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PI3K SIGNALLING

Topic Editors
Klaus Okkenhaug, Martin Turner and
Michael R. Gold

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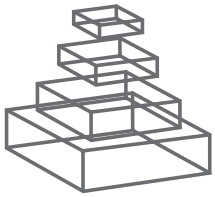

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PI3K SIGNALLING

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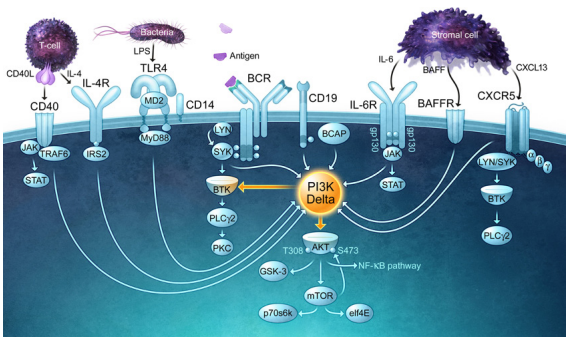

PI3Kδ is a central signaling enzyme that mediates the effects of multiple receptors on B cells. PI3Kδ signaling is important for B-cell survival, migration, and activation, functioning downstream of the B-cell antigen receptor (BCR) and its co-receptor CD19, chemokine receptors (CXCR5), and activating/co-stimulatory receptors such as CD40 and Toll-like receptors (TLRs). Cytokines derived from lymphoid stromal cells (BAFF, IL-6) and T cells (IL-4) that are essential for the expansion and survival of B cells also require PI3Kδ for their actions and bind receptors that activate PI3Kδ. The receptors depicted in this figure use a variety of kinases and adaptor proteins to recruit PI3Kδ to the plasma membrane, where it can produce the lipid second messenger PIP3. By binding to PH domain-containing proteins, PIP3 promotes the activation of multiple signaling enzymes including PLCγ2 and Akt, both of which control key signaling networks. Note that the connecting arrows may represent multiple intermediate signaling reactions. Copyright: Puri and Gold, 2012.

The PI3Ks control many key functions in immune cells. PI3Ks phosphorylate PtdIns(4,5)P2 to yield PtdIns(3,4,5)P3. Initially, PI3K inhibitors such as Wortmannin, LY294002 and Rapamycin were used to establish a central role for Pi3K pathway in immune cells. Considerable progress in understanding the role of this pathway in cells of the immune system has been made in recent years, starting with analysis of various PI3K and Pten knockout mice and subsequently mTOR and Foxo knockout mice. Together, these experiments have revealed how PI3Ks control B cell and T cell development, T helper cell differentiation, regulatory T cell development and function, B cell and T cell trafficking, immunoglobulin class switching and much, much more. The PI3Kδ inhibitor idelalisib has recently been approved for the treatment of B cell lymphoma. Clinical trials of other PI3K inhibitors in autoimmune and inflammatory diseases are also in progress.

This is an opportune time to consider a Research Topic considering when what we have learned about the PI3K signalling module in lymphocyte biology and how this is making an impact on clinical immunology and haematology.

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EDITORIAL
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PI3K signaling in B cell and T cell biology

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Keywords: PI3K/AKT/mTOR, PI3K pathway inhibitors, T cell, B cell, signal transduction

The drug Idelalisib is the first PI3K inhibitor to be approved by the FDA for clinical use. It is therefore timely to take stock of our current understanding of the role of PI3Ks in the immune system. The phosphoinositide 3-kinases (PI3Ks) control many key functions in immune cells (1). PI3Ks phosphorylate PtdIns(4,5)P_2 to yield PtdIns(3,4,5)P_3, which acts as a second messenger signaling molecule that controls the activation of Akt and other proteins with PH domains. Initially, PI3K inhibitors such as Wortmannin, LY294002, and Rapamycin were used to establish a central role for PI3K pathway in immune cells. More recent progress in understanding the role of this pathway in cells of the immune system has been made through the generation of wide range of gene-targeted mouse models as well as with the development of highly selective small molecule inhibitors, culminating in the FDA approval of the PI3Kδ inhibitor Idelalisib in 2014. Together, lab experiments, preclinical, and clinical trials have revealed how PI3Ks control B cell and T cell development, T helper cell differentiation, regulatory T cell (Treg) development and function, B cell and T cell trafficking, immunoglobulin class switching, and much more.

The class I PI3Ks are heterodimers composed of a catalytic subunit and a regulatory subunit. The p110α, p110β, and p110δ catalytic subunits form heterodimers with either of the p85α, p55α, p50α, p85β, or p55γ regulatory subunits, each of which contains SH2 domains that are engaged by tyrosine phosphorylated proteins. By contrast, the p110γ catalytic subunit forms heterodimers with either the p84 or p101 regulatory subunits that are recruited to the Gβγ subunits released by G-protein coupled receptors. The respective heterodimers are often referred to as PI3Kα, PI3Kβ, PI3Kδ, or PI3Kγ, respectively. Progress has been made in understanding the often non-redundant roles of each of these PI3K isoforms in immunity (2). The majority of these efforts have been directed at PI3Kδ and PI3Kγ, which are highly expressed in cells of the immune system.

mTOR inhibitors such as rapamycin are already established as among the most commonly used drugs to prevent transplant rejection and are increasingly also evaluated for the use in cancer (3). Most recently, the PI3Kδ-selective inhibitor Idelalisib (CAL-101) has received FDA approval for the treatment of chronic lymphocyte leukemia and indolent non-Hodgkin's lymphoma following on from successful clinical trials showing dramatic improvements in progress-free survival (4, 5). Many other PI3K inhibitors are going through clinical trials with the aim to treat cancers, and inflammatory and autoimmune diseases.

This eBook contains 11 chapters that cover different roles of the PI3K and mTOR pathways, primarily in B cells and T cells. Limon and Fruman review the role of PI3Ks in B cell development and activation and also consider how the related Akt, Foxo, and mTOR pathways affect B cell biology (6). Marshall and colleagues present a complementary view, focusing on the different mechanisms of activation of PI3K in B cells, and the roles of the different phosphatases as well as individual role for additional PIP_3 effectors such as Bam32, Tapp1, and Tapp2 (7). Puri and Gold pick up on this theme and further explore the clinical utility of blocking PI3K activity in B cells, both in the context of autoimmune diseases, but also by summarizing the early clinical trials that led to accelerated approval of Idelalisib for the treatment of B cell lymphomas (8). Bhatt and Damania consider the roles of PI3K and mTOR in the transformation of B cells by Kaposi sarcoma viruses and how PI3K and/or mTOR inhibitors should be considered for the treatment of virally induced lymphomas (9).

Lewings and colleagues consider how PI3K signaling strength affects T cell differentiation events. Their data show that high levels of PI3K signaling can antagonize the differentiation of Treg in favor of the differentiation of effector T cells (10). Okkenhaug and colleagues present a somewhat different perspective, reviewing evidence that low PI3K activity is also detrimental to Treg function and attempt to reconcile some of the different conclusions regarding the role of PI3K in Treg function (11). Newton and Turka consider the role of the PIP_3 phosphatase Pten in T cells and how unrestrained PI3K signaling can lead to T cell transformation (12). They further discuss the differential roles of Pten on PI3K signaling and genome stability. Gamper and Powell discuss data that challenge the common assumption that PI3K and mTOR lie on a common pathway, by providing a detailed discussion of similarities but importantly also key differences in the phenotypes observed when either PI3K or mTOR is inhibited in CD4 T cells (13). Similarly, Finlay discusses recent data showing that the PI3K is in fact dispensable for mTOR activation in CD8 T cells and that a Pdk1–mTOR axis regulates T cell metabolism independently of PI3K and Akt (14).

These studies indicate that we need to start considering other signaling pathways regulated by PI3K. Venigalla and Turner summarize recent evidence suggesting a key role for PI3K, often in concert with p38, in regulating mRNA stability and translation (15). Some of these effects may also be mediated by mTOR.

Drugs against PI3K have become a clinical reality. However, not all PI3K isoforms can be targeted as specifically as Idelalisib inhibits

PI3Kδ. Blunt and Ward summarized the progress in developing PI3K inhibitors, with particular focus on Idelalisib, but then go on to summarize the roles of the PIP_3 5-phosphatases SHIP1 and SHIP2 (16). Moreover, they review the development of allosteric drugs that activate SHIP as an alternative strategy to inhibit PI3K signaling in immune cells.

Altogether, these reviews summarize the remarkable progress in our understanding of how PI3Ks regulate many facets of the adaptive immune response, but also help highlight many unresolved and even controversial areas of research. We are grateful for the considerable efforts that the authors have made to help us compile this ebook for Frontiers in Immunology.

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Akt and mTOR in B cell activation and differentiation

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Activation of phosphoinositide 3-kinase (PI3K) is required for B cell proliferation and survival. PI3K signaling also controls key aspects of B cell differentiation. Upon engagement of the B cell receptor (BCR), PI3K activation promotes Ca^{2+} mobilization and activation of NFκB-dependent transcription, events which are essential for B cell proliferation. PI3K also initiates a distinct signaling pathway involving the Akt and mTOR serine/threonine kinases. It has been generally assumed that activation of Akt and mTOR downstream of PI3K is essential for B cell function. However, Akt and mTOR have complex roles in B cell fate decisions and suppression of this pathway can enhance certain B cell responses while repressing others. In this review we will discuss genetic and pharmacological studies of Akt and mTOR function in normal B cells, and in malignancies of B cell origin.

Keywords: B cells, proliferation, differentiation, antibody, PI3K, Akt, mTOR, kinase

OVERVIEW OF PI3K EFFECTORS IN B CELLS

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinase enzymes that produce 3′-phosphorylated phosphoinositides (Okkenhaug and Fruman, 2010; Vanhaesebroeck et al., 2010; So and Fruman, 2012). These lipids act as second messengers to redirect cytoplasmic proteins to cellular membranes. The production of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) by class I PI3Ks is a shared response downstream of a variety of receptors in all mammalian cell types. In B cells, class I PI3K activation is initiated when the B cell receptor (BCR) recognizes antigen and is augmented by CD19, a component of the B cell co-receptor. Class I PI3K activity is necessary for BCR-dependent proliferation and is sufficient for BCR-dependent tonic survival signaling. T cell-derived cytokines including interleukin-4 (IL-4) augment and sustain PI3K activity during B cell growth and clonal expansion. Toll-like receptor (TLR) engagement, chemokine signaling, and cytokines (e.g., BAFF) also trigger class I PI3K activation. The central role of PI3K activation in B cell function has prompted detailed studies of signaling mechanisms downstream of PI3K.

Proteins recruited to the membrane through binding to PI3K lipid products are generally termed PI3K effectors (Fruman, 2004; Lemmon, 2008). Most effectors of class I PI3K have a pleckstrin homology (PH) domain that binds selectively to PIP_3 and/or phosphatidylinositol-3,4-bisphosphate (PtdIns-3,4-P_2). Btk and Tec are closely related protein tyrosine kinases whose PH domains bind with high affinity to PIP_3. These kinases function in a large protein assembly called the BCR signalosome, whose primary output is activation of phospholipase-C-gamma (PLCγ), leading to production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3; Fruman et al., 2000; Fruman, 2004). Together these second messengers promote Ca^{2+} mobilization, protein kinase C (PKC) activation, and ultimately the nuclear translocation of NFκB transcription factors to drive B cell proliferation (**Figure 1**). Loss of Btk or blockade of its binding to PIP_3 reduces the Ca^{2+} response, diminishes NFκB activation, and prevents productive B cell activation. Conversely, forced activation of PKC or NFκB restores BCR-dependent responses in B cells lacking PI3K or Btk function. These findings suggest that signalosome assembly and NFκB activation are the most important functional outcomes of PI3K signaling during BCR-stimulated B cell activation. Moreover, genetic inactivation of class I PI3K in both humans and mice causes a B cell deficiency similar to the Btk-loss phenotype (Fruman et al., 1999; Suzuki et al., 1999; Conley et al., 2012). Apart from the Ca^{2+} signalosome, PI3K activation triggers the membrane recruitment of additional protein assemblies that might also have key functions.

Two PH domain-containing proteins linked to PI3K activity in all cells, including B cells, are the serine/threonine kinases Akt and phosphoinositide-dependent kinase-1 (PDK-1; Fayard et al., 2010). The three related Akt kinases (Akt1, Akt2, Akt3; also known as PKBα,β,γ) each contain a threonine residue in the activation loop (T308 in Akt1) that is phosphorylated by PDK-1 in a manner dependent on PI3K activity (**Figure 1**). Subsequent phosphorylation of a serine in a hydrophobic motif (S473 in Akt1) by a distinct kinase is required for maximal Akt activation. All three Akt isoforms are expressed in B lineage cells and their functions appear to be partially redundant (Calamito et al., 2010). Of the many Akt substrates reported, the Forkhead Box, Subgroup O (FOXO) transcription factors are of particular importance for B cell biology as discussed below. Akt-mediated FOXO phosphorylation suppresses their transcriptional activity and causes their nuclear export and sequestration or degradation (Burgering, 2008).

The mTOR kinase is encoded by a single gene in mammals but is the active enzyme in two distinct multi-protein complexes called mTORC1 and mTORC2 (**Figure 2**; Laplante and Sabatini, 2012). mTORC1 is defined by the raptor subunit and mTORC2 by the rictor subunit. The main function of mTORC1 is to sense nutrients and mitogenic signals; when conditions are favorable, mTORC1

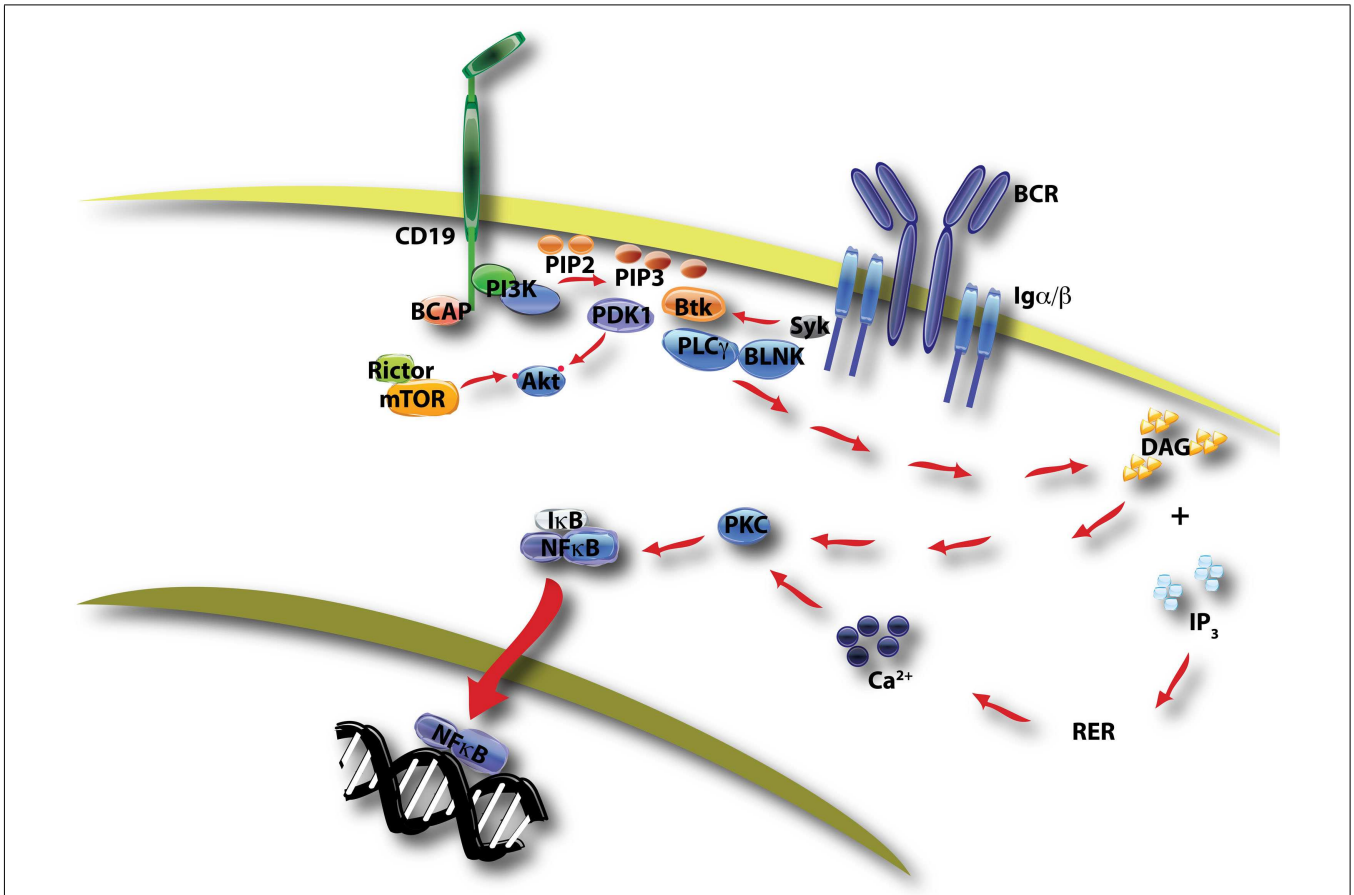

FIGURE 1 | This diagram of BCR/CD19-initiated signaling shows two key outcomes of PI3K activation. PIP3 production by class IA PI3K promotes formation of a signalosome containing Btk, BLNK, and PLCγ, leading to hydrolysis of PI-4,5-P2 by PLCγ. The products of this reaction, DAG and IP3, promote the activation of PKC and ultimately the nuclear translocation of NFκB transcription factors. This pathway is essential for proliferation following antigen recognition. A second outcome of PIP3 production is the membrane recruitment of PDK-1 and Akt, with subsequent phosphorylation of Akt on two distinct sites by PDK-1 and mTORC2 (mTOR in complex with rictor, see **Figure 2**).

