



## Phosphoinositide 3-kinase in immunological systems

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*Phosphoinositide 3-kinases (PI3Ks) are an evolutionarily conserved family of signal transducing enzymes. A great variety of stimuli activate PI3K, leading to the transient accumulation of its lipid products in cell membranes. These lipids serve as second messengers to regulate the location and activity of an array of downstream effector molecules. In cells of the mammalian immune system, PI3K is activated by receptors for antigen, cytokines, costimulatory molecules, immunoglobulins and chemoattractants. Signaling via PI3K regulates immune cell proliferation, survival, differentiation, chemotaxis, phagocytosis, degranulation, and respiratory burst. Here we review our current understanding of PI3K signaling in leukocytes.*

**Key words:** phosphoinositide / kinase / PI3K / signal transduction / lymphocyte

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### Introduction

Phosphoinositide 3-kinase (PI3K) was first identified as an activity associated with various oncoproteins and growth factor receptors, and evidence has accumulated that PI3K can provide a critical signal for cell proliferation and survival. It is now clear, however, that PI3K signaling influences many other aspects of cell function. The diverse roles of PI3K are the result of several factors. First, many extracellular ligands engage receptors that can activate PI3K. Second, there are three classes of PI3K enzyme, two

of which are represented by multiple isoforms. Third, many potential effector proteins can interact with PI3K lipid products. Depending on the cell type, the receptor(s) engaged and the PI3K isoform(s) involved, different effectors respond to the PI3K signal and collaborate with other pathways to produce distinct responses.

The structure of different PI3K classes, the lipids they produce, and the types of phosphoinositide-binding domains have been extensively reviewed elsewhere.<sup>1–3</sup> This review will focus on class I PI3Ks, whose role in leukocyte signaling pathways is understood in most detail. All class I PI3Ks consist of a catalytic subunit and a tightly associated regulatory subunit (Figure 1). Tyrosine kinase-based signaling pathways primarily activate the class IA subgroup of PI3Ks, of which there are three catalytic isoforms (p110 $\alpha$ , p110 $\beta$  or p110 $\delta$ ). There are five class IA regulatory isoforms (p85 $\alpha$ , p85 $\beta$  and p55 $\gamma$  are encoded by distinct genes; p55 $\alpha$  or p50 $\alpha$  are produced from alternate transcripts of the p85 $\alpha$  gene), each possessing two Src Homology 2 (SH2) domains that mediate binding to phosphotyrosine residues in the sequence context pYXXM. Together with other protein-interaction domains in the catalytic and regulatory subunits (Figure 1), the SH2 domains help recruit class IA PI3Ks to membrane-associated signaling complexes following tyrosine kinase activation. Heterotrimeric G protein-based signaling pathways mainly activate the single class IB PI3K, p110 $\gamma$ . This catalytic isoform associates with a unique regulatory subunit, p101, which contributes to its regulation by G protein  $\beta\gamma$  dimers.

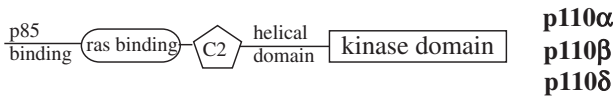
*In vitro*, class I PI3Ks phosphorylate the 3-hydroxyl of phosphatidylinositol (PtdIns) and its derivatives to produce PtdIns(3)P, PtdIns(3,4)P<sub>2</sub>, PtdIns(3,5)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>.<sup>3</sup> *In vivo*, the major products of class I PI3Ks appear to be PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub>, as these are transiently induced following cell stimulation. PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> bind selectively to certain pleckstrin homology (PH) domains, modular segments of about

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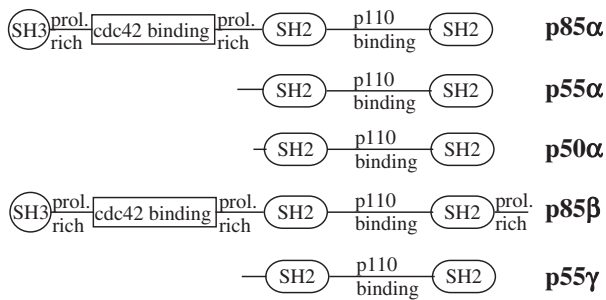
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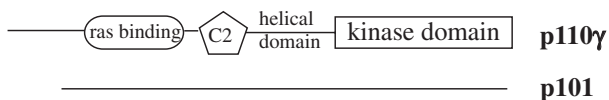
### Class IA Catalytic Isoforms



### Class IA Regulatory Isoforms



### Class IB Catalytic and Regulatory



**Figure 1.** Schematic diagram of the domain structure of mammalian class IA and class IB PI3Ks. The class IA catalytic isoforms associate interchangeably with the different regulatory isoforms. p85 $\alpha$ , p55 $\alpha$  and p50 $\alpha$  are encoded by alternative transcripts of a single gene.

100 amino acids found in many signaling proteins.<sup>4</sup> The ability of PH domains within different proteins to discriminate PtdIns(3,4)P<sub>2</sub> and PtdIns(3,4,5)P<sub>3</sub> from each other and from other phosphoinositides is a central basis for specificity in PI3K signaling.<sup>4</sup> Proteins containing other modular protein domains, including PX domains and SH2 domains, may interact with PI3K lipid products and serve as specific effectors in certain signaling pathways.<sup>5-9</sup>

There are two major lipid phosphatases that modulate PI3K signaling by dephosphorylating PI3K products. The tumor suppressor PTEN is a 3-phosphoinositide phosphatase that converts PtdIns(3,4,5)P<sub>3</sub> to PtdIns(4,5)P<sub>2</sub>. SHIP (SH2-containing inositol phosphatase) is a 5-phosphoinositide phosphatase that converts PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>.

