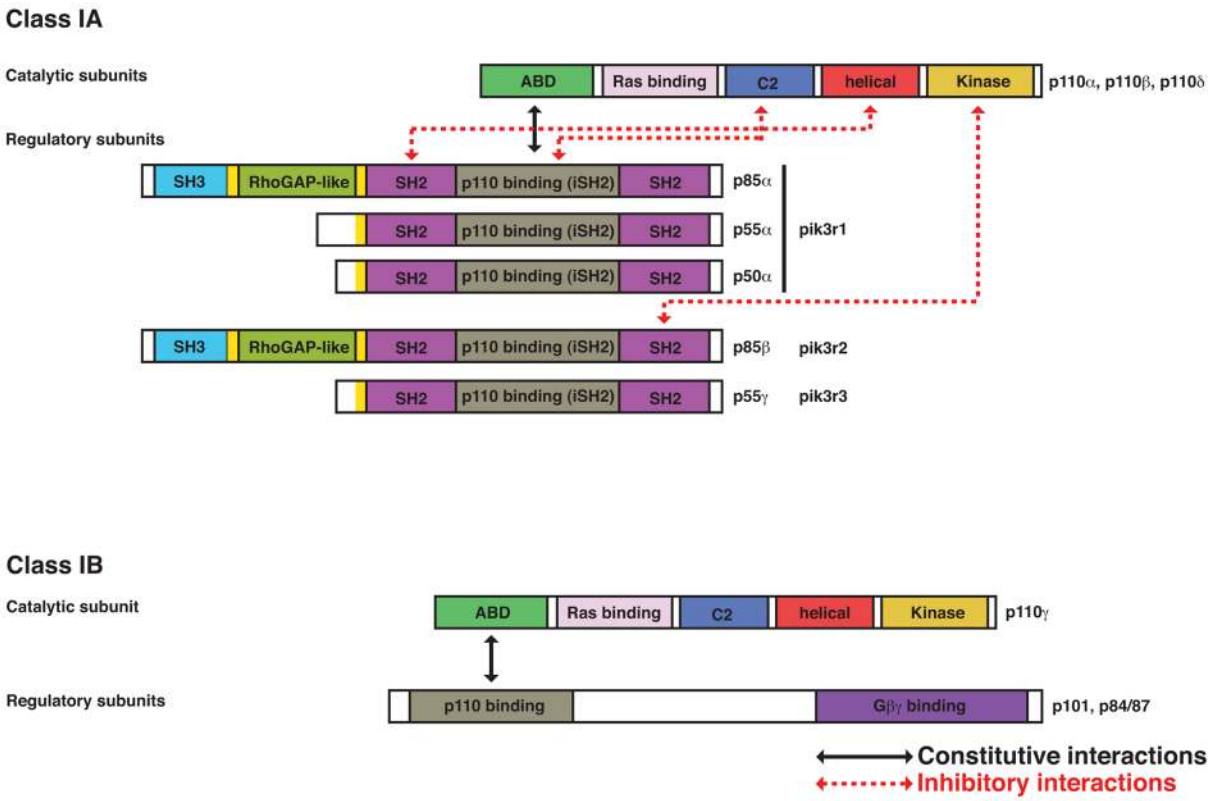

Figure 1. Domain structure of class IA and class IB PI3K isoforms

Black arrows indicate constitutive interactions of the heterodimers. Dashed red arrows indicate inhibitory interactions between the regulatory and catalytic isoforms of class IA PI3K that maintain low basal activity of the enzyme. Phosphorylation of tyrosine (Y) residues on the conserved pY-X-X-M motifs on various receptors or adaptor proteins (CD19/BCAP for B cells, CD28/ICOS for T cells) recruits the regulatory isoforms via the two SH2 domains and this binding releases the inhibitory interactions. Other protein:protein interactions also contribute to class IA and IB recruitment and activation.

