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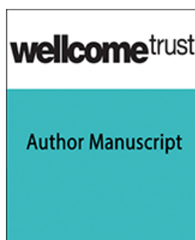

PRINCIPLES OF PI3K BIOLOGY AND ITS ROLE IN LYMPHOMA

Ralitsa R. Madsen[1]

TAKE HOME POINTS

1. Both PI3K signaling quality (interaction partners, effectors) and quantity (strength, kinetics) differ across contexts, and are under dynamic regulation.
2. Rather than as a simple ON/OFF cellular switch, the PI3K signaling pathway should be viewed as a highly sensitive dial, whose accurate tuning is a prerequisite for a given cell's optimal function.
3. Information transmission within the PI3K signaling pathway is not linear but features complex network properties whose understanding is necessary for successful pharmacological targeting in cancer.
4. Despite the relatively small number of known (epi)genetic alterations in the PI3K pathway in hematological malignancies, leukemic cells often hijack its functionality for enhanced survival, uncontrolled proliferation and reprogramming of their microenvironment.

1.1. OVERVIEW

The coordinated phenotypes of mammalian cells rely on complex signaling networks, which are comprised of a highly conserved set of signal transduction pathways. The so-called PI3K signaling pathway, featuring activation of class I phosphoinositide 3-kinases (PI3Ks) and the serine/threonine kinases AKT, is among the best-studied due to its involvement in numerous human disorders. Originally a primary nutrient storage pathway, it subsequently evolved into a key homeostatic signaling mechanism that senses and integrates myriad external signals, including hormones, growth factors, cytokines and chemokines (Fruman *et al.*, 2017; Manning and Toker, 2017). Far from being a linear information relay, this pathway features complex feedback loops, which ensures response accuracy as well as remarkable robustness in the face of perturbation. While these properties are essential for the high fidelity of information transmission in normal cells, they also make effective pharmacological targeting of PI3K signaling in disease settings very challenging (Madsen and Vanhaesebroeck, 2020). This is compounded by the many subtle ways in which the biochemical wiring of the pathway may differ

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depending on cell type, developmental stage, and environmental input. The aim of this Chapter is to provide a flavor of this complexity, with a particular focus on lymphocyte (patho)physiology. Emphasis is put on appreciating the exquisite quantitative regulation of the PI3K signaling pathway, with both "too much" and "too little" activity causing debilitating human disease.

1.2. FOUR DECADES OF PI3K SIGNALING RESEARCH

Research into the PI3K signaling pathway spans more than three decades and can broadly be subdivided into three phases. Phase I (1980-2000), spurred by the preceding molecular biology revolution, saw the discovery of the basic building blocks of the canonical PI3K pathway, most importantly the different class I PI3K enzymes, as well as PTEN, AKT and mTOR. The remarkable phylogenetic conservation of these components hinted at the fundamental importance of the PI3K signaling pathway in metazoans, and its likely evolution from a rudimentary version with key roles in chemotaxis and metabolic remodeling in response to nutrient availability (Kriplani *et al.*, 2015).

With the advent of large-scale DNA sequencing, the second phase of PI3K signaling research (year 2000-2010) firmly established the PI3K pathway as one of the most commonly perturbed in human cancers, in line with the discovery of many of its basic components in the context of cellular transformation. In parallel, discoveries of monogenic disorders caused by mutations in PI3K pathway components, alongside ever-more sophisticated genetically engineered mouse models, revealed a fundamental requirement for PI3K signaling in organismal growth, metabolic and immune homeostasis (Madsen, Vanhaesebroeck and Semple, 2018; Nunes-Santos, Uzel and Rosenzweig, 2019).

Enthused by these discoveries and the druggability of key pathway components, many academic and pharmaceutical teams subsequently invested in the development and trialing of chemical compounds targeting one or more nodes in the PI3K network, particularly in the context of cancer. This third phase (year 2010-2020) of PI3K signaling research was not without disappointments, however. Except for regulatory approval of PI3Kδ-selective inhibition in certain blood cancers, and PI3Kα-selective inhibition in a subset of breast cancers, efforts to target the PI3K pathway in disease settings have often led to underwhelming results due to issues with tolerability and drug resistance (Castel *et al.*, 2021a; Vanhaesebroeck *et al.*, 2021).

Perhaps the most important lesson learned from the first three phases of PI3K signaling research is that successful pharmacological targeting of a fundamental signaling network with pleiotropic organismal functions requires an in-depth understanding not only of the signaling "hardware" (the constituent pathway components) but also of the signaling "software" (the flow of biochemical information between individual components). Different cell types employ the PI3K signaling "hardware" in different ways, giving rise to myriad cell-specific "software" versions, as illustrated by the distinct wiring of the pathway in non-lymphoid versus lymphoid cell types. It is therefore likely that the fourth phase (year 2020 onwards) of PI3K signaling research will feature the systematic disentangling of the context-dependent wiring of the network, with the resulting discoveries used to guide the rational optimization of existing therapeutic modalities (Madsen and Vanhaesebroeck, 2020).

1.3. CLASS I PI3K ENZYMES

1.3.1. Isoforms

Class I PI3Ks are heterodimeric enzymes characterized by their ability to synthesize the phospholipid second-messenger phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol-(4,5)-bisphosphate, an event that predominantly occurs at the cytosolic face of the plasma membrane as well as at some endomembranes (Manning and Toker, 2017). This class is further divided into two subfamilies, class IA and IB, based on interactions with different regulatory subunits (Figure 1.1). Class IA enzymes consist of either one of three catalytic p110 isoforms (α, β and δ encoded by *PIK3CA*, *PIK3CB* and *PIK3CD*, respectively), coupled to

either one of five regulatory subunits (p85α/p55α/p50α, p85β and p55γ encoded by *PIK3R1, PIK3R2, PIK3R3*, respectively). Class IB PI3K consists of a single catalytic subunit (p110γ encoded by *PIK3CG*), complexed with either one of two regulatory subunits (p101 and p84 encoded by *PIK3R5* and *PIK3R6*, respectively) (Bilanges, Posor and Vanhaesebroeck, 2019).

Mammalian cells express two additional classes of PI3Ks: class II (PI3K-C2α, β, γ) and the highly evolutionarily conserved, single class III PI3K (VPS34). Class II and III PI3Ks have important functions in intracellular membrane dynamics, vesicular trafficking, and autophagy, enabling them to influence class I PI3K signaling indirectly (Bilanges, Posor and Vanhaesebroeck, 2019). The remainder of this chapter will focus exclusively on signaling downstream of class I PI3Ks (henceforth referred to as PI3K signaling).

1.3.2. Structural organization

The catalytic p110 subunits share a common domain structure, composed of an N-terminal adaptor binding domain (ABD), a RAS binding domain (RBD), a C2 domain, a helical domain, and a C-terminal kinase domain (Bilanges, Posor and Vanhaesebroeck, 2019). Collectively, these domains orchestrate the catalytic activity of the p110 subunit in time and space, both through intramolecular interactions as well as interactions with the regulatory subunits and other cellular components.

The regulatory subunits of class IA PI3Ks contain two SRC homology 2 domains (nSH2 and cSH2), separated by an intervening iSH2 domain that mediates tight binding to the catalytic p110 subunit. The longer regulatory isoforms, p85α and p85β, have additional N-terminal domains, including an SH3 domain, a BAR cluster region homology (BH) domain, and two proline-rich regions (Bilanges, Posor and Vanhaesebroeck, 2019). The regulatory subunits of class IA PI3Ks are important for stabilizing the catalytic p110 subunit, for inhibiting its baseline kinase activity, and for coupling the catalytic subunit to sites of receptor activation (Dornan and Burke, 2018). In addition, there is evidence for the existence of p110-free regulatory subunits with independent functions as well as indirect effects on class IA PI3K activity (Cheung *et al.*, 2015; Tsolakos *et al.*, 2018).

