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Phosphoinositide 3-kinase and its targets in B-cell and T-cell signaling

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Phosphoinositide 3-kinase (PI3K) activation is essential for lymphocyte proliferation driven by receptors for antigen, costimulatory ligands and cytokines. The lipid products of PI3K contribute to the assembly of membrane-associated signaling complexes by promoting recruitment of selected proteins from the cytoplasm. Many proteins possess domains that are able to bind selectively to PI3K products. Different 'PI3K effector' proteins are coupled to distinct biological responses, depending on cell type and on the receptor that is engaged. In B cells and T cells, Tec-family tyrosine kinases and Akt serine/threonine kinases are emerging as crucial mediators of proliferation and survival signals downstream of PI3K. Of particular interest is recent evidence that PI3K signaling controls increases in lymphocyte size and metabolic activity that accompany cell cycle progression.

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Abbreviations

BCR	B-cell receptor
FOXO	Forkhead Box, Subgroup O
GSK-3	glycogen synthase kinase-3
IKKα	inhibitory NF-κB (IκB)-kinase-α
IL	interleukin
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
NFκB	nuclear transcription factor κB
PH	pleckstrin homology
PIP5K	phosphatidylinositol-4-phosphate-5-kinase
PKC	protein kinase C
S6K	S6 kinase
TCR	T-cell receptor

Introduction

Productive activation of naïve lymphocytes generally requires engagement of the antigen receptor and a costimulatory receptor. In T cells, co-engagement of the T cell receptor (TCR) and CD28 by MHC (major histocompatibility complex)-peptide and B7 ligands provides the requisite combination of signals to promote survival, as well as production of interleukin (IL)-2 and other cytokines. Subsequently, IL-2 provides strong proliferation and survival signals that enable clonal T-cell expansion. In B cells, signals that are initiated by the B cell receptor (BCR) are greatly enhanced by co-ligation of a coreceptor complex that includes CD19. Subsequent proliferation and survival are further enhanced by T-cell help, in the form of CD40 ligation and/or IL-4. Each of the receptor complexes described above is known to activate PI3K [1–3]. Furthermore, lymphocyte proliferation and survival driven by these receptors is attenuated in the presence of PI3K inhibitors and in cells lacking specific PI3K isoforms [1–3]. Conversely, mice with enhanced PI3K signaling in lymphocytes show lymphadenopathy and increased incidence of autoimmunity and leukemia/lymphoma [4,5]. These findings have spurred interest in understanding the molecular mechanisms by which PI3K activation controls lymphocyte proliferation.

A general model of the way that PI3K functions in signal transduction pathways is illustrated in Figure 1 (reviewed further in [6]). PI3K in resting cells is a cytoplasmic enzyme, the substrates of which are membrane phospholipids (phosphatidylinositol and its phosphorylated derivatives). Receptor crosslinking leads to tyrosine phosphorylation of receptor tails and associated proteins, leading to recruitment of PI3K to a signaling complex. These interactions increase PI3K enzyme activity and position the lipid kinase near its substrates to allow local production of PI3K products, termed 3-phosphoinositides. These lipids then promote further assembly of signaling complexes at the membrane by recruiting specific proteins with domains that selectively bind 3-phosphoinositides. This membrane-targeting signal is reversible and is opposed by specific lipid phosphatases, including PTEN (phosphatase and tensin homolog deleted on chromosome ten) and SHIP (SH2 domain-containing inositol 5-phosphatase).

The most widely studied 3-phosphoinositide binding domain is the pleckstrin homology (PH) domain [6] (Figure 1). Many proteins possess PH domains that are selective for PI3K products and are candidates for propagating signals downstream of PI3K. Putative PI3K effectors have been identified from several protein classes, including the serine/threonine kinases, tyrosine kinases, guanine nucleotide exchange factors, GTPase-activating proteins, and scaffolding proteins. This review focuses on recent advances in the study of effector proteins that link

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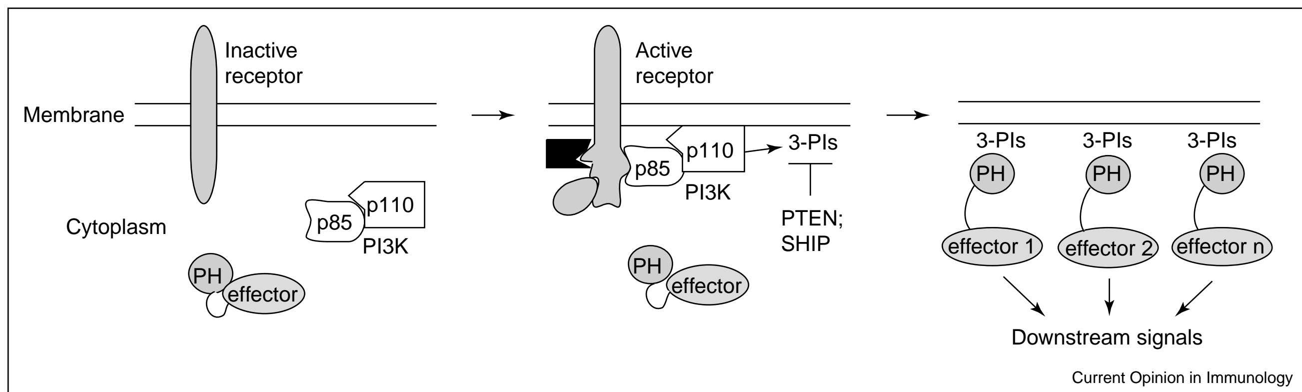
Figure 1



Overview of the PI3K signaling mechanism. Receptor activation recruits PI3K from the cytoplasm to the membrane, primarily via interactions involving the regulatory subunit (p85 here). Other signaling proteins are recruited to the receptor and can contribute to PI3K activation. The catalytic subunit (p110 here) then produces 3-phosphoinositides (3-PIs) in the inner leaflet of the membrane. These lipids are metabolized by phosphatases, including PTEN and SHIP. Regulated production of 3-PIs allows transient recruitment of cytoplasmic effector proteins to the membrane, via phosphoinositide-binding domains, such as the PH domain. For more detail, see [6].

PI3K activation to lymphocyte proliferation and survival. Other recent reviews provide thorough discussion of the mechanisms of PI3K activation and the roles of specific PI3K isoforms in lymphocyte function [1–3,6].

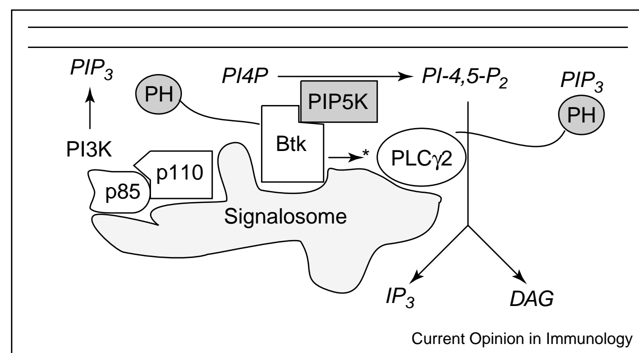
Tec-family tyrosine kinases and phospholipase C

Crosslinking of antigen receptors triggers sustained Ca^{2+} mobilization, which is required for lymphocyte proliferation. In B cells, Ca^{2+} flux, elicited by the BCR, is attenuated by PI3K inhibitors or deletion of specific PI3K genes [1–3,7]. Similar defects in BCR-driven Ca^{2+} flux and proliferation have been reported in murine B cells that are deficient for a host of other signaling proteins, including the Tec-family tyrosine kinase Btk and its substrate phospholipase C-gamma-2 (PLCγ2) [7]. These findings support biochemical data indicating that a large complex of signaling proteins assembles at the membrane to promote activation of PLCγ2, the enzyme responsible for generating inositol trisphosphate (Ins(1,4,5)P_3) to initiate Ca^{2+} mobilization (Figure 2).

It is likely that this assembly of proteins, together termed the 'signalosome', depends on PI3K products for proper membrane targeting [1–3,7,8]. Both Btk and PLCγ2 possess domains that bind selectively to PI3K products. Tyrosine phosphorylation of PLCγ2 by Btk augments PLCγ2 activity and Ins(1,4,5)P_3 generation [9,10]. The PH domain of Btk appears to be essential for function of the protein because a point mutation in the PH domain produces a phenotype similar to that of a null allele [11]. This point mutation, R28C, abrogates selective binding to the PI3K product phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3) [12]. On the basis of these findings and other evidence, it has been proposed that PtdIns(3,4,5)P_3 production is essential for Btk kinase activation and subsequent phosphorylation of PLCγ2. In this model, membrane-targeting of Btk allows phosphorylation of its activation-loop tyrosine residue by Src family kinases; however, this view has been challenged by recent findings. In particular, treatment with general PI3K inhibitors or deletion of specific PI3K isoforms does not inhibit the phosphorylation or activation of Btk, and has little effect on PLCγ2 phosphorylation [13,14,15••].

These unexpected results support a revision of the signalosome model in which PtdIns(3,4,5)P_3 binding

Figure 2



Proposed function of PI3K and its product PtdIns(3,4,5)P_3 in the signalosome. BCR engagement triggers assembly of a large group of kinases, scaffolding proteins and other signaling components to facilitate PLCγ2 activation. Phosphorylation of PLCγ2 (denoted by *) by Btk and other kinases increases its ability to generate Ins(1,4,5)P_3 and diacylglycerol (DAG). Efficient production of Ins(1,4,5)P_3 requires that PLCγ2 can access its substrate phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2 or PtdIns(4,5)P_2). The PI3K product PtdIns(3,4,5)P_3 helps to position the complex at the membrane by binding to PH domains in Btk and PLCγ2. To allow sustained Ins(1,4,5)P_3 output, the substrate for PLCγ2 must be replenished from phosphatidylinositol-4-phosphate (PI4P). This step can be mediated by the enzyme PIP5K that is associated with Btk.

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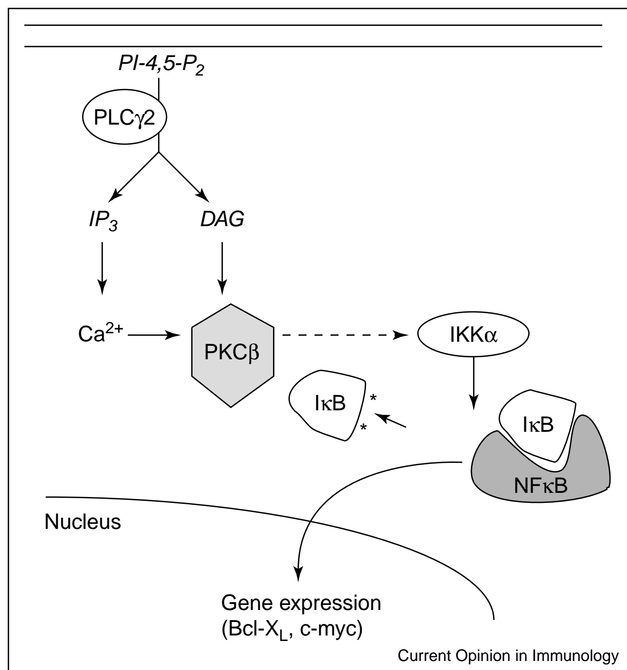
is necessary not for Btk or PLCγ2 enzyme activation *per se*, but for targeting of the signalosome complex to the membrane location where the substrate for PLCγ2 resides. This view is supported by the recent discovery of a kinase-independent function of Btk in the signalosome. Btk was shown to associate with phosphatidylinositol-4-phosphate-5-kinase (PIP5K), the enzyme that generates the substrate (phosphatidylinositol-4,5-bisphosphate; PtdIns(4,5)P_2) for PLCγ2 [16••]. Various lines of evidence were presented, supporting the proposition that one function of Btk is to ferry PIP5K to the membrane to resupply the substrate that is necessary for Ins(1,4,5)P_3 generation (Figure 2). This could explain the observation that kinase-dead Btk is able to enhance the generation of Ins(1,4,5)P_3 and Ca^{2+} flux [10,16••]; however, the function of PIP5K in the signalosome has not yet been tested by knockout or knockdown studies.

Which Ca^{2+}-dependent enzymes are responsible for propagating the proliferation signal in B cells? Conventional protein kinase C (PKC) isoforms are intriguing candidates because full activation requires both Ca^{2+} and diacylglycerol, another second messenger produced by PLCγ2 (Figure 3). PKC inhibitors or deletion of the PKCβ isoform blocks BCR-initiated cell cycle progression [17,18]. Conversely, pharmacological activation of PKC can restore proliferation in Btk-deficient B cells [19]. Recent data indicate that PKCβ functions downstream of the BCR by promoting the activation of NFκB (nuclear transcription factor κB)-dependent transcription. Specifically, PKCβ is essential for phosphorylation of IκB-kinase-α (IKKα) [20•,21•]. Phosphorylation of IKKα and IκB is also blocked in cells treated with PI3K inhibitors or lacking the catalytic isoform p110δ [13,22••]. The anti-apoptotic protein Bcl-X_L is a target gene of NFκB, the upregulation of which is impaired in B cells lacking p110δ, the PI3K regulatory isoform p85α, or Btk [13,15••]. Importantly, overexpression of Bcl-X_L restores BCR-dependent proliferation in B cells lacking p85α or Btk [15••,23]. Together, these findings suggest that a central function of the PI3K-dependent signalosome is to trigger the activation of PKCβ and the NFκB pathway, leading to transcription of crucial pro-survival genes like Bcl-X_L (Figure 3).

Figure 3



A pathway leading from PKC to activation of the NFκB pathway has emerged as a crucial conduit of the growth and survival signal, provided by PI3K-dependent PLCγ2 activation in B cells. A member of the conventional PKC subgroup, PKCβ, is activated by Ca^{2+} and DAG. This leads, via undefined steps (dashed arrow), to activation of IKKα and phosphorylation of IκB. This releases NFκB transcription factors to migrate to the nucleus and drive transcription of crucial target genes.

In T cells, the importance of PI3K activation to sustained Ca^{2+} mobilization is unclear. Treatment with PI3K inhibitors has little effect on Ca^{2+} entry that is triggered by contact with peptide-loaded APCs [24••]. T cells express three Tec-family kinases: Itk, Tec and Rlk [25]. Itk and Tec have PH domains with apparent selectivity for PtdIns(3,4,5)P_3, whereas Rlk is tethered to the membrane by a covalent lipid modification and might, therefore, be PI3K-independent. Both Itk and Rlk contribute to TCR-dependent Ca^{2+} flux [25]; however, the role of the membrane-targeting domains of these proteins in the Ca^{2+} response has not yet been evaluated. Itk is involved in TCR-dependent actin polymerization [26,27] and it has been suggested that this function could be mediated by Itk PH domain-mediated targeting of PIP5K to the membrane [28]. This idea is based on the Btk PH–PIP5K association [16••] and the established roles of both PIP5K and its product PtdIns(4,5)P_2 in actin polymerization in platelets and other cell types.

Akt

Akt, also known as protein kinase B (PKB), is a ubiquitously expressed serine/threonine kinase with a PH domain that is selective for 3-phosphoinositides. Akt is well established as a PI3K effector in many cell types, both normal and transformed [29,30]. Indeed, phosphorylation and activation of Akt is often used as a surrogate readout of PI3K activity, because direct measurement of 3-phosphoinositide levels is technically more difficult. In most cases examined, Akt activation promotes various cell responses that are associated with cell division, including increased cell size, suppression of apoptosis, inactivation of cell cycle inhibitors, and induction of cyclin and cytokine gene expression. In T and B cells, Akt activation can be observed following antigen receptor engagement and is greatly boosted by co-stimulation

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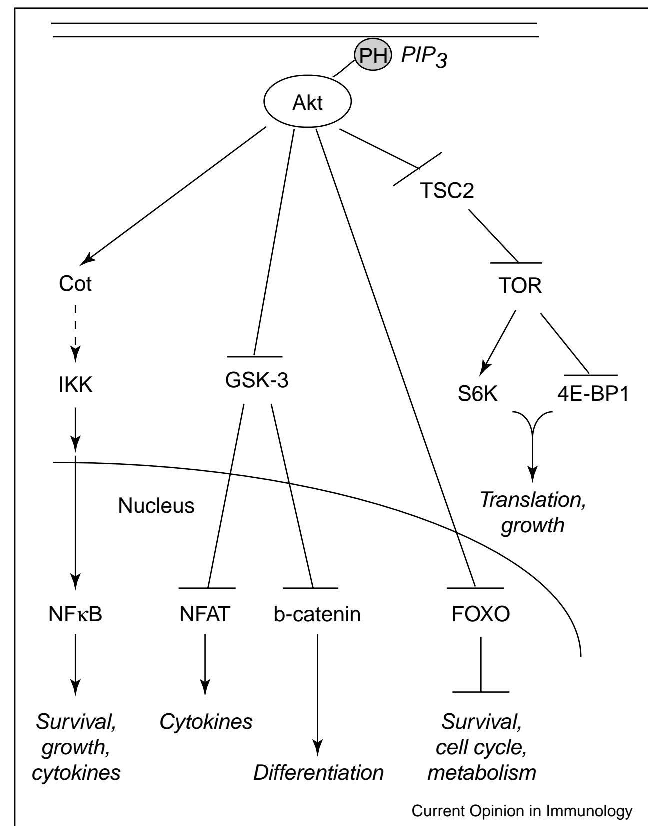
[29,31]. Cytokines such as IL-2 and IL-4 also activate Akt [29].

Akt was originally identified as the cellular counterpart of a viral oncogene (v-Akt), in which the PH domain is replaced by viral GAG sequences, leading to constitutive membrane-targeting. Subsequently, transgenic models have confirmed that constitutive membrane-targeting of Akt in T cells is sufficient to cause lymphoma and is associated with altered lymphocyte homeostasis and autoimmunity [4,32,33,34•]. At the molecular level, expression of activated Akt in T cells correlates with augmented NFκB function, including the upregulation of Bcl-X_L [32,35]. Membrane-targeted Akt could also restore antigen-mediated production of IL-2 and interferon-gamma in T cells lacking CD28 [35]. However, it has been noted that membrane-targeted alleles of Akt might not accurately reflect the functions of endogenous Akt, which associates only transiently with the plasma membrane following antigen receptor stimulation [29]. To date, no lymphocyte phenotypes have been reported in mice lacking Akt function, perhaps because of functional redundancy of three Akt isoforms that are encoded by distinct genes.

Recent experiments have illustrated that Akt has several substrates that mediate distinct aspects of the downstream response. These substrates reside in different cellular compartments, including the cytoplasm, nucleus and mitochondrial membrane. Many proteins on the list of Akt substrates have been studied, primarily, in nonlymphoid cells and lymphoid cell lines. Here, the focus is on those Akt substrates and downstream effectors whose function has been most extensively evaluated in primary, nontransformed lymphocytes (Figure 4).

Glycogen synthase kinase-3 (GSK-3) is a substrate for Akt in many cell types and is rapidly phosphorylated upon lymphocyte activation in a PI3K-dependent manner [29,36]. Phosphorylation by Akt suppresses GSK-3 kinase activity in certain insulin-responsive cells – this is important for augmenting glycogen synthesis. Nuclear factor of activated T cells (NFAT) is likely to be an important GSK-3 substrate in resting lymphocytes. GSK-3 phosphorylation of NFAT promotes its nuclear exit, thus preventing transcription of NFAT target genes (Figure 4). It has been speculated that PI3K-Akt activation releases this break, contributing to NFAT nuclear accumulation [29,36]. In support of a possible negative role for GSK-3 in T cell activation, transgenic expression of constitutively active GSK-3β interferes with T cell proliferation [37]. Furthermore, pharmacological inhibition of GSK-3 in T cells enhanced activation while augmenting NFAT nuclear accumulation [37]. However, the link between Akt, GSK-3 and NFAT is contradicted by the findings that activated Akt does not enhance transcription of an NFAT reporter gene in Jurkat T cells

Figure 4



Akt substrates and downstream targets with likely or suspected functions in lymphocyte activation. Activating events are indicated by arrows, inactivating events by inverted T shapes. Note that, although in many cases Akt phosphorylation inactivates the substrate, the net result for the cell is activation because these substrates oppose cell proliferation. An example that is not discussed in the text is TSC2, a substrate for Akt that has been defined (thus far in nonlymphoid cells only) as an inhibitor of mTOR function [45]. See text for details on other pathways depicted in this figure.

or the endogenous IL-4 gene in primary T cells [35]. GSK-3 also regulates β-catenin (Figure 4), the function of which, in T cell development and activation, has recently come to be appreciated [38,39]. Whether PI3K-Akt signaling regulates β-catenin via GSK-3 inactivation remains to be determined.

Transcription factors of the Forkhead Box, Subgroup O (FOXO) family are an important group of Akt substrates [40]. Akt phosphorylation inactivates FOXO factors by inducing their release from DNA, sequestration in the cytoplasm, and degradation. Akt-mediated FOXO inactivation has an evolutionarily conserved role in growth factor-driven increases in cellular metabolism, and is important for cell proliferation and survival in vertebrates (Figure 4). FOXO factors increase transcription of many target genes that are involved in cell-cycle arrest, quiescence and apoptosis [for example, $p27^{kip}$, Rb2/p130, Bim,

TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), Fas ligand]. FOXO1 is expressed in naïve T cells and B cells and is phosphorylated following mitogenic stimulation in a PI3K-dependent manner [41•,42]. Forced expression of FOXO variants that cannot be phosphorylated by Akt promote cell-cycle arrest and apoptosis in activated lymphocytes, correlating with induction of $p27^{kip}$ and Bim [41•,42]. These findings support the view that FOXO inactivation in lymphocytes is central to the ability of PI3K-Akt signaling to initiate the transition from quiescence into the cell cycle, and to sustain both cell-cycle progression and survival in activated cells.

Although probably not a direct Akt substrate, the mammalian target of rapamycin (mTOR) is an important downstream mediator of PI3K-Akt signaling. mTOR is a kinase that controls a crucial cellular switch, allowing cells to increase translation of a subset of mRNAs that are important for growth and proliferation [43]. This function of mTOR is mediated, in part, by its ability to activate serine/threonine kinases of the S6 kinase (S6K) family, leading to phosphorylation of the S6 ribosomal protein (Figure 4). The complex mechanisms by which PI3K controls mTOR and S6K activation have been reviewed elsewhere [44,45]. In the context of lymphocyte biology, the potent immunosuppressive effects of rapamycin highlight the importance of mTOR/S6K function for lymphocyte activation; moreover, in both T cells and B cells, stimulation via the antigen receptor and other mitogenic receptors causes phosphorylation and activation of S6K in a PI3K-dependent manner [3].

Under certain conditions, Akt can contribute to the activation of NFκB [36]. As mentioned previously, transgenic expression of active Akt in T cells augments the ability of mitogens to stimulate the NFκB pathway [32]. Work from Kane, Weiss and colleagues [35] has delineated a pathway in which Akt and PKCθ cooperate to activate the IKK complex, leading to IκB degradation. In this model, PKCθ is activated by TCR crosslinking, whereas Akt activation is primarily initiated by CD28 costimulation. A possible link between Akt and the IKK complex is Cot/Tpl2, a member of the MAP3K family of kinases (Figure 4). Cot was shown to be a direct Akt substrate; cotransfection studies in T cell lines and 293 cells further demonstrated that Cot promotes NFκB pathway activation in a manner that is dependent on Akt phosphorylation [46•]. The pathway leading from Akt to Cot, and to IKK activation remains to be confirmed by gene ablation in primary T cells. It also remains to be determined whether Akt contributes to NFκB activation in B cells; this might be most relevant in B cells that are stimulated with lipopolysaccharide or anti-CD40, mitogens that trigger activation of Akt and NFκB without initiating Ca^{2+} mobilization.

Size Matters

Naive lymphocytes are small quiescent cells with low rates of glycolysis, oxidative phosphorylation and macromolecular synthesis [47]. In preparation for cell division, activated T and B cells grow dramatically in size, alter their energy metabolism and increase rates of RNA and protein synthesis. Recent work has established a central role for PI3K in promoting molecular events that are integral to the lymphocyte growth response.

Treatment of B cells with PI3K inhibitors prevents mitogen-induced increases in cell size [22••,48]. The role of PI3K in B-cell growth has been linked to NFκB-dependent induction of c-myc [22••] (Figure 3), the role of which in B-cell growth and proliferation is well established. Some of the PI3K-dependent growth signal also appears to be mediated by mTOR signaling, as rapamycin partially inhibits B-cell growth [22••]. Significantly, withdrawal of mitogens or addition of PI3K inhibitors to activated B cells arrests further cell growth [48], even at late time points after c-myc expression has peaked and returned to baseline [22••]. Growth arrest under these conditions correlates with S6K inactivation [48], suggesting that sustained S6K function might be essential for activated B cells to complete the growth program in preparation for cell division. In T cells that are activated by APC, a late requirement for PI3K signaling is supported by inhibitor time-course studies and by the observation of sustained PtdIns(3,4,5)P_3 production at the site of APC contact [24••,49••].

In T cells, an intriguing link has been suggested between the PI3K-Akt signaling pathway and activation-induced increases in metabolic activity [50••]. In human T cells, activated via the TCR–CD3 complex, costimulation through CD28 was shown to be required for upregulation of the glucose transporter Glut1 and for increased glycolysis. These effects correlate with the ability of CD28 to promote maximal Akt activation. Furthermore, expression of membrane-targeted Akt in a hematopoietic cell line increases glucose metabolism – a finding that was later reproduced in Akt-transgenic T cells [34•]. Interestingly, the glycolytic switch is not triggered by CD28 engagement alone, nor by IL-2 or strong TCR crosslinking, stimuli that induce considerable Akt activation. It appears that Akt can only promote increased glucose metabolism in the context of T-cell costimulation via the TCR and CD28 (or when Akt is constitutively targeted to the membrane). Notably, Akt-transgenic T cells also exhibit a basal increase in cell size [34•]. The mechanisms by which Akt promotes growth and glucose metabolism in T cells are not yet clear. On the basis of B-cell studies, one might speculate that cell size increases are mediated by activation of mTOR/S6K and/or the NFκB pathway. The role of Akt in energy metabolism might be the result of inactivation of GSK-3 and FOXO proteins, as in other cell types.

Future directions

It has been well documented, first by inhibitor studies and later by gene knockouts in mice, that PI3K activation is essential for lymphocyte proliferation. In recent years, the molecular mechanisms by which PI3K and its lipid products promote the clonal expansion of lymphocytes have begun to come into focus. This review has emphasized the fact that Tec-family kinases and Akt are two crucial groups of PI3K effectors that, together, coordinate many of the downstream actions of PI3K signaling in T and B cells. An emerging theme is that PI3K signaling controls cell growth and metabolic changes, as well as survival signals, which allow progression through the cell cycle, in preparation for cell division. Although more work is certainly necessary to unravel the complexities of Tec-kinase and Akt signaling in lymphocytes, it is important to consider the roles of other putative PI3K effectors in lymphocyte activation. Of particular interest is the recent demonstration that the adaptor protein Bam32, the PH domain of which is selective for 3-phosphoinositides, is required for optimal B-cell proliferation [51].

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