### Antigen receptors

The B cell receptor (BCR), T cell receptor (TCR) and high affinity IgE receptor (Fc $\epsilon$ RI) recognize

antigen (Ag) in different forms and mediate distinct functional responses. However, early signal transduction triggered by these three types of receptor shares many common features.<sup>10-12</sup> Src-family tyrosine kinases help initiate signaling by phosphorylating immunoreceptor-based tyrosine activation motifs (ITAMs) in the cytoplasmic tails of receptor signaling chains. This leads to the recruitment and activation of the SH2 domain-containing tyrosine kinase Syk, or the related kinase ZAP-70 in T cells. A host of further phosphorylation events ensues, leading to the activation of Tec family tyrosine kinases. Syk and Tec family kinases cooperate to phosphorylate and activate phospholipase C $\gamma$  (PLC $\gamma$ ), resulting in production of the second messengers diacylglycerol and Ins(3,4,5)P<sub>3</sub> with subsequent calcium (Ca<sup>2+</sup>) flux and protein kinase C activation. PI3K is also activated by Ag receptor engagement and its lipid products regulate a number of downstream events.

The mechanism of PI3K function is perhaps best defined in the context of BCR-mediated Ca<sup>2+</sup> elevation.<sup>13</sup> PtdIns(3,4,5)P<sub>3</sub> binds with high affinity to the PH domain of the Tec family kinase Btk, recruiting it to the membrane. There it can be activated by membrane-associated Src family kinases that phosphorylate Y551 in the activation loop.<sup>14,15</sup> PtdIns(3,4,5)P<sub>3</sub> also directly increases Btk activity, apparently by relieving an inhibitory interaction between the PH and kinase domains.<sup>16</sup> A critical substrate of Btk is PLC $\gamma$ 2. Tyrosine phosphorylation of the scaffolding protein BLNK by Syk provides docking sites for the SH2 domains of both Btk and PLC $\gamma$ 2, facilitating phosphorylation of the latter.<sup>17,18</sup> PI3K may also contribute to recruitment of PLC $\gamma$ 2 via direct interactions of PtdIns(3,4,5)P<sub>3</sub> with the SH2 and/or PH domains of PLC $\gamma$ 2.<sup>19,20</sup> As for Btk, PLC $\gamma$ 2 activity *in vitro* was shown to be enhanced by PtdIns(3,4,5)P<sub>3</sub>.<sup>21</sup> The end result of this pathway is maximal activation of PLC $\gamma$ 2, production of Ins(3,4,5)P<sub>3</sub>, and generation of a sustained increase in intracellular Ca<sup>2+</sup> concentration.

This model is supported by studies showing that overexpression of Btk and PI3K enhances PLC $\gamma$ 2 phosphorylation and the sustained phase of Ca<sup>2+</sup> flux.<sup>22</sup> Conversely, treatment of primary B cells with wortmannin attenuates BCR-mediated Ca<sup>2+</sup> flux.<sup>23</sup> Co-engagement of the BCR with the inhibitory receptor Fc $\gamma$ RIIB also diminishes sustained Ca<sup>2+</sup> flux, an important mechanism for limiting B cell activation in later phases of an immune response when Ags are coated with

specific IgG. Fc $\gamma$ RIIB coligation inhibits signaling in part by recruiting SHIP to the membrane in the vicinity of the BCR,<sup>24</sup> resulting in conversion of PtdIns(3,4,5)P<sub>3</sub> to PtdIns(3,4)P<sub>2</sub>, a lipid that does not bind to the Btk PH domain. Finally, mutation of arginine 28 in the Btk PH domain eliminates selective binding to PtdIns(3,4,5)P<sub>3</sub>, reduces the Ca<sup>2+</sup> response, and is the genetic lesion in X-linked immunodeficiency (*Xid*) in mice and some cases of X-linked agammaglobulinemia (XLA) in humans.<sup>25</sup> *Xid* mice with a R28C mutation show B cell defects comparable to mice completely lacking Btk.<sup>26</sup> However, the B cell development defects in *Xid* or Btk-null mice are less severe than in human XLA patients. In the mouse, Btk may be partially redundant with the related kinase Tec. Deletion of Tec alone has no effect on B cells, but deletion of both Tec and Btk results in a more severe block in B cell development compared to loss of Btk alone.<sup>27</sup>

Additional observations point to the importance of PI3K in BCR function. The PI3K inhibitors, wortmannin and LY294002 inhibit BCR-mediated proliferation of primary B cells from humans and mice.<sup>28,29</sup> SHIP-deficient B cells show enhanced sensitivity to BCR signals, even in the absence of inhibitory receptor engagement.<sup>30</sup> Deletion of the gene encoding p85 $\alpha$  causes a partial block in murine B cell development and completely abrogates BCR-mediated proliferation.<sup>29,31</sup> The spectrum of defects in p85 $\alpha$ -deficient B cells is similar to the phenotypes of B cells deficient in Btk, BLNK or PLC $\gamma$ 2.<sup>13</sup> Although this genetic correlation suggests that the Btk/PLC $\gamma$ 2 pathway is a critical effector of PI3K signaling in B cells, PI3K influences other pathways as well. This conclusion is supported by microarray studies showing that PI3K and Btk share transcriptional targets but that additional genes are regulated by PI3K alone.<sup>133</sup>

A central mediator of PI3K signaling is phosphoinositide-dependent kinase 1 (PDK1).<sup>32</sup> This ubiquitously expressed protein has a PH domain that binds selectively to PI3K lipid products. This interaction is thought to position the kinase domain in proximity to relevant substrates in the membrane. Two key substrates for PDK1 are PKB (also known as Akt) and S6 kinase, each of which is activated by BCR engagement in a PI3K-dependent manner.<sup>33-35</sup> PKB family members regulate cell growth and survival via the phosphorylation of a number of critical substrates (see section on cytokine receptors). S6 kinases phosphorylate the S6 ribosomal protein and regulate translation. Given that the PH domains of PKB and

PDK1 bind with similar affinity to PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub>, it was proposed that coligation of the BCR with Fc $\gamma$ RIIB may not abrogate PKB activation.<sup>36</sup> However, Fc $\gamma$ RIIB coligation reduces the accumulation of both PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub> and blocks BCR-stimulated increases in PKB kinase activity.<sup>33,37</sup>

The mechanism of PI3K function downstream of the TCR is less clear than for the BCR. A major problem in the study of PI3K signaling in T cells is that a widely used model system has been Jurkat cells. This human T leukemia line was recently shown to lack expression of both PTEN and SHIP and to have constitutive membrane association of PKB and Itk, a kinase of the Tec/Btk family.<sup>38</sup> Hence, studies of Jurkat cells may underestimate the contribution of PI3K to T cell signaling. Experiments using primary T cells have clearly established a role for PI3K. Wortmannin or LY294002 abolish TCR-dependent proliferation and IL-2 production by primary human and mouse T cells.<sup>29,39,40</sup> In addition, T cell-specific deletion of PTEN enhances proliferation triggered by TCR crosslinking and disrupts both negative and positive selection of thymocytes.<sup>41</sup>

The pathway linking TCR engagement to Ca<sup>2+</sup> flux involves an array of kinases and adapter proteins that are mostly distinct from those in B cells.<sup>11</sup> For example, the scaffolding functions of BLNK appear to be distributed between LAT and SLP-76, and the target phospholipase is the PLC $\gamma$ 1 isoform. Instead of Tec and Btk, T cells express Tec, Itk and Rlk. Although the first two possess PH domains with likely specificity for PtdIns(3,4,5)P<sub>3</sub>, Rlk lacks a PH domain and is tethered to the membrane by palmitoylation.<sup>42</sup> Gene targeting in mice has shown that TCR-mediated sustained Ca<sup>2+</sup> flux is impaired in Itk-deficient T cells, which also show moderate proliferation defects.<sup>43,44</sup> Deletion of both Itk and Rlk results in a severe block in both Ca<sup>2+</sup> flux and proliferation.<sup>42</sup> Combined with biochemical studies, these findings support the model that PI3K-dependent Itk activation cooperates with Rlk to mediate TCR-dependent phosphorylation of PLC $\gamma$ 1 and sustained Ca<sup>2+</sup> flux. As for the BCR, other PI3K-dependent targets of TCR signaling include PKB and S6 kinase. Mice expressing a membrane-targeted PKB in the T lineage show enhanced survival of thymocytes and peripheral T cells.<sup>45</sup>

Surprisingly, deletion of the p85 $\alpha$  gene has no discernable effect on T cell development or TCR-mediated proliferation.<sup>29</sup> The p85 $\beta$  isoform, though expressed at lower levels in T cells, may be more

important than p85 $\alpha$  or redundant in function. It is also possible that class IB PI3K is the relevant enzyme downstream of the TCR. One of three groups that disrupted the mouse gene encoding p110 $\gamma$  observed diminished TCR-mediated proliferation of peripheral T cells.<sup>46</sup> Heterotrimeric G protein  $\alpha$  subunits have been reported to associate with the TCR,<sup>47</sup> suggesting a possible mechanism for class IB PI3K activation.

Wortmannin and LY294002 inhibit granule exocytosis triggered by Fc $\epsilon$ RI crosslinking of mouse bone marrow-derived mast cells (BMMC) or the rat basophilic leukemia line RBL-2H3.<sup>48-50</sup> As for the BCR, Fc $\epsilon$ RI engagement triggers a sustained increase in intracellular Ca<sup>2+</sup> that is attenuated by pretreatment with PI3K inhibitors.<sup>51-53</sup> The finding of enhanced Ca<sup>2+</sup> flux in SHIP-deficient BMMC provides additional evidence for PI3K regulation of this pathway.<sup>52</sup> As in B cells, an important PI3K effector is likely to be Btk, as BMMC from Xid mice show diminished degranulation.<sup>51,54</sup> Microinjection studies of RBL-2H3 cells have implicated the p110 $\beta$  and p110 $\delta$  catalytic isoforms in Fc $\epsilon$ RI-mediated sustained Ca<sup>2+</sup> flux.<sup>21</sup> Consistent with a role for class IA PI3K in mast cell activation, mice lacking Gab2, an adapter for tyrosine kinase-dependent PI3K recruitment, have defective Fc $\epsilon$ RI-dependent responses (see later).<sup>55</sup> However, we found that Fc $\epsilon$ RI-triggered degranulation of fetal liver-derived mast cells does not require p85 $\alpha$ , p55 $\alpha$  or p50 $\alpha$ .<sup>50</sup>

An emerging concept in Ag receptor research is that receptors and critical signaling molecules are reversibly recruited to lipid rafts.<sup>56</sup> An interesting question is whether PI3K regulates this process. In human pre-B cell lines, class IA PI3K can be detected in a pre-formed signaling complex in rafts.<sup>57</sup> In the A20 mouse B cell line, BCR engagement triggers the transient recruitment of PLC $\gamma$ 2 to lipid rafts, and disruption of raft integrity abrogates sustained Ca<sup>2+</sup> flux.<sup>58</sup> Vav1 and Vav2 may link PI3K to cytoskeletal changes and raft aggregation. These modular proteins possess PH domains and a Dbl-homology domain with guanine nucleotide exchange activity for Rac. Interaction of the PH domain of Vav1 with PtdIns(3,4,5)P<sub>3</sub> was shown to enhance exchange activity *in vitro*,<sup>59</sup> but the relevance of this observation for Ag receptor-dependent Vav recruitment and activation is uncertain. Interestingly, Ca<sup>2+</sup> flux is impaired in B cells lacking Vav1 and Vav2.<sup>60,61</sup>

How does PI3K become activated following Ag receptor engagement? Although interactions of p85-SH2 domains with Ag receptor signaling chains have

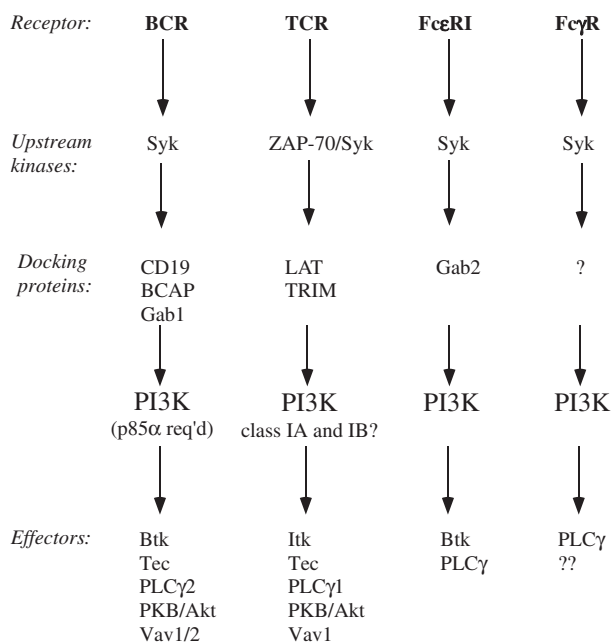
been reported, the absence of consensus YXXM motifs in the tails of these proteins suggests that such associations may be indirect. Src family kinases may play a direct role in membrane recruitment and activation of class IA PI3K. The SH3 domains of Lyn, Fyn and Lck are able to bind to the proline-rich motifs of p85 $\alpha$  *in vitro* and enhance activity of the associated PI3K catalytic subunit.<sup>62-64</sup> No single Src family kinase has been established as critical for PI3K activation in primary cells. Indeed, mice lacking Lyn have hyperresponsive B cells and develop B cell-mediated autoimmunity that can be attenuated by loss of Btk function.<sup>65</sup> Lyn-deficient B cells also show increased PKB activation, implying that PI3K signaling is enhanced in the absence of Lyn.<sup>66</sup>

There is good evidence that Syk functions upstream of PI3K in B cells. Deletion of Syk in the DT40 chicken B cell line abolishes BCR-dependent increases in PI3K lipid products and PKB activation, and expression of a kinase-dead Syk in A20 mouse B cells attenuates PI3K activation.<sup>66,67</sup> A plausible mechanism involves the phosphorylation of adapter/scaffolding proteins and coreceptor molecules by Syk (or ZAP-70), providing docking sites for PI3K (Figure 2). Candidate docking proteins are discussed in the ensuing sections.

### Costimulatory receptors

CD19 is a B cell-specific transmembrane protein that is loosely associated with the BCR in unstimulated cells. Coligation of CD19 with the BCR greatly lowers the threshold for BCR-mediated proliferation. Notably, the cytoplasmic tail of CD19 contains tandem YXXM motifs that become tyrosine phosphorylated following BCR crosslinking and associate with the SH2 domains of class I PI3K regulatory subunits.<sup>68</sup> Experiments in cell lines have supported the idea that the CD19-PI3K interaction is important for BCR-mediated activation of PI3K. A CD19-negative variant of A20 mouse B cells shows greatly reduced PKB activation following BCR cross-linking.<sup>69</sup> In addition, a mouse plasmacytoma cell line expressing human CD19 shows increased PI3K activity and sustained Ca<sup>2+</sup> elevation relative to vector control cells or to cells expressing CD19 with mutations in the tyrosine residues critical for PI3K binding.<sup>23</sup> Deletion of CD19 in mice has yielded some conflicting data concerning the importance of the CD19-PI3K interaction *in vivo*. One group reported that CD19<sup>-/-</sup> showed reduced

### PI3K Signaling Downstream of Antigen/IgG Receptors



**Figure 2.** Pathways of PI3K activation and downstream effectors in Ag receptor signaling. Activation of Syk/ZAP-70 tyrosine kinases results in phosphorylation of coreceptors and/or adaptor molecules. Some of these recruit and activate class IA PI3K via the SH2 domains of the regulatory subunit. Other PI3K interacting molecules (Src family kinases, Ras) may contribute to PI3K activation. Class IB PI3K may play a role downstream of the TCR. Phosphoinositide-binding effectors of PI3K with likely functional roles in Ag receptor signaling are listed.

activation of PI3K, Btk, PKB, and diminished  $Ca^{2+}$  elevation following BCR engagement.<sup>23,69,70</sup> On the other hand, another group studying CD19<sup>-/-</sup> mice reported normal PI3K activation and  $Ca^{2+}$  mobilization.<sup>71</sup> It is worth noting that p85αKO mice and CD19<sup>-/-</sup> mice differ in their antibody responses to T-dependent and T-independent Ags. Overall, the evidence suggests that CD19 contributes to PI3K activation, but that CD19 has other functions and PI3K has additional modes of activation in B cells.

The T cell costimulatory receptor CD28 contains various protein interaction motifs in its cytoplasmic tail, including a YXXM sequence (Y170) that becomes phosphorylated upon CD28 crosslinking and mediates association with class IA PI3K.<sup>72</sup> In contrast to CD19 on B cells, CD28 is not phosphorylated by Ag receptor engagement alone, so it may not contribute to PI3K activation under these conditions. However, the interaction of CD28 with its

ligands on Ag presenting cells plays a critical role in TCR-dependent responses *in vivo*. Early transfection studies of T cell lines yielded conflicting results on the importance of the CD28-PI3K association for costimulation.<sup>72</sup> Moreover, enhancement of TCR-dependent proliferation by CD28 signaling in normal mouse T cells is only partially inhibited by PI3K inhibitors and does not require p85α or its splice variants.<sup>29</sup> Three recent papers examined T cell responses in CD28 knockout mice expressing transgenic or retroviral CD28 constructs.<sup>73–75</sup> Each group found that mutation of the critical tyrosine (Y170F) does not eliminate the ability of CD28 to enhance TCR-dependent proliferation or IL-2 production *in vitro*. Two of the papers reported that Y170 is required for CD28-dependent upregulation of the pro-survival protein Bcl-X<sub>L</sub>.<sup>74,75</sup> As both PI3K and PKB have been implicated in Bcl-X<sub>L</sub> induction in T cells, the primary role of the CD28-PI3K interaction may be to promote survival. The relevance of these findings *in vivo* are not yet clear: one paper found that CD28(Y170F) T cells are completely incapable of mediating an acute graft *versus* host response,<sup>73</sup> whereas another found that CD28(Y170F) functions comparably to wild-type CD28 in the prevention of anergy.<sup>75</sup> A caveat to these studies is that tyrosine phosphorylation of Y170 creates a binding site for the adapter protein Grb2 in addition to PI3K. Furthermore, the conclusions are seemingly inconsistent with a report that retroviral expression of constitutively active PKB rescues IL-2 production in T cells of CD28-deficient mice.<sup>76</sup> Thus, the question of whether the PI3K pathway mediates CD28-dependent enhancement of IL-2 production and/or survival is still open for debate.

### Adapter/scaffolding proteins

Adapter/scaffolding proteins are critical for the localization, propagation and integration of signals from Ag receptors and costimulatory receptors. Although they do not generally possess enzymatic activity, these proteins have multiple modular domains, sequence motifs and/or phosphorylated residues that serve as docking sites for signaling proteins.

There is good evidence that the scaffolding protein BCAP plays a role in PI3K activation in B cells.<sup>77</sup> BCAP possesses four YXXM motifs and associates with p85 proteins following BCR stimulation of chicken or mouse B cells. Deletion of BCAP in chicken DT40 cells reduces BCR-triggered PtdIns(3,4,5)P<sub>3</sub>

production and PKB activation, though not as completely as wortmannin treatment. Transfection of these cells with wild-type BCAP, but not a version mutated in each of the four YXXM motifs, restores PKB activation. BCAP phosphorylation requires Syk and is enhanced in the absence of Lyn, consistent with the positive and negative roles of these kinases in PI3K activation. Interestingly, sustained BCAP phosphorylation requires Btk, suggesting a possible amplification of PI3K signaling by one of its own effectors. This could explain the observation that both Syk and Btk are required for optimal PKB activation.<sup>34</sup>

Gab1 is a member of a family of docking proteins, including IRS-1, IRS-2, c-Cbl and Cbl-b, which possess N-terminal PH domains and multiple tyrosine phosphorylation sites. Like BCAP, Gab1 is tyrosine phosphorylated following BCR stimulation and interacts with class IA PI3K.<sup>78</sup> Overexpression of Gab1 enhances BCR-mediated PKB activation in the WEHI-231 mouse B cell line.<sup>79</sup> Both Gab1 and BCAP become enriched in membrane fractions following BCR engagement.<sup>77,78</sup> Interestingly, Gab1 membrane recruitment was reported to require its PH domain as well as PI3K activation.<sup>79</sup> Thus, Gab1 may respond to the PI3K signal by amplifying or extending the duration of PI3K activation.

Gab2 is a protein closely related to Gab1, and appears to play a role in PI3K activation downstream of various cytokine receptors (see later). Analysis of Gab2 knockout mice has revealed a required function for Gab2 in activation of PI3K following FcεRI engagement.<sup>55</sup> In Gab2-deficient mast cells, PtdIns(3,4,5)P<sub>3</sub> production and PKB phosphorylation are dramatically reduced; PLCγ1 phosphorylation, IP<sub>3</sub> production, sustained Ca<sup>2+</sup> flux are also diminished. Importantly, the cells show deficient mast cell function *in vitro* and allergic responses *in vivo*, suggesting that the Gab2/PI3K interaction may be a useful target for therapy.