The different regulatory subunits also shape the functional specificity of individual class I PI3K isoforms through their ability to interact with distinct signaling components, possibly targeting the catalytic p110 subunits to spatially defined signaling domains. This is best illustrated with the two regulatory subunits of class IB PI3K, where loss of p84 in neutrophils leads to selective defects in p110γ-dependent reactive oxygen species (ROS) generation, in contrast to defects in p110γ-dependent migration in neutrophils lacking p101 (Deladeriere *et al.*, 2015).

Figure 1.1. On the left: the different class I PI3K subunit isoforms, their structural organization, and the combinatorial diversity arising upon heterodimerization (e.g., there are three catalytic p110 subunits that can heterodimerize with either one of the two regulatory p85 subunits, resulting in 3x2 combinations). Note that the regulatory p84 subunit of class IB PI3K is also known as p87; neither p101 nor p84/p87 have a recognizable domain structure are therefore depicted as grey rods. On the right: the enzymatic reaction catalyzed by class I PI3Ks is phosphorylation of PI(4,5)P_2 to PI(3,4,5)P_3, with PTEN acting as the opposing phosphatase. Note that

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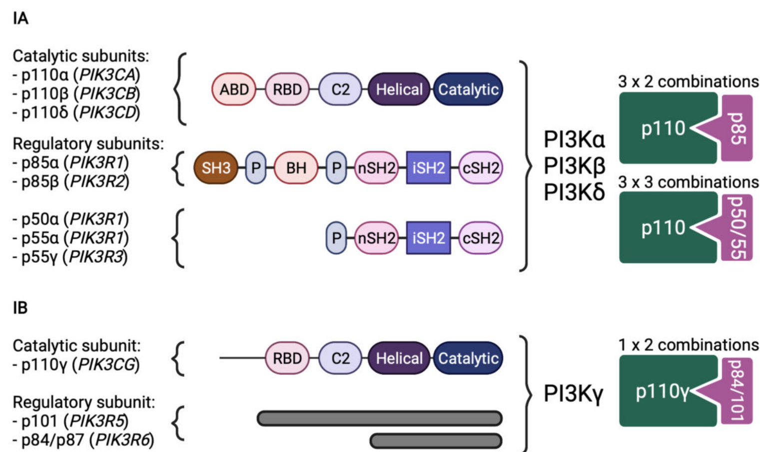
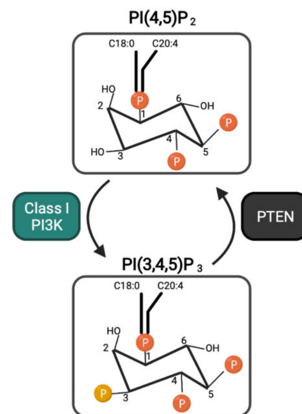
these phosphoinositides are embedded in the plasma membrane via their fatty acyl moieties, most commonly stearoyl (C18:0) and arachidonoyl (C20:4). Created with BioRender.com



1. Class I PI3K isoforms.



2. Class I PI3K function.

1.3.3. Isoform-specific functions

Unique biological functions of the different class I PI3K isoforms are well established based on evidence from targeted gene inactivation in mice, studies with isoform-selective pharmacological inhibitors and the clinical phenomenology of monogenic disorders caused by mutations in individual PI3K subunits. An appreciation of isoform-specific functions is a critical for interpreting the efficacy and on-target toxicity profiles of the numerous PI3K-targeted inhibitors in clinical development.

The differential expression of the catalytic p110 subunits partly explains known isoform-specific functions (Figure 1.2). The p110α and p110β subunits, alongside the regulatory p85α and p85β subunits, are ubiquitously expressed, whereas p110δ and p110γ are enriched in cells of the hematopoietic system. This distinction is not absolute, however, with p110α and p110β contributing to immune cell biology in specific contexts (Okkenhaug, 2013). Conversely, p110δ and p110γ can also be expressed in non-hematopoietic cells. Adding to this complexity, the interactions between individual catalytic and regulatory subunits exhibit subtle but important differences (Burke and Williams, 2015), which may further determine the selective involvement of a given class I PI3K isoform in a biological process (Dornan and Burke, 2018; Tsolakos *et al.*, 2018).

Another factor that contributes to isoform-specific biological functions is the differential ability of class I PI3Ks to be activated downstream of unique combinations of receptor- and non-receptor tyrosine kinases (RTKs and nRTKs, respectively), tyrosine-phosphorylated adaptor proteins, G protein-coupled receptors (GPCRs) and small GTPases from the RAS superfamily (Figure 1.3a). The key determinants for individual interaction preferences reside with the specific domains of each regulatory and catalytic isoform. Thus, the SH2 domains of the regulatory subunits enable all class IA PI3K isoforms (α, β, δ) to be activated downstream of tyrosine-phosphorylated YXXM motifs (Bilanges, Posor and Vanhaesebroeck, 2019). The RBD of p110α, p110δ and p110γ couples to RAS family GTPases (mainly RAS), whereas the equivalent domain in p110β enables its activation downstream of RHO family GTPases (mainly RAC1 or CDC42). The various upstream inputs often act in synergy, with both RAS and phosphorylated RTKs contributing to the enhanced membrane interaction and activation of p110α and p110δ. Conversely, p110β integrates inputs from RTKs as well as GPCRs, and p110γ from GPCRs and RAS (Bilanges, Posor and Vanhaesebroeck, 2019). The different regulatory subunit isoforms may also couple to upstream receptors in a context-dependent manner (Fos *et al.*, 2008), yet this is less well understood compared to the catalytic subunits. In summary, isoform-specific functions of the different class I PI3Ks are highly dependent on levels of expression, intrinsic catalytic activity, and spatial localization as determined by differential interactions with upstream regulators and downstream effectors.

In the following sections, "PI3K" will be used to refer to class I PI3Ks, and where relevant individual heterodimers will be denoted as PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ as determined by the catalytic p110 subunit, irrespective of the associate regulatory subunit unless specified.

Figure 1.3. a. Modular overview of the PI3K signaling pathway, emphasizing the combinatorial complexity at the level of upstream regulators and downstream effectors. Generic receptor tyrosine-kinase (RTK) input into class IA PI3Ks is shown, with more specific details related to BCR and TCR signaling included in Figure 1.4. The FOXO1 targets shown are mostly lymphocyte-specific and include components involved in class switch recombination and somatic hypermutation (AID, RAG1/2) as well as lymphocyte homing (CD62L). For a more general list of FOXO1 targets, the reader is referred to (Manning and Toker, 2017). Also note the examples of crosstalk with other signaling pathways such as RAS/MAPK (ERK), NFAT and NFkB. b. Non-exhaustive examples of reported negative feedback loops in the PI3K signaling pathway in lymphocytes. Additional negative feedback loops have been studied more extensively in non-lymphoid contexts and are not covered here. Such

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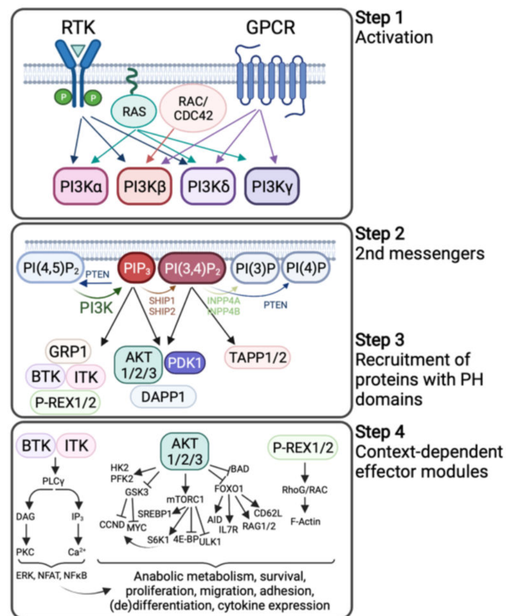
loops endow the PI3K signaling pathway with remarkable robustness in the face of perturbations, ensuring that the signaling output remains "just right" in physiological settings. Created with BioRender.com



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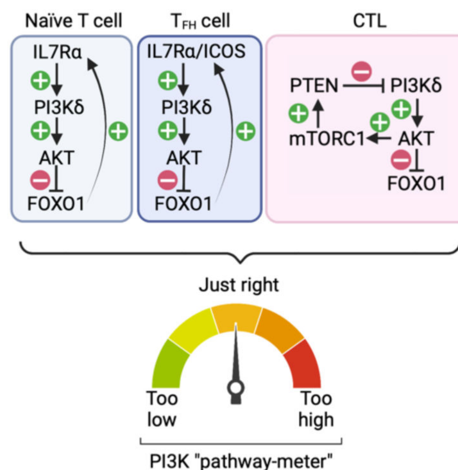

b.