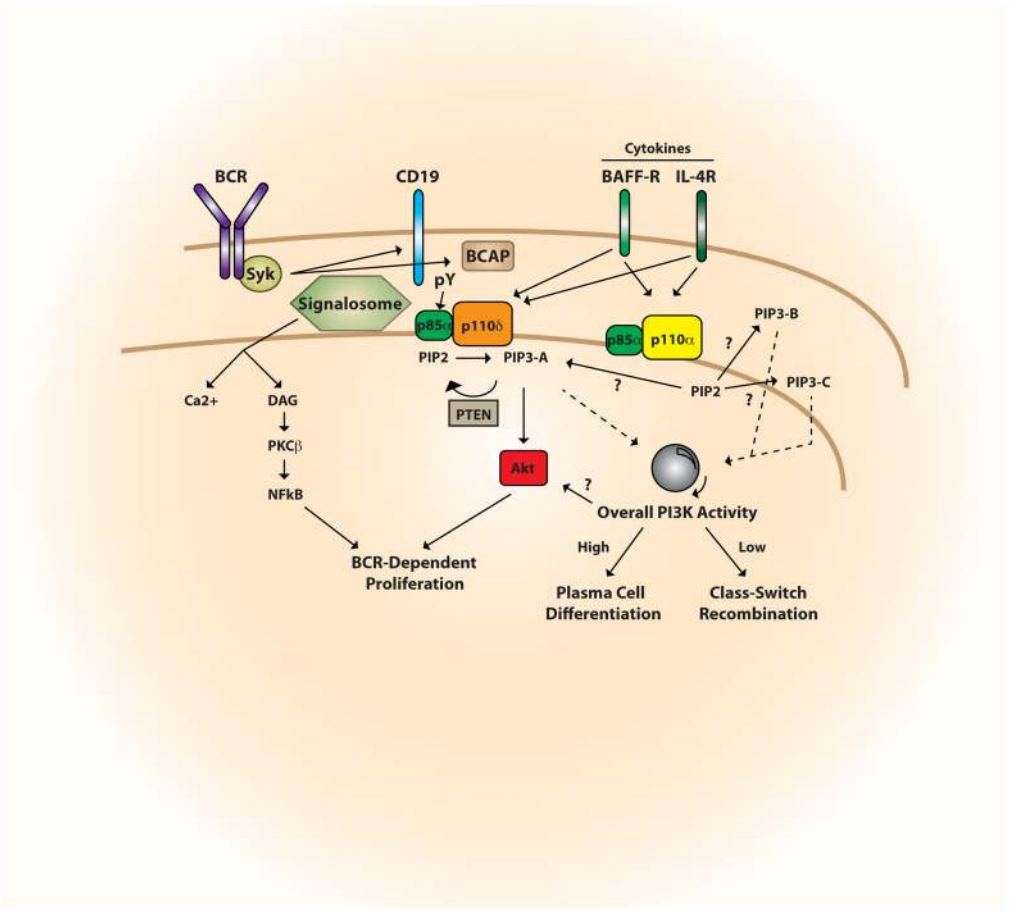

Figure 2. PI3K engagement in B lymphocytes and the rheostat concept

BCR engagement triggers tyrosine (Y) phosphorylation on CD19 and BCAP to recruit PI3K dimers mainly consisting of p85α and p110δ. PI3K activation promotes signalosome assembly for Ca2+ mobilization and diacylglycerol (DAG) production, and increases activity of AKT. BCR dependent Ca^{2+} flux, AKT activation and proliferation is mainly dependent on PIP_3 pools generated by dimers of the p110δ catalytic isoform with the p85α regulatory isoform. Cytokine (BAFF and IL-4) dependent survival signals require p110α as well, which might generate distinct pools of PIP_3 as shown. Two parallel membranes drawn in light brown represent a 3-dimensional cell surface rather than distinct membranes. *In vivo*, overall PI3K activity serves as a 'rheostat' (gray circle) whose signal output strength determines the nature of the response. In B cells, high PI3K activity opposes class switch recombination and promotes plasma cell differentiation. Low PI3K activity promotes class switch recombination, with p110δ inhibition selectively augmenting IgE production in mice. A similar rheostat concept applies in CD4 T cells to generate the variety of different subsets required depending on the immune context.

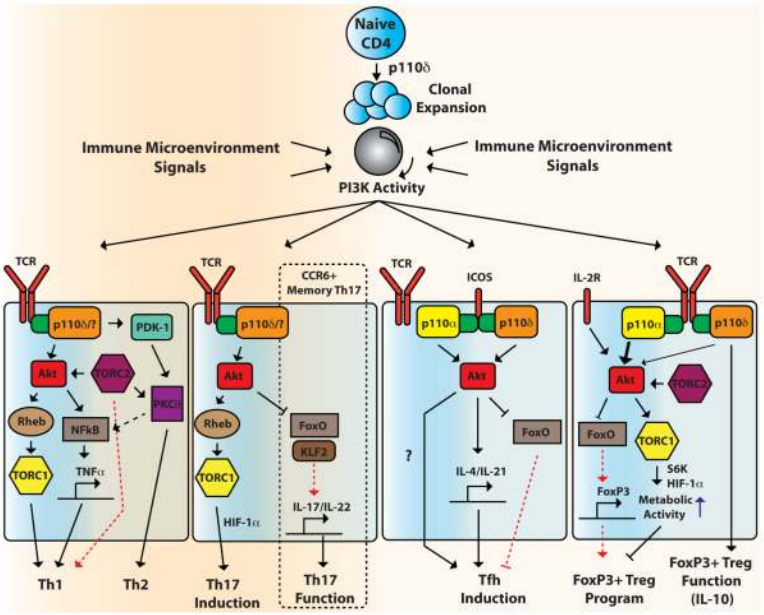

Figure 3. Role of PI3K and downstream effectors in the differentiation of activated CD4 T lymphocytes