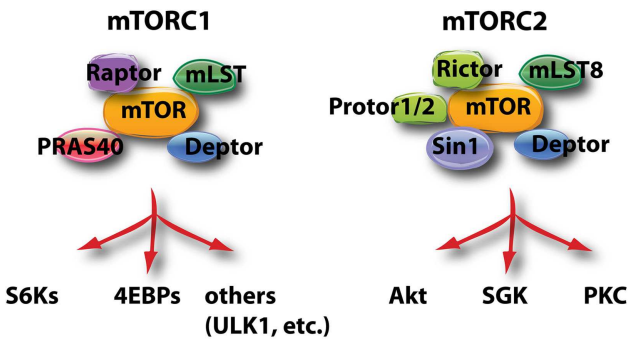

FIGURE 2 | This schematic diagram shows the components of mTORC1 and mTORC2, and the most well known substrates of the two complexes. ULK1 (Unc51-like kinase-1) is involved in the control of autophagy.

triggers biosynthetic pathways essential for cell growth and proliferation. Akt can promote mTORC1 activation through several mechanisms, as described in more detail below. mTORC2 activity can be stimulated by growth factors but is nutrient-independent. The best-described function of mTORC2 is to phosphorylate Akt on S473. However, other kinases can phosphorylate this site in certain conditions (Fayard et al., 2010) and mTORC2 has other important substrates as well, including serum- and glucocorticoid-induced kinase (SGK) and PKC as shown in **Figure 2**. SGK family members have some overlapping properties with Akt isoforms, for example the ability to phosphorylate FOXO transcription factors (Brunet et al., 2001).

Overall, the signaling network defined by Akt and the two mTOR complexes is a central driver of cell growth, metabolism, and proliferation, and the activity of this network is elevated in nearly all human cancers (Engelman et al., 2006; Liu et al., 2009). Consequently, there has been great interest in targeting the pathway for therapeutic benefit in cancer. In parallel, Akt and mTOR signaling has been actively studied in the context of normal lymphocyte function (So and Fruman, 2012). Indeed, mTOR was first discovered and named as the target of rapamycin, an immunosuppressive drug that is now used clinically to prevent organ transplant rejection (Guertin and Sabatini, 2009). Rapamycin suppresses T cell proliferation, promotes regulatory T cell differentiation, and

modulates the function of innate immune cells. In this review we describe our current knowledge of Akt and mTOR functions in B lymphocytes.

THE Akt-FOXO AXIS IN B CELL DEVELOPMENT, ACTIVATION, AND DIFFERENTIATION

Akt was first defined as a key PI3K effector in 1995 (Franke et al., 1995). Within a few years, several groups had shown that Akt is recruited to the membrane and activated downstream of the BCR and CD19, in a manner dependent on PI3K (Aman et al., 1998; Astoul et al., 1999; Pogue et al., 2000; Otero et al., 2001). Subsequently, we reported that BCR signaling through PI3K downregulates expression of FOXO target genes *Rbl2* and *Ccng2* (Fruman et al., 2002; Yusuf et al., 2004). These genes encode the proteins p130 and cyclin G2, both implicated in cell cycle arrest in non-lymphoid cells (Kops et al., 2002; Martinez-Gac et al., 2004). Consistent with a role for FOXO factors in opposing cell cycle progression, Akt-dependent inactivation of FOXO transcription factors is important for optimal B cell proliferation in response to lipopolysaccharide (LPS; Yusuf et al., 2004). It is likely that Akt has many other substrates that play key roles in B cell biology. However, the Akt-FOXO axis has emerged as a key control point for various aspects of B cell function.

FOXO transcription factors (FOXO1, FOXO3a, FOXO4, FOXO6) are an evolutionarily conserved family of proteins whose activity is tightly controlled by growth factors (Burgering, 2008). In the absence of mitogenic signals, FOXO proteins are mainly nuclear and direct a transcriptional program that blocks cell cycle progression and promotes stress resistance and longevity (**Figure 3**). FOXO factors can also promote expression of pro-apoptotic genes (Fu and Tindall, 2008). Growth factor receptor signaling inactivates FOXO through Akt-dependent phosphorylation on three conserved serine or threonine residues. These phosphorylation events trigger the release of FOXO from DNA, nuclear export, and sequestration or degradation in the cytoplasm (**Figure 3**). Some of the consensus sites for Akt phosphorylation are also substrates for SGKs, whose activity is not as tightly coupled to PI3K signaling (Brunet et al., 2001). Also, FOXO function is regulated further by acetylation and by the status of cooperating transcription factors (Calnan and Brunet, 2008). Nevertheless, PI3K/Akt activation plays a dominant role in regulation of FOXO activity. Both FOXO1 and FOXO3 are controlled by Akt-mediated phosphorylation and both isoforms are expressed in B lineage cells (Dengler et al., 2008; Hinman et al., 2009; Lin et al., 2010).

Foxo1 is an essential component of a transcription factor network in pro-B cells that also includes E2A and EBF1 (Lin et al., 2010). This study showed that E2A binds to regulatory elements upstream of the *Foxo1* gene, and that FOXO1 protein functions together with E2A and EBF1 to induce transcription of the *Pax5* gene to drive B cell commitment. An unanswered question is how FOXO1 retains a required nuclear function in B cell progenitors, which are continuously exposed to cytokines and other signals that activate PI3K/Akt signaling. Gene knockout studies have confirmed that the *Foxo1* gene is essential for proper B cell

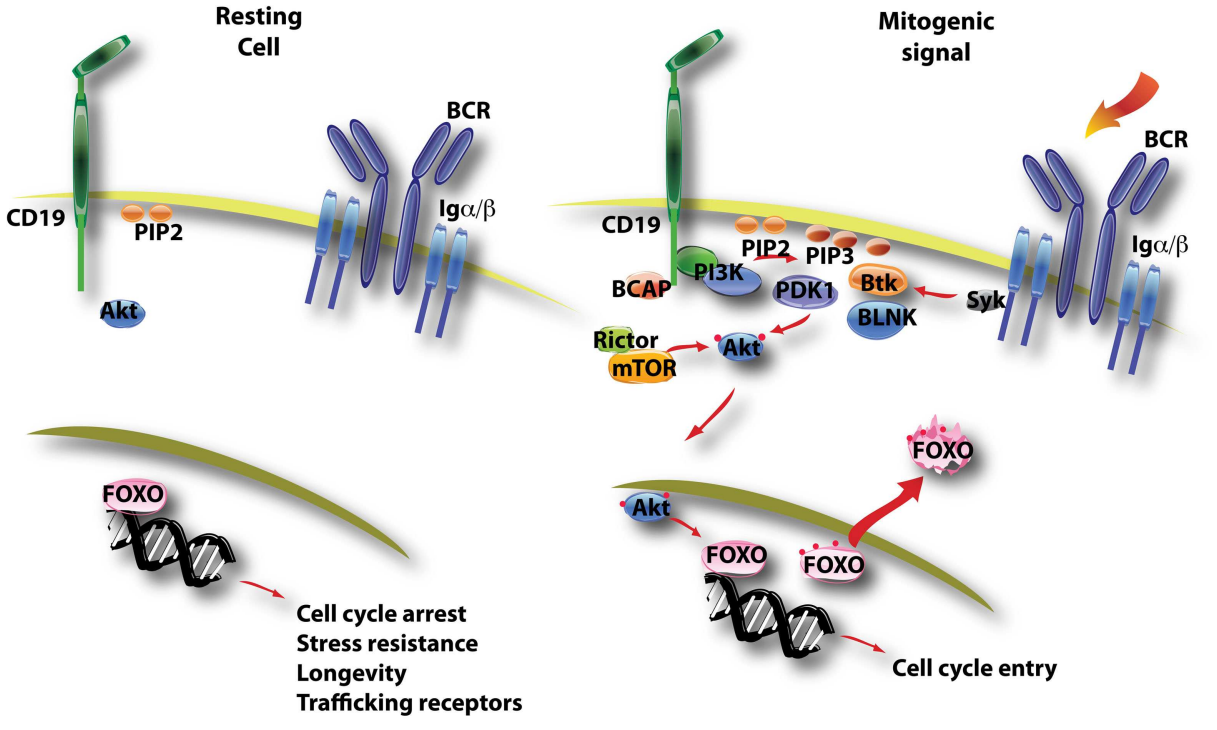

FIGURE 3 | This diagram illustrates the control of FOXO function by PI3K/Akt activation. In resting B cells, FOXO factors are mainly nuclear and direct a gene expression program favoring quiescence (cell cycle arrest, longevity) and recirculation (trafficking through blood and lymphoid tissue). B cell activation triggers PI3K/Akt activity, and active Akt enters the nucleus to phosphorylate FOXO proteins on several conserved sites (noted by red dots). This leads to nuclear export of FOXO factors that are then sequestered and/or degraded in the cytoplasm.

development. Using mice with a conditional allele of *Foxo1*, Rickert and colleagues analyzed FOXO1 function at various stages using different Cre deleter strains (Dengler et al., 2008). Deletion at an early stage using *Mb1*-Cre causes a partial block at the pro-B cell stage that can be attributed to impaired expression of the interleukin-7 receptor. Deletion in late pro-B cells using *Cd19*-Cre causes a block at the pre-B cell stage owing to reduced expression of the recombination activating genes (*Rag-1* and *Rag-2*). Similarly, FOXO3a-deficient mice have reduced numbers of pre-B cells (Hinman et al., 2009). Others have shown that *Rag* genes are direct targets of FOXO1 and FOXO3a (Amin and Schlissel, 2008; Herzog et al., 2008). Successful rearrangement of heavy and light chain genes produces functional pre-BCR and mature BCR expression, and it is likely that basal (tonic) signaling through these receptors acts through PI3K and Akt to suppress FOXO function and turn off *Rag* gene expression to achieve allelic exclusion. Consistent with this model, mice lacking PI3K function in pre-B cells have an elevated fraction of cells with two rearranged heavy chain alleles (Ramadani et al., 2010).

PI3K also regulates the fate of immature B cells. Successful light chain gene recombination at the pre-B cell stage results in expression of surface immunoglobulin (the mature BCR) and transition to the immature B cell stage. Tonic signaling through the BCR is required to extinguish *Rag* gene expression, exit the pre-B cell stage and positively select immature B cells for further differentiation (Tze et al., 2005). Three reports showed that PI3K activity is required for basal BCR signals at the immature B cell stage, with a selective role for p85α and p110δ isoforms (Tze et al., 2005; Llorian et al., 2007; Verkoczy et al., 2007). However, the available data indicate that PI3K suppresses *Rag* gene expression at this stage via phospholipase C-γ rather than through the Akt-FOXO axis (Verkoczy et al., 2007).

Deletion of *Foxo1* with *Cd19*-Cre does not fully block the formation of mature B cells (Dengler et al., 2008; Chen et al., 2010). However, peripheral lymphoid organs from these mice show aberrant representation of B cell subsets. There is a large fraction of $B220^+$ cells lacking surface Ig (Dengler et al., 2008; Chen et al., 2010), which may represent B cell progenitors escaping the bone marrow due to altered expression of trafficking receptors. There is also a significant increase in the marginal zone (MZ) B cell subset (Dengler et al., 2008; Chen et al., 2010). The opposite phenotype, a reduced MZ B cell compartment, is observed upon deletion of PI3K catalytic (p110δ) or regulatory (p85α) subunits or inactivation of both Akt1 and Akt2 (Okkenhaug et al., 2002; Suzuki et al., 2003b; Donahue et al., 2004; Calamito et al., 2010). A similar loss of MZ B cells occurs in mice lacking CD19, but not in mice lacking Btk (Donahue et al., 2004). Furthermore, the MZ B cell defect in CD19-deficient mice can be reversed by combined deletion of *Foxo1* (Chen et al., 2010). Together these observations suggest that commitment to the MZ fate in transitional B cells is driven by CD19 signaling through PI3K and Akt to inactivate FOXO factors (**Figure 4**). The FOXO target genes that restrain MZ B cell commitment have not been established. The Notch signaling pathway can promote MZ B cell development even in the absence of CD19 (Hampel et al., 2011), suggesting that FOXO proteins might oppose Notch signaling. However, in other cellular systems Notch and FOXO were shown to cooperate (Kitamura et al., 2007).

In the T cell lineage, a major function of FOXO proteins is to maintain expression of the lymph node homing receptor CD62L and other trafficking receptors necessary for proper recirculation of quiescent cells through blood and lymphoid tissues (Fabre et al., 2008; Kerdiles et al., 2009; Ouyang et al., 2009). Similarly, deletion of *Foxo1* in late transitional B cells (using *Cd21*-Cre) impairs CD62L expression on mature B cells (Dengler et al., 2008; **Figure 3**). This results in altered homing with fewer B cells detected in the lymph nodes (Dengler et al., 2008; Chen et al., 2010). In wild-type B cells, BCR-dependent downregulation of CD62L is partially dependent on PI3K activity (Hess et al., 2004). FOXO activity probably controls CD62L transcription indirectly via Krüppel-like factors (KLFs) in B cells, as in T cells (Hart et al., 2012).

Mature B cells lacking FOXO1 show reduced surface expression of the BCR and significantly reduced BCR signaling responses including Ca^{2+} mobilization and phosphorylation of Akt and ERK (Dengler et al., 2008). The mechanism for signal attenuation in the absence of FOXO1 has not been established. However, there is a potential connection to cancer cell lines in which PI3K/Akt inhibition leads to FOXO-dependent upregulation of receptor tyrosine kinase expression and function (Hay, 2011). Therefore, FOXO1 in B cells might maintain expression of signaling proteins necessary for activation, such that BCR-PI3K-Akt signaling would inactivate FOXO1 to trigger a built-in negative feedback mechanism. BCR signaling through PI3K also suppresses *Foxo1* expression at the transcriptional level (Hinman et al., 2007).

At the mature B cell stage, exposure to the cytokine BAFF and continuous expression of the surface BCR are essential to maintain survival (Lam et al., 1997; Schiemann et al., 2001). Mouse genetic models have shown that PI3K activity is both necessary and sufficient to maintain survival of mature peripheral B cells (Srinivasan et al., 2009; Ramadani et al., 2010). Rescue of BCR-negative cells by expression of constitutively active PI3K or deletion of PTEN correlates with low but detectable levels of Akt phosphorylation (Srinivasan et al., 2009). It is likely that Akt activity has an important function in peripheral B cell survival, as B cells lacking both Akt1 and Akt2 show reduced fitness compared to wild-type in a competitive repopulation assay (Calamito et al., 2010). In addition, deletion of *Foxo1* partially rescues survival of BCR-negative peripheral B cells, though the rescued cells have low CD62L expression and do not accumulate in lymph nodes (Srinivasan et al., 2009).