1.3.4. The essential phospholipid second messenger PIP_3

Understanding why PI3Ks are of such fundamental importance to human physiology, and conversely, why they are so prominent in diverse human disorders, requires appreciation of the powerful function of their product: the second messenger PIP_3 (Figure 1.1b). This phospholipid is mainly generated at the inner leaflet of the plasma membrane and is estimated to comprise <0.05% of cellular polyphosphoinositides, being almost undetectable in quiescent cells (Dickson and Hille, 2019). In physiological settings, PIP_3 synthesis is very rapid and usually transient, attesting to the importance of keeping levels of this second messenger low. In addition to spatial localization, quantitative and temporal differences in PIP_3 are key determinants of downstream biological responses. For example, both B-cell negative selection and anergy depend on the magnitude of the PIP_3 signal (Browne *et al.*, 2009). In most cases, however, the detailed mechanisms whereby these factors contribute to the

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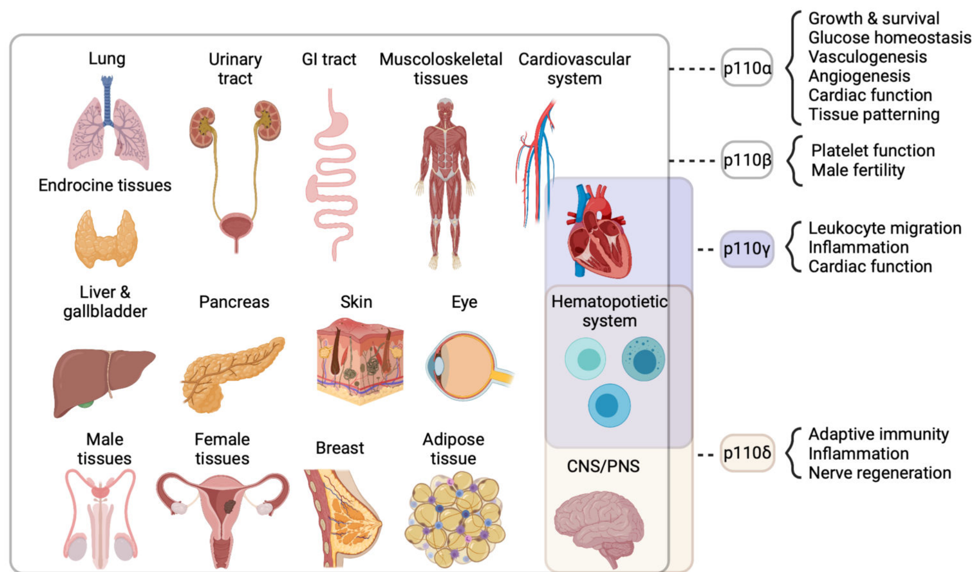

Figure 1.2. Overview of tissue-specific expression and physiological functions of class I PI3K catalytic subunits. Whereas p110α and p110β are ubiquitously expressed (outer box), p110δ and p110γ are enriched for in specific tissues (highlighted inner boxes) and overlap in the hematopoietic lineage. Only the boxes and their colors, not the position of their names with respect to the shown tissues, are used to specify relative expression levels of the catalytic p110 subunits. CNS, central nervous system; PNS, peripheral nervous system. Created with BioRender.com

translation of a given signaling input into a specific biological output remain unknown, yet their importance for the fidelity of cellular information transmission cannot be understated (Madsen and Vanhaesebroeck, 2020).

Tight regulation of PIP_3 levels is ensured by several lipid phosphatases, with the tumor suppressor PTEN as the best known. PTEN is a lipid 3-phosphatase that catalyzes the conversion of PIP_3 back to $PI(4,5)P_2$, and acts as the main break to PI3K pathway activation (Figure 1.1b). PIP_3 can also be metabolized into $PI(3,4)P_2$ by 5-phosphatases such as SHIP1 or SHIP2 (Bilanges, Posor and Vanhaesebroeck, 2019). As $PI(3,4)P_2$ retains key signaling functions, full inactivation is achieved through its dephosphorylation by PTEN and/or the 4-phosphatases INPP4A and INPP4B (Malek *et al.*, 2017) (Figure 1.3a).

The range of cellular phenotypes regulated by PIP_3, and to some extent $PI(3,4)P_2$, is extraordinary. This is achieved through the ability of these second messenger to recruit proteins with pleckstrin homology (PH) domains (Figure 1.3a). The list of PH-domain-containing proteins is long (>250), however not all of them bind PIP_3 or $PI(3,4)P_2$. PDK1 and AKT are among the best known PI3K signaling effectors able to bind both phosphoinositides. The PH domains of TEC family members, including BTK (Bruton's tyrosine kinase) and ITK (interleukin-2-inducible T-cell kinase), have high affinity for PIP_3, whereas other adaptor proteins such as BAM32 (B-cell adapter molecule of 32 kDa) and TAPP1/2 (tandem PH domain-containing protein 1 and 2) exhibit selectivity for $PI(3,4)P_2$ (So and Fruman, 2012) (Figure 1.3a).

1.4. PI3K PATHWAY EFFECTORS

Conventionally, textbooks represent the PI3K signaling pathway as a linear signal transduction, originating at the plasma membrane, followed by intracellular information transmission via AKT, mTORC1 and their myriad substrates. However, the relative importance of individual effectors can be highly context-dependent, including numerous non-linear feedback and feedforward loops. Such loops are key to understanding so-called adaptive resistance to PI3K pathway inhibitors, a phenomenon that has been studied mostly in the context of non-lymphoid cancers. This should be borne in mind when reading the following generic outline of PI3K signaling effectors, with lymphocyte-specific examples highlighted where relevant. Cell type-specific differences

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notwithstanding, activation of the PI3K pathway typically promotes anabolic metabolism, cell proliferation, survival, and migration (Figure 1.3a). Activation of this pathway can also shape cell fate decisions, either promoting or opposing differentiation depending on context (Madsen, 2020). Collectively, all these cellular processes are dysregulated in cancer, and thus it is not surprising that PI3K signaling is frequently activated across human malignancies.

1.4.1. AKT, FOXO and mTORC1

AKT is comprised of three isoforms – AKT1, 2 and 3 – which exhibit overlapping as well as non-redundant functions. The three isoforms are ubiquitously expressed (Uhlén *et al*., 2015), though relative levels in any given cell type will differ and thus contribute to reported isoform-specific functions (Manning and Toker, 2017). Irrespective of AKT isoform, the PH domain recruits this serine-threonine kinase to sites of PI3K activation, alongside PDK1. The latter is required for AKT activation via direct phosphorylation of T308/T309/T305 (AKT1/AKT2/AKT3) in the activation loop. Maximal activation requires phosphorylation of S473/S474/S472 (AKT1/AKT2/AKT3) in the hydrophobic motif (Manning and Toker, 2017), with mammalian target of rapamycin complex 2 (mTORC2) often considered to be the primary kinase although the exact mechanisms, including the potential for AKT autophosphorylation, may differ depending on cell type and context (Toker and Newton, 2000; Feng *et al*., 2004; Baffi *et al*., 2021).

AKT is suggested to have over 100 substrates and downstream effectors, with the FOXO family of transcription factors and upstream components of mTORC1 among the best studied. Upon AKT-mediated phosphorylation of three conserved residues, FOXO isoforms are excluded from the nucleus and become sequestered by 14-3-3 proteins in the cytosol. AKT thus acts to suppress FOXO transcriptional targets, many of which are involved in induction of apoptosis, cell cycle arrest, catabolism, ROS regulation, and growth inhibition (Manning and Toker, 2017). In lymphocytes, the AKT-FOXO axis is important for regulating cell type-dependent differentiation programs, effector cell activation and trafficking. The exact nature of the cellular response often depends on integration of the strength of the upstream stimulus. For example, mechanistic studies in T cells have revealed that the strength of AKT activation may dictate the pattern of chemokine receptors and adhesion molecules expressed on immune-activated T cells (Waugh *et al*., 2009). In B cells, an important consequence of AKT-mediated FOXO inhibition is suppression of class switch recombination (Omori *et al*., 2006; Sander *et al*., 2015).