Antigen encounter triggers clonal expansion of naïve CD4 T cells that is mainly dependent on p110δ. Depending on the immune microenvironment (cytokines, etc.), primed CD4 T cells differentiate into different CD4 T cell subsets (Th1, Th2, Th17, Tfh, and iTregs). TORC2-dependent AKT activation promotes Th1 differentiation through TORC1 and NFκB, and TORC2-dependent PKCθ activation promotes Th2 differentiation. PDK-1 also triggers NFκB activation through a complex with PKCθ. AKT increases Th17 differentiation through TORC1 and HIF-1α activation. Already established CCR6+ human memory Th17 cell function (IL-17/IL-22 secretion) also depends on PI3K/AKT activity via modulation of FOXO and its target genes including KLF2. ICOS stimulation triggers Tfh differentiation through a p110δ-dependent pathway, with a speculative role for p110α binding to ICOS. The total PI3K output acts as a rheostat (gray circle) with decreased PI3K/AKT activity favoring iTreg induction. Isoforms other than p110δ including p110α probably suppress iTreg induction in this context. PI3K/AKT blockade results in the induction of FOXO-mediated FoxP3 expression and low TORC1 activity leads to decreased metabolic activity via S6K and HIF-1α. Established FoxP3+ Tregs also depend on p110δ activity for proper suppressive function such as IL-10 secretion. Due to space constraints, the nucleus is not depicted where all of the genes are regulated.

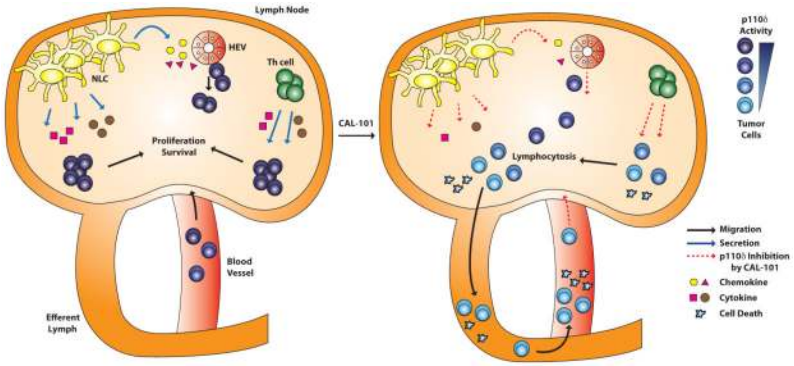

Figure 4. A model for the efficacy of a p110δ inhibitor in CLL

The two illustrations show a lymph node containing CLL tumor cells before treatment (*Left* panel) or after treatment with the p110δ inhibitor, CAL-101 (*Right* panel). Chemokines drive the entry of CLL cells and other lymphocytes into the lymph node from blood vessels via the high endothelial venules (HEV). Cells exit the lymph node via the efferent lymph. The legend at the lower right explains the arrows and symbols. The scale at the upper right depicts the differential p110δ enzyme activity in cells shown in the figure. NLC = nurse-like cells. Th = T helper. Other stromal cells also interact with CLL cells in the tumor cell niche.

Table 1

Selected PI3K inhibitors currently available for basic research or in clinical trials[1]

Targeted Isoform(s)	Compound Name	Status
Pan-class I	GDC-0941	Basic and clinical
	ZSTK474	Basic and clinical
	XL147	Basic and clinical
p110α high selectivity	A66	Basic
	INK1117	Clinical
	BYL-719	Clinical
p110α moderate selectivity	PIK-75	Basic
p110α/γ/δ	PIK-90	Basic
p110β	TGX-115, TGX-221	Basic
	AZD6482	Basic and clinical
p110γ	AS252424, AS604850	Basic
p110δ	IC87114	Basic
	CAL-101/GS1101	Clinical
	CAL-263	Clinical
	AMG-319	Clinical
Dual p110γ/p110δ	IPI-145	Clinical

[1]Dual PI3K/TOR inhibitors not included in the table