Following B cell clonal selection by antigen, activated B cells commit to one of two distinct differentiation pathways. Some cells undergo rapid differentiation into antibody-secreting plasma cells that secrete mostly IgM antibodies of low affinity. Other cells commit to the germinal center (GC) fate, and surviving clones emerge 1–2 weeks later as memory or plasma cells making high affinity class switch antibodies (**Figure 5**). There is accumulating evidence that the choice between rapid plasmablast differentiation versus GC entry is determined by the degree of PI3K-Akt signaling, and thus by the level of FOXO activity (**Figure 5**). Elevation of PI3K-Akt signaling through loss of PTEN increases antibody-secreting cell (ASC) differentiation and strongly suppresses class switch recombination (CSR; Suzuki et al., 2003a; Omori et al., 2006). The mechanism is directly linked to the Akt-FOXO axis, since CSR can be restored in PTEN-deficient cells by expression

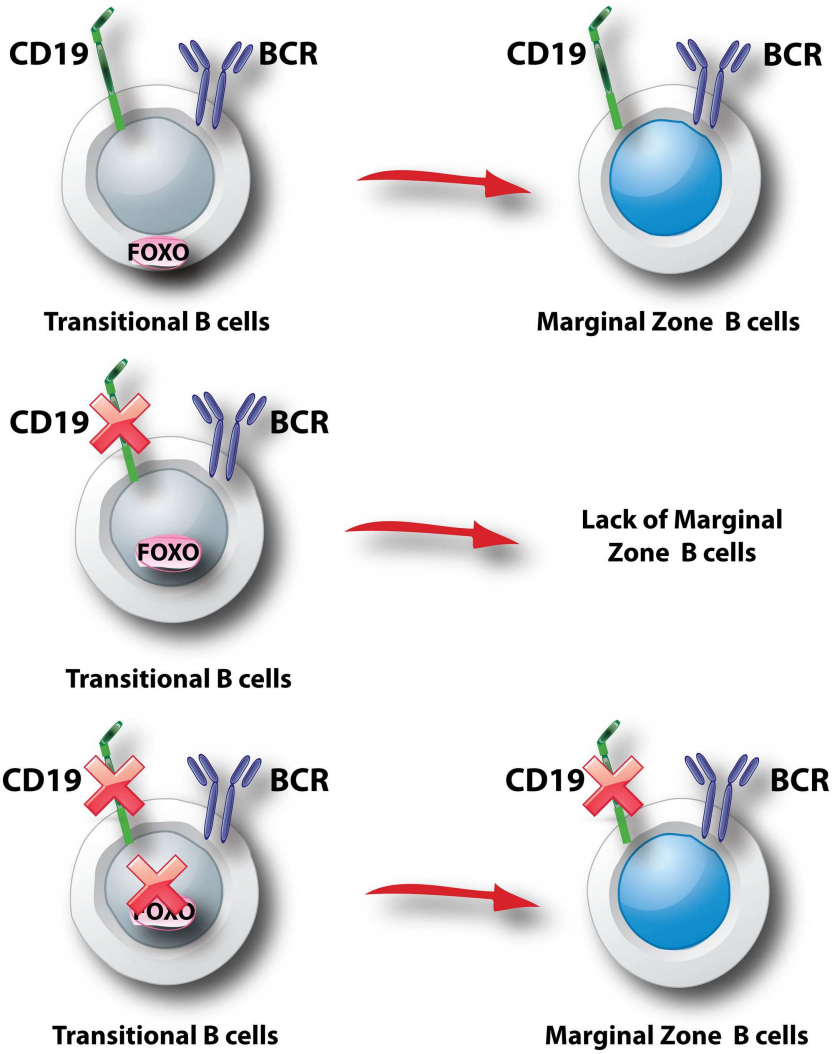

FIGURE 4 | Model for control of marginal zone B cell development. It is thought that transitional B cell selection into the MZ lineage is driven by interactions of the BCR with self antigens (Wen et al., 2005). In addition, CD19 associates with BCR signalosomes and plays a key role in recruiting PI3K to the membrane and activating Akt following BCR engagement (Otero et al., 2001; Wang et al., 2002; Aiba et al., 2008; Depoil et al., 2008). Genetic studies support the model that commitment to the MZ lineage requires a signal initiated by CD19 and propagated by PI3K (p85α/p110δ) to Akt (not shown in this figure). This signal blocks nuclear entry of FOXO (top panel). In the absence of CD19, the signal is interrupted and Foxo1 can program a block to MZ development (middle panel). In the absence of Foxo1, CD19 is dispensable for MZ B cell development (bottom panel).

of constitutively active FOXO proteins (Omori et al., 2006). Furthermore, mature B cells lacking *Foxo1* have severe defects in class switching due to a failure to upregulate the gene *Aicda* encoding the AID mutator protein (Dengler et al., 2008). In contrast, PI3K inhibitors enhance AID expression and class switching and oppose ASC differentiation *in vitro* (Omori et al., 2006). Genetic or pharmacological blockade of the p110δ isoform of class I PI3K specifically enhances the production of IgE (Zhang et al., 2008), but this appears to be independent of Akt (Zhang et al., 2012). It is important to note that whereas PI3K is essential for proliferation triggered by BCR engagement, this requirement can be bypassed when B cells are activated through other receptors such as TLRs, CD40, and IL-4. Indeed, B cells stimulated with CD40L + IL-4 proliferate in the presence of PI3K inhibitors and it is these conditions that have revealed the role for PI3K-Akt signaling in opposing CSR (Fruman et al., 1999; Omori et al., 2006). Similarly, LPS-stimulated B cells maintain the ability to proliferate and show elevated plasmablast differentiation in the presence of PI3K inhibitors (Omori et al., 2006). Further complicating the issue, class I PI3K plays a positive role in the ability of BCR engagement to enhance CSR initiated by TLR ligands (Pone et al., 2012). This phenomenon is linked to NFκB activation, and might be independent of the Akt-FOXO axis.

In the context of T cell-dependent antibody responses, PI3K inhibition can limit GC responses by suppressing the differentiation and function of T follicular helper (Tfh) cells (Rolf et al., 2010). PI3K function in Tfh cells might depend on Akt activation and FOXO inhibition, since deletion of *Foxo1* in T cells enhances

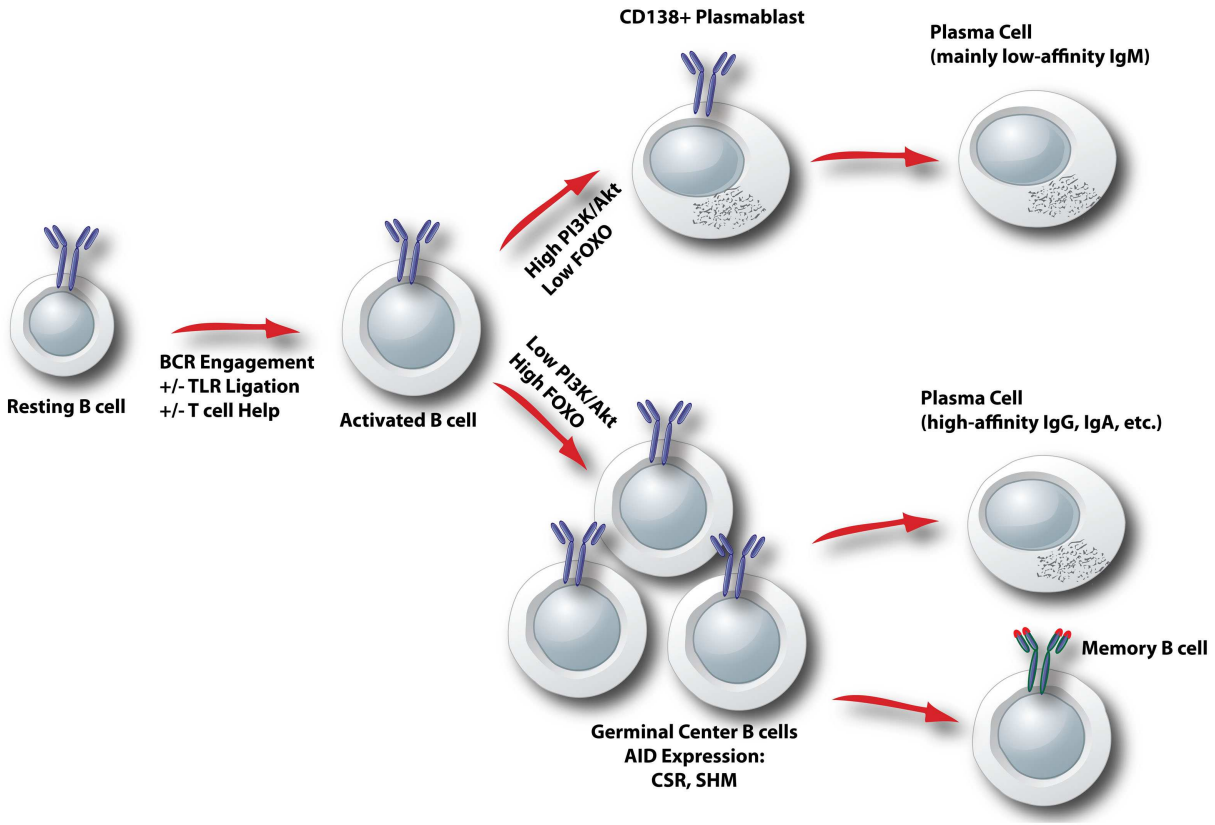

FIGURE 5 | Model for control of activated B cell differentiation fate by the Akt-FOXO axis. In activated B cells, the degree of ongoing PI3K/Akt signaling determines the relative nuclear activity of FOXO. When PI3K/Akt is high, FOXO activity is low and plasmablast differentiation is favored. When PI3K/Akt is low, FOXO activity is elevated and a germinal center B cell fate is programmed. CSR, class switch recombination; SHM, somatic hypermutation.

Tfh differentiation and expands GCs (Kerdiles et al., 2010). This phenotype might be an indirect effect of reduced Treg function in mice with *Foxo1*-deficient T cells (Kerdiles et al., 2010), since Tregs suppress GC responses (Alexander et al., 2011; Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Nevertheless, at a global level the Akt-FOXO axis directs opposing outcomes in T and B cells with respect to GC differentiation. In T cells, FOXO loss enhances GCs whereas in B cells FOXO loss reduces GC differentiation. Therefore it will be interesting to determine how inhibitors targeting the PI3K/Akt network at different levels will affect antibody responses to different types of pathogens and vaccines.

RAPAMYCIN AND THE TWO mTOR COMPLEXES IN B CELLS

mTORC1 is composed of the mTOR enzyme, raptor, PRAS40, mLST8, and DEPTOR (**Figure 2**; Laplante and Sabatini, 2012). Raptor is essential for phosphorylation of mTORC1 substrates, whereas PRAS40 and DEPTOR suppress kinase activity. The mechanisms of mTORC1 activation and repression are complex and have been worked out mainly in non-lymphoid cell types. Nutrients sustain mTORC1 activity through amino acid-dependent recruitment to a complex of proteins containing Rag GTPases, termed the Ragulator (Sancak et al., 2010), whereas glucose metabolism prevents the activation of AMP kinase, a negative regulator of mTORC1. However, nutrient availability alone is not sufficient to fully activate mTORC1; growth factor inputs (antigen receptors and costimulatory receptors in lymphocytes) are also required. Mitogenic signals activate mTORC1 through several mechanisms. First, Akt and/or Erk phosphorylate and suppress the tuberous sclerosis complex (TSC1/TSC2), relieving its GAP activity toward the Rheb small GTPase that is an essential upstream activator of mTORC1. Akt can also phosphorylate and inactivate PRAS40, one of the intrinsic negative regulators of the mTORC1 complex. Production of phosphatidic acid also occurs during cell activation and this can contribute to mTORC1 activation. One of the surprising findings of the last several years has been that mTORC1 activation in lymphocytes can occur even when PI3K/Akt activity is undetectable. This was first observed in B cell lymphoma lines (Wlodarski et al., 2005). Later we reported that the mechanism of mTORC1 activation in normal mature B cells depends on the stimulus (Donahue and Fruman, 2007). BCR-dependent mTORC1 activation is PI3K-dependent whereas basal and LPS-dependent mTORC1 activity is partly PI3K-independent. Similarly, mTORC1 activity is sustained in activated T cells lacking detectable PI3K/Akt activity (Deane et al., 2007). Other Akt-independent inputs are likely to regulate downstream mTORC1 substrates in lymphocytes, as has been shown for the S6 ribosomal protein in CD8 T cells (Salmond et al., 2009).

mTORC2 is composed of three components essential for activity (rictor, Sin1, mLST8) along with regulatory components DEPTOR and PROTOR (**Figure 2**; Laplante and Sabatini, 2012). Based on studies mainly done in non-lymphoid cell types, current models indicate that mTORC2 has some basal activity in cells and phosphorylates sites in Akt and certain PKC isoforms that are important for folding and stability (Cybulski and Hall, 2009). Growth factor stimulation promotes increased activity of mTORC2 through association with ribosomes (Zinzalla et al., 2011), and subsequent mTORC2-dependent phosphorylation of the hydrophobic motif of various AGC family kinases – most notably the S473 site on Akt. Thus, mTORC2 acts upstream of Akt whereas mTORC1 acts downstream of Akt. Recent data indicate that PI3K lipid products stimulate mTORC2 activity (Gan et al., 2011; Tato et al., 2011; Shanmugasundaram et al., 2012); the degree of basal mTORC2 activity maintained in PI3K-deficient lymphocytes is not known.

Rapamycin was first identified in a screen for natural products with anti-fungal activity but was later shown to suppress lymphocyte proliferation (Sehgal et al., 1975; Martel et al., 1977; Calne et al., 1989). Although most studies of rapamycin mechanism have focused on T cells, it is often overlooked that rapamycin suppresses B cell proliferation more completely. Three papers in the early 1990s established that rapamycin has profound effects on B cell proliferation and differentiation (Wicker et al., 1990; Kay et al., 1991; Aagaard-Tillery and Jelinek, 1994). Pretreatment with rapamycin completely blocked murine B cell proliferation induced by anti-IgM (±IL-4), and reduced by 50–60% the response to LPS (Wicker et al., 1990; Kay et al., 1991). Rapamycin prevented B cell growth but did not impact survival (Wicker et al., 1990). In human B lymphocytes stimulated with the polyclonal activator *S. aureus*, rapamycin reduced cell proliferation by 60–80% and completely blocked differentiation into ASCs (Aagaard-Tillery and Jelinek, 1994). Notably, rapamycin suppressed T cell receptor (TCR)-dependent proliferation of CD4 T cells to a lesser extent than BCR-dependent cell proliferation (Kay et al., 1991), and more recent work showed that genetic deletion of mTOR in T cells delays but does not block clonal expansion (Delgoffe et al., 2009). Rapamycin does not strongly suppress CD8 T cell expansion (Slavik et al., 2001) and actually enhances generation of memory CD8 cells (Araki et al., 2009). Therefore, the profound effect on BCR-driven proliferation is unusual and highlights that rapamycin could be an effective approach for treatment of B cell-driven autoimmune diseases. Indeed, rapamycin reduces pathogenic antibody accumulation and ameliorates disease in mouse models of lupus (Warner et al., 1994; Lui et al., 2008).

The mechanisms by which rapamycin blocks B cell cycle entry and differentiation remain unclear. mTORC1 has many substrates, of which the most well-studied are the ribosomal S6 kinases (S6K1 and S6K2) and the eIF4E binding proteins (4EBPs; **Figure 2**; Laplante and Sabatini, 2012). S6Ks promote protein and lipid synthesis and their activity is completely dependent on mTORC1-mediated phosphorylation (Magnuson et al., 2012). In contrast, the phosphorylation of 4EBPs by mTORC1 is an inhibitory event that blocks the ability of 4EBPs to suppress eIF4E function in cap-dependent translation (Silvera et al., 2010). Overall, the phosphorylation of S6Ks and 4EBPs along with the suppression of autophagy by active mTORC1 are essential for cell growth in preparation for division. Phosphorylation of S6Ks and 4EBPs occurs rapidly following BCR engagement (Donahue and Fruman, 2007), but whether these signals are required for successful B cell growth and proliferation has not been determined. Rapamycin strongly suppresses S6K phosphorylation in all cell types, but has limited and variable effects on 4EBP phosphorylation (Choo et al., 2008; Thoreen and Sabatini, 2009). Rapamycin does not acutely inhibit mTORC2 but chronic treatment with rapamycin can suppress mTORC2 assembly in some cell types, including lymphocytes (Sarbassov et al., 2006; Lazorchak et al., 2010; Delgoffe et al., 2011). Further work is necessary to establish whether inhibition of mTORC1 and/or mTORC2 underlies the effects of rapamycin in B cells. It is also possible that rapamycin has targets other than mTOR in lymphocytes, or that a kinase-independent scaffolding function of mTOR is disrupted upon rapamycin exposure.

Surprisingly little is known about the unique functions of the mTOR complexes in B cells. Rapamycin is not an optimal tool for addressing this question, since as noted above the compound is only a partial inhibitor of mTORC1 (Choo et al., 2008; Thoreen and Sabatini, 2009) and its effects on mTORC2 are dependent on concentration and time of treatment (Sarbassov et al., 2006; Lazorchak et al., 2010; Delgoffe et al., 2011). To date there have been no publications describing the phenotype of mature B cells lacking essential components of mTORC1 or mTORC2. One report described a B cell-specific knockout of Sin1 but the analysis focused on progenitor B cell development in the bone marrow (Lazorchak et al., 2010). Interestingly, Sin1-deficient cells showed elevated expression of IL-7 receptor and RAG proteins, consistent with increased FOXO activity when mTORC2-Akt (and SGK) signaling is reduced. In Sin1-deficient pre-B cells cultured from bone marrow *in vitro*, expression of constitutively active Akt2 led to FOXO1 phosphorylation and reduced RAG expression (Lazorchak et al., 2010). Akt2 might not be essential for FOXO regulation *in vivo*, as B cell development is not impaired in mice lacking Akt2 or in chimeric mice lacking both Akt1 and Akt2 in B cell progenitors (Calamito et al., 2010). Notably, prolonged rapamycin treatment suppressed mTORC2-dependent Akt phosphorylation leading to elevated expression of FOXO1 and RAG proteins (Lazorchak et al., 2010).

Although B cells lacking mTORC1 function have not yet been described, one study analyzed the consequences of B cell-specific loss of the negative regulator TSC1 (Benhamron and Tirosh, 2011). As expected, mTORC1 activity was increased as judged by phosphorylation of S6 protein downstream of S6Ks. A striking phenotype was a reduced percentage of MZ B cells. The most likely explanation is that elevated mTORC1 activity in transitional B cells engages negative feedback loops that reduce activity of upstream PI3K/Akt signaling. Well-established negative feedback mechanisms include S6K-dependent phosphorylation of insulin receptor substrate (IRS) proteins and mTORC1-dependent phosphorylation of the adaptor protein Grb10 (Yea and Fruman, 2011; Laplante and Sabatini, 2012). As discussed earlier, reduced PI3K/Akt activity is known to diminish MZ B cell development. However, the status of PI3K/Akt signaling in TSC1-deficient B cells was not assessed in this study.

An interesting report described the phenotype of mice with a hypomorphic allele of *Mtor* that reduces mTOR protein

expression and diminishes the activity of both mTORC1 and mTORC2 (Zhang et al., 2011). These mice have a greatly reduced number of peripheral B cells and a partial block in early development at the large pre-B cell stage. Whether this block is associated with altered FOXO function or RAG expression was not investigated. B cells with reduced mTOR expression have greatly impaired proliferative responses to anti-IgM or anti-CD40, whereas the LPS response is largely intact. Curiously, B cells from these mice display elevated phosphorylation of Akt on S473 following LPS stimulation. This correlates with increased expression of DNA-PK, an alternative kinase for Akt-S473, and can be reduced by a selective DNA-PK inhibitor. These findings highlight that the effect of mTOR inhibition on Akt-S473 phosphorylation is highly context-dependent. Whether chronic treatment with mTOR catalytic inhibitors would also lead to DNA-PK upregulation in B cells is not known.

Akt AND mTOR IN B CELL MALIGNANCIES

Activation of the PI3K/Akt/mTOR signaling network is a common feature of most human cancers (Engelman et al., 2006; Liu et al., 2009; Hanahan and Weinberg, 2011). Malignancies of B cell origin are no exception. Many groups have documented high basal levels of Akt and mTOR activation in B cell leukemias, B cell lymphomas, and multiple myeloma (MM) cells. For example, the BCR-ABL oncoprotein strongly activates PI3K/Akt signaling and mTOR activity in Philadelphia Chromosome-positive (Ph^+) leukemias (Skorski et al., 1997; Kharas et al., 2008; Janes et al., 2010). mTOR is activated in a Syk-dependent manner in follicular lymphoma cells (Leseux et al., 2006; Fruchon et al., 2012). In diffuse large B cell lymphoma (DLBCL), the microRNA miR-155 is often overexpressed leading to reduced amounts of the SHIP phosphatase that can dephosphorylate the 5'-phosphate of PIP_3 (Pedersen et al., 2009). The activated B cell subset of DLBCL displays constitutive Akt signaling through chronic active BCR signaling (Davis et al., 2010). Interestingly, a fraction of MM tumors shows elevated expression of DEPTOR, an endogenous inhibitor of mTORC1 and mTORC2 (Peterson et al., 2009). In this case, DEPTOR appears to be important for limiting mTORC1-dependent negative feedback on PI3K/Akt activity.

The central role of PI3K/Akt/mTOR signaling in B cell neoplasms has led many investigators to test the efficacy of small molecule inhibitors of this network. Proof-of-concept was provided first by clinical trials showing significant responses to temsirolimus (CCI-779; an orally active rapamycin analog) in patients with mantle cell lymphoma (Hess et al., 2009). Such rapalogs have shown generally more limited success in other clinical trials of leukemia, lymphoma, and myeloma (Kelly et al., 2011). ATP-competitive compounds that target of the active site of mTOR show more promise. In preclinical studies, dual-targeted agents that directly inhibit both PI3K and mTOR (e.g., PI-103, NVP-BEZ235) have shown efficacy in Ph^+ pre-B cell acute leukemia (Kharas et al., 2008), chronic lymphocytic leukemia (CLL; Niedermeier et al., 2009), various B cell lymphomas (Bhatt et al., 2010; Bhende et al., 2010), and MM (Baumann et al., 2009).

Selective mTOR kinase inhibitors appear to be as effective as panPI3K/mTOR inhibitors in models of Ph^+ leukemia (Carayol et al., 2010; Janes et al., 2010) and MM (Maiso et al., 2011), with lesser toxicity (Janes et al., 2010). However, there might be some B cell malignancies in which dual PI3K/mTOR inhibition or PI3K inhibition alone might be most effective. Recent studies have revealed that selective inhibition of the p110δ isoform of PI3K produces remarkable clinical responses in CLL patients (Fruman and Rommel, 2011). Similarly, Btk inhibitors in clinical development have shown great promise in clinical trials of CLL treatment (Winer et al., 2012). Thus, the connection of PI3K and Btk is not limited to BCR-mediated activation of normal B cells, but seems to represent a key signaling axis for CLL cell proliferation, survival, and migration. Although antibody-mediated B cell depletion (anti-CD20; rituximab) often provides benefit for the treatment of B cell malignancies, PI3K/Btk-targeted small molecules might have some advantages. Such agents would be more rapidly reversible than long-lived antibodies upon cessation of treatment, allowing prompt resolution of adverse immunosuppressive effects. Small molecule orally active compounds might also be more convenient and less expensive to administer. It is also possible that PI3K/Btk inhibitors will be useful as adjuncts to rituximab, as suggested by preliminary reports of combination trials in non-Hodgkin's lymphoma (Fruman and Rommel, 2011; Winer et al., 2012). Ultimately, the optimal PI3K/mTOR inhibitors and combinations for different malignancies will require careful comparison of efficacy and tolerability in clinical trials.

SUMMARY AND FUTURE DIRECTIONS

In B cells activated through BCR crosslinking, treatment with either PI3K inhibitors or rapamycin profoundly blocks B cell proliferation. This suggests a direct function of mTOR downstream of PI3K in BCR signaling. However, subsequent studies of PI3K, Akt, and mTOR signaling in B cells have led to a number of surprises. Whereas rapamycin completely blocks differentiation of B cells stimulated with TLR ligands or T cell-derived helper factors (i.e., CD40L + IL-4), PI3K inhibition has the distinct effect of enhancing CSR while suppressing terminal differentiation to plasma cells. Deletion of *Foxo1*, which might have been predicted to lower the threshold for B cell activation, actually attenuates B cell proliferation and differentiation. We propose a model in which two key downstream PI3K effector arms in B cells have distinct functions. In simple terms, the Ca^{2+} signalosome drives proliferation, whereas the Akt-FOXO axis controls differentiation. Following antigen recognition, BCR signaling through PI3K leads to signalosome assembly to drive cell cycle progression primarily through NFκB activation (**Figure 1**). The subsequent differentiation path of the activated B cell is controlled by the kinetics and magnitude of PI3K activation through the BCR and other signals including TLR engagement and T cell help (**Figure 5**). High PI3K/Akt activity suppresses FOXO function to promote rapid production of plasma cells secreting mainly IgM. Low PI3K/Akt activity allows FOXO function to be re-established, and programs the cell to express AID and commit to the GC B cell fate. This mechanism makes sense in that it allows the host to tailor the antibody response to the antigen. When there is a high affinity or abundant antigen, the goal is to make antibodies quickly. This is achieved through sustained PI3K/Akt signaling that drives plasma cell differentiation. When the antigen is of low affinity or not abundant, eradication of the antigen requires high affinity class

switched antibodies. This would be achieved because the reduced antigen-derived signals limit PI3K/Akt activity, allowing FOXO factors to program the GC B cell fate.

A question that arises from this model is why mTOR inhibition with rapamycin is such a potent inhibitor of both B cell proliferation and differentiation, regardless of the stimulus. This finding suggests an essential function of mTORC1 in these processes. One possibility is that mTORC1 activity is required in B cells and that sufficient mTORC1 function is maintained even when PI3K/Akt activity is suppressed. However, the mTORC1 substrates whose function is essential in B cells have not been established. Future work should investigate the functions of S6Ks, 4EBPs, and other mTORC1 substrates in B cells. Complicating matters, we have shown that ATP-competitive mTOR kinase inhibitors have distinct effects on B cells compared to rapamycin. At concentrations that strongly suppress phosphorylation of mTORC1 and mTORC2 substrates, mTOR kinase inhibitors only partially reduce proliferation of activated mature splenic B cells (Janes et al., 2010). Ongoing work in our laboratory is focused on resolving this paradox.

Another key question to be resolved is whether Akt in B cells has other important substrates besides FOXO transcription factors. The activation of PI3K and Akt has been linked to increased glycolysis in activated B cells (Doughty et al., 2006), but the relevant components linking Akt to metabolic changes have not been fully worked out. At the organismal level, Akt2 is primarily responsible for insulin/glucose homeostasis whereas Akt1 has a more dominant role in cell proliferation and differentiation (Fayard et al., 2010). Whether a similar division of labor occurs in B cells will be interesting to determine. It is also worth testing whether mTORC2 and its non-Akt substrates regulate B cell motility and chemotaxis, as reported in other systems (Masri et al., 2007; Gulhati et al., 2011). Identification of the Akt and mTOR substrates that control various aspects of B cell function could lead to novel targets for therapeutic intervention in immune diseases and cancer.

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The phosphoinositide 3-kinase signaling pathway in normal and malignant B cells: activation mechanisms, regulation, and impact on cellular functions

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The phosphoinositide 3-kinase (PI3K) pathway is a central signal transduction axis controlling normal B cell homeostasis and activation in humoral immunity. The p110δ PI3K catalytic subunit has emerged as a critical mediator of multiple B cell functions. The activity of this pathway is regulated at multiple levels, with inositol phosphatases PTEN and SHIP both playing critical roles. When deregulated, the PI3K pathway can contribute to B cell malignancies and autoantibody production. This review summarizes current knowledge on key mechanisms that activate and regulate the PI3K pathway and influence normal B cell functional responses including the development of B cell subsets, antigen presentation, immunoglobulin isotype switch, germinal center responses, and maintenance of B cell anergy. We also discuss PI3K pathway alterations reported in select B cell malignancies and highlight studies indicating the functional significance of this pathway in malignant B cell survival and growth within tissue microenvironments. Finally, we comment on early clinical trial results, which support PI3K inhibition as a promising treatment of chronic lymphocytic leukemia.

Keywords: antibody, antigen receptor, B lymphocyte, germinal center, inositol phosphatase, isotype switch, leukemia and lymphoma, phosphatidylinositol 3-kinase

INTRODUCTION: THE PHOSPHOINOSITIDE 3-KINASE PATHWAY IN LYMPHOCYTES

Phosphoinositide 3-kinases (PI3K) are a family of enzymes that selectively phosphorylate the D3 hydroxyl group of the inositol head group of phosphoinositide (PI) lipids. PI3K enzymes can be divided into three families based on their structure and PI specificity. Class I PI3K enzymes are heterodimeric complexes composed of regulatory (p85α, p85β, p55γ, p55α, p50α, and p101) and catalytic (p110α, p110β, p110δ, or p110γ) subunits. Class I PI3K enzymes can generate two major species of D3 phosphoinositides, $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Class II PI3K enzymes selectively phosphorylate PI and PI(4)P to produce PI(3)P and $PI(3,4)P_2$, whereas class III only produce PI(3)P (Deane and Fruman, 2004). Little is known about the role of class II and III PI3Ks in lymphocytes and they will not be reviewed here. The accumulation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are further controlled by PI phosphatases, including phosphatase and tensin homolog (PTEN) and SH2 domain-containing inositol-5′-phosphatase 1 (SHIP1 or SHIP). Specific binding of intracellular signaling enzymes and scaffold proteins to $PI(3,4,5)P_3$ and $PI(3,4)P_2$ are thought to mediate the functional outcomes triggered by these second messengers molecules. Collectively we refer to PI3K enzymes, PI phosphatases, D3 phosphoinositides, and their direct binding partners as the PI3K pathway (**Figure 1**). The cellular responses triggered by the PI3K pathway are highly influenced by integration with other signals that can directly impact PI-binding proteins and also modify downstream events.

Substantial evidence now exists indicating that development and activation of B lymphocytes is highly dependent on the PI3K pathway. Mature B cell survival and maintenance requires continuous expression of a signaling-competent B cell antigen receptor (BCR) which is thought to support a basal level of "tonic" signaling (Lam et al., 1997; Tze et al., 2005). Enforced PI3K activity was found to promote survival of mature B cells in the absence of BCR expression (Srinivasan et al., 2009). Abundant evidence has emerged indicating that activation of the PI3K pathway is required for B cell survival and differentiation into mature B cell subsets, as well as a number of mature B cell immune functions. In addition to functions in normal B cells, several types of B cell malignancy show evidence of deregulation of the PI3K pathway. Moreover, some malignant B cells appear to be dependent on this pathway for survival and retention in protective lymphoid tissue niches.

The functional and potential clinical importance of this pathway continues to drive research into molecular regulation of this pathway in B cells. While many components of are conserved among cell types, lymphocytes appear to have a few unique modifications of the core pathway. While the p110α and β catalytic subunits are ubiquitously expressed, the p110δ and p110γ are largely restricted to leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997). Class IA PI3K enzymes such as p110δ

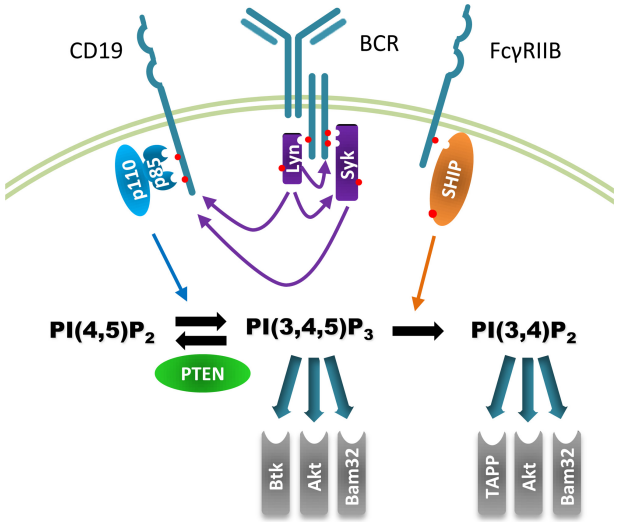

FIGURE 1 | Major components of the PI3K pathway in B cells discussed in this review. PI3K pathway activation via B cell antigen receptor is depicted schematically. Lyn and Syk are recruited to the engaged BCR where they are activated by phosphorylation and initiate the downstream signaling cascade. Class I PI3K such as p110δ or p110α binds phosphorylated CD19 and catalyzes the production of PIP_3. Alternative PI3K recruitment mechanisms not shown include binding to BCAP, TC21, or IRS-2/IL-4R. Several PH domain-containing signaling proteins bind to PIP_3 including enzymes such as Btk and Akt and adaptor proteins such as Bam32. PIP_3 levels are down-regulated by phosphatases PTEN and SHIP. PTEN directly opposes the PI3K reaction while SHIP, classically activated downstream of FcγRIIB co-aggregation with the BCR, produces a novel second messenger $PI(3,4)P_2$ which can recruit its own set of PH domain proteins. Recruited PI-binding proteins are enzymatically activated and/or serve to form membrane-associated protein complexes that regulate downstream signaling and cytoskeletal rearrangement.

are classically thought to be activated by tyrosine kinase-linked receptors whereas class 1B PI3K (p110γ) is activated through G protein-coupled receptors (Deane and Fruman, 2004). Consistent with this model, the weight of evidence suggests that p110γ is not essential for B cell signaling via antigen (Ag) and cytokine receptors (Reif et al., 2004; Beer-Hammer et al., 2010). However, a more complex picture has emerged regarding p110δ which seems to have some involvement in signaling via G protein-coupled chemokine receptors. Immune cells also selectively express the PI phosphatase SHIP1, and some PI-binding proteins such as Bam32/DAPP1 and Btk. These molecules appear to have regulatory functions specific to immune cells and clearly function in B cells. The goal of this review is to bring together for discussion the current evidence on molecular regulation and cellular functions of the PI3K pathway in the context of normal and malignant B cells.

MECHANISMS OF PI3K PATHWAY ACTIVATION IN NORMAL B CELLS

CONTROL OF PI3K ENZYMATIC ACTIVITY

Class IA PI3Ks at rest exist predominantly in an inhibited conformation. The p110 catalytic subunit constitutively associates with the regulatory subunit via its interstitial SH2 domain (iSH2). This iSH2 bridges two other SH2 domains, the N-terminal and C-terminal SH2 domains (nSH2 and cSH2, respectively), which also associate weakly with p110 and promote an inhibited conformation (Yu et al., 1998b). Activation of catalytic activity involves binding of nSH2 and cSH2 to phosphopeptide motifs such as pTyr-x-x-Met. This binding both recruits the complex to the membrane and dissociates these SH2 domains from p110, relieving their inhibitory effect (Carpenter et al., 1993; Yu et al., 1998b). Intriguingly, the precise mechanism of regulation by p85 may differ between p110 isoforms (Burke et al., 2011). It was previously shown for p110α that the minimal p85 domains required for inhibition are the nSH2 plus iSH2, where nSH2 is responsible for reversible inhibition in the absence of phosphopeptide binding (Yu et al., 1998a). More recently, it has been demonstrated that p110δ and p110β have a distinct mechanism of inhibition involving cSH2 in addition to nSH2 (Burke et al., 2011; Zhang et al., 2011), suggesting differential regulation of these isoforms by cSH2-binding phosphopeptide ligands. Recent evidence suggests that binding of p110 itself to anionic lipids in the cell membrane contributes to enhancement of catalytic activity for all Class IA isoforms. Access to these lipids is regulated by p85 and, at least for p110α, seems to confer a global conformational change (Hon et al., 2011). Binding of the catalytic subunit to certain Ras family GTPases at the membrane also contributes to activation (Rodriguez-Viciana et al., 1994, 2004; Jimenez et al., 2002). Interestingly, p110 isoforms show selectivity in activation by distinct Ras family members, with p110δ showing selective activation by R-Ras1 and R-Ras2 (Rodriguez-Viciana et al., 2004). TC21/R-Ras2 has recently been shown to act by mediating an interaction between the BCR and both p110δ and p85α, thereby recruiting P13K to the cell membrane (Delgado et al., 2009).

Phosphoinositide 3-kinase is activated downstream of BCR ligation in a manner dependent on the src family kinase Lyn and sustained by the tyrosine kinase Syk (Kurosaki et al., 1994; Beitz et al., 1999). Ag binding to the BCR induces tyrosine phosphorylation of the cytoplasmic tails of the associated signaling chains, Ig-α and Ig-β by Lyn and Syk (Kurosaki et al., 1994; Beitz et al., 1999). It has been proposed that both a conformational change in the receptor and a change in the local lipid environment may be required for this signal initiation step (Tolar et al., 2005). Lyn and Syk also phosphorylate CD19 (Tuveson et al., 1993; Fujimoto et al., 2000) and B cell adaptor protein BCAP (Okada et al., 2000; Yamazaki et al., 2002; Aiba et al., 2008). These both co-aggregate with the BCR complex in the cell membrane and directly bind p85 via YxxM motifs. CD19 and BCAP are reported to make complementary and functionally important contributions to PI3K activation in B cells (Aiba et al., 2008), with CD19 ligation via the complement receptor functioning to reduce the threshold of Ag required to trigger B cell activation (Carter and Fearon, 1992; Buhl et al., 1997; Wang et al., 2002). The guanine nucleotide exchange factor Vav is required for PI3K pathway activation downstream of BCR–CD19 co-ligation but not BCR ligation alone (Vigorito et al., 2004). Recent studies suggest the adaptor protein Grb2 is required for efficient PI3K signaling in B cells, however the mechanism is not yet clear (Ackermann et al., 2011).

Receptors other than the BCR can also induce PI3K signaling in B cells. PI3K is clearly activated by IL-4R and p110δ is required for functional responses to IL-4 (Bilancio et al., 2006). PI3K recruitment and activation downstream of the IL-4 receptor

seems to be mediated primarily by insulin receptor substrate (Zamorano et al., 1996). Activation downstream of the IL-3 receptor has been shown in some systems to involve binding of p85 to the adaptor protein Gab2 which localizes to activated cytokine receptors via a Shc–Grb2–Gab2 complex (Gu et al., 2000). Activity downstream of Toll-like receptors has also been reported and seems to be mediated by BCAP (Ni et al., 2012; Troutman et al., 2012). PI3K activation downstream of CD40 ligation depends on casitas B-lineage lymphoma (c-CBL), acting as an adaptor protein (Arron et al., 2001). B cell activating factor (BAFF) binding to its receptor on B cells is reported to induce p110δ activation (Patke et al., 2006; Henley et al., 2008), however the mechanism is yet to be characterized.

CONTROL OF PI PHOSPHATASE ACTIVITY

PTEN activity is regulated by a variety of mechanisms, including interactions with lipids and proteins, regulation of protein levels, serine/threonine phosphorylation, and catalytic inactivation by reactive oxygen species (ROS). *In vitro* studies revealed that dynamic binding of the N-terminus of PTEN to anionic lipids, especially the PTEN substrate $PI(4,5)P_2$, leads to a conformational change and an increase in PTEN phosphatase activity (McConnachie et al., 2003). PTEN protein is sensitive to regulation by ubiquitination followed by proteasomal degradation (Wang et al., 2007). Interestingly, monoubiquitination has also been reported and was shown to promote nuclear import (Trotman et al., 2007). PTEN levels can also be controlled post-transcriptionally by the microRNA cluster miR-17-92 (Rao et al., 2011). Serine/threonine phosphorylation appears to be a double edged sword in terms of PTEN regulation, with the outcome depending on the precise site. Deletion of the C-terminal tail of PTEN, which contains several phosphorylation sites, revealed a role both in dampening catalytic activity and increasing protein stability (Vazquez et al., 2000). The effect on catalytic activity was later proposed to be the result of a conformational change induced by phosphorylation (Vazquez et al., 2001; Odriozola et al., 2007), while the effect on stability is due to protection from proteasomal degradation (Torres and Pulido, 2001). A study in Jurkat T cells provided evidence for a feedback loop involving phosphorylation of Thr366 by GSKβ, a downstream effector of PI3K, which was reported to inhibit PTEN activity (Al-Khouri et al., 2005). Thr366 phosphorylation was also found to decrease PTEN stability in glioblastoma cell lines (Maccario et al., 2007). One particularly interesting PTEN-binding protein that seems to directly promote its enzymatic activity is p85α, the regulatory subunit classically associated with the PI3K p110 subunit (Taniguchi et al., 2006; Chagpar et al., 2010).

A number of reports show that PTEN activity can be regulated by ROS. Specifically, oxidation by either exogenous or endogenous H_2O_2 leads to the formation of a disulfide bond between cysteine 124, found in the active site, and cysteine 71 (Lee et al., 2002). ROS can be produced in B cells by NADPH oxidase activity triggered downstream of various receptors (Hancock et al., 1990; Lee and Koretzky, 1998). Our group demonstrated that treatment of B cell lines with H_2O_2 lead to selective accumulation of $PI(3,4)P_3$-specific pleckstrin homology (PH) domains at the cell membrane. A synergistic effect was observed with co-stimulation through the BCR. This is consistent with a role for H_2O_2 in the catalytic inactivation of PTEN but not SHIP (Cheung et al., 2007). Since hydrogen peroxide has been proposed as a significant "second messenger" for B cell activation (Reth, 2002), and ROS production by neutrophils and macrophages is a ubiquitous component of inflammation, oxidative inactivation of PTEN may be an important mechanism contributing to PI3K pathway activation in infectious disease and chronic inflammatory disease. All of these regulatory mechanisms, including binding to lipid and protein partners, degradation, post-transcriptional repression, Ser/Thr phosphorylation, and inactivation by ROS, have been described; however their relative importance in B cells remains to be determined.

SHIP phosphatase activity is regulated at the levels of expression, sub-cellular localization, phosphorylation, and conformation. Expression levels can be altered either by translational inhibition mediated by the microRNA miR-155 (Costinean et al., 2009) or by ubiquitin-mediated proteasomal degradation (Ruschmann et al., 2010). The principal activation mechanism of SHIP catalytic function seems to be recruitment to the cell membrane where it can access its substrate, $PI(3,4,5)P_3$ (Phee et al., 2000). Classically, this is accomplished by binding of its SH2 domain to phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail of the inhibitory receptor FcγRIIB when this receptor is co-engaged with the BCR (Tridandapani et al., 1997). In mice, another C-terminal tyrosine residue outside the ITIM in FcγRIIB is reported to be required for SHIP binding as it allows the formation of a stabilized tri-molecular complex that includes Grb2 and/or Grap (Isnardi et al., 2004). In human FcγRIIB, the corresponding C-terminal sequence contains no tyrosine residue, however the segment itself is required for SHIP binding (Isnardi et al., 2006). These studies support earlier reports that the C-terminus of SHIP, containing both a proline rich region as well as several phosphorylation sites, is required in addition to the minimal phosphatase domain to efficiently mediate downstream inhibition of Ca^{2+} flux (Aman et al., 2000).

Although SHIP mediates the inhibitory functions of FcγRIIB in B cells (Isnardi et al., 2006), SHIP can also act independently of FcγRIIB to regulate signaling via the BCR and other receptors (Brauweiler et al., 2000). In many cases, the proteins responsible for recruitment of SHIP to the membrane are uncharacterized, however there are reports that SHIP can bind ITAMs in receptors such as FcγRIIA (Maresco et al., 1999), FcεRI (Kimura et al., 1997), and perhaps the Ig-α chain of the BCR under some conditions (Mukherjee et al., 2012). Once recruited to the membrane, SHIP is phosphorylated on tyrosine residues in its C-terminus by Lyn kinase (Tridandapani et al., 1997; Phee et al., 2000). Although the C-terminal segment is required for full enzymatic activation (Aman et al., 2000) and tyrosine phosphorylation, especially at Tyr1020 has been frequently used as a functional readout, it seems that it does not directly promote 5′-phosphatase activity (Phee et al., 2000). The main function of phosphorylation at Tyr1020 may be to allow association with SHC and DOK1 (Tridandapani et al., 1997; Tamir et al., 2000), however it is unclear how these associations can affect the membrane association or catalytic activity of SHIP. On the other hand, the role of specific interactions in promoting adaptor functions of SHIP have been

described (Tridandapani et al., 1997; Tamir et al., 2000). Moreover, phosphorylation of Tyr1020 in response to long-term IL-4 stimulation has been shown to promote proteasomal degradation of SHIP (Ruschmann et al., 2010).

Contrary to tyrosine phosphorylation, phosphorylation on serine 440 in the phosphatase domain of SHIP by protein kinase A can directly enhance catalytic activity in B cells (Zhang et al., 2009a, 2010a). A recently described model proposes that the SHIP SH2 domain can bind its tyrosine-phosphorylated C-terminal tail potentially generating an auto-inhibited conformation. This implies that other phosphopeptide motifs must compete with the SHIP C-terminus for binding to the SHIP SH2 domain (Mukherjee et al., 2012). Another proposed regulatory mechanism is allosteric activation induced by PI(3,4)P2 binding through SHIP's C2 domain (Ong et al., 2007). This model suggests a potential "feed-forward" activation of SHIP by its product that can be exploited by SHIP-activating compounds (Ong et al., 2007). All of these regulatory mechanisms, including control of protein expression, localization, phosphorylation on tyrosine and serine, and conformational state, likely contribute to SHIP function in B cells.

PHOSPHOINOSITIDE DYNAMICS AND REGULATION BY PI PHOSPHATASES

PI3K activation through BCR ligation leads to a transient increase in $PI(3,4,5)P_3$ (PIP_3) and $PI(3,4)P_2$ in B cell lines (Gold and Aebersold, 1994; Fruman and Bismuth, 2009). PIP_3 levels peak after approximately 1 min and return to baseline after 10 min in primary mouse B cells (Clayton et al., 2002). The PI3K reaction producing PIP_3 can be directly antagonized by PTEN (Maehama and Dixon, 1998; Anzelon et al., 2003). Later, it was confirmed that $PI(3,4)P_2$ can be generated either from direct phosphorylation of PI(4)P by PI3K or from dephosphorylation of PIP_3 by SHIP (Brauweiler et al., 2000). Brauweiler et al. (2000) showed that PIP_3 is transiently produced after BCR ligation while $PI(3,4)P_2$ levels increase steadily over a much longer period of time, consistent with earlier studies in B cell lines (Gold and Aebersold, 1994). In SHIP-deficient B cells, however, PIP_3 levels are dramatically increased and sustained while $PI(3,4)P_2$ production is significantly abrogated (Brauweiler et al., 2000). In the human BJAB B cell line we found that BCR-induced PIP_3 responses are rapid and transient, while $PI(3,4)P_2$ responses are relatively delayed and sustained (Marshall et al., 2002) and verified that stimulation of B cell lines under conditions that promote SHIP activity results in reduced PIP_3 levels and a dampening of downstream pathways dependent on PIP_3 (Krahn et al., 2004).

Although the action of both SHIP and PTEN antagonizes PI3K signaling by consuming PIP_3, the SHIP product $PI(3,4)P_2$ is itself a second messenger subject to further regulation by inositol 4′-phosphatases (Norris and Majerus, 1994; Gewinner et al., 2009). While PTEN can also efficiently dephosphorylate $PI(3,4)P_2$ *in vitro*, limited evidence exists for PTEN regulation of $PI(3,4)P_2$ levels *in vivo*. Stimulation of cells with H_2O_2, which inactivates PTEN and 4-phosphatases but not SHIP (Leslie et al., 2003; Ross et al., 2007), leads to a selective accumulation of $PI(3,4)P_2$ (Van der Kaay et al., 1999). Thus differential regulation of PIP_3 and $PI(3,4)P_2$ dynamics under different stimulation conditions may specify distinct cellular outcomes based on recruitment of differentially regulated effector molecules.

PIP_3 AND $PI(3,4)P_2$ BINDING PROTEINS

Phosphoinositides generated by active PI3Ks provide binding sites for some signaling proteins containing PI-binding domains such as PH domains (Dowler et al., 2000; Lemmon and Ferguson, 2000) and phox homology (PX) domains (Kanai et al., 2001; Sato et al., 2001). Akt phosphorylation is often used as a readout of PI3K activity, as D3 phosphoinositides are required to recruit Akt itself to the cell membrane along with its activator, PDK (Burgering and Coffer, 1995; Anderson et al., 1998). The PH domain of Akt binds to both PIP_3 and $PI(3,4)P_2$ (Frech et al., 1997). While $PI(3,4)P_2$ has been implicated in Akt phosphorylation and activation (Frech et al., 1997; Ma et al., 2008) some studies suggest that PIP_3 is the limiting factor for Akt membrane recruitment and activation (Astoul et al., 1999). Notably, Akt activation appears to be increased by selective deregulation of PIP_3 via loss of SHIP (Liu et al., 1998) or selective deregulation of $PI(3,4)P_2$ by loss of INPP4 (Gewinner et al., 2009). Thus Akt phosphorylation may integrate to some extent both PIP_3 and $PI(3,4)P_2$ levels. Direct evidence for the function of Akt in BCR signaling is scarce, however a recent study reported that combined deletion of Akt1 and Akt2 in B cells affects B cell maturation and survival (Calamito et al., 2010).

Another key process triggered by BCR-induced PIP_3 production is the formation of a "signalosome" involving the tyrosine kinase Btk which contributes to PLCγ2 activation and Ca^{2+} flux (O'Rourke et al., 1998; DeFranco, 2001; Engels et al., 2001; Chiu et al., 2002). Btk contains a PH domain that binds to PIP_3 with a high degree of selectively (Salim et al., 1996), and loss of PIP_3 binding leads to loss of Btk function (Rawlings et al., 1993). BCR stimulation leads to a rapid, transient rise in PIP_3 levels that temporally correlates with the membrane recruitment of Btk (Marshall et al., 2002). Both PIP_3 levels and Btk PH recruitment can be inhibited by PI3K inhibition or by conditions that promote SHIP activity (Krahn et al., 2004). Bypassing PI3K-dependent recruitment of Btk by a membrane targeted Btk construct overcomes the inhibitory effect of SHIP on Ca^{2+} flux (Bolland et al., 1998).

B cells also express a number of adaptor proteins and guanine nucleotide exchange factors that contain PI-binding domains with various degrees of selectivity for PI3K products. The PH domain adaptor protein Bam32 is selectively expressed in immune cells and has high affinity and high selectivity for binding to PIP_3 and $PI(3,4)P_2$, while TAPP adaptor proteins selectively bind $PI(3,4)P_2$ (reviewed in Zhang et al., 2009b). TAPP adaptors show delayed and sustained membrane localization after BCR stimulation which correlated well with the timing of the PIP_3 to $PI(3,4)P_2$ conversion (Marshall et al., 2002; Krahn et al., 2004). H_2O_2 induced selective recruitment of TAPP proteins in B cells (Cheung et al., 2007). Membrane recruitment of TAPP1-PH is significantly impaired when PTEN is re-expressed in PTEN-null B cells (Cheung et al., 2007) or when inositol 4′-phosphatase is over-expressed in non-immune cells (Ivetac et al., 2009), suggesting that $PI(3,4)P_2$-dependent responses can be regulated by both of these phosphatases. Given the differential control of PIP_3

and PI(3,4)P_2 by phosphatases and the existence of distinct binding proteins, it seems likely that these phosphoinositides impact different aspects of B cell activation.

ROLES OF THE PI3K PATHWAY IN NORMAL B CELL FUNCTION

B CELL DEVELOPMENT

B cell development and maintenance are dependent on "positive selection" signals initiated by the pre-BCR and BCR and the PI3K pathway has clearly been demonstrated to be a critical component of these developmental and homeostatic signals. We will provide only a brief overview of this area since excellent reviews covering this in detail are available (Okkenhaug and Fruman, 2010).

Mutations targeting p85α or p110δ have been shown to retard B cell maturation at the pro-B cell stage within the bone marrow and lead to a reduction in mature B cells within the spleen and lymph nodes (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002). Disruption of BCR-induced PI3K activation in CD19/BCAP double knock-out mice also led to severe impairment in the generation of immature and mature B cell subsets within the spleen and bone marrow (Aiba et al., 2008). In contrast, deletion of p110γ did not affect B cell development; however it did have a profound impact on thymocyte development and mature T cell activation (Sasaki et al., 2000). It was found that combined deletion of p110δ and p110γ impairs B cell development to a greater extent than p110δ deficiency alone, however it is not clear to what extent this reflects a B cell-intrinsic requirement for p110γ (Beer-Hammer et al., 2010). While deficiency in p110α did not affect B cell development or BCR signaling, combined deletion of p110α and p110δ led to a nearly complete block in B cell development (Ramadani et al., 2010). This result suggests that p110α may make an important contribution to tonic pre-BCR and BCR signaling, but is less important for signaling induced by acute BCR cross-linking. p110β appears to be dispensable for both B cell development and activation (Ramadani et al., 2010).

At the pro/pre-B cell transition, PI3K signaling appears to be essential to turn off recombination-activating gene (RAG) expression to ensure allelic exclusion (Tze et al., 2005; Verkoczy et al., 2007). RAG expression must again be down-regulated once B cells reach the immature stage and this process has been shown to specifically depend on p110δ (Llorian et al., 2007). The mechanism for PI3K regulation of RAG expression depends on Akt, which phosphorylates and inactivates the transcription factor FOXO1. FOXO1 is able to directly bind to RAG gene promoters, facilitating the transcription of both RAG1 and RAG2; thus, pre-BCR-dependent PI3K signaling turns off RAG expression by inactivating FOXO1 (Amin and Schlissel, 2008). FOXO1 is however required for early B cell development, since FOXO1 deficiency leads to impaired expression of IL-7Rα and RAG in pro-B cells (Dengler et al., 2008). These results point to dual roles of PI3K signaling in promoting and repressing different aspects of B cell differentiation, which is also observed in late B cell differentiation in germinal centers (GC; see below).

Studies in mice with SHIP deletion suggest that deregulation of PIP_3 levels can impact B cell maturation. SHIP-deficient mice had decreased numbers of pre- and immature B cells but increased number of total B cells in the spleen. Further study provided evidence for accelerated B cell development and quicker emigration from the bone marrow in the deficient cells, implying that SHIP restrains developing B cells from moving through immature and transitional stages to the mature stage (Brauweiler et al., 2000).

INNATE-LIKE B CELL POPULATIONS

The PI3K signaling pathway appears particularly critical for generation and/or maintenance of innate-like B cell populations (B1 and marginal zone B cells). B1 and MZ populations are nearly absent in p85- or p110δ-deficient mice (Clayton et al., 2002; Donahue et al., 2004). Subsequent studies found that B cell specific deletion of p110δ led to loss of both MZ and B1 B cells, indicating an intrinsic requirement for PI3K in the development of these cells (Rolf et al., 2010). In contrast, follicular B cells numbers are not markedly diminished in p110δ deficient mice; however dual loss of p110δ and CD19 does substantially reduce FO B cell numbers (Kovesdi et al., 2010), suggesting other p110 isoforms and/or other pathways activated by CD19 are sufficient to maintain FO B cells in the absence of p110δ. p110δ mutant mice also showed reduced natural antibodies and reduced T-independent responses characteristic of innate-like B cell populations (Okkenhaug et al., 2002; Durand et al., 2009). Conversely, B1 and MZ populations were increased in mice with B cell specific deletion of PTEN (Anzelon et al., 2003). Combined deficiency of p110δ and PTEN restored the B1 population to normal numbers, but only partially reduced the MZ B cell population, suggesting that additional PI3K isoforms may promote MZ B cell development (Janas et al., 2008).

Interestingly, SHIP-deficient mice show a defect in MZ B cell development; however this was reported to be secondary to a defect in marginal zone macrophages rather than a B cell-intrinsic defect (Karlsson et al., 2003). This suggests that increased PIP_3 levels may not be sufficient for innate-like B cell expansion: deregulation of both PIP_3 and PI(3,4)P_2 (i.e., PTEN deficiency) may be required. The effect of B cell specific deletion of SHIP on innate-like B cell development has not been reported to our knowledge. Combined deficiency of Akt1 and Akt2 led to selective reduction in B1 and MZ populations (Calamito et al., 2010), indicating that Akt is one of the PI3K effectors required for innate-like B cell development. The FOXO1 transcription factor, which is a direct target of Akt, is also implicated in MZ B cell development (Chen et al., 2010).

Several studies indicate that the PI3K pathway is also involved in both homeostatic maintenance and activation of innate-like B cell subsets. Treatment of normal mice with the p110δ inhibitor IC87114 leads to a striking depletion of MZ B cells from the spleen (Durand et al., 2009), suggesting that continuous PI3K tonic signals may be required to maintain the MZ compartment. Consistent with this study, we have found that oral treatment with IC87114 leads to significant MZ B cell depletion within 4 days and appears to be reversible upon discontinuation of treatment. Since integrin blockade was also found to deplete MZ B cells from the spleen (Lu and Cyster, 2002), it is possible that acute p110δ inhibition may impact on the MZ B cell compartment by antagonizing the adhesive interactions of MZ B cell within this microenvironment. This would seem to parallel the findings in B cell leukemia

where acute p110δ inhibition leads to release of leukemic cells from lymphoid tissues into circulation (see below). However more work will be required to determine the contributions of B cell-intrinsic de-adhesion versus indirect effects of p110δ inhibition on the microenvironment and/or induction of apoptosis due to loss of tonic BCR signaling.

p110δ mutation or pharmacological blockade inhibited the ability of splenic B cells to produce IL-10 after stimulation with TLR ligands (Dil and Marshall, 2009), consistent with findings that marginal zone B cells are a major source of IL-10 and may have a unique capacity to generate regulatory B cells (Lund, 2008). p110δ blockade also impairs TLR-induced proliferation and chemokine-induced adhesion and migration responses of MZ B cells *in vitro* (Durand et al., 2009). The latter findings contradict the paradigm which suggests that only class 1B PI3Ks are linked to G protein-coupled receptors such as chemokine receptors; however they are consistent with findings in neutrophils which clearly show that p110δ functions in migration responses and respiratory burst triggered by GPCRs (Sadhu et al., 2003; Condliffe et al., 2005).

ANTIGEN PRESENTATION

B cell Ag presentation involves a number of complex cell biological processes, including BCR–Ag endocytosis and intracellular trafficking, formation of specialized compartments for Ag degradation and peptide loading onto MHC molecules, and formation of stable cell–cell conjugates with Ag-binding T cells (**Figure 2**). Several early studies using high doses of less specific PI3K inhibitors found that PI3K activity was required for optimal B cell Ag presentation function *in vitro* (Granboulan et al., 2003). Some studies suggested PI3K-dependent signal are required at the level of BCR endocytosis (Phee et al., 2001); however we and others have found that BCR endocytosis of soluble Ag does not require PI3K (Al-Alwan et al., 2007). Studies using Ags tethered to artificial membranes found that Ag uptake is associated with a cell spreading and contraction response that requires p110δ PI3K, CD19, Vav, and Rac (Arana et al., 2008; Depoil et al., 2008). These studies suggest that physiological "Ag gathering" from cell surfaces likely requires 3-phosphoinositide-dependent activation of Rac GTPases (**Figure 2**).

We found that pharmacological or genetic inactivation of p110δ impairs BCR-mediated Ag presentation function, and this was associated with a defect in generation of polarized conjugates with cognate T cells (Al-Alwan et al., 2007). It remains to be determined whether PI3K-dependant signals impact intracellular Ag trafficking or generation of the MHCII peptide loading compartment; however we found no effect of PI3K inhibitors on generation of the MHCII–peptide complexes in a murine cell line. On the other hand, p110δ is clearly required for efficient B cell adhesion mediated by LFA1–ICAM and chemokine-induced cell spreading and migration (Durand et al., 2009). In contrast with p110δ inhibition, p110δ deletion was not found to impair BCR-mediated Ag presentation (Rolf et al., 2010). The reasons for this discrepancy are unclear, but may reflect compensatory mechanisms in the deletion model, or other differences in systems used (e.g., different BCR expression/affinity in transgenic versus non-transgenic systems). The SLAM family receptor CD150, known to have a selective role in B:T cell adhesion (Cannons et al., 2010), was shown to activate the PI3K pathway (Mikhalap et al., 2004), suggesting another possible PI3K-dependant adhesion mechanism. Together these studies suggest that PI3K signaling may primarily impact B:T conjugate formation at the level of cell migration and adhesion.

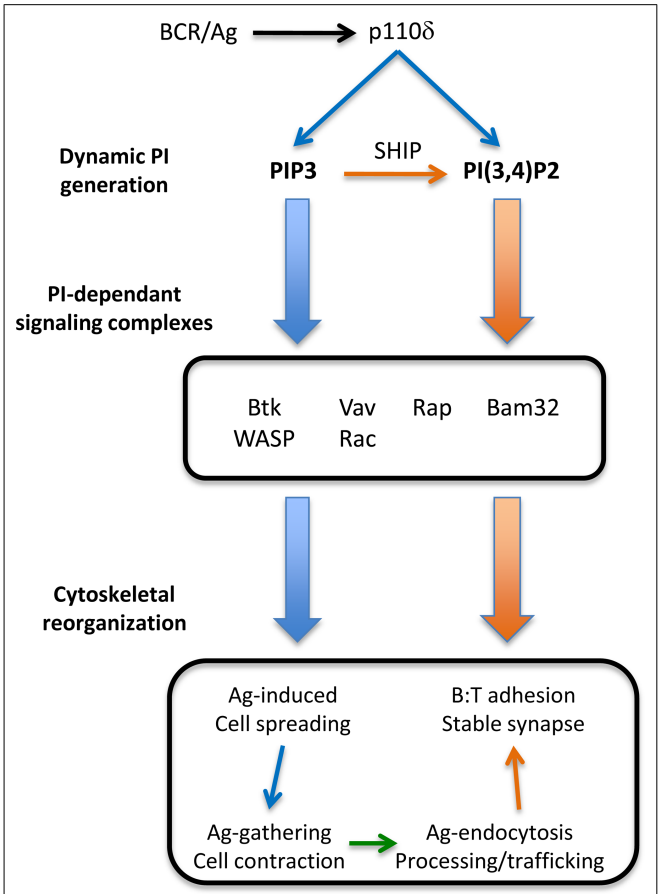

FIGURE 2 | Role of the PI3K pathway in B cell antigen presentation functions. The Figure outlines the D3 phosphoinositides and PI-dependant signaling complexes implicated in cytoskeletal reorganization associated with B cell Ag uptake and presentation. The involvement of different components in different steps of Ag presentation is discussed in the text.

A few PI-binding proteins have been implicated in B cell migration, adhesion, and cell:cell conjugate formation (**Figure 2**), however the mechanisms remain incompletely understood. The Rac-GEF Vav, which has a PIP_3-binding PH domain, is important in B cell spreading and contraction responses (Arana et al., 2008). We found that the Bam32/DAPP1 adaptor promotes efficient Rac activation and B cell spreading on integrin ligands (Al-Alwan et al., 2010). The activation of Rap GTPase, which is important for cytoskeletal rearrangements occurring in B cell adhesion, spreading, and migration responses (Lin et al., 2008), was found to be dependent on p110δ (Durand et al., 2009).

While the relative importance of PIP_3 and $PI(3,4)P_2$ in B cell Ag gathering and conjugate formation is not clear, SHIP appears to play both positive and negative roles. SHIP-deficient B cells show

increased spreading responses and increased F-actin accumulation, presumably due to increased Btk recruitment, but impaired centripetal movement and growth of BCR clusters (Liu et al., 2011). This study suggests that while PIP_3 drives Btk-dependent F-actin polymerization required for Ag-induced B cell spreading, conversion to $PI(3,4)P_2$ by SHIP may then promote "gathering" of BCR–Ag microclusters into large aggregates for internalization. In other cell systems, SHIP has been found to promote certain cellular responses related to cytoskeletal dynamics (Nishio et al., 2007; Severin et al., 2007; Harris et al., 2011). For example, although knock-down of SHIP in primary human T cells resulted in increased total F-actin, it also paradoxically led to loss of actin-rich microvilli projections (Harris et al., 2011). Although incompletely understood, recruitment of $PI(3,4)P_2$-specific effectors by SHIP such as TAPP2 (Dowler et al., 2000) and lamellipodin (Krause et al., 2004) may be central to these and other positive regulatory mechanisms.

CLASS SWITCH RECOMBINATION

We and others have found that PI3K regulates Ig class switch recombination (CSR), with either pharmaceutical blockade or genetic deficiencies in PI3K leading to markedly enhanced switch to IgG1 or IgE isotypes (Omori et al., 2006; Zhang et al., 2008). CSR, also known as isotype switching, is a specific DNA recombination mechanism that replaces the currently expressed immunoglobulin heavy chain constant region gene (C_H) with one downstream C_H gene. Switch (S) regions are highly repetitive sequences upstream of each C_H gene which are regulated by sterile germline transcripts (GLTs). Specific GLTs are induced by cytokines and T cell-derived signals, such as IL-4Ra and CD40 (Stavnezer et al., 1988; Nambu et al., 2003). In addition, these signals induce the expression of the enzyme AID, which serves a critical catalytic function for CSR.

Several lines of evidence indicate that PI3K signaling can suppress CSR through Akt-dependant inactivation of FOXO transcription factors which drive expression of AID (**Figure 3**). PI3K blockade markedly increased expression of AID and this was reversed by constitutively active Akt (Omori et al., 2006). Moreover, we found that inhibition of Akt activity is sufficient to deregulate AID expression (Zhang et al., 2012). Conversely PTEN deficiency leads to reduced isotype switch associated with reduced AID expression (Suzuki et al., 2003). Constitutively active FOXO1 activated AID transcription (Omori et al., 2006) while FOXO1-deficient B cells showed reduced AID expression (Dengler et al., 2008), indicating that Akt regulates AID by inactivating FOXO. While IL-4 is an important driver of CSR, mice with a targeted mutation in the insulin receptor substrate-2 binding site of the IL-4Ra chain, which impairs IL-4 induced PI3K activation, were reported to paradoxically show increased CSR to the IgE isotype (Blaeser et al., 2003). This suggests that PI3K activation via the IL-4R may modulate CSR *in vivo*.

We found that PI3K blockade has a particularly potent effect on CSR to the IgE isotype. Genetic or pharmaceutical inactivation of p110δ resulted in markedly increased IgE levels *in vivo* despite reduced GC responses and reduced production of IL-4 by T cells (Nashed et al., 2007; Zhang et al., 2008). Increased IgE switch was associated with elevated epsilon GLTs (εGLTs) in addition to increased AID expression (Zhang et al., 2008). Production of εGLTs is controlled by the promoter of Iε exon, which contains binding sites for many transcription factors such as STAT6, BCL6, NF-κB, PU.1, PAX5, AP1, and E-box binding sites (Oettgen and Geha, 2001). All of these transcription factors promote the transcription of εGLTs except BCL6, which is a negative regulator that competes with STAT6 for promoter binding (Harris et al., 1999). We recently found that blockade of p110δ PI3K decreases the expression of BCL6, providing a potential mechanism for PI3K regulation of IgE (**Figure 3**). Re-expression of BCL6 was sufficient to reverse excess εGLT expression and IgE switch (Zhang et al., 2012). Interestingly, selective blockade of Akt markedly enhanced AID expression as expected, but had no effect on BCL6 or εGLT expression (Zhang et al., 2012), suggesting other PI3K-dependent signals may regulate BCL6.

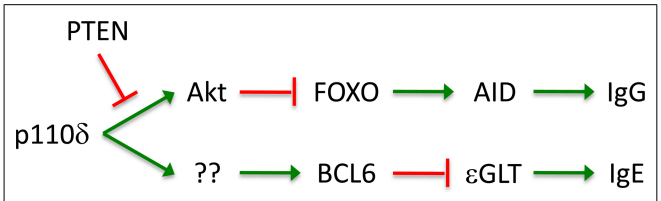

FIGURE 3 | Role of the PI3K pathway in Ig isotype switch. The figure depicts the regulatory linkages through which PI3K impacts on Ig isotype switch to IgG and IgE. PI3K-dependent activation of Akt leads to phosphorylation and inactivation of FOXO transcription factors, which drive expression of AID, an enzyme required for all isotype switch. PI3K-dependent, Akt-independent enhancement of BCL6 expression (through an unknown mechanism) promotes the GC B cell gene expression program and also suppresses germline transcription of the IgE locus (eGLT), selectively repressing switch to IgE.

T CELL-DEPENDANT ANTIBODY RESPONSES

The T cell-dependent (TD) antibody response and associated B cell selection in GC is a fascinating system for generation of high affinity isotype-switched antibodies as well as B cell memory. The PI3K pathway acts at multiple levels to regulate these responses generated by follicular B cells. Inactivation of p85 or p110δ or treatment with p110δ-selective PI3K inhibitors significantly perturb functional responses of follicular B cells *in vitro*, including reduced BCR-induced proliferation, increased susceptibility to apoptosis, and impaired adhesion and migration responses (Clayton et al., 2002; Okkenhaug et al., 2002; Durand et al., 2009). It should be noted that some responses are not decreased by p110δ blockade, including proliferative responses induced by CD40 and isotype switch as described above.

In vivo, p85 or p110δ mutant mice show dramatically reduced antibody production after immunization with TD Ags (Suzuki et al., 1999; Clayton et al., 2002; Okkenhaug et al., 2002; Okkenhaug and Vanhaesebroeck, 2003). Impairment of BCR-induced PI3K activation by deletion or mutation of CD19 was also found to significantly impair GC differentiation and Ab responses to TD Ags (Rickert et al., 1995). Dual inactivation of PTEN and CD19 was found to restore the ability to mount GC responses (Anzelon et al., 2003). Deletion of SHIP results in enhanced Ab responses to TD Ags (Helgason et al., 2000). However SHIP deletion affects many immune cell types resulting in severe systemic

pathology (Helgason et al., 1998), and the impact of B cell specific deletion of SHIP on TD responses has not been reported to our knowledge.

Consistent with failure to induce TD Ab responses, many of the above studies also found that p110δ deficiency virtually abrogates the generation of GC. Similarly we have found that treatment with IC87114 strongly reduces GC responses, without the associated decreased serum Ig levels seen in p110δ mutant mice (Zhang et al., 2008, 2012). We found that IC87114 treatment of mice with a pre-established GC response significantly reduced the number of GC B cells, suggesting that continuous p110δ signaling is required for GC maintenance since. As discussed above p110δ appears to have an ancillary function in maintaining expression of BCL6, a key transcriptional regulator for the GC B cell genetic program, providing a potential mechanism linking p110δ to GC B cell maintenance.

Given the substantial evidence for a B cell-intrinsic requirement for p110δ, it was surprising when Rolf et al. (2010) found that B cell specific deletion of p110δ did not markedly impair TD Ab or GC responses. This result may suggest that other PI3K isoforms such as p110α can functionally compensate for loss of p110δ in the context of the GC response. Since follicular B cell responses to CD40 and TLR ligands seem to be less dependent on the PI3K pathway, it is possible that, under immunization conditions generating abundant T cell help and other adjuvant-induced activation signals, the D3 phosphoinositide levels may not be a limiting factor controlling GC size. Interestingly, Rolf et al. (2010) also found compelling evidence that p110δ plays an important role in follicular helper T cell (T_{FH}) function. Thus, the dramatic loss of GC responses associated with p110δ blockade is likely due to the combined impact on GC B cells and T_{FH} cells.

Accumulating evidence indicates that selection of high affinity B cell clones within the GC is driven largely by cognate interactions with T_{FH}, which deliver co-stimulatory signals required for continued GC B cell survival (King et al., 2008). Given the evidence outlined above regarding the roles of the PI3K pathway in B cell migration, adhesion and Ag presentation to T cells, PI3K signaling would be expected to have an important role in affinity maturation. However, there is currently limited evidence supporting this proposition. As noted above, p110δ-deficient B cells show only subtle reductions in affinity maturation (Rolf et al., 2010) while p110α/δ double deficiency abrogates B cell development (Ramadani et al., 2010), precluding affinity maturation studies.

We found that the PI-binding adaptor protein Bam32/DAPP1 is required for optimal B cell Ag presentation, and Bam32-deficient mice showed premature dissolution of the GC response and impaired affinity maturation (Zhang et al., 2010b). It should be noted that the GC defect in Bam32-deficient mice was observed under conditions of relatively low precursor frequency of Ag-specific B and T cells (i.e., no Ag receptor transgenes) and with relative mild adjuvant conditions (OVA/alum). Bam32-deficient mice were able to generate relatively normal GC responses after immunization with sheep red blood cells, known to activate large numbers of lymphocytes and to contain a potent adjuvant (hemin). It is tempting to speculate that, under conditions of limiting T cell numbers in GC, BCR-induced activation of PI3K may recruit PI-binding proteins such as Bam32 and Vav to the plasma membrane and provide a selective advantage to GC B cells by enhancing their ability to form cognate interactions. In this context it would be interesting to examine the ability of p110δ-deficient B cells to compete with wild-type B cells within a GC response.

CONTROL OF B CELL HOMEOSTASIS AND ANERGY

Three studies performed in mice harboring B cell-targeted deletions of PTEN have shed light on the specific roles of this phosphatase in B cell homeostasis (Anzelon et al., 2003; Suzuki et al., 2003; Browne et al., 2009). Both immature and mature PTEN-deficient B cells exhibited hyper-activation and hyper-proliferation in response to various stimuli (Anzelon et al., 2003; Suzuki et al., 2003; Browne et al., 2009). Mature B cell populations also displayed enhanced migration and resistance to apoptotic signals (Suzuki et al., 2003). Two of the studies report an increase in autoantibody titer (Suzuki et al., 2003; Browne et al., 2009), which one study attributed to abnormal generation of innate-like B cells (Suzuki et al., 2003). In the context of chronic self-Ag stimulation, anergic BCR transgenic B cells showed increased expression of PTEN and showed reduced PIP_3 levels after BCR cross-linking, suggesting that dampening of the PI3K pathway via PTEN may be a significant component of anergy induction (Browne et al., 2009). Consistent with this idea, PTEN-deficient BCR transgenic cells failed to develop anergy in presence of self-Ag (Browne et al., 2009). Notably, PTEN can also act as a protein phosphatase (Myers et al., 1997). The extent to which the effect of PTEN on B cell anergy is due its PI-hydrolyzing function is unclear, and the relative contributions of innate-like B cell populations and GC defects to autoimmunity development remain to be determined.

Deregulation of PI dynamics via deletion of SHIP has also been reported to impact B cell anergy. SHIP-deficient B cells were shown to exhibit heightened *in vitro* responsiveness, including increased phosphorylation of Akt and MAPKs as well as enhanced proliferation, survival and cell cycling upon stimulation through the BCR (Helgason et al., 2000). $SHIP^{-/-}$ mice also display elevated serum Ig levels and increased Ag-specific IgG in response to a T cell-independent Ag (Helgason et al., 2000). Another group found that SHIP-deficient B cells were more sensitive to induction of CD86 expression upon BCR ligation and more sensitive to BCR-induced apoptosis (Brauweiler et al., 2000). Recently, B cell-targeted SHIP-deficient mice were characterized (O'Neill et al., 2011). These mice displayed a severe lupus-like autoimmune phenotype featuring increased autoantibodies produced against nuclear components and IgG deposition in glomeruli of the kidneys. The authors also showed that SHIP phosphorylation is increased in primary anergic B cells from wild-type mice, suggesting that increased SHIP activity may contribute to low PIP_3 levels. The authors hypothesize that chronic monophosphorylation of Igα/β (CD79a/b) ITAMs by Src family kinases in anergic B cells leads to constitutive activation of SHIP, which is essential for the maintenance of B cell anergy (O'Neill et al., 2011).

We have recently completed a study on mice bearing mutations in the PH domains of TAPP1 and TAPP2 (Landego et al., 2012).

These mutations effectively uncouple TAPP adaptors from the SHIP product $PI(3,4)P_2$ (Wullschleger et al., 2011). It was found that TAPP mutant B cells display exaggerated proliferative responses to BCR cross-linking which was associated with increased Akt phosphorylation. Strikingly these mice show several additional similarities to SHIP-deficient mice, including increased basal Ig levels, autoantibodies and development of lupus-like disease. Given the similar phenotypes observed in TAPP KI and SHIP-deficient mice, it is tempting to speculate that TAPP-$PI(3,4)P_2$ interactions may in part mediate the regulatory effects of SHIP in B cells. The mechanism by which TAPPs antagonize Akt activity is currently unclear, but may involve competition for $PI(3,4)P_2$ or recruitment of a regulatory phosphatase.

Consistent with the reported role of PIP_3 phosphatases in maintaining anergy, haploinsufficiency of PI3K p110δ was very recently reported to partially attenuate the autoimmune phenotype of Lyn-deficient mice (Maxwell et al., 2012). Plasma cell numbers were reduced, as were titers of antibodies and autoantibodies in the serum. The hyper-proliferative B cell phenotype was also moderated. $Lyn^{-/-}p110\delta^{+/KD}$ B cells maintained high basal and BCR-stimulated Akt and MAPK phosphorylation as well as increased surface expression of CD80 and CD86 characteristic of Lyn-deficient B cells. Since myeloid cells and T cells likely contribute to the reduction of disease severity (Maxwell et al., 2012), the contribution of B cell-intrinsic p110δ signaling in this autoimmune mouse model is not yet clearly defined.

MECHANISMS OF PI3K ACTIVATION IN MALIGNANT B CELLS

The finding that BCR signaling via PI3K is critical for mature B cell homeostasis and function has driven interest in understanding the role of the PI3K pathway in malignant B cells. The PIK3CA gene is mutated in many cancers but rarely in hematological disorders, as a recent study on multiple myeloma (MM) confirmed (Ismail et al., 2010). Together with the finding that PTEN mutations are relatively rare in B cell malignancies (Leupin et al., 2003; Georgakis et al., 2006), the initial conclusion was that PI3K signaling was less critical in these diseases compared to solid tumors. However, constitutive PI3K activity is significantly increased in chronic lymphocytic leukemia (CLL) relative to normal B cells (Herman et al., 2010; Ringshausen et al., 2002). Enhanced basal and stimulated Akt phosphorylation is observed in a subset of CLL patients, with increased phosphorylation associated with progressive disease (Barragan et al., 2006; Longo et al., 2007) and decreased phosphorylation associated with an "anergic" phenotype (Muzio et al., 2008). p110δ was reported to be marginally over-expressed in CLL B cells (B-CLL) compared to normal B cells (Herman et al., 2010), and as discussed below, recent results indicate that this isoform is functionally important for Akt activation and survival of malignant B cells. Current findings collectively indicate that elevated PI3K pathway activity in B cell malignancies is driven by altered BCR signaling (**Figure 4**) together with other co-stimulatory signals present in lymphoid tissues such as chemokines and cytokines. Below we review recent studies that identify molecular alterations in malignant B cells affecting the PI3K signaling pathway. We focus particularly on CLL, where there is the most information available and promising clinical results of PI3K inhibitors have been reported in early trials.

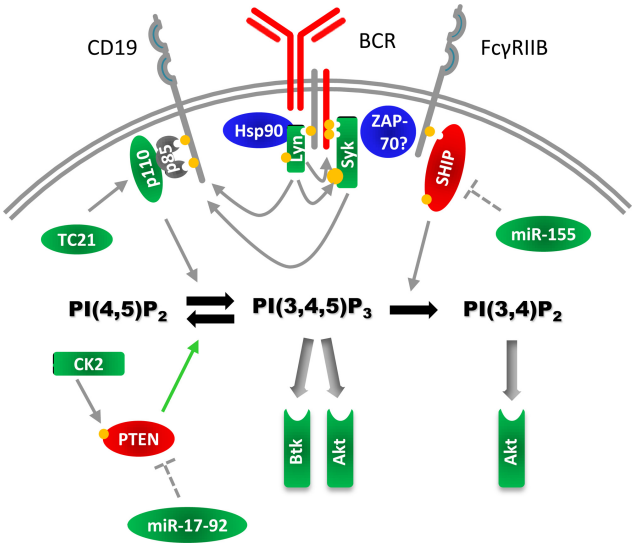

FIGURE 4 | Alterations in the PI3K pathway in malignant B cells. In malignant B cells, several pathway players have been found to be over-expressed or hyper-phosphorylated. The figure illustrates signaling molecules whose expression, phosphorylation or activity are reported to be altered in malignant B cells (green indicates increased activity, red indicates reduced activity). Altered molecules discussed in the text include p110 subunits, Lyn, Syk, Btk, Akt, TC21, and the BCR signaling chain CD79b. The formation of a non-physiological complex between Lyn and Hsp90 in malignant B cells is indicated. Increased expression of microRNA species that target PTEN and SHIP are reported to reduce expression of these phosphatases. Moreover, the PTEN kinase CK2 is reported to be over-expressed which may diminish PTEN activity. ZAP-70 is expressed in some B cell malignancies, however the impact on the PI3K pathway is currently not clear.

BCR AND ASSOCIATED PROTEIN TYROSINE KINASES

Many B cell malignancies show evidence of altered BCR signaling (**Figure 4**), which is likely relevant to increased cell survival and accumulation in blood and tissues. Compared to normal B cells, B-CLL exhibit low surface expression of Ig and CD79b, associated with low levels of mRNA encoding for CD79b (Thompson et al., 1997). CD79b mRNA level was however found to be higher in the more severe CLL prognostic group associated with unmutated Ig heavy chain variable (V_H) genes (Cajiao et al., 2007), suggesting a possible link between CD79b expression and disease severity. Some authors have compared this BCR-low phenotype to "anergic" B cells which, in animal models, down-modulate surface BCR in response to chronic BCR stimulation by low affinity or soluble self-Ags. Despite low BCR expression, B-CLL show evidence of constitutively elevated PI3K pathway activity that is dependent on protein kinases normally activated by the BCR, including Lyn and Syk.

Lyn is over-expressed in CLL (Contri et al., 2005; Trentin et al., 2008) and Hodgkin lymphoma (HL; Martin et al., 2011) and is anomalously present in the cytosol where it forms an aberrant complex with Hsp90 (Contri et al., 2005; Trentin et al., 2008). In contrast with normal B cells, Lyn's activity does not appear to change with anti-IgM stimulation; however Lyn inhibition induced apoptosis in CLL (Contri et al., 2005). Although no Syk

mutations were found in CLL (Brown et al., 2008; Philippen et al., 2010), Syk was found to be over-expressed at the protein level (Buchner et al., 2009). In HL, Syk expression was associated with shorter failure-free survival (Martin et al., 2011), while in plasma cell-like Waldenström's macroglobulinemia, BLNK and Syk genes were reported to be up-regulated (Gutierrez et al., 2007). Constitutive phosphorylation and activation of Syk has been observed in acute lymphocytic leukemia (ALL), follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL), and CLL, as reviewed elsewhere (Efremov and Laurenti, 2011). The mechanisms underlying aberrant expression and activity of these kinases remain unknown.

A subset of B-CLL can also express the Syk-family kinase ZAP-70, which has been proposed to influence BCR signaling. Although its role in leukemic B cells is not fully understood, ZAP-70 expression is used as a clinical marker predictive of aggressive clinical course (Crespo et al., 2003; Orchard et al., 2004; Rassenti et al., 2004). While ZAP-70 activation through the BCR is inefficient in CLL and lymphoma cell lines and appears to be negligible compared to Syk activation, ZAP-70 can still recruit downstream signaling molecules following BCR stimulation (Gobessi et al., 2007). B cells from ZAP-70 positive CLL patients expressed more phosphorylated Syk, PLCγ, and BLNK than ZAP-70 negative B cells only after anti-IgM stimulation (Chen et al., 2005). Introduction of ZAP-70 into ZAP-70 negative B cells also enhanced Akt phosphorylation (Gobessi et al., 2007), suggesting the effects on tyrosine kinase activation can leads to elevated PI3K activity. In transducing B-CLL with intact or mutant ZAP-70, Chen et al. (2008) identified ZAP-70 SH2 domain but not its kinase activity as necessary to induce calcium flux after BCR stimulation, suggesting that ZAP-70 can function as an adaptor protein in BCR signaling. Interestingly, ZAP-70 expression was reported to both prolong Syk activation and delay BCR internalization (Gobessi et al., 2007; Calpe et al., 2011), suggesting that it could help reduce BCR down-modulation in response to chronic stimulation by self-Ags.

Besides altered protein tyrosine kinase activity, other mechanisms potentially affecting PI3K pathway activity in malignant B cells have been proposed. The GTPase TC21, which promotes PI3K activity presumably by recruiting p110δ to the BCR, is over-expressed in DLBCL and HL compared to healthy donors (Delgado et al., 2009), providing another potential gain-of-function mechanism. Notably, the relative importance of classical modes of PI3K recruitment downstream of BCR signaling, such as through CD19 or BCAP binding, versus other mechanisms such as TC21- or insulin receptor substrate-dependent recruitment, is unknown in malignant B cells. The adaptor p66Shc, which can inhibit Akt phosphorylation and promote apoptotic signaling via the BCR (Pacini et al., 2004), was profoundly impaired in B-CLL compared to normal B cells, with lower expression in the unfavorable prognosis group (Capitani et al., 2010). The protein tyrosine phosphatase receptor-type O (PTPRO) has been implicated as a significant regulator of protein tyrosine kinase activity in B cell malignancy. Following the identification of Syk as a target for the truncated form of PTPRO (PTPROt) in a BCR stimulation-independent manner (Chen et al., 2006), ZAP-70 and Lyn activities were shown to be inhibited by PTPROt in leukemic cells (Motiwala et al., 2010). In CLL, extensive methylation of the CpG island in the gene encoding PTPROt was detected in 82% of patients (Motiwala et al., 2007). These findings suggest that Lyn, Syk, and ZAP-70 hyper-activity could be due in part to epigenetic silencing of their negative regulator and that this system regulates tonic signaling from BCR.

PI PHOSPHATASES

Although PTEN is frequently mutated in several kinds of cancers, it is rarely mutated in B cell malignancies (Leupin et al., 2003; Georgakis et al., 2006). PTEN mutations were found in 5% of primary lymphomas (Gronbaek et al., 1998; Sakai et al., 1998) and in two primary effusion lymphoma cell lines but not in primary cells (Boulanger et al., 2009). However PTEN expression and function are regulated at transcriptional and post-transcriptional levels via microRNAs, phosphorylation, ubiquitination and oxidation, and substantial evidence now indicates that malignant B cells frequently alter PTEN protein expression and function through such mechanisms.

Several studies showed a reduction or loss of PTEN expression in DLBCL (Abubaker et al., 2007; Liu et al., 2010) and CLL (Leupin et al., 2003). We found that the BJAB B cell lymphoma has no detectable PTEN protein and highly elevated generation of PIP_3 and $PI(3,4)P_2$ (Marshall et al., 2002; Cheung et al., 2007), however PTEN mRNA appeared to be expressed normally in these cells and contained no mutations. The miR-17-92 has emerged as an important negative regulator of PTEN expression. This microRNA cluster is over-expressed in several leukemias and lymphomas (Lenz et al., 2008; Rao et al., 2011) providing a potential mechanism for PTEN down-regulation. Signaling via NOTCH1 is reported to activate the PI3K pathway by inhibiting PTEN transcription (Palomero et al., 2007) and NOTCH1 was recently found to be mutated in CLL (Rosati et al., 2009; Fabbri et al., 2011; Balatti et al., 2012; Rossi et al., 2012) and is a predictor of survival (Rosati et al., 2009; Del Giudice et al., 2012; Rossi et al., 2012). Moreover, PTEN enzymatic activity is reported to be deficient in CLL (Shehata et al., 2010), suggesting that PTEN post-translational regulation is also altered in hematological cancer. In HL cell lines, reduced PTEN function was suggested to be due to its phosphorylated status (Georgakis et al., 2006). A well-known PTEN regulator CK2 is over-expressed and hyper-activated in CLL and CK2 blockade decreased PTEN phosphorylation, restoring PTEN activity (Shehata et al., 2010; Martins et al., 2011).

Since SHIP is also an important regulator of PI3K signaling, one might expect it to have tumor suppressor properties in the immune cells. Indeed, this has been demonstrated for some hematological malignancies (Fukuda et al., 2005; Vanderwinden et al., 2006). Recent evidence, including data from human samples as well as mouse models and tumor cell lines, supports a tumor suppressor role in certain B cell malignancies as well. SHIP was identified as a target of miR-155 (Costinean et al., 2009; Pedersen et al., 2009), which is over-expressed in several B cell lymphomas (Eis et al., 2005; Kluiver et al., 2005). One group studying DLBCL found that the more aggressive disease type (non-GC DLBCL) was associated with higher levels of miR-155, and consequently lower levels of SHIP, compared to GC DLBCL. Patients with the least SHIP expression also had the worst survival outcome

(Pedersen et al., 2009). Another group independently confirmed that SHIP is often down-regulated in DLBCL patients (Miletic et al., 2010). Interestingly, SHIP down-regulation occurs more frequently in patients that have also down-regulated PTEN (Miletic et al., 2010). Moreover, B cells from ZAP-70 positive CLL patients exhibited decreased expression of SHIP as well as decreased SHIP phosphorylation both basally and induced by BCR cross-linking (Gabelloni et al., 2008).

Animal models support a role for SHIP as a tumor suppressor in B cells. Transgenic mice overexpressing miR-155 develop a mixed tumor phenotype with characteristics of ALL and high grade lymphoma (Costinean et al., 2006). Further study revealed that the highest miR-155 transgene expression in these mice occurred in the bone marrow and, specifically, in pre-B cells. The authors identified SHIP as one of the miR-155 targets which showed decreased expression in pre-B lymphocytes and declined further during progression to leukemia. Another mouse model highlighting a protective role for SHIP in B cell cancers is a B cell-specific double knock-out of PTEN and SHIP (Miletic et al., 2010). The authors found that unlike with B cell-specific deletion of either phosphatase, lethal B cell neoplasms arose spontaneously in double-deficient mice. Since SHIP deletion is sufficient to substantially deregulate PIP_3 levels in B cells, its seems likely that additional functions of PTEN, such as regulation of $PI(3,4)P_2$ or protein phosphatase activity may be required to poise B cells for proliferative expansion.

Several studies have explored the impact of small molecule agonists or antagonists of SHIP in B cell malignancies. When MM cell lines were treated with a specific allosteric activator of SHIP, AQX-MN100 (Ong et al., 2007), cell viability was significantly reduced, at least in part due to induction of apoptosis (Kennah et al., 2009). Surprisingly, another group provide evidence that treatment with compounds designed to inhibit rather than activate SHIP reduced tonic and agonist-induced Akt activation and decreased viability of blood cancer cells, including human MM cell lines (Brooks et al., 2010). The authors suggest that these effects reflect that Akt requires $PI(3,4)P_2$ in addition to PIP_3 for full activation (Scheid et al., 2002). This study also provided some *in vivo* evidence of the therapeutic potential of SHIP inhibition using a tumor xenograft model. Interestingly, mice that were resistant to treatment were found to have up-regulated SHIP2 in their tumor cells (Fuhler et al., 2012).

The suggestion that both activation and inhibition of SHIP in MM cells have potential therapeutic benefits is puzzling. Certainly the use of different cell lines is a major limitation in determining the relevance of these studies, and off-target effects of these compounds cannot be ruled out. It is conceivable, however, that both activation and inhibition of SHIP could independently lead to apoptosis by different mechanisms. For example, activation of SHIP leads to reduction of PIP_3 which helps dampens effector functions including cell proliferation, thus limiting tumor growth. On the other hand, SHIP inhibition could reduce $PI(3,4)P_2$ levels, affecting Akt activation or impacting other PI(3,4)P2-binding proteins such as the TAPP proteins. It is also possible that SHIP inhibition leads to chronically elevated PIP_3 levels which could trigger activation-induced cell death. Future studies in primary human MM cells will hopefully shed light on which approach has the most therapeutic potential and in what disease context.

PI3K PATHWAY INHIBITION AS A THERAPEUTIC STRATEGY FOR B CELL MALIGNANCIES

As described above, the PI3K pathway plays pivotal roles in B cell responses such as survival, activation, proliferation, cytoskeleton dynamics, migration, and adhesion. Since the PI3K pathway is deregulated at multiple levels in malignancies, this has become a major target for new therapies. In fact, as PI3K inhibitors enter clinical trials, some of the first success stories have come from B cell malignancies. Given that PI3K enzymes do not act as a classical mutated oncogene in these diseases, this early success has come as somewhat of a surprise to many in the field. In this section we will focus on clinical results in CLL, which have taught us much about therapeutic considerations and mechanisms of PI3K inhibitors.

IN VITRO PI3K INHIBITOR STUDIES

The PI3K inhibitors LY294002 and wortmannin have both been shown to have activity against CLL *in vitro*. LY294002 helped to identify the PI3K pathway as the major pathway responsible for IL-4 and plasma albumin-induced protection from apoptosis (Wickremasinghe et al., 2001; Barragan et al., 2002; Jones et al., 2003). Phorbol 12-myristate 13-acetate (PMA)-induced Akt activation is relatively insensitive to LY294002 (Barragan et al., 2006). This PI3K inhibitor was suggested to induce B-CLL apoptosis by reducing X-linked inhibitor of apoptosis protein (XIAP) expression (Ringshausen et al., 2002), caspase 8 cleavage (Plate, 2004), and Mcl-1 expression (Ringshausen et al., 2002; Spagnuolo et al., 2011). Wortmannin was found to inhibit B-CLL migration to stromal cells mediated by CXCR4–CXCL12 (Burger et al., 1999) and CXCR5–CXCL13 (Burkle et al., 2007). Moreover, LY294002 treatment enhanced B-CLL apoptosis induced by Fludarabine (DNA synthesis inhibitor) or Dexamethasone (corticosteroid; Barragan et al., 2002), identifying the PI3K pathway as a good candidate for combination drug therapy. LY294002 and wortmannin failed to enter clinical trials: LY294002 because of dermal toxicity and low bioavailability (Hu et al., 2000); wortmannin, due to liver and hematologic toxicity as well as instability of the molecule (Ihle et al., 2004). Both of these compounds have also been found to have significant off-target effects (Knight et al., 2006).

Recent drug development efforts have focused on generating compounds with improved specificity and bioavailability, as well as targeting specific PI3K catalytic subunits (Marone et al., 2008). Two more recently developed inhibitors, PI-103 and PIK-90, are defined as p110α multi-target inhibitors (Knight et al., 2006), meaning they behave as pan-PI3K pathway inhibitors at high doses (Raynaud et al., 2007) but can be used as p110α-specific inhibitors at low doses (Niedermeier et al., 2009). Our group used PI-103 to study the role of the PI3K pathway in B-CLL adhesion to stromal cells, previously shown to provide efficient B-CLL protection (Kurtova et al., 2009). We observed that PI-103 abrogated B-CLL binding to stromal cells and inhibited B cell survival. PI3K inhibition blocked adhesion of both ZAP-70 positive and ZAP-70 negative B-CLL and reversed enhanced adhesion induced

by CD40L + IL-4, IL-6, or IL-8 (Lafarge et al., in preparation). Moreover, PI3K multi-target inhibitors were found to inhibit B-CLL migration toward stromal cells via CXCL12, as well as inhibit Akt and S6 phosphorylation more efficiently than specific p110δ (IC87114) and p110β/δ (TGX115) inhibitors (Niedermeier et al., 2009). These drugs also reversed stromal cell protection and enhanced Fludarabine-induced apoptosis (Niedermeier et al., 2009). These results indicate that PI3K inhibition is a promising strategy to reverse B-CLL protection from apoptosis mediated by stromal cell interactions.

Recent studies also indicate that specific inhibitors of p110δ can have activity against CLL *in vitro*. CAL-101/GS-1101 is a potent, orally bioavailable PI3K inhibitor highly selective for the p110δ isoform. It has been shown to have activity against multiple B cell malignancies (Lannutti et al., 2011). *In vitro*, CAL-101 reduced B-CLL survival, associated with inhibition of Akt (Herman et al., 2010; Hoellenriegel et al., 2011) and ERK (Hoellenriegel et al., 2011) pathways, more efficiently than LY294002 (Herman et al., 2010). CAL-101 was also shown to induce B-CLL apoptosis despite addition of protective factors such as CD40L, BAFF, TNF-α, anti-IgM, fibronectin, nurse-like cells (NLC) co-culture, or stromal cells. Notably, CAL-101 did not affect IL-4-induced survival (Herman et al., 2010; Hoellenriegel et al., 2011), suggesting that IL-4 in this context signals survival through another pathway or another PI3K isoform. In addition to CLL, recent studies suggest CAL-101 may have therapeutic activity in MM (Ikeda et al., 2010) and HL cells (Meadows et al., 2012).

In B-CLL co-cultures with NLC, CAL-101 inhibited the production of many cytokines and chemokines (CCL7, CCL17, CCL22, CXCL13, CD40L, and TNF-α). Interestingly, IL-6 levels were unaltered following CAL-101 treatment in this co-culture system (Hoellenriegel et al., 2011), implying that production of this cytokine alone was p110δ-independent. Moreover, CAL-101 inhibited B-CLL migration toward CXCL12, CXCL13, and stromal cell lines (Hoellenriegel et al., 2011). In normal cells, CAL-101 showed low toxicity for T and NK cells, but significantly reduced production of IFN-γ by NK cells and production of several T cell cytokines (IL-6, IL-10, TNF-α, and CD40L mRNA). Consistent with this finding, we have found that the p110δ inhibitor IC87114 markedly inhibits cytokine production by human T cells, while inducing minimal apoptosis (Lotoski et al., submitted). The production of these protective cytokines was also reduced in NK and T cells from CLL patients (Herman et al., 2010). Lastly, CAL-101 enhanced the cytotoxic activity of Fludarabine, Dexamethasone, and Bendamustine (DNA synthesis inhibitor; Hoellenriegel et al., 2011). Together these results suggest that p110δ inhibition has potential to selectively block both intrinsic (BCR tonic) and extrinsic signals from the lymphoid tissue microenvironment promoting B-CLL survival and proliferation.

EARLY CLINICAL TRIAL RESULTS

Early clinical trial results have revealed potential efficacy as well as insights into the *in vivo* mechanisms of action of p110δ inhibitors. After 28 days of CAL-101 treatment, plasma from CLL patients showed lower levels of CCL3/4 (Hoellenriegel et al., 2011) and CXCL13 (Brown et al., 2011; Hoellenriegel et al., 2011) and B-CLL from these patients showed lower levels of Akt phosphorylation (Brown et al., 2011; Hoellenriegel et al., 2011). Strikingly, these patients presented with increased numbers of B-CLL in the peripheral blood (absolute lymphocyte count, ALC), presumably reflecting release of the malignant cells from lymphoid tissues (Hoellenriegel et al., 2011). Patients treated with CAL-101 plus Rituximab or Bendamustine did not increase their ALC, potentially reflecting more efficient killing of B-CLL after release from protective tissue microenvironments (Castillo et al., 2012). Brown et al. (2011) reported marked lymph node shrinkage and found that ALC increased shortly after treatment with either CAL-101 alone or combination therapies. With CAL-101 alone, ALC stayed high for an extended period, whereas with CAL-101 plus Bendamustine ALC rapidly decreased over time.

Together, current data suggests that p110δ inhibition releases B-CLL from their protective microenvironment but does not by itself efficiently induce their apoptosis *in vivo*. Since CLL is characterized by the progressive accumulation of B-CLL in the peripheral blood, lymph nodes, spleen, and bone marrow (Cheson et al., 1996; Hallek et al., 2008), and these microenvironments play a major role in providing survival signals from supporting cells, this "tissue release" action seems likely to provide clinical benefit. This finding was somewhat unexpected, however it is consistent with accumulating data from *in vitro* models showing that PI3K inhibitors can disrupt B-CLL-stromal interactions and with mouse models showing that p110δ inhibition can release marginal zone B cells from the spleen. Mechanistically, it is currently unclear whether CAL-101 acts by directly antagonizing B-CLL intrinsic chemotactic/adhesion responses required for tissue retention or by altering production of chemotactic/adhesive factors produced within other lymphoid tissue cells. Clinical trials with CAL-101 and other PI3K inhibitors are on-going and have expanded to other B cell malignancies including MM, NHL, FL, HL, small lymphocytic lymphoma, and acute myeloid lymphoma.

An interesting analogy was observed between CAL-101 and the Btk inhibitor PCI-32765 (Ibrutinib). Briefly, this Btk inhibitor induced apoptosis in B-CLL but not in normal cells (Herman et al., 2011) and reversed the microenvironment-induced survival (Herman et al., 2011), signaling (Herman et al., 2011; de Rooij et al., 2012), adhesion (de Rooij et al., 2012), and migration (Herman et al., 2011; Ponader et al., 2012). Moreover, in an adoptive transfer TCL1 mouse model of CLL, PCI-32765 was shown to slow decrease progression (Chen et al., 2011; Ponader et al., 2012). In early clinical trials, reduced lymph node size and increased ALC were observed in almost all patients, as reviewed in (Ma and Rosen, 2011). It seems that inhibiting p110δ or Btk have similar outcomes: releasing B-CLL from their protective niche, leading to clinical improvement.

CONCLUDING REMARKS

Studies of PI3Ks and the phosphatases that regulate their products have revealed the complex system of checks and balances that control phosphoinositide accumulation. Clearly B cells can integrate multiple signaling inputs to control the pathway appropriately under various biological circumstances. Interpretation of the literature is complicated by the fact that most functional studies

looking at the roles of PI3K, PTEN, or SHIP do not include PI lipid measurements. Given the discovery of various PI phosphatase-independent functions of PTEN and SHIP, further work is needed to verify conclusions about the roles of different PI species in B cell biology. Of particular interest for the future is understanding the signaling mechanisms linked to is the independently regulated $PI(3,4)P_2$ and their roles in normal and malignant B cell functions.

The emerging importance PI3K pathway in B cell malignancies seems to derive not from not from classical oncogenic or tumor suppressor mutations, but from more subtle re-wiring of the BCR-linked activation mechanisms present in normal B cells. Thus, understanding the regulation and functions of the pathway in normal B cells will continue to inform studies in B cell malignancy as clinical applications move forward. Despite a significant literature on aberrant PI3K signaling in malignancy, the impact of these changes on PI lipid dynamics remains largely unstudied, representing a major gap in our understanding. Finally, as clinical results have highlighted the importance of external influences on the PI3K pathway such as factors in the lymphoid tissue microenvironment, the contribution of this pathway in cell:cell interactions influencing normal and malignant B cell biology is an important area for future study.

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Selective inhibitors of phosphoinositide 3-kinase delta: modulators of B-cell function with potential for treating autoimmune inflammatory diseases and B-cell malignancies

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The delta isoform of the p110 catalytic subunit (p110δ) of phosphoinositide 3-kinase is expressed primarily in hematopoietic cells and plays an essential role in B-cell development and function. Studies employing mice lacking a functional p110δ protein, as well as the use of highly-selective chemical inhibitors of p110δ, have revealed that signaling via p110δ-containing PI3K complexes (PI3Kδ) is critical for B-cell survival, migration, and activation, functioning downstream of key receptors on B cells including the B-cell antigen receptor, chemokine receptors, pro-survival receptors such as BAFF-R and the IL-4 receptor, and co-stimulatory receptors such as CD40 and Toll-like receptors (TLRs). Similarly, this PI3K isoform plays a key role in the survival, proliferation, and dissemination of B-cell lymphomas. Herein we summarize studies showing that these processes can be inhibited *in vitro* and *in vivo* by small molecule inhibitors of p110δ enzymatic activity, and that these p110δ inhibitors have shown efficacy in clinical trials for the treatment of several types of B-cell malignancies including chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL). PI3Kδ also plays a critical role in the activation, proliferation, and tissue homing of self-reactive B cells that contribute to autoimmune diseases, in particular innate-like B-cell populations such as marginal zone (MZ) B cells and B-1 cells that have been strongly linked to autoimmunity. We discuss the potential utility of p110δ inhibitors, either alone or in combination with B-cell depletion, for treating autoimmune diseases such as lupus, rheumatoid arthritis, and type 1 diabetes. Because PI3Kδ plays a major role in both B-cell-mediated autoimmune inflammation and B-cell malignancies, PI3Kδ inhibitors may represent a promising therapeutic approach for treating these diseases.

Keywords: phosