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PI3Ks IN LYMPHOCYTE SIGNALING AND DEVELOPMENT

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Abstract

Lymphocyte development and function are regulated by tyrosine kinase and G-protein coupled receptors. Each of these classes of receptors activates PI3K. Here we summarize current understanding of how PI3K contribute to key aspects of the adaptive immune system.

2 Introduction

The adaptive immune response protects the host from recurring infections by viruses, bacteria and parasites. The enormous diversity and exquisite specificity of the antigen receptors expressed by B cell and T lymphocytes ensures that any pathogen can be recognized while clonal expansion and the generation of long-lived memory cells ensures effective clearance and, in many cases, life-long immunity from re-infection by the same pathogen. In addition, natural killer (NK) lymphocytes provide more immediate protection and can, in some cases, serve as a back-up mechanism to kill pathogens that evade immune recognition by T cells by suppressing major histocompatibility complex (MHC) expression. The diversity of the antigen receptor repertoire is generated by homologous recombination of gene components in B cell and T cell precursors. After gene recombination, each clone is selected so that clones bearing non-functional or autoreactive antigen receptors are eliminated. In the case of T cells, clones bearing antigen receptors capable of recognizing MHC structures are positively selected. This system originally evolved in teleost fishes and is required for protection against a wide variety of common pathogens, many of which have co-evolved mechanisms to evade the adaptive immune response. The extreme susceptibility of severe combined or acquired immune deficiency patients to common infections illustrates how dependent we are on the adaptive immune responses for survival. However, the evolution of the adaptive immune system has also come at a cost, particularly in long-lived animals such as humans. Self-nonself discrimination is an imperfect process and several common autoimmune diseases are caused by autoreactive T cells and/or B cells. In addition, the gut, vaginal tract, respiratory tract and skin are host to a rich microbial flora of mostly harmless or beneficial microbes. Inappropriate activation of the immune system against commensal bacteria causes unwarranted inflammation and disease. Moreover, the immune system can respond inappropriately to innocuous substances such as pollen and cause allergic reactions. Finally, the rejection of transplanted organs is mediated by the adaptive

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immune response. Therefore, therapeutic strategies are aimed at dampening unwanted immune responses without rendering the patient unprotected from infections.

Currently, the most widely used immunosuppressive drugs are corticosteroids, cyclosporine, and cytotoxic or cytostatic drugs such as methotrexate or rapamycin. In addition, various monoclonal antibody therapies have been developed; the most widely used of these have been the anti-tumor necrosis factor-α drugs used to treat rheumatoid arthritis and other autoimmune diseases [1]. Abatacept (CTLA4-Ig) and Rituximab (anti-CD20) are more recent examples of agents that target T cells and B cells specifically and help treat autoimmunity [2, 3]. Yet, there remains significant clinical need for additional drugs that either suppress or modulate adaptive immune responses as not all patients respond favorably to currently available drugs. The phosphoinositide 3-kinase (PI3K) enzymes, which are the focus of this series, have received considerable interest in this context [4, 5]. PI3Ks play central roles in the signaling not only by antigen receptors, but also by various costimulatory, cytokine and chemokine receptors that control lymphocyte biology [6, 7]. In general, the PI3K isoforms p110α and p110β transmit signals from tyrosine kinases and G protein coupled receptors (GPCRs), respectively. However, in lymphocytes, tyrosine-kinase associated receptors signal primarily through p110δ whereas GPCRs signal primarily through p110γ, although there are notable exceptions that we will highlight in this review. Since p110δ and p110γ are expressed at low or undetectable levels in most organs, inhibitors against p110δ and p110γ should, in theory, be selective for the immune system and non-toxic. In this chapter, we will consider the roles played by PI3K during development and activation of lymphocytes and also consider both the advantages and liabilities associated with pharmacological targeting of these PI3K isoforms.

Our understanding of the roles of PI3Ks in lymphocyte biology has been advanced both by the development of small molecule inhibitors that target the PI3Ks with high selectivity, and by gene mutation through homologous recombination in mice (for a list of gene targeting studies, see table 1). Initial studies demonstrated that B cell and T cell proliferation could be blocked by the broad-spectrum PI3K inhibitors wortmannin and LY294002 [8]. However, these compounds have many off-target effects and a specific role for PI3K could therefore not always be established unequivocally. The demonstration that p85α knockout mice showed impaired B cell development and humoral immune responses provided the clear evidence that PI3Ks are intimately involved in signal transduction by the B cell antigen receptor (BcR) [9, 10]. These phenotypes were also evident in mice where p110δ had been inactivated by deletion or by point mutations in the genes [11-13]. Thus a picture emerged, in which p85α would recruit p110δ to antigen and coreceptor complexes at the plasma membrane and that the PI3K activity thus generated was essential for B cell proliferation. The situation for T cells was more complicated. While p85α was dispensable for normal T cell proliferation and cytokine production, p110δ did contribute to these responses - especially when T cells were stimulated with peptide-MHC rather than antibodies [12, 14]. This apparent discrepancy was in part resolved subsequently when it was demonstrated that p85α and p85β play mutually redundant roles in transmitting signals from the T cell antigen receptor (TcR) [15]. Nonetheless, in terms of development and proliferative responses by purified cells, the T cells appeared to be less severely affected by PI3K deficiency than B cells. However, while the development of various mature subsets and their proliferative

responses are the most immediately obvious measurements to make, both T cells and B cells live complicated lives and recently a more detailed appreciation of how PI3Ks can not only enhance, but also suppress certain B cell and T cell responses. The challenge of understanding the apparently contradictory roles of PI3K in adaptive immune function is a significant one. Understanding of these roles is going to have a significant impact on the development of small molecules against the PI3Ks expressed in lymphocytes.

3 PI3K in B cells

3.1 B cell development

B cells develop from lymphoid progenitors in the fetal liver and bone marrow [16]. At an early stage, a lineage split occurs resulting in two general subsets: B-1 B cells that reside in body cavities, and B-2 B cells that reside in secondary lymphoid organs. The B-2 subset is further sub-divided into marginal zone (MZ) B cells, which occupy a unique anatomical and functional niche in the marginal zone of the spleen, and follicular (FO) B cells, which recirculate through blood, lymph and secondary lymphoid tissues. Mice lacking p85α or p110δ have markedly fewer B-1 and MZ B cells as well as reduced numbers of FO cells [9-13, 17, 18].

Developing B cells undergo an ordered series of gene rearrangement steps [19]. First, heavy chain rearrangement occurs at the pro-B cell stage. If successful, light chain rearrangement proceeds at the pre-B cell stage. Cells that make productive heavy and light chain rearrangements express surface IgM and are classified as immature B cells. These cells are then screened for self-reactivity in the bone marrow and spleen, and autoreactive cells are either anergized, deleted, or induced to re-express recombination-activating gene (RAG) proteins and undergo receptor editing. In the absence of self-antigen, immature B cells suppress RAG expression via tonic BcR signaling and advance to the mature B cell state. Mice lacking p85α or p110δ have reduced numbers of cells making it through the pro-B to pre-B transition, as well as defects in tonic signaling at the immature stage [9-12, 20, 21]. In addition, PI3K inhibitors can lead to de-differentiation of immature B cells in vitro [22].

Activation of naive B cells by foreign antigen, in the presence of T cell help or Toll-like receptor (TLR) costimulation, results in clonal expansion and differentiation. Some activated B cells develop rapidly into antibody-secreting plasma cells to provide an early source of antibody. Other B cell clones continue differentiation within the germinal centers within lymphoid follicles, where the antibody constant region is changed by isotype switching, and the variable region is modified by somatic hypermutation. B cells surviving the germinal center reaction then develop either into plasma cells secreting high affinity antibody, or memory B cells capable of mounting rapid secondary responses. B cells lacking p85α or p110δ mediate reduced T-independent antibody responses in response to polymeric antigens in vivo [9-13].

3.2 BcR signaling, antigen presentation and metabolism

Each of the B cell fate decisions outlined above is controlled by signaling through the BcR (Fig 1). Considering the various defects in PI3K-deficient B cells; it is therefore not surprising that PI3K activation is a fundamental aspect of BcR signaling. The mechanism

through which the BcR is coupled to PI3K signaling has recently been resolved. Two proteins with YxxM motifs (providing the optimal binding site for the Src homology-2 (SH2) domains of p85) are known to be important for B cell signaling: CD19 and B cell adaptor protein (BCAP). Whereas deleting either of these alone is insufficient to ablate PI3K signaling in B cells, combined deficiency of CD19 and BCAP reduces PI3K signaling below a detectable threshold and strongly impacts B cell development at the stage where the B cell progenitors first express a pre-BcR (composed of an immunoglobulin heavy chain and a surrogate light chain) [23]. As CD19 and BCAP are both substrates for BcR dependent Syk activity, we can conclude that we now have a detailed model of how the BcR connects with PI3K activity. However, it should be noted that the guanine exchange factor Vav and its substrate Rac have also be implicated in the activation of PI3K downstream of the BcR [24-26]. In recognition of this complexity, we have proposed a “signalosome” model of BcR signaling in which PI3K, its lipid products, and other components function as an integrated molecular machine in which all parts are functionally interconnected [27].

A primary role of PI3K in BcR signaling is to promote signalosome assembly and membrane localization, leading to phospholipase C-gamma (PLCγ) activation and phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis [7, 17]. Thus, signaling events controlled by PLCγ such as elevation of cytoplasmic calcium and activation of protein kinase C-beta (PKCβ) are also impaired in PI3K-deficient B cells. B cells lacking PI3K or CD19 exhibit defective accumulation of protein aggregates, termed microsignalosomes, at or near the plasma membrane [28, 29]. Consequently, PI3K-deficient B cells proliferate poorly in responses to antigen receptor triggering. This response can be rescued by the provision of signals via CD40 or TLR4, which may explain why humoral immune responses can be realized in PI3K-deficient mice, albeit at reduced levels. Engagement of CD40 or TLRs provides a mechanism for activating the NFκB pathway, whose induction by BcR signaling is attenuated in PI3K-deficient cells. Interestingly, a B cell-intrinsic mechanism to limit activation involves engagement of the inhibitory receptor FcγRIIb1, which recruits the inositol lipid phosphatase SHIP1 and reduces phosphatidylinositol-3,4,5-trisphosphate (PIP_3) levels. Mice lacking FcγRIIb1develop a lupus-like syndrome, and some human lupus patients show decreased expression or function of FcγRIIb1 which indirectly suggests a role for SHIP in preventing autoimmunity [30-32]. Of interest, BcR-mediated PIP_3 production is suppressed in anergic B cells, providing another example of how intrinsic attenuation of PI3K activation limits B cell responsiveness [33].

BcR clustering promotes antigen internalization, processing and presentation of the antigen to T cells. Several studies using anti-Ig antibodies as model antigens suggested a role for PI3K signaling in BcR aggregation, internalization and early stages of antigen presentation since internalization was inhibited by coligation of FcγRIIB [34, 35]. A more recent study suggested that p110δ is required for efficient antigen presentation, but through regulation of a later step in antigen processing [36]. Although defective antigen presentation may contribute to the reduced T-dependent antibody responses seen in p110δ-deficient mice [11-13], other functions for p110δ in B and T cells are also likely to determine this phenotype.

In addition to providing "core" signals required for proliferation and differentiation, PI3Ks contribute to the fitness of B cells by increasing cellular growth and metabolism. These functions are mediated in part by the Akt serine/threonine kinases, which are activated by BcR and/or CD19 engagement in a PI3K-dependent manner. As in most cells, Akt contributes to B cell size increase through promoting activation of mammalian target of rapamycin (mTOR) complex-1 (mTORC1) [37]. However, this mechanism is stimulus-dependent, with lipopolysaccharide (LPS) inducing mTORC1 activity in a PI3K-independent manner. MZ cells exhibit high basal mTORC1 activity that might contribute to the larger size and more rapid activation of these cells compared to FO cells. Akt is also thought to mediate changes in glucose metabolism in activated B cells. BcR engagement induces a glycolytic switch and this can be mimicked by expression of an inducible, constitutively active Akt construct [38]. Activation of the PI3K/Akt signaling axis also contributes to survival in response to interleukin-4 (IL-4), B cell activation factor of the tumor necrosis factor family (BAFF), and other cytokines [39, 40]. Thus PI3Ks contribute to diverse signaling events that control B cell development and antibody production.

3.3 Immunoglobulin gene rearrangement and isotype switching

A surprising observation was that SH2 domain containing protein of 65kDa (SLP-65) (otherwise known as BLNK), an adaptor protein important for signalosome assembly, appears to suppress PI3K signaling in pre-B cells [41]. The mechanism by which SLP-65 suppresses PI3K signaling is not known. However, a picture of why it may be important to suppress PI3K signaling at various stages of development has recently emerged.

The only group of transcription factors whose activity is entirely dependent on PI3K signaling in diverse cell types is the Forkhead Box Subgroup O (Foxo) proteins [42]. There are four Foxo isoforms; among these, Foxo1 and Foxo3a appear to be the most important in B cells and T cells [43-47]. In its unphosphorylated form, Foxo translocates to the nucleus where it drives the transcription of a number of genes whose products regulate apoptosis and cell cycle progression such as Bim, p27 and FasL [42]. More recently, it was found that Foxo promotes expression of the *Rag-1* and *Rag-2* genes [43, 44]. When phosphorylated by Akt, Foxo is found in the cytoplasm associated with 14-3-3 proteins and unable to regulate gene transcription. Thus, active PI3K/Akt signaling inhibits Foxo-dependent transcription. With respect to the *Rag* genes, it has been proposed that suppression of PI3K signaling is important for recombination of the immunoglobulin light chain locus to occur. Furthermore, it seems that subsequent reactivation of PI3K is also important to suppress Rag expression and to prevent inappropriate expression of a second light chain [20, 41, 44]. Concurrently, tonic signaling by surface IgM on immature B cells acts through PI3K to positively select cells for further differentiation into mature B cell subsets and to promote survival of mature B cells [21, 22, 48].

Immunoglobulin isotype switching is also regulated by the suppression of Foxo function, and hence indirectly by PI3K. Class-switch recombination was enhanced in the presence of p110δ inhibitors and increased further by constitutively active Foxo proteins, but suppressed in Pten-deficient B cells where PI3K signaling was elevated [49]. These results seemed to contradict other studies with p85α or p110δ deficient mice that showed reduced switching to

other isotypes after immunization. However, further analysis revealed increased propensity for both p85α and p110δ deficient B cells to produce immunoglobulins of the IgE subclass, both in vitro and in vivo [50, 51]. A similar effect was observed in mice that were treated with the p110δ-selective inhibitor IC87114 [50]. An increased percentage of cells showed sequential switching from IgG1 to IgE, consistent with unrestrained class-switch recombination in the absence of p110δ activity. The excessive IgE production in p85α$^{-/-}$, p110δ^{D910A} and IC87114-treated mice suggest that therapeutic use of p110δ inhibitors could lead to increased allergic responses. In part, this should be compensated by diminished mast cell function, but only as long as the inhibitor is administered [52, 53]. Thus, while in general PI3Ks are thought to integrate signals from the BcR, costimulatory and cytokine receptors to promote B cell activation and differentiation, PI3Ks also negatively regulate the expression of Rag proteins and genes involved in immunoglobulin class-switch recombination and may thus paradoxically also limit aspects of B cell development and differentiation.

3.4 B cell chemotaxis and trafficking

B cell trafficking is strongly influenced by PI3K signaling. Treatment of B cells with wortmannin reduces chemokine-driven migration in vitro, and alters homing after adoptive transfer to mice [54-56]. B cell chemotaxis is partially dependent on the class IA isoform p110δ, an exception to the rule that class IB (p110γ) functions downstream of GPCRs in lymphocytes. Wortmannin-treated B cells and p85α-deficient B cells also display reduced basal motility in lymph nodes [56]. Proper recirculation of B cells requires the lymph node homing receptor L-selectin (CD62L), whose expression is controlled by Foxo1 [43]. BcR stimulation down-regulates L-selectin expression, and this is partially prevented in p85α-deficient B cells [57].

Figure 1A outlines key actions of PI3K in B cell development and function, as highlighted in this section.

4 PI3K in T cells

4.1 T cell development and differentiation

T cells develop in the thymus from CD4^{-}CD8^{-}TcR^{-} precursors that arrive from the bone marrow. These precursors are referred to as double negative (DN) cells (due to the lack of expression of CD4 or CD8) and are subdivided into DN1-4 based on the differential expression of CD44 and CD25. At the DN3 stage, T cells express RAG enzymes that rearrange the TcR β-chain genes so that a TcRβ chain can be expressed on the cell surface. T cells that express a productively rearranged TcRβ chain are selected to become CD4^{+}CD8^{+} double-positive (DP) cells that rearrange and express TcRα chains. Concurrent with this developmental stage, TcRαβ+ DP T cells are positively selected to become CD4 or CD8 single positive mature thymocytes if they bind self-peptide presented by MHC class I or II molecules with intermediate affinity. DP cells are negatively selected though apoptosis if they bind peptide-MHC (pMHC) with high affinity. Most DP thymocytes die by neglect, however, as they show no apparent affinity for pMHC molecules. Selected CD4+ or CD8+

thymocytes then migrate through the blood and lymph to populate the lymph nodes and spleen. PI3K controls T cell biology at multiple junctions of their development (see Fig. 1b).

4.1.1 A surprising redundancy between p110δ and p110γ during T cell development—

Both p110δ and p85 double knockout (p85α deleted in T cells, p85β germline deleted)(dko) mice produce near normal number of thymocytes whereas p110γ-deficient mice have reduced numbers of thymocytes, which was attributed to increased apoptosis of DP thymocytes [12, 15, 58, 59]. Strikingly, p110δ-p110γ double-deficient mice show a dramatic reduction in thymocyte numbers caused by a block at the DN3-DN4 stage of development in addition to reduced DP cell survival [60, 61]. Pre-TcR signaling as this stage was mostly controlled by p85α and p110δ [62]. The unexpected redundancy between p110δ and p110γ therefore suggested that GPCRs may play a greater role at this developmental stage than previously appreciated. Indeed, further investigations demonstrated that coordinated activation of p110δ by the pre-TcR and p110γ by the chemokine receptor CXCR4 is essential for optimal development of double positive T cells [63]. A key role for PI3K signaling during thymocyte development has also been revealed from mice with T cell-specific deletions of Akt or Pdk1 [64-67]. In each case, DP cell numbers were dramatically reduced. By contrast, deletion of Pten in pre-T cells led to the development of double positive T cells in the absence of pre-TcR expression, suggesting that unrestrained PI3K signaling can substitute for the signals that normally drive β-selection [68]. One key observation was that the transition from DN3 to DN4 stage was accompanied by increased metabolic activity, increased cell size and by the expression of nutrient receptors such as CD71 (the transferrin receptor) and CD98 (an amino acid transporter), presumably required to support the increased growth and proliferative burst [69, 70]. $Pdk1^{-/-}$ thymocytes failed to upregulate these receptors and $Pdk1^{-/-}$ DN4 thymocytes were smaller than normal [70]. There is also a requirement for Notch signaling at the DN3 to DP thymocyte transition and PI3K and Akt were required for Notch-dependent increase in metabolism and cell growth at this stage [65, 69, 70]. Notch is unlikely to directly regulate the recruitment of PI3K to the plasma membrane. Instead, it has been proposed that notch can increase PI3K signaling by suppressing the expression of Pten [71]. This would be consistent with the observation that deletion of Pten was sufficient to promote DP thymocyte development in the absence of TcR signaling [68]. Interestingly, Pten deficiency was also permissive for DP T cell development in absence of Pdk1, thus implicating additional PI3K-dependent signaling pathways in early T cell development [72].

4.2 TcR signaling and costimulation

The mature T cell receptor activates PI3K signaling by recruiting p85/p110 to the immune synapse [73]. Precisely how PI3K becomes recruited to the TcR is less clear than it is in the case of BcR signaling. The major TcR-associated tyrosine kinase substrates CD3 and Lat lack obvious p85 docking sites. T cells do express the transmembrane adapter protein TRIM, which contains YxxM motifs that become phosphorylated by ZAP-70 and to which PI3K could dock [74]. However, Akt phosphorylation was unimpaired in TRIM-deficient T cells [75]. CD28 has often been considered to play an analogous role in T cells as CD19 does in B cells; that is by acting as a docking site for PI3K after phosphorylation by Lck or other TcR associated kinases [76]. Consistent with this model is the presence of a p85 SH2 binding

motif (Y^{170}MNM) in the CD28 cytoplasmic domain. However, while $CD28^{-/-}$ T cells showed reduced PI3K activity in response to stimulation by pMHC on antigen presenting cells (APCs), retrogenic expression of a $CD28^{Y170F}$ mutant rescued CD28-dependent PI3K activity despite the lack of association between p85-SH2 and $CD28^{Y170F}$ [77]. In addition, recent imaging experiments that followed the segregation of the TcR and CD28 into different microclusters revealed that in the mature synapse, PI3K co-localised with the TcR whereas, surprisingly, protein kinase C-theta (PKCθ) co-localised with CD28 [78]. Indeed, PKCθ recruitment to the immune synapse is more diffuse in T cells lacking CD28 [77, 79, 80]. Thus, while initial models proposed that CD28-dependent PI3K activity would complement TcR dependent PKC activity, recent genetic and imaging experiments implicate the TcR as the main activator of PI3K (helped by CD28 through undefined mechanisms) and that CD28 instead contributes to sustained or enhanced PKC activity (again as initiated by the TcR). It remains possible that subsequent to initial activation and in certain T cell lines, CD28 may act more independently of the TcR which may have contributed to the original notion that CD28 is a major activator of PI3K. Indeed, CD28-dependent migration of memory T cells to peripheral tissues was dependent both on the PI3K binding motif in CD28 and on p110δ [81, 82]. Where CD28 does bind p85, there appears to be a preference for the association with the p85β isoform [83]. The CD28-related protein ICOS, expressed mainly by primed T cells ,is also an important PI3K activator. ICOS contains a p85-SH2 binding motif that shows a higher affinity than CD28 for p85 [84]. Recent evidence also indicates that ICOS may recruit the smaller p50α subunit, which appears to correlate with greater PI3K enzyme activation [85]. A tyrosine to phenylalanine mutation in ICOS mimics the ICOS deficient phenotype showing that PI3K engagement is essential for ICOS function. Thus $ICOS^{Y181F}$ mice lacked follicular helper T cells which support the germinal cell reaction that leads to class switching and high affinity antibody production [86]. Mice lacking p110δ activity have a similar phenotype to ICOS deficient mice with regards to reduced IL-10 production by T cells, reduced Th2 responses and impaired germinal centre formation in the spleen [12, 14, 87-90](see section 4.3). Thus, PI3K may be an important component of T cell costimulatory signaling, but the rules of engagement are different than initially assumed.

In addition to the tyrosine-based recruitment mechanisms described here, p110δ has recently also been shown to bind the atypical Ras family member Rras2 (also known as Tc21) which localizes to the T cell and B cell antigen receptor complexes [91]. Thus p85α and p110δ recruitment to the BcR and TcR was impaired in $Rras2^{-/-}$ B cells and T cells and aspects of the $Rras2^{-/-}$ phenotype, such as the lack of MZ B cells, was reminiscent of that observed in the context of p85α or p110δ-deficiency [91]. To what extent Rras2 complements or supplements tyrosine-based recruitment mechanisms remains to be further explored.

4.2.1 PI3K regulates some, but not all, TcR-dependent signaling pathways—

The activation of Akt is entirely dependent on the p85s and p110δ in peripheral T cells stimulated through the antigen receptor with or without CD28 [12, 14, 15, 77]. PI3K deficiency also has a particularly strong impact on Erk phosphorylation in T cells, but a more modest effect on Ca^{2+} release into the cytosol [12, 15]. The modest impact on Ca^{2+} flux suggest that PI3K may act on pathways downstream of, or in parallel to, PLCγ. In this

context, it is of interest to note that the PI3K effector Bam32 was required for full Erk activation in T cells [92]. PI3K may also contribute to NF-κB nuclear translocation, though this is a subject of considerable debate. Several mechanisms exist for stimulating the nuclear translocation and activation of NF-κB [93]. In response to TcR signaling, the main pathway leading to NF-κB activation involves the activation of PKCθ and sequential engagement of Carma, Bcl10 and Malt1 leading to the activation of IκB kinase [94]. Where PI3K has been proposed to contribute, it has been suggested to be either via the capacity of Pdk1 to interact with and phosphorylate PKCθ or by Akt-dependent phosphorylation and activation of Cot (also known as Tpl2 or Map3k8) [95-98] . The capacity of Pdk1 to phosphorylate AGC kinases, including PKCs, is generally thought to be PI3K-independent so a requirement for Pdk1 in activating PKCθ does not necessarily imply a requirement for PI3K. Indeed, a knockin mutation in the PH domain of Pdk1 which renders Pdk1 insensitive to PI3K signaling and which attenuates Akt phosphorylation, did not appear to have any effect on PKCθ activation [99]. In addition, the extent to which the putative Pdk1 phosphorylation site in PKCθ regulates downstream signaling events has been questioned [100]. Although Akt-dependent phosphorylation of Cot may be critical in some cells, NF-κB activation and IL-2 production are unimpaired in Map3k8-deficient T cells suggesting that Akt-dependent Cot phosphorylation is also non-essential in this context [101, 102]. Pertinently, PKCθ recruitment to the synapse and NF-κB translocation to the nucleus were intact in antigen-stimulated p110δ-deficient T cells [14, 77]. These data suggest that PI3K contribution to NF-κB signaling in T cells is non-essential. However, PI3K signaling does play a key role in regulating the Foxo family of transcription factors as these are direct targets for phosphorylation by Akt. Although this may in part relieve T cells from anti-proliferative and apoptosis inducing signals [42], more recent data suggest a key role for Foxo family proteins in the regulation of T cell trafficking which will be discussed further in section 4.6.

4.3 T helper cell differentiation

Both p110δ-deficient and p85 dko T cells show reduced proliferation and cytokine production [12, 14, 15, 87, 90, 103-105]. In general, the defect in cytokine production is more pronounced for the effector cytokines interferon-gamma (IFN-γ) and IL-4 that are mainly produced by antigen experienced differentiated T-helper type-1 (Th1) or T-helper type-2 (Th2) cells than it is for interleukin-2 (IL-2) which can be produced by naïve T cells. Thus, PI3K may contribute to Th lineage-specific differentiation programs. In addition, p110δ-inhibitors blocked cytokine production by both mouse and human memory T cells, suggesting that even after a T helper cell has differentiated, it remains dependent on PI3K activity for optimal cytokine secretion [106]. However, defective cytokine secretion has not been observed in all experimental models. For instance, under Th2 skewing conditions in vitro or in vivo, IFN-γ production by p110δ-deficient T cells was enhanced, perhaps reflecting reduced interleukin-10 (IL-10) production that strongly antagonizes IFN-γ production [50, 87]. Similarly, mice with a T cell-specific deletion of SHIP showed enhanced Th1 but reduced Th2 responses under Th2 skewing immunization conditions [107]. In addition, both p110δ-deficiency and Ship deficiency are associated with reduced IL-17 production by T cells [106, 108]. These are curious observations, as one would anticipate that p110δ-deficiency and SHIP-deficiency would have opposing effects on T cell function as the former is associated with reduced PIP3 levels whereas the latter is associated

with elevated PIP3 levels. However, it should be noted that PtdIns(3,4)P_2 produced by Ship-mediated hydrolysis of PIP3 may contribute to T cell activation by mechanisms that have yet to be fully appreciated [109].

As a consequence of impaired T cell development in the thymus, p110δ-p110γ double deficient mice were lymphopenic, particularly with respect to T cell numbers [60, 61]. The T cells found in the periphery secreted elevated amounts of Th2 cytokines. The serum from these mice was also enriched in IgE, had normal levels of IgG1 and reduced levels of IgG2a [103]. The enhanced Th2-like phenotype in the p110γ-p110δ double deficient mice seems paradoxical considering the reduced Th2 responses observed in p110δ-deficient mice [87], but is most likely explained by stress caused by lymphopenia rather than any role for p110δ or p110γ in suppressing Th2 responses. The requirement for PI3K in promoting Th1 differentiation may be mitigated by a role for PI3K in the negative regulation of TLR signaling in dendritic cells (DCs). Thus, PI3K-deficient DCs secreted larger amounts of IL-12, which promoted Th1 differentiation in p85α knockout mice [110]. Thus, while PI3K seems to play a positive and T cell intrinsic role in promoting cytokine production, in the context of additional stimuli this defect may not always be apparent.

4.4 Survival and glucose homeostasis in mature T cells

PI3K can also regulate glucose uptake in T cells by stimulating Akt, which controls Glut1 translocation to the membrane [111]. This was put forward as an alternative mechanism for CD28 to promote costimulation as it was known that CD28 has minimal impact on the transcription profile of T cells [112, 113]. However, there is no evidence that the CD28 PI3K binding motif was required for this response and more recent evidence might suggest that CD28 contributes in concert with the TcR to trigger optimal PI3K and Akt activity required for glucose uptake. CD28 and PI3K were also required for the upregulation of Bcl-x_L which promotes survival of T cells [114, 115]. The serine/threonine Pim kinases, whose expression is regulated by IL-2 in T cells, may also play a partial and PI3K-independent role in stimulating T cell metabolism and/or survival through mechanisms that remain to be fully defined [116]. In this context it is important to note that both Pim kinases and mTOR can be inhibited by the commonly used tool compound LY294002 – thus some studies using this inhibitor alone to probe PI3K function need to be reinterpreted [117]. Therefore, while PI3K may promote cellular fitness by augmenting metabolic and pro-survival responses, other pathways such as Pim and PI3K-independent activation of mTOR may also contribute which may explain why it is possible to stimulate PI3K-deficient T cells to grow and proliferate in the absence of detectable PI3K activity [15].

4.5 PI3K controls the development and suppressive function of regulatory T cells (Tregs)

Tregs play an essential life preserving function by reining in the adaptive immune system [118, 119]. Tregs can develop either in the thymus from immature progenitors or in the periphery from mature CD4 T cells. Both peripheral and thymic development of Treg require TGF-β signaling [120]. p110δ-deficient mice showed increased proportions of Treg in the thymus, whereas over-expression of myristoylated Akt in thymocytes inhibited Treg development, suggesting that PI3K negatively regulates thymic development of Tregs via Akt [90, 121]. By contrast, Treg were found in reduced numbers in the spleen and lymph

nodes of p110δ-deficient mice, suggesting a possible role for PI3K in the homeostatic maintenance or peripheral development of Tregs. Consistent with this notion, $Ship^{-/-}$ mice harbored increased proportions of Treg in the spleen [108]. Curiously, treatment of naïve T cells with PI3K and mTOR inhibitors 18 hours after activation through the antigen receptor led to the induction of Foxp3 expression in the absence of TGF-β [122]. In addition, genetic deletion of mTor in T cells led to enhanced Treg development at the expense the Th lineages [123]. The physiological relevance of how interrupted PI3K and mTOR signaling could lead to induction of Foxp3 expression remains to be defined; nonetheless, these results, together with the $p110\delta^{D910A}$ and Akt over-expressing thymocytes, show that PI3K signaling is intimately linked to the production and maintenance of Foxp3+ Tregs.

Treg from p110δ mice are defective in their capacity to suppress T helper cell proliferation in vitro and to secrete IL-10 [90]. One apparent consequence of reduced Treg function in p110δ mice is that they were shown to clear Leishmania infections more efficiently than WT mice despite impaired Th1 responses [104]. However, acute or chronic depletion of Treg cells can also lead to massive lymphoproliferation, lymphocyte tissue infiltration and multiple organ failure [124]. More subtle defects in Treg function or Treg depletion in the absence of intact adaptive immunity, frequently leads to colon inflammation stimulated by commensal flora in the gut [125]. Indeed, p110δ-deficient Treg failed to prevent disease in an experimental model of colitis [90]. Accordingly, $p110\delta^{D910A}$ mice showed signs of inflammatory bowel disease but no other autoimmune symptoms were evident [12]. By contrast, p85 dko mice showed symptoms reminiscent of Sjogren's syndrome, including dry eyes, lacrimal gland destruction and autoantibodies [105]. Whether the different symptoms described in the different mice represent real differences in how p85 vs p110δ deficiency affects Treg function or whether other environmental or tissue-specific factors explain the different symptoms is not yet clear. PI3K signaling in response to IL-2 stimulation was reduced in Treg compared to conventional CD4 T cells [126]. Deletion of Pten resulted in elevated PI3K signaling and enhanced proliferation of Treg in response to IL-2 – even in absence of TcR signaling which is required for the proliferation of conventional Treg [127]. However, deletion of Ship or Pten had no effect on Treg-mediated suppression [108, 127]. However, one study showed that the expression of transgenic Akt in mouse Treg enhanced suppression [128] whereas another study showed that in human Tregs basal PI3K signaling was also lower than in conventional T cells and that ectopic expression of myristoylated Akt interfered with Treg-mediated suppression [129]. Thus, PI3Ks regulate many facets of Treg development, homeostasis and suppression through mechanisms that are still incompletely understood. What seems clear is that the timing and magnitude of the PI3K and mTOR signals have strong impacts on the homeostasis and function of Treg.

4.6 T cell chemotaxis and migration in lymph nodes: not all about PI3K

The role of p110γ in peripheral T cells is debated. While some investigators observe reduced proliferation and cytokine production by $p110\gamma^{-/-}$ T cells [58, 130], others observe that $p110\gamma^{-/-}$ T cells proliferate normally [131, 132]. Recent evidence suggests that the TcR can form complexes with chemokine receptors, especially in memory T cells, and may hence influence TcR-dependent events under some circumstances [133, 134]. Other factors such as the health status of the mice, cell purification protocols and media components may also

influence to what extent p110γ contributes to T cell activation by antigen receptor stimulation in vitro by altering the levels of available GPCR ligands. Perhaps surprisingly, chemotactic responses to CCL19, CCL21 and CXCL12 were only moderately affected by p110γ deficiency despite the capacity of these agonists to stimulate PI3K activity in a p110γ-dependent manner [54, 55, 81]. Instead, the Rac guanine nucleotide exchange factor (GEF) Dock2 was required for T cell chemotaxis in a mostly PI3K-independent manner [55, 135]. More recently, the role of PI3Ks in regulating T cell migration within the lymph nodes has been investigated using intravital two-photon microscopy or by fluorescent microscopy of lymph node slices [56, 135, 136]. Constitutive migration of T cells within lymph nodes is promoted by chemokines secreted by and adhered to fibroblastic reticular cells [137]. However, the effects of inhibiting PI3K were more subtle. Interestingly, by some criteria, the loss of the p85 adapter subunits had a stronger effect than inhibition by wortmannin. These observations suggest that p85 may regulate some aspects of cell motility independently of PI3K in T cells as had previously been shown in fibroblasts [56, 138].

In addition to promoting chemotaxis, CCL19 and CCL21 are important for T cell homeostasis and survival [139]. Thus an intriguing possibility is that chemokines use p110γ in T cells to promote survival or metabolic fitness rather than to detect chemotactic gradients. Consistent with this interpretation is the observation that p110γ-deficiency could alleviate autoimmunity caused by the overexpression of an activating form of p85 (referred to as p65) and that this alleviation was correlated with reduced accumulation of memory T cells rather than by reduced infiltration of T cells into affected organs [140]. Chemokines can also provide costimulatory signals to T cells to enhance proliferation, but this is not p110γ or p110δ dependent [141]. More recent data has provided evidence for a key role for p110γ in mediating chemotactic signaling in previously activated T cells in response to inflammatory chemokine CCL5 or the lipid chemotractant leukotriene B4 [131]. This was correlated with a defective recruitment of $p110\gamma^{-/-}$ T cells to the site of vaccinia virus infection and diminished ability to resist infection. Similarly, $p110\gamma^{-/-}$CD4 T cells showed reduced response to the chemokine CCL22 [132].

4.7 T cell trafficking

Several lines of evidence have indicated key roles for class IA PI3Ks in regulating lymphocyte motility and trafficking. p85-deficient T cells show reduced motility in intact lymph nodes and p110δ-deficient T cells show reduced recruitment to sites of inflammation or skin allografts [56, 81]. These defects do not appear to reflect a role for p85 or p110δ in chemokine signaling as PI3K plays a minor role in T cell chemotaxis. Where there is a role for PI3K, p110γ appears to be the relevant isoform. Instead, PI3K, via its activation of Akt controls the exclusion of Foxo from the nucleus and hence prevents Foxo from driving the transcription of Krüppel-like factor-2 (KLF2) and a number of genes that are critical for lymphocyte trafficking and homing, such as L-selectin (CD62L), CCR7 and receptors for sphingosine-1-phosphate (S1P) and interleukin-7 (IL-7) [45, 47, 142, 143]. L-selectin, CCR7 and S1P receptor expression can also be inhibited by rapamycin, suggesting a novel capacity of mTORC1 to inhibit transcription [142]. CD62L is required for the movement of lymphocytes across the high endothelial venules during entry into lymph nodes. CCR7 contributes to the recruitment of T cells into the T cell areas of lymph nodes, whereas S1P

promotes the exit of T cells from lymph nodes. IL-7 is a key regulator of homeostatic survival of T cells and it found in limiting amounts in lymph nodes. Because the effects of the defined Foxo targets are pleiotropic and can promote recruitment or exit from lymph nodes, the effect of PI3K inhibition (and hence sustained expression of these trafficking molecules) may not always be easily predicted.

In memory T cells, the TcR and CD28 can independently stimulate T cell trafficking to peripheral tissues by mechanisms that remain to be defined but which are absolutely dependent on p110δ activity [81, 82]. The cells used in these studies had been generated by repeated immunizations followed by in vitro cultures, hence both CCR7 and CD62L expression was low also on the $p110\delta^{D910A}$ T cells resulting in few T cells of either genotype being recruited to the lymph nodes in these studies [81]. Circulating T cells interact with the capillary and post-capillary endothelium and translocate through the endothelial barrier if they detect the expression of foreign antigen or presence of costimulatory ligands. How PI3K is required for this process is presently unclear but may involve the clustering or otherwise alteration of selected integrin affinity or avidity [82] or additional Foxo or KLF2 targets that have yet to be fully characterized. In this context it is of interest to note that KLF2 deficient lymphocytes have been found to accumulate preferentially in non-lymphoid tissues [144]. Given that PI3K suppresses KLF2 [142], this suggests that p110δ-deficient cells may lack the expression of certain genes that would facilitate entry into non-lymphoid organs. These observations have potentially important clinical implications as they indicate that p110δ inhibitors could reduce the recruitment of T cells to sites of inflammation where the T cells otherwise might increase the inflammatory response.

4.8 Nonessential role for PI3K in cytotoxic responses

p85 or p110δ do not appear to be essential for CD8 T cell-mediated killing in vivo of either virally infected cells or of hematopoietic allografts [15, 77]. Also, the capacity of NK cells to kill targets was either unaffected or modestly affected in p110δ or p110γ deficient mice [145-148]. These results were of particular interest as the NKG2D receptor, which recognizes proteins on cancer and virally infected cells, is linked to PI3K via the adapter DAP10 [149]. PI3K-deficient NK cells showed defective IFN-γ secretion and both p110γ and p110δ played an important role in the recruitment of NK cells to sites of infection and to tumors [145, 146, 150]. The precise role of PI3K in CD8 T cells and NK cells remain to be fully understood, but the retention of significant cytotoxicity in the absence of PI3K activity does suggest that while PI3K inhibitors may limit inflammation and autoimmune responses, they will not necessarily compromise resistance to common viral infections.

Figure 1B outlines key actions of PI3K in T cell development and function, as highlighted in this section.

5 Prospects for PI3K inhibitors in inflammation and autoimmunity

As will be discussed in other chapters in this book, PI3K inhibitors have been used with notable success in preclinical models of lupus, arthritis, asthma and allergy. While much of this effort has focused on the important capacity of PI3K inhibitors to reduce neutrophil

recruitment and reactive oxygen species generation, p110δ inhibitors may block T cell and B cell dependent inflammation, autoimmunity or graft rejection with minimal impact on PI3K signaling in other tissues. By a similar vein, the therapeutic potential of p110γ inhibitors may in part be mediated by suppressing the survival of memory T cells or they may prevent T cells from being recruited to sites of inflammation. To what extent p110δ or p110γ inhibition will compromise resistance to infection is currently not known, but this will be an important area of investigation as inhibitors of these isoforms enter clinical trials. In addition, attention needs to be paid to the possibility that PI3K inhibition can enhance certain immune responses by blocking regulatory T cells or by enhancing DC function or B cell class switch recombination.

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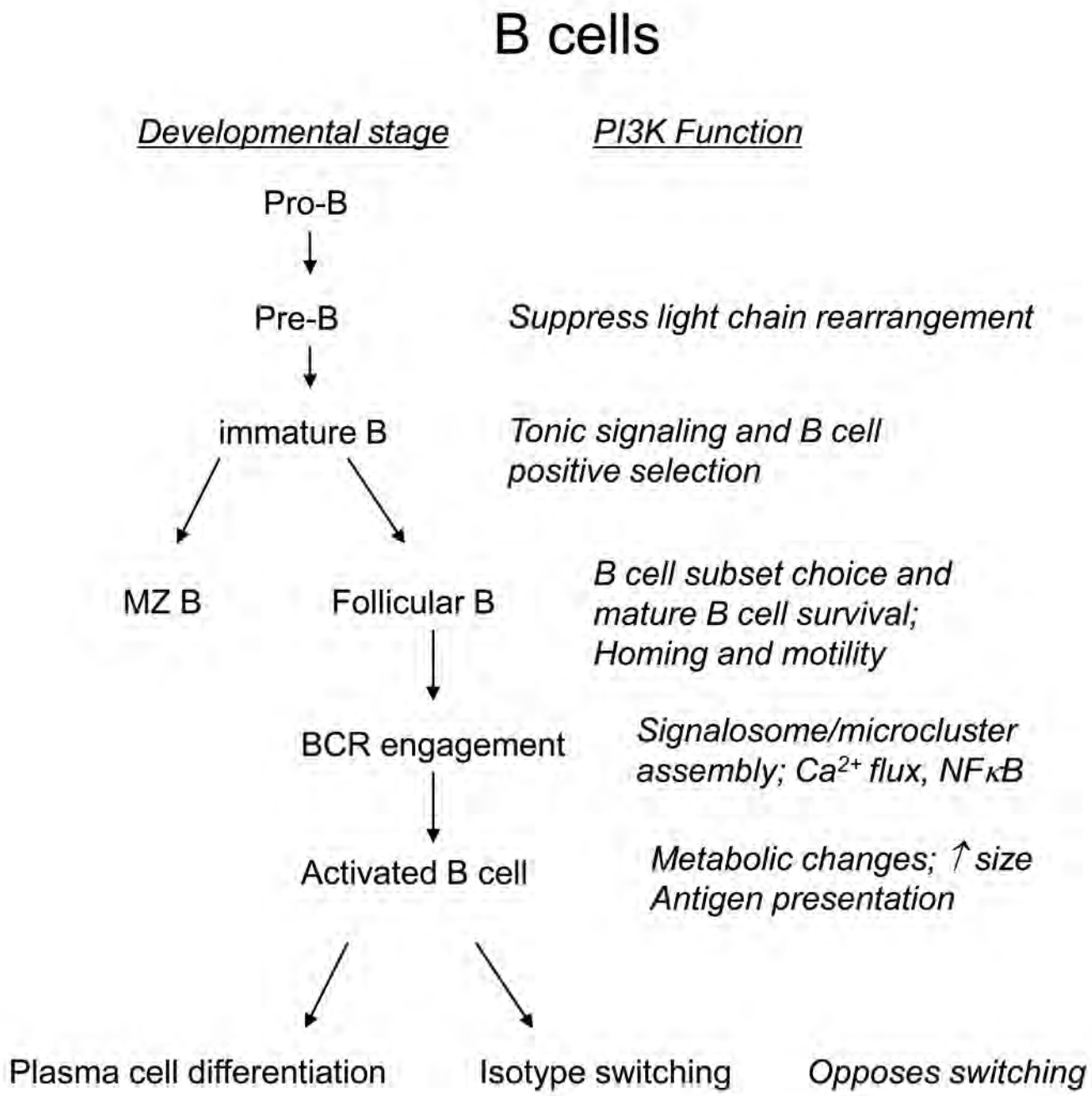

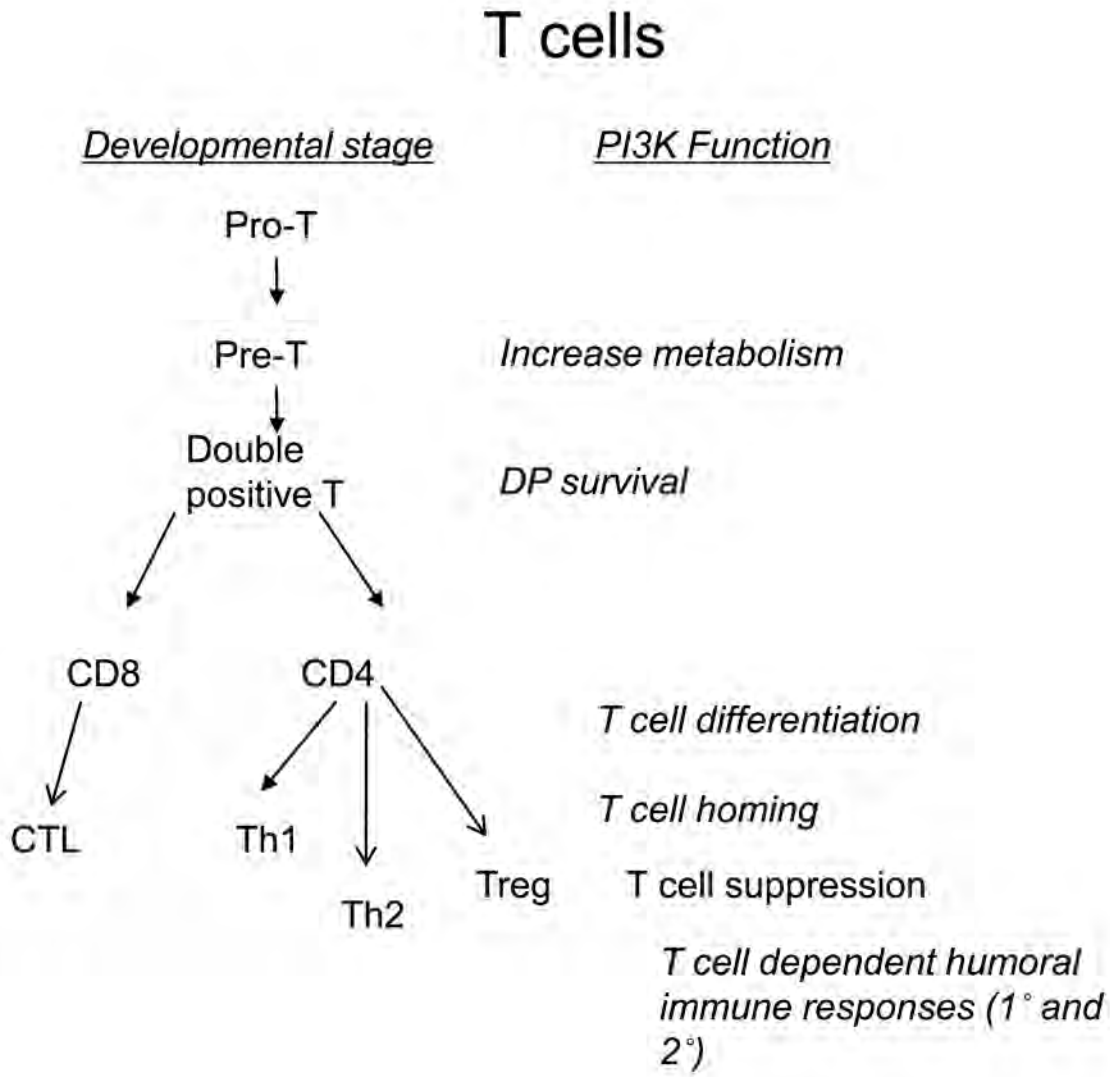

Figure 1.
Simplified flow charts of lymphocyte development and function, showing key points of action of PI3K in B cells (A) and T cells (B).

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Table 1

PI3K isoform(s) targeted	Genetic or pharmacological	Cell Type or animal model	References
p110δ	Null allele	T and B cells B cells NK cells	[11, 13] [20, 151] [145, 147, 152]
p110δ	Kinase-dead D910A knock-in	T and B cells T cells B cells NK or NKT	[12, 54] [14, 77, 81, 82, 87, 90, 104, 106, 142] [36, 39] [148, 152, 153]
p110δ	Selective inhibitor (IC87114)	B cells In vivo (asthma)	[39, 50] [154]
p110γ	Null or kinase-dead	T and B cells T cells NK	[54, 55, 58, 155, 156] [77, 130-132] [146, 148, 152]
p110γ	Selective inhibitors	In vivo (Arthritis, lupus)	[157, 158]
p110δ/p110γ	Double knockout or knockout/knock-in	T cells NK cells In vivo (arthritis)	[60, 61, 63, 103] [146] [159]
p85α	Null	B cells T cells	[10, 17, 18, 37, 57] [62]
p85α/p55α/p50α	Null	B cells	[9]
p85α/p55α/p50α	T cell-specific conditional	T cells	[15]
p85α/p55α/p50α	B cell-specific conditional	B cells	[160]
p85β	Null	T and B cells T cells	[161] [162]
p85α/p55α/p50α/p85β	p85 dko = double knockout (conditional p85α/p55α/p50α, null p85β)	T cells B cells	[15, 105] [160]