T cells express two membrane-anchored adapters that have been implicated in PI3K activation. LAT is a palmitoylated protein that localizes to lipid rafts and becomes tyrosine phosphorylated following TCR stimulation. p85 has been detected in LAT immunoprecipitates, although it is not certain that the interaction is direct.<sup>80</sup> The importance of LAT for PI3K activation *in vivo* has been difficult to assess genetically because LAT-deficient mice have an early block in T cell development and the only other model is LAT-deficient Jurkat cells. TRIM is a transmembrane scaffolding protein that

associates with the TCR and contains a YXXM motif in its cytoplasmic tail.<sup>81</sup> TCR stimulation of HPB-ALL human leukemia cells causes tyrosine phosphorylation of TRIM and association with p85.

c-Cbl and Cbl-b are multifunctional proteins with complex roles in signal transduction. They are among the most highly tyrosine phosphorylated proteins following Ag receptor engagement, and associate with numerous other signaling proteins including class IA regulatory subunits.<sup>82</sup> PI3K associates with c-Cbl and Cbl-b via interactions involving both the SH2 and SH3 domains of p85.<sup>83,84</sup> Disruption of the c-Cbl or Cbl-b genes in mice, or a Cbl homolog in *C. elegans*, indicated that this protein family negatively regulates tyrosine kinase signals.<sup>85</sup> It is now known that Cbl proteins possess E3-ubiquitin ligase activity that helps target associated signaling proteins for ubiquitination and degradation.<sup>86</sup> In Jurkat cells, Cbl-b was shown to interact with p85 proteins and induce their ubiquitination.<sup>84</sup> Interestingly, primary T cells lacking Cbl-b show enhanced activity of Vav, a putative PI3K effector.<sup>87,88</sup> In addition, deletion of Cbl-b overcomes the requirement for CD28 in costimulation.<sup>87,88</sup> A plausible model is that one function of the CD28-PI3K interaction is to prevent activated PI3K from being targeted for ubiquitination by Cbl-b.

## Fc receptors

Whereas the inhibitory IgG receptor FcγRIIB attenuates PI3K signaling in B cells by recruiting SHIP, the Fcγ receptors on phagocytes activate PI3K. PtdIns(3,4,5)P<sub>3</sub> was shown to accumulate in human NK cells stimulated via FcγRIIIA (CD16),<sup>89</sup> in platelets activated through FcγRIIA,<sup>90</sup> and in mouse macrophages exposed to IgG-coated particles.<sup>91</sup> PI3K activation appears to play an important role in Fcγ receptor function. Wortmannin and/or LY294002 block IgG-dependent phagocytosis in neutrophils, macrophages and a monocytic cell line, and inhibit antibody-mediated cellular cytotoxicity by natural killer cells.<sup>91-93</sup> Expression in COS cells of the human FcγRIIA, FcγRI or FcγRIIIA (the latter two cotransfected with the obligate gamma signaling chain) confers phagocytic function that can be blocked by wortmannin.<sup>94</sup> In addition, transfection of fibroblasts with a chimeric receptor composed of the extracellular and transmembrane domains of mouse FcγRI fused to p85α is sufficient to induce phagocytosis of IgG-coated particles.<sup>95</sup>

Fc $\gamma$  receptors possess ITAM motifs in their cytoplasmic tail (human Fc $\gamma$ RIIA) or in the associated gamma signaling chain (mouse Fc $\gamma$ RI and Fc $\gamma$ RIII, human Fc $\gamma$ RIA and Fc $\gamma$ RIIA). Src family kinases and Syk are both required for Fc $\gamma$  receptor function,<sup>96,97</sup> suggesting that PI3K is activated by a mechanism analogous to the BCR system. The critical scaffolding proteins that recruit and activate PI3K downstream of Fc $\gamma$  receptors are not yet established. In different cellular contexts, Fc $\gamma$  receptor signaling may activate distinct PI3K effectors and downstream pathways. In platelets, PI3K activation is required for Fc $\gamma$ RIIA-mediated PLC $\gamma$ 2 activation, suggesting further similarity to BCR signaling.<sup>90</sup> In phagocytes, PI3K is required for maximal pseudopod extension and closure of the phagocytic vesicle but appears to be dispensable for initial phases of actin polymerization.<sup>91,98</sup> Consistent with this idea, PtdIns(3,4,5)P<sub>3</sub> accumulates in the area of the phagocytic cup, as detected by microscopy of cells expressing GFP-PH domain fusion proteins.<sup>99</sup>

### Receptors for mitogenic cytokines

Inhibitor and transfection studies have established that PI3K and its effector PKB link multiple cytokine receptors to both cell cycle entry and cell survival.<sup>100</sup> Phosphorylation by PKB reduces the pro-apoptotic function of several substrates including BAD, caspase-9 and forkhead family transcription factors.<sup>100</sup> The PI3K/PKB pathway also contributes to the induction of the anti-apoptotic protein Bcl-X<sub>L</sub>.<sup>45,101</sup> In the IL-2-dependent cell line Kit225, PKB promotes cell cycle entry by inducing E2F activity.<sup>102</sup> PKB has been shown to negatively regulate p27kip, in part by phosphorylating the transcription factor FKHL1 and reducing p27kip mRNA levels.<sup>103</sup> PKB phosphorylation and inactivation of GSK-3 may also accelerate cell cycle entry, as GSK-3 phosphorylation of D-type cyclins promotes their degradation.<sup>104</sup>

Other PI3K effectors are likely to be important for cytokine signaling. PI3K contributes in several ways to the cytokine-dependent activation of S6 kinases. Although S6 kinases do not interact directly with PI3K lipid products, PDK1 phosphorylates S6 kinases at a site critical for activation.<sup>32,100</sup> PDK1 also contributes to the activation of various PKC isoforms that phosphorylate other sites within S6 kinases.<sup>32,100</sup> The mammalian target of rapamycin (mTOR), a critical regulator of S6 kinase activation, is also influenced by PI3K signaling and may be

a direct substrate for PKB.<sup>105</sup> Although cytokines generally do not induce Ca<sup>2+</sup> flux, it is possible that activation of Tec family kinases by PI3K lipid products plays a role in cytokine-driven mitogenesis. Indeed, Btk-deficient B cells show diminished proliferative responses to IL-5.<sup>106</sup> On the other hand, mast cell proliferation driven by Kit ligand requires PI3K but not Btk.<sup>50</sup>

Different cytokine receptors use distinct mechanisms for activating class IA PI3K. CSF-1R and c-Kit are members of a receptor tyrosine kinase family, including PDGF receptors, which interact directly with PI3K. Ligand-induced activation of these receptor tyrosine kinases leads to phosphorylation of YXXM motifs in their kinase-insert regions and association with SH2 domains of class IA regulatory subunits.<sup>107,108</sup> Most cytokine receptors lack intrinsic enzyme activity and initiate signaling by activating Jak family tyrosine kinases. The receptors for IL-3, IL-5, and GM-CSF share a signaling chain (the  $\beta$ -common chain) that is phosphorylated by Jak kinases, leading to the recruitment of signaling effectors. Activation of PI3K downstream of this class of receptors appears to proceed by a complex mechanism involving the successive recruitment of the adapter proteins Shc, Grb2 and Gab2, the last of which becomes tyrosine phosphorylated and binds PI3K.<sup>109</sup> A similar mechanism for PI3K activation appears to operate downstream of the receptors for IL-2 and IL-15, which share a signaling chain (IL-2R $\beta$ ) that recruits Shc.<sup>109</sup> Still another mechanism applies to activation of PI3K by IL-4. Binding of IL-4 leads to phosphorylation of the IL-4R cytoplasmic tail by Jak kinases, and subsequent recruitment and phosphorylation of IRS-1/2 docking proteins.<sup>110</sup> As in the insulin signaling system, phosphorylated YXXM sites in IRS proteins then recruit SH2 domains of class IA PI3K. Finally, the IL-7 receptor activates PI3K by two routes. The tail of the IL-7R $\alpha$  chain contains a YXXM site that is phosphorylated after activation and binds PI3K directly.<sup>111</sup> In addition, as for the IL-4R, the IL-7R mediates IRS-1 recruitment and phosphorylation.<sup>112</sup>

Knockout studies have revealed a unique function for p85 $\alpha$  (or p55 $\alpha$ /p50 $\alpha$ ), in some cytokine signaling systems. Loss of p85 $\alpha$  blocks IL-4-dependent survival of mouse splenic B cells to a similar extent as treatment with LY294002.<sup>29</sup> p85 $\alpha$ -deficient mast cells show partially reduced proliferation driven by c-Kit ligand.<sup>50</sup> However, p85 $\alpha$  is not required for IL-2-dependent T cell proliferation<sup>29</sup> or IL-3-dependent mast cell proliferation.<sup>50</sup>

## Receptors for inflammatory cytokines/microbial products

A role for PI3K in inflammatory responses and innate immunity has begun to emerge from studies of signaling by receptors of the IL-1R and TNFR superfamilies. PI3K inhibitors have been reported to block NF $\kappa$ B activation triggered by IL-1, TNF $\alpha$ , and anti-CD40 (a TNF receptor family member).<sup>113-115</sup> These compounds, or loss of p85 $\alpha$ , also block B cell proliferation in response to anti-CD40 or LPS,<sup>29</sup> whose receptor TLR4 is a member of the IL-1R/TLR superfamily.

A variety of cell lines, many non-hematopoietic, have been used to investigate the mechanism of PI3K activation and its role in NF $\kappa$ B regulation downstream of the IL-1R, TNFR and related receptors. In 3T3-L1 adipocytes, TNF $\alpha$  treatment leads to phosphorylation of IRS-1 and recruitment of PI3K.<sup>116</sup> In 293 human embryonic kidney cells, TNF $\alpha$ -dependent activation of NF $\kappa$ B requires PI3K and PKB.<sup>114</sup> In this system, PKB phosphorylates I $\kappa$ B-kinase, leading to I $\kappa$ B phosphorylation and degradation. In osteoclasts and dendritic cells, the TNFR family member TRANCE-R activates PI3K via a signaling complex that includes Src and TRAF6.<sup>117</sup> Src family kinases may also contribute to PI3K activation in IL-1R/TLR family receptors. The Src inhibitor PP1 blocks PKB activation by IL-1 and LPS in dendritic cells,<sup>117</sup> and Lyn associates with class IA PI3K following LPS stimulation of monocytes.<sup>118</sup>

The IL-1R has been reported to associate both directly and indirectly with PI3K. The tail of IL-1R contains a YXXM site and binds to p85 *in vitro*.<sup>113,119</sup> In transfections of Saos2 osteosarcoma cells, a mutated IL-1R lacking the critical tyrosine failed to recruit or activate PI3K, and was deficient in promoting NF $\kappa$ B-dependent transcription.<sup>119</sup> PI3K has also been reported to associate with the IL-1 receptor accessory protein in HepG2 cells.<sup>120</sup> Further investigation in this system indicated that PI3K signaling via PKB leads to phosphorylation of the p65/RelA subunit of NF $\kappa$ B, rather than the sequestering protein I $\kappa$ B. TLR2, a receptor for components of gram-positive bacteria, activates PI3K in 293 cells via a pathway requiring the small G protein Rac1.<sup>121</sup> Subsequent PKB activation leads to p65/NF $\kappa$ B-dependent transcription but does not affect I $\kappa$ B degradation, as in the IL-1R system.

LPS activates S6 kinase in murine macrophages and the atypical PKC $\zeta$  (another PDK1 substrate) in human monocytes.<sup>122,123</sup> In addition, Btk-deficient

B cells show diminished proliferation in response to LPS.<sup>26,29</sup> Thus, PKB may not be the only important PI3K effector in TLR signaling. Since BCR-stimulated NF $\kappa$ B activation is also reduced by the *Xid* mutation,<sup>124</sup> it is worth investigating whether Btk or other Tec family kinases contribute to NF $\kappa$ B regulation downstream of IL-1R/TLR and TNFR family members.

## Chemoattractant receptors

Another role for PI3K in innate immunity is in responses to chemoattractants. Some of the earliest studies of wortmannin and LY294002 described inhibition of chemotaxis and superoxide production in neutrophils treated with fMLP.<sup>125,126</sup> Like the endogenous chemoattractants C5a and IL-8, fMLP acts through a G protein-coupled receptor (GPCR). Studies of mice lacking the class IB PI3K, p110 $\gamma$ , have shown that this isoform is essential for PtdIns(3,4,5)P<sub>3</sub> production and PKB activation in neutrophils exposed to fMLP, C5a or IL-8.<sup>46,127,128</sup> *In vitro*, superoxide production was severely compromised and chemotaxis was partially reduced in the absence of p110 $\gamma$ . *In vivo*, migration of inflammatory cells was also impaired. This work has established p110 $\gamma$  as a critical PI3K isoform linking GPCR ligands to oxidative burst and chemotaxis.

The role of PI3K in superoxide production has been clarified by the recent discovery that the cytosolic component of the oxidative burst complex directly binds to lipid products of PI3K. The p40phox and p47phox proteins have in common a domain called the PX or phox domain that is also found in a variety of other proteins. Several of these PX domains have recently been shown to bind to phosphoinositides.<sup>6-9</sup> The p40phox PX domain binds to PtdIns(3)P while the p47phox PX domain binds preferentially to PtdIns(3,4)P<sub>2</sub>.<sup>8</sup> Reconstitution of an oxidative burst response requires the cytosolic components (p40phox/p47phox/p67phox and GTP rac2) along with the membrane components (gp91phox and p22phox) and the incorporation of PtdIns(3)P into the membrane.<sup>9</sup> Interestingly, the preference of the p47phox PX domain for binding to PtdIns(3,4)P<sub>2</sub> raises the possibility that the complex is preferentially activated in membranes that have both PtdIns(3,4)P<sub>2</sub> and PtdIns(3)P.<sup>8</sup> The former lipid is likely to be derived from activation of a class I PI3K at the plasma membrane while the latter lipid is generally

found in endosomes. This lipid requirement may insure that superoxide production is only activated when the phagosome is internalized and both PtdIns(3,4)P<sub>2</sub> and PtdIns(3)P are present. Further studies are needed to investigate the pathways for phosphoinositide generation in the phagosome.

The role of PI3K in chemotaxis is also complex and poorly understood. In fMLP-stimulated neutrophils, PtdIns(3,4,5)P<sub>3</sub> is found at the leading edge of the migrating cell,<sup>129</sup> as judged by localization of PH domain-GFP fusion proteins. Inhibition of PI3K with drugs or, as indicated earlier, disruption of p110gamma impairs lamellipodia formation and cell migration.<sup>130</sup> Production of PtdIns(3,4,5)P<sub>3</sub> at the leading edge appears to precede actin rearrangement and may initiate cortical actin polymerization, in part by activating Rac. The mechanism by which production of PtdIns(3,4,5)P<sub>3</sub> remains polarized to the leading edge of cells migrating in a shallow gradient of chemoattractant is not yet clear but presumably involves a positive feedback loop whereby products of PI3K enhance local activation of this enzyme.

### Concluding thoughts

The study of PI3K signaling in leukocytes has advanced considerably from early experiments defining responses sensitive to PI3K inhibitors. In many receptor systems, important PI3K effectors have been identified and linked to particular functional responses (Figure 2). More needs to be learned about the mechanisms of PI3K activation, particularly in TCR and FcγR signaling. Future work is also likely to focus on understanding the precise spatio-temporal regulation of PI3K activation and recruitment of relevant effectors. The use of PH domain-GFP fusion proteins has already been instrumental in defining the membrane microdomains at which PI3K lipid products accumulate. It is also important to understand the role of molecules that may serve as endogenous modulators of PI3K signaling, such as the PH domain protein Bam32/DAPP1<sup>131</sup> and the CD2AP/Ruk family.<sup>132</sup> To achieve the eventual goal of therapeutic targeting of the PI3K pathway will require a detailed knowledge of the PI3K isoforms, effectors, and regulators that operate in different signaling systems.

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