AKT leads to activation of the serine/threonine kinase mTOR within mTOR complex 1 (mTORC1), primarily through phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2, also known as tuberin). This phosphorylation inhibits the GAP activity of the TSC complex towards the small GTPase RHEB. In its GTP-bound form, RHEB promotes mTORC1 activation at lysosomes (Liu and Sabatini, 2020). Proline-rich AKT substrate of 40 kDa (PRAS40), a component of the mTORC1 complex, is also an AKT substrate, however it is often considered less important for mTORC1 activation downstream of AKT (Manning and Toker, 2017). Importantly, in lymphocytes, mTORC1 activity can be uncoupled from PI3K/AKT activation and is instead more dependent on nutrient signals such as amino acids (Fruman *et al*., 2017). Once activated, mTORC1 phosphorylates numerous protein substrates, collectively resulting in upregulation of nutrient uptake, cell proliferation, as well as the associated biosynthetic activities including protein, lipid, and nucleotide synthesis (Hukelmann *et al*., 2016; Liu and Sabatini, 2020). Key mTORC1 substrates include the ribosomal S6 kinases and a family of mRNA translation inhibitors known as eIF4E-binding proteins (4E-BPs) (Figure 1.3a). Phosphorylation of the latter by mTORC1 enhances the formation of the eIF4F translational initiation complex required for cap-dependent mRNA translation. Through this mechanism, mTORC1 increases the synthesis of activation-induced cytidine deaminase (AID) in activated B cells, thereby promoting antibody class switching from IgM to IgG and other isotypes (Chiu *et al*., 2019). This is opposite to the overall negative effect of PI3K pathway activation on this process (Omori *et al*., 2006; Sander *et al*., 2015), and serves as an important example of uncoupling between PI3K/AKT and mTORC1 activity.

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1.4.2. TEC tyrosine kinases

In lymphocytes, the TEC family of tyrosine kinases are important mediators of PI3K pathway activation, in addition to AKT. Among these, BTK and ITK are key signal transducers in B and T cells, respectively. Both contain PH domains that are highly selective for PIP_3, in addition to SH2 and SH3 domains that can aid their recruitment to specific protein complexes and sites of PI3K activation at the plasma membrane (Fruman *et al.*, 2017). Within the B cell receptor (BCR) signalosome, PI3Kδ and BTK are required for maximal signaling output, with BTK contributing to activation of phospholipase Cγ (PLCγ), which drives the formation of diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Figure 1.3a). These second messengers are key for triggering Ca^{2+} mobilization as well as activation of NFkB, NFAT and the RAS/MAPK signaling cascade (Fruman *et al.*, 2017). The function of ITK in T cells is similar to that of BTK in B cells (Wang, Hills and Huang, 2015).

1.4.3. Network topology and signal robustness

An appreciation of network topology is central to understanding some of the difficulties associated with successfully targeting the PI3K pathway with available pharmacological inhibitors. Network topology is another term for network structure, which in the context of signal transduction refers to individual signaling components and the connections between them. The dynamic properties of signal transduction networks are thus determined by their topologies, and these are often built by recurrent motifs such as positive and negative feedback loops (Kolch *et al.*, 2015). A feedback loop is a network motif in which a downstream molecule (B) affects the activity of its upstream regulator (A). Negative feedback loops, where B inhibits A, are important not only for attenuating the output of a signaling pathway, but also for conferring robustness to perturbations such as pharmacological inhibitors (Kolch *et al.*, 2015).

Although the PI3K pathway features numerous negative feedbacks, most of them have been uncovered in non-lymphoid settings and rely on molecules that are not necessarily expressed in lymphocytes. Nevertheless, some observations are likely generalizable. Known negative feedbacks within the PI3K pathway operate across a range of time scales: those mediated by post-translational modifications occur within minutes, whereas those involving transcription and translation take hours and even days to be observed. A detailed understanding of fast and slow feedback loops is therefore critically dependent on temporal studies across the relevant time scales.

A common transcriptional feedback loop within the PI3K pathway involves the AKT-FOXO axis, with inhibition of FOXO leading to reduced expression of genes encoding for upstream activators of PI3K signaling (Figure 1.3b). In naïve T cells, this negative feedback operates at the level of IL7Ra, and homing receptor expression, and may act as a protective mechanism against avaricious T cells that would otherwise dominate the peripheral population (Kerdiles *et al.*, 2009; Stone *et al.*, 2015). In T follicular helper (T_{FH}) cells, FOXO1 also controls the expression of inducible T-cell co-stimulator (ICOS) (Stone *et al.*, 2015), a co-receptor that promotes PI3K activation. Similar to their non-lymphoid counterparts (Mukherjee *et al.*, 2021), lymphocytes may also rely on mTORC1 for negative regulation of the upstream PI3K signal via control of PTEN translation – as demonstrated in cytotoxic T lymphocytes (CTLs) (Hukelmann *et al.*, 2016) (Figure 1.3b). The signaling ramifications of breaking this negative feedback loop can be profound. Thus, CTLs treated with an inhibitor of both mTORC complexes, initially exhibit decreased phosphorylation of AKT T308 (PDK1 site) and S473 (mTORC2 site) (Hukelmann *et al.*, 2016). This is consistent with the known ability of S473 phosphorylation to promote docking of AKT to PDK1 – as a result, loss of S473 phosphorylation would also decrease phosphorylation at T308. However, inhibition of mTORC1 activity results in reduced PTEN translation, eventually causing a compensatory increase in PIP_3 levels and a resurgence in AKT T308 phosphorylation, paralleled by re-phosphorylation of FOXO1. Accordingly, catalytic mTOR inhibitors are not efficient at curbing AKT activity in this context, with increased PIP_3 synthesis seemingly bypassing the need for mTORC2-mediated S473 phosphorylation (Hukelmann *et al.*, 2016).

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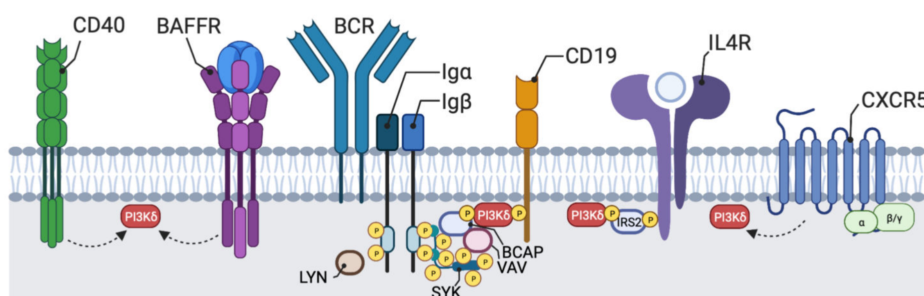
In contrast to their negative counterparts, positive feedback loops enable signal amplification and are often important for driving irreversible cell fate decisions. Positive feedback loops within the PI3K pathway remain relatively enigmatic, although recent studies have begun to characterize them, specifically in a lymphoid context. Pending independent validation, the small antiviral membrane protein IFITM3 was recently found to function as a scaffold for PIP_3 and mediator of a PI3K signaling amplification loop in B cells (Lee *et al.*, 2020). Moreover, a study of $CD8^+$ effector T cells has also demonstrated a positive feedback loop between PI3K-driven glycolytic activity and subsequent ATP-mediated enhancement of PI3K activation (Xu *et al.*, 2021).

1.5. DYNAMIC PI3K SIGNALING IN LYMPHOCYTE BIOLOGY

The following sections contain important examples of dynamic PI3K pathway regulation in B and T cells, illustrating how the differential usage and wiring of the PI3K "hardware" translates into a highly specialized cellular "software", appropriate for the task at hand. The attentive reader will not fail to notice that corruption of this software is what underpins the myriad immune system pathologies of aberrant PI3K activity, as well as the challenges faced upon pharmacological targeting of this pathway.

Figure 1.4. In both B (a) and T (b) lymphocytes, PI3Kδ is recruited to signalosomes formed upon clustering of the B-cell receptor (BCR) and T-cell receptor (TCR), respectively. A phosphorylation cascade is triggered by SRC-like non-receptor tyrosine kinases (LYN in B cells, LCK in T cells), followed by SYK (B cells) and ZAP70 (T cells) – members of the SYK tyrosine kinase family. The BCR co-opts the co-receptor CD19 or the adaptor protein BCAP for recruitment of PI3Kδ via interactions of p85 with YXXM phosphotyrosines within these molecules. How exactly PI3Kδ gets recruited to the TCR signalosome remains under investigation (Luff et al., 2021). In both B and T cells, various co-receptors (e.g., CD40, OX40), cytokine (e.g., IL4R) and chemokine receptors (e.g., CXCR5) also contribute to PI3K pathway activation as indicated. IL2 was previously thought to induce PI3Kδ activation in T cells, however more recent work in CTLs has forced a revision of this model, and it has therefore been omitted from this diagram (Ross et al., 2016). While PI3Kδ is shown as the main isoform responsible for PI3K signaling initiation downstream of antigen-dependent receptor activation, other PI3K isoforms can be involved depending on context. For example, chemokine receptors activate PI3Kδ in B cells but PI3Kγ in T cells. Created with BioRender.com



a. PI3Kδ activation in B cells.

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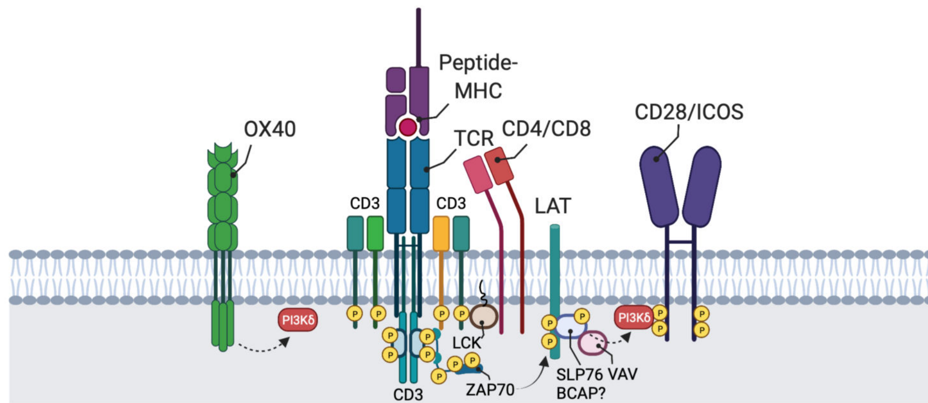

b. PI3Kδ activation in T cells.

1.5.1. B cell development and survival

The PI3K pathway is essential for the survival of pre-B and mature B cells (Srinivasan *et al.*, 2009; Ramadani *et al.*, 2010). In murine pre-B cells, both PI3Kα and PI3Kδ have been shown to contribute PI3K activity, with important roles in the dynamic suppression of *Rag* expression and VDJ recombination during B cell development (Ramadani *et al.*, 2010). Moreover, PI3Kα appears able to substitute for PI3Kδ inactivation in the context of tonic BCR signaling, which is likely to be sufficient to allow the development and survival of follicular B cells (Srinivasan *et al.*, 2009). In contrast, PI3Kδ is essential where BCR crosslinking is involved (Figure 1.4a), for example in the development of B1 and marginal zone B cells, and for overall antigen-dependent activation of mature B cells (Fruman *et al.*, 1999; Suzuki *et al.*, 1999; Clayton *et al.*, 2002; Jou *et al.*, 2002; Okkenhaug, 2002; Oak *et al.*, 2009; Ramadani *et al.*, 2010). Thus, peripheral B cell maturation mainly depends on the p85α-p110δ heterodimer as the predominant PI3K isoform, consistent with the immunodeficiency phenotypes observed in people with pathological PI3Kδ activation, caused either by mutations in *PIK3CD* (p110δ) or *PIK3R1* (p85α/p55α/p50α) (see below and (Lucas *et al.*, 2016)).

Adequate regulation of the magnitude and duration of PI3K signaling throughout B cell development averts the generation, activation, and persistence of abnormal cells, including autoreactive clones. Conversely, sustained PIP_3 signaling during B cell development converts what is normally a tolerogenic response into a mitogenic response, resulting in impaired tolerance induction (Browne *et al.*, 2009). Moreover, while baseline survival of resting mature B cells depends on tonic BCR signaling through the PI3K-AKT-FOXO1 branch, on its own this is not sufficient and requires engagement of additional signaling pathways via co-receptors such as BAFFR (Srinivasan *et al.*, 2009). Pathological activation of PI3Kδ enhances the survival of B cells, likely by amplifying or mimicking such crosstalk (Preite *et al.*, 2019).

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1.5.2. The germinal center (GC) reaction

Appropriate B cell selection is critical during the GC reaction. The GC is the site where B cells undergo somatic hypermutation (SMH), affinity maturation, and class-switch recombination, ultimately leading to positive selection of B cells capable of producing high-affinity IgG antibodies. Given its importance for humoral immunity, GC dysregulation is associated with immunodeficiency, autoimmune disease, and cancer. For example, B cell-derived lymphomas, the most common lymphoid malignancies, typically originate from GC or post-GC B cells, as indicated by their somatically mutated immunoglobulin genes (Klein and Dalla-Favera, 2008). Correct function of the GC depends on its patterning into light (LZ) and dark (DZ) zones, in addition to exquisite spatiotemporal control of interactions between B cells and $CD4^+$ T_{FH} cells within these topologically and functionally distinct areas (Figure 1.5).

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Compared to naïve B cells, physiological BCR-mediated PI3K signaling is attenuated in GC B cells, including both lower magnitude and less sustained activation. Moreover, CD40 ligation by T_{FH} cells no longer contributes to PI3K activation in GC B cells, in contrast to their naïve counterparts. This attenuated PI3K signal is nevertheless sufficient for the selective inactivation of FOXO1 downstream of AKT-mediated phosphorylation (Luo, Weisel and Shlomchik, 2018). Its inactivation downstream of PI3K/AKT thus licenses GC B cells to enter and/or stay in the LZ where they interact with T_{FH} cells and undergo positive selection. Here, dual BCR and CD40 engagement is required for induction of c-MYC expression and S6 phosphorylation (Luo, Weisel and Shlomchik, 2018), ultimately coordinating cell cycle reentry and anabolic metabolism for successful positive selection (Ersching *et al.*, 2017). This is a biphasic and transient regulatory phenomenon, with positively selected GC B cells either undergoing differentiation to plasma and memory cells, or re-entering the DZ for another cycle of somatic hypermutation and proliferation. The sequence of these events is critically dependent on the correct spatiotemporal activation of PI3K signaling versus FOXO1 (Dominguez-Sola *et al.*, 2015; Sander *et al.*, 2015) (Figure 1.5). An essential step in the selection of cells expressing high-affinity antibody mutants is their recruitment from the GC LZ back into the DZ. This cyclical process depends on the timely expression of the chemokine receptor CXCR4. In mice, enforced PI3K pathway activation or FOXO1 ablation results in failure to express CXCR4, thus disrupting the GC architecture and impairing positive selection (Dominguez-Sola *et al.*, 2015; Sander *et al.*, 2015). Moreover, CSR and affinity selection but not SHM is selectively hampered in these cells (Dominguez-Sola *et al.*, 2015; Sander *et al.*, 2015). Thus, getting the PI3K signaling balance in GC B cells right is essential for functional humoral immune responses.

Similarly, the dynamic regulation of PI3K versus FOXO1 activity underpins the successful development of mature, resting B cells from their pre-B cell counterparts (Okkenhaug, 2013). The picture that emerges is one where PI3K pathway activation can promote either cellular proliferation, survival and/or differentiation, depending on the stage of B cell development and the strength of the stimulus.

1.5.3. T_{FH} cell function

Like the BCR in B cells, the TCR in T cells is typically the focal point of PI3K activation, augmented further by co-receptors such as CD28 and ICOS (Figure 1.4b). ICOS expression increases on activated T cells and is particularly important for differentiation of the follicular B helper T (T_{FH}) subtype, a T cell population that drives and sustains GC formation (Figure 1.5). This function depends on PI3Kδ activation and the timely augmentation of helper cytokine expression, including IL21 and IL4 (Gigoux *et al.*, 2009; Rolf *et al.*, 2010). Consequently, knock-in mice with selective loss of p85 binding to ICOS present with severe defects in T_{FH} cell generation, GC reaction, antibody class switching and affinity maturation (Gigoux et al. 2009). Moreover, several transcription factors controlling T_{FH} cell differentiation and function are modulated by PI3K signaling. Among these, the master T_{FH} transcription factor, BCL6, is under direct repression by FOXO1, and is upregulated upon PI3K/AKT activation (Stone *et al.*, 2015). Finally, both mTORC2 and mTORC1 are required for T_{FH} cell differentiation downstream of ICOS engagement, enabling the coordination of anabolic metabolism, proliferation, and transcriptional activity in response to immune signals (Yang *et al.*, 2016; Zeng *et al.*, 2016). While these studies support the notion that PI3K activation promotes T_{FH} cell differentiation, it is worth emphasizing some of the parallels to GC B cells when it comes to balancing FOXO1 activation and inactivation. To initiate T_{FH} cell differentiation, PI3K signaling and FOXO1 inactivation must take place transiently, according to a poorly understood temporal code, whereas final specification of GC-T_{FH} cells appears to occur in a FOXO1-dependent manner (Stone *et al.*, 2015).

1.5.4. Naïve and effector T cells

Consistent with the notion that PI3K/AKT activation does not simply function as an on/off switch, its signaling strength has emerged as important in determining the expression pattern of chemokine receptors and adhesion molecules on immune-activated T cells (Waugh *et al.*, 2009). Whereas relatively weak PI3K/AKT activation is

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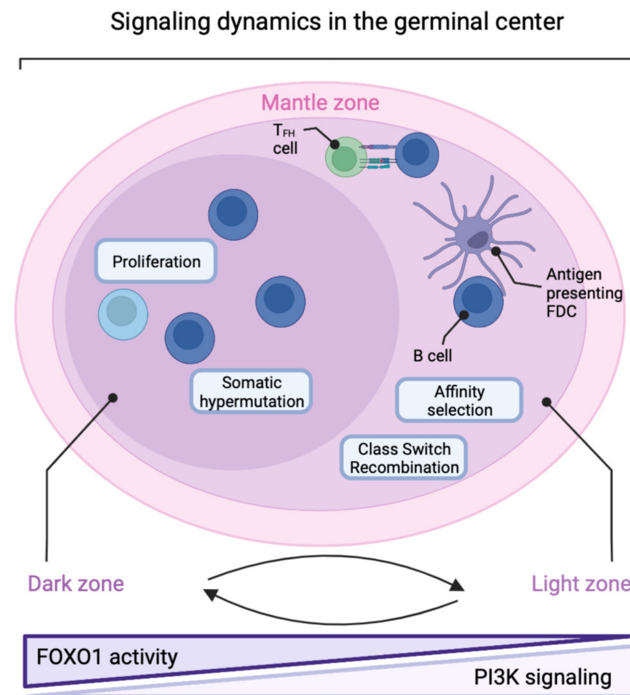

Figure 1.5. The germinal center is a transient microanatomical structure in which mature B lymphocytes under repeated rounds of clonal expansion and genetic diversification of their immunoglobulin genes, ultimately resulting in the generation of high-affinity B cells destined to become either memory B cells or plasma cells. The GC is organized into two topologically and functionally distinct compartments known as the dark zone (DZ) and the light zone (LZ). Proliferative expansion and Ig somatic hypermutation (SHM) occur in the DZ, whereas affinity selection and class-switch recombination take place in the LZ where B cells expressing high-affinity antibodies interact with follicular dendritic cells (FDCs) and T follicular helper (T_{FH}) lymphocytes. As part of this process, B cells undergo repeated cycles of LZ-DZ migration, with B cells in the different zones exhibiting a characteristic PI3K signaling polarity. The transcription factor FOXO1 is essential for the development of the proliferative GC DZ, consistent with low or absent PI3K activity in this area (Dominguez-Sola et al., 2015; Sander et al., 2015). FOXO1 instructs the DZ gene program directly and by licensing the activity of BCL6, a GC master regulator (Dominguez-Sola et al., 2015). Created with BioRender.com

compatible with T-cell survival and proliferation, strong and sustained activation is required to downregulate homing of naïve T cells to secondary lymphoid tissues *in vivo* (Finlay *et al*., 2009; Waugh *et al*., 2009). As a result, loss of PI3Kδ or AKT activity impairs T cell exit from lymphoid organs and migration to peripheral sites of infection (Liu and Uzonna, 2010; Macintyre *et al*., 2011).

Unbiased comparisons of PI3Kδ signaling in naïve CD8$^+$ T cells versus CTLs have further emphasized the importance of context. Both the quality and the quantity of the PI3Kδ response differs in these otherwise closely related cell types (Spinelli *et al*., 2021). For example, naïve CD8$^+$ cells but not their effector cytotoxic counterparts show altered expression of cytolytic effector molecules (decreased) and ICOS (increased) upon PI3Kδ inhibition. Furthermore, many more TCR-regulated transcripts depend on PI3Kδ activity in naïve T cells than in CTLs (Spinelli *et al*., 2021). Among other changes, PI3Kδ inhibition in CTLs enhances gene expression of three important inhibitory receptors: *Ctla4*, *Slamf6* and *Lag3*. Not all of these and other transcriptional changes in PI3Kδ-inhibited CTLs are mediated by the AKT/FOXO1-axis, with evidence for a substantial contribution by PI3Kδ-dependent activation of the RAS/MAPK signaling pathway (Spinelli *et al*., 2021). When it comes to understanding the effects of pharmacological PI3K inhibition in cancer, one important conclusion from his work is that the dominant targets for PI3Kδ in both naïve and effector CD8$^+$ T cells are the secreted molecules and cell surface receptors that orchestrate paracrine communication between CD8$^+$ T lymphocytes and other immune cells (Spinelli *et al*., 2021).

Other large-scale and in-depth analyses of context-dependent PI3K signaling are also emerging as the required omics technology matures. A recent study looked at differences in the p110δ interactome in murine, primary

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naïve $CD4^+$ T cells and in differentiated T cell blasts, demonstrating substantial differences in adaptor protein usage and a myriad of previously documented p110δ interactors (Luff *et al.*, 2021), indicating that these complexes might be more extensive than has been appreciated to date. Moving forward, such studies will be instrumental for improving current understanding of how the PI3K pathway fine-tunes immunological responses, a prerequisite for the optimal usage of PI3K-targeted therapies.

1.6. LESSONS FROM MONOGENIC DISORDERS

While experimental models based on cultured cells and research animals are instrumental for dissecting the detailed cellular and organismal effects of PI3K signaling, their relevance must always be evaluated against direct evidence from human physiology. In contrast to cancers which often harbor numerous genetic and epigenetic changes, human diseases caused by single-gene mutations are powerful experiments of nature, against which to calibrate results from experimental model systems. The importance of quantitative control of PI3K signaling in immune cells is therefore best illustrated by the discovery of human immunodeficiencies caused by either loss- or gain-of-function mutations in the PI3Kδ enzyme (Lucas *et al.*, 2016).

1.6.1. Genetic PI3Kδ inactivation

Biallelic loss-of-function mutations in either p85α or p110δ have been reported in rare cases of human immunodeficiency. The affected individuals present with B cell lymphopenia, hypo- or agammaglobulinemia, recurrent infections, and several autoimmunity-related pathologies (Conley *et al.*, 2012; Zhang *et al.*, 2013; Tang *et al.*, 2018; Swan *et al.*, 2019). These observations are important for several reasons.

Firstly, the loss of p85α is selective, with p55α and p50α isoform expression from the same allele (*PIK3R1*) remaining intact, yet p110δ expression was shown to be markedly lower in primary hematopoietic cells (T cells, neutrophils, dendritic cells) from one of the examined patients (Conley et al. 2012). Loss of p85α expression would manifest in most if not all cells in the body, yet rather remarkably the clinical phenomenology in these patients is mainly caused by B cell defects. This suggest that human B cells are selectively dependent on the p85α-p110δ heterodimer, with the remaining isoforms unable to compensate. Moreover, primary B cells from healthy controls have been shown to only express p85α and not p55α/p50α, which may explain the selective dependence of human B cells on the p85α isoform (Conley *et al.*, 2012).

Secondly, the developmental B cell block in individuals with loss-of-function of PI3Kδ is far more severe than expected from the respective mouse models (Fruman *et al.*, 1999; Suzuki *et al.*, 1999; Clayton *et al.*, 2002; Jou *et al.*, 2002; Okkenhaug, 2002; Oak *et al.*, 2009; Ramadani *et al.*, 2010), a clear example of species-specific differences and the need for calibration against human biology. It is worth noting, however, that although the clinical case reports mainly emphasize the primary B cell defect, the human autoimmunity phenotypes associated with PI3Kδ loss-of-function may be caused by dysfunctional T regulatory (T_{REG}) cells given substantial evidence that PI3K signaling is required for $FOXP^+$ T_{REG} cell homeostasis and function in mice (Patton *et al.*, 2006; So and Fruman, 2012). Thus, PI3Kδ-deficient mice develop colitis due to inappropriate suppression of effector T cells by their regulatory counterparts (Okkenhaug, 2002), consistent with colitis being a frequent side-effect of PI3Kδ inhibitors tested in the clinic (Lucas *et al.*, 2016).

1.6.2. Genetic PI3Kδ hyperactivation

Immunodeficiency can also arise from heterozygous mutations that hyperactivate the PI3Kδ enzyme. These mutations are found either in the *PIK3CD* (p110δ) or *PIK3R1* (p85α/p55α/p50α) gene, giving rise to the clinical entities APDS1 and APDS2, respectively. APDS stands for Activated PI3K Delta Syndrome, an autosomal dominant combined immunodeficiency, featuring functional and developmental defects in both T and B cells (Angulo *et al.*, 2013; Lucas *et al.*, 2014a; Lucas *et al.*, 2014b; Deau *et al.*, 2014). It is notable that these diseases largely phenocopy each other, given that the ubiquitous expression of the *PIK3R1* gene and the ability of p85α/

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p55α/p50α to pair with all three class IA catalytic subunits would have suggested more widespread organismal dysfunction due to potential activation of all three class IA p110 subunits (Figure 1.1). Structural biology work has demonstrated that p110δ is activated more strongly by the most common APDS2 *PIK3R1* mutation (Dornan *et al.*, 2017), an exon-skip variant resulting in a deletion in the iSH2 domain of p85α/p55α/p50α, thus offering a potential explanation for the selective immune cell defects in this context. Recent quantitative analyses have also shown a preferential association of p110δ with p85α over p85β in mouse embryonic fibroblasts, spleen and bone marrow extracts (Tsolakos *et al.*, 2018).

Collectively, APDS phenotypes mimic an exaggerated version of the normal biology of PI3Kδ activation in immune cells. Patients often present with recurrent infections, hypogammaglobulinemia (often with increased IgM levels), reduced class-switch memory B cells and impaired vaccine responses, an increase in transitional B cells and an increase in effector T cells (particularly the T_{FH} subset) (Lucas *et al.* 2016). Freshly isolated peripheral blood cells from APDS patients are prone to apoptosis upon TCR restimulation – in stark contrast to the pro-survival phenotype typically associated with PI3K pathway activation. Moreover, $CD8^+$ T cells from APDS patients have reduced telomeres and increased expression of senescence markers, suggestive of functional exhaustion. This phenotype is poorly mimicked in available mouse models owing to their long telomeres (Lucas *et al.* 2016). Finally, benign lymphoproliferation (lymphadenopathy, hepatosplenomegaly and focal nodular lymphoid hyperplasia) is a common feature of APDS, in addition to increased risk of lymphoma (Lucas *et al.* 2016). A milder form of APDS-like immunodeficiency has also been observed in patients with Cowden disease, caused by heterozygous loss of the tumor suppressor *PTEN* (Lucas *et al.* 2016).

1.7. CORRUPTED PI3K SIGNALING IN CANCER

Large scale, multi-omic molecular profiling by The Cancer Genome Atlas (TCGA) has enabled identification of the most frequently altered cell signaling pathways in malignancy. Consistently, the PI3K signaling pathway tops the list in solid tumors, predominantly due to activating mutations/copy number changes in *PIK3CA* (p110α), or inactivation of *PTEN* (Zhang *et al.*, 2017; Sanchez-Vega *et al.*, 2018). In contrast, genetic PI3K pathway activation is rarely observed in hematological malignancies, with few notable exceptions. Pediatric T-acute lymphoblastic leukemia (T-ALL) features relatively frequent somatic alterations in *PIK3R1*, and less commonly in *PIK3CA* and *PIK3CD* (Ma *et al.*, 2018). Increased PI3K pathway activation in T-ALL is common and may also arise from post-translational inactivation of PTEN lipid phosphatase activity due to high levels of ROS (Silva *et al.*, 2008). Moreover, loss of PTEN expression has been found in over 50% of germinal center B-cell-like (GCB) diffuse large B cell lymphomas (DLBCL), compared to less than 15% of non-GCB DLBCL patient samples (Pfeifer *et al.*, 2013). Accordingly, GCB DLBCLs are addicted to PI3K pathway activation for their survival (Pfeifer *et al.*, 2013).

Despite the relatively small number of direct (epi)genetic alterations in the PI3K pathway in blood cancers, it is well-established that leukemic cells hijack its functionality for enhanced survival, uncontrolled proliferation and reprogramming of their microenvironment. Similar to their normal counterparts, PI3K signaling in leukemic cells is mainly mediated by PI3Kδ, with a subset of B cell malignancies showing particular dependence on constitutive BCR signaling via PI3Kδ. It is worth noting that cancer cells may corrupt both positive and negative branches of the PI3K pathway to enhance their survival, with FOXO1 presenting as a prominent example. In contrast to loss-of-function FOXO1 mutations in a variety of solid tumors and Hodgkin's lymphoma, a significant fraction (10-50% depending on subtype) of GC-derived, aggressive variants of B-cell non-Hodgkin lymphomas (B-NHL) present with recurrent pro-oncogenic missense mutations that partially disrupt FOXO1 inactivation downstream of PI3K/AKT while also reducing the affinity of FOXO1 for specific target genes (Roberto *et al.*, 2021). Due to transcriptional rewiring and disruption of signaling feedback, mutant B cells with oncogenic FOXO1 activity also exhibit concomitant hyperactivation of the PI3K/AKT and stress-activated protein kinase (SAPK) pathways, a protective mechanism that promotes cancer cell stress resistance (Roberto *et al.*, 2021). Consistent with FOXO1's critical role in the physiological GC response reaction, expression of the B-

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NHL-associated oncogenic FOXO1 mutations in mice confers competitive advantage to mutant B cells by mimicking the positive selection signals that promote GC B cell expansion (Roberto *et al.*, 2021).

1.7.1. The success of PI3Kδ inhibition in lymphoid malignancies

The prominent role of PI3K signaling in cancer maintenance and progression inspired the development of numerous PI3K-targed inhibitors (Castel *et al.*, 2021b; Vanhaesebroeck *et al.*, 2021). However, the ubiquitous requirement for this pathway in normal cell biology and organismal function presents a formidable challenge for the successful application of PI3K-targeted therapy in the clinic. While some issues can be addressed by the development of highly isoform-selective inhibitors, there are still substantial toxicity if the targeted isoform is as ubiquitously expressed as the cancer-associated p110α. It is therefore not surprising that most success in this area has been in the context of hematological malignancies and selective inhibitors of p110δ. The anti-cancer efficacy of these agents can be attributed both to cell-intrinsic and cell-extrinsic mechanisms, as demonstrated in studies of chronic lymphocytic leukemia (CLL) and follicular lymphoma (FL) (Vanhaesebroeck *et al.*, 2021). Thus, in addition to a cancer-selective pro-apoptotic effect, PI3Kδ inhibition also alters cytokine and chemokine production both by CLL cells and the associated stromal cells, thereby influencing the microenvironmental stimuli that support cancer growth (Herman *et al.*, 2010; Hoellenriegel *et al.*, 2011; Okkenhaug and Burger, 2016). Consistent with the normal function of the PI3K pathway in lymphocyte migration and homing, PI3Kδ inhibition in CLL triggers an early redistribution of the cancer cells from their protective tissue environment and into the blood, leading to rapid and sustained lymph node size reduction and a transient lymphocytosis (Furman *et al.*, 2010). A similar effect is observed in patients treated with inhibitors of SYK, BTK, and mTOR, in line with a shared signaling mechanism of action (Okkenhaug and Burger, 2016). It has also been suggested that some of the beneficial clinical activity of PI3Kδ inhibitors such as idelalisib (Zydelig) may be due to breaking of T_{REG}-mediated immune tolerance, as demonstrated in mouse models of solid tumors (Ali *et al.*, 2014; Lim *et al.*, 2018).

Although PI3Kδ is considered the functionally dominant isoform in lymphocytes, the context-dependent contribution of other PI3K isoforms should not be ignored. For example, inhibition of the other leukocyte-enriched isoform, PI3Kγ, has been shown to reduce CLL cell adhesion to stromal cells to an extent similar to that observed with the PI3Kδ inhibitor idelalisib, with the dual and clinically-approved PI3Kδ/γ inhibitor duvelisib giving rise to a greater reduction in CLL migration compared to the use of the respective single isoform-selective inhibitors (Ali *et al.*, 2018). Conversely, PI3Kδ inhibition has been shown to elicit rapid reactivation of BCR signaling via PI3Kα in cell line models of the activated B cell-like (ABC) subtype of DLBCL, arguing for evaluation of dual PI3Kα/δ inhibition in this context (Pongas, Annunziata and Staudt, 2017). Co-targeting of PI3Kα/δ has also been suggested for a subset of mantle cell lymphomas with increased expression of the p110α protein (Iyengar *et al.*, 2013). Moreover, a subgroup of B-ALL have a gene expression profile similar to normal pre-BCR^{+} B cells (pre-B ALL) and exhibit an exquisite dependence on SYK for PI3K pathway activation and proliferation (Köhrer *et al.*, 2016). Given evidence from mice that normal pre-B cells rely both on PI3Kα and PI3Kδ (Ramadani *et al.*, 2010), dual inhibition of these isoforms may also be relevant in this context. However, in addition to the considerations outlined in the subsequent section, a caveat of this approach relates to the substantial side-effects associated with systemic PI3Kα inhibition, with on-target metabolic toxicities as the most prominent (Castel *et al.*, 2021b; Vanhaesebroeck *et al.*, 2021). Finally, primary resistance to PI3Kδ inhibition in CLL has been linked to the emergence of activating mutations in the RAS/MAPK pathway which can be targeted by available pharmacological agents such as Trametinib (Murali *et al.*, 2018).

It is rare for cancer cells to be so reliant on a single signaling pathway and PI3K isoform as observed in some of the aforementioned B cell malignancies. It therefore remains to be established whether PI3Kδ inhibitors will show similar efficacy in the context of other hematological cancers with PI3K pathway hyperactivation, for example ALL (Sanchez *et al.*, 2019). PI3Kδ inhibitors are also receiving attention as potential immunomodulatory agents in solid tumors (Vanhaesebroeck *et al.*, 2021).

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1.7.2. Quantitative biology and therapeutic considerations

While it is true that constitutive PI3K pathway activation is a common and often necessary event in malignancy, cancer cells must still ensure control of the exact magnitude and kinetics of this activation for optimal survival. Substantial evidence, particularly in immune cells, suggests that overactivation of oncogenic signaling can be deadly in certain contexts, leading to paradoxical rescue of cancer cells upon treatment with the targeted inhibitors that were designed to kill them (Madsen & Vanhaesebroeck 2020). Thus, pharmacological hyperactivation of pre-BCR signaling components, including SYK, engages the deletional checkpoint for removal of self-reactive B cells and selectively kills the cancer cells in a mouse model of Ph^+ B-ALL (Chen *et al.*, 2015). Similarly, even modest dose reduction of *Pten* in mouse models of pre-B ALL has been shown to result in rapid cancer cell death and clearance of leukemia from transplant recipient mice. This toxicity is specific to pre-B ALL cells and is attributable to PI3K pathway hyperactivation (Shojaee *et al.*, 2016), consistent with the physiological function of this pathway in determining the outcome of early B cell selection in the bone marrow. It therefore appears that pre-B ALL cells cannot tolerate PI3K pathway activity beyond a specific threshold, in line with lack of evidence for activating, genetic hits within the PI3K pathway in human pre-B ALL cohorts (Shojaee *et al.*, 2016).

Recent work in mice has also demonstrated that terminally differentiated B cells, or plasma cells (PCs), with an APDS-related activating PI3Kδ variant have compromised survival due to disrupted endoplasmic reticulum proteostasis and autophagy (Al Qureshah *et al.*, 2021). Mechanistically, this is caused mainly by increased mTORC1 activity, which promotes protein synthesis and inhibits autophagy. Given their ability to produce and secrete high levels of antibodies, PCs already have a high baseline protein synthesis load, and have developed unique mechanisms to deal with the resulting ER stress in a physiological context. These mechanisms fail in the face of genetic PI3Kδ activation, thus compromising survival upon secondary antigen challenge (Al Qureshah *et al.*, 2021).

The counterintuitive observation that overactivation of an otherwise oncogenic component can elicit cell death is not new, and is best established for the oncoprotein MYC. Within a limited expression range unique to each tumor, MYC appears to have a paradoxical, pro-apoptotic function (Harrington *et al.*, 2021). Given evidence that the PI3K pathway activation can boost MYC protein levels in B cell malignancies, it has been suggested that transient manipulation of the pathway may allow for MYC stabilization beyond a threshold compatible with cancer cell survival (Harrington *et al.*, 2021).

Finally, the notion of intermittent pharmacological inhibition is gaining traction, with evidence that PI3K-targeted inhibitors administered in this manner lead to fewer adverse effects while retaining their therapeutic efficacy (Vanhaesebroeck *et al.*, 2021). A better quantitative understanding of PI3K signaling dynamics across biological contexts will be necessary to enhance the rational implementation of this therapeutic strategy (Madsen and Vanhaesebroeck, 2020).

1.8. CONCLUDING REMARKS

Studies of PI3K signaling, particularly in immune cells, support the notion that regulation of this pathway must follow the Greek maxim "nothing in excess". Normal cell physiology thus requires a golden mean of PI3K signaling, with both too much and too little resulting in pathology. The challenge now is to determine this golden mean in any given cell type and (patho)physiological context, and to integrate the resulting knowledge in predictive models that can guide the optimal use of PI3K-targeted therapies. While conventional reductionist approaches have been tremendously useful for mapping the individual components of the PI3K pathway, on their own the resulting maps fall short of predicting the extensive non-linear and often unintuitive information transmission that governs PI3K signaling output. Moving forward, such understanding will likely be led by a

combination of the new and the old – a symbiotic relationship between emerging computational modeling approaches and unbiased experimental studies of the complex perturbation-response relationship.

Whether studying this pathway in normal or cancer cells, however, two key conclusions apply: 1) both PI3K signaling quality (interaction partners, effectors) and quantity (strength, kinetics) differ across contexts; 2) rather than as a simple ON/OFF switch, the PI3K pathway should be viewed as a highly sensitive dial, whose accurate tuning is a prerequisite for a given cell's optimal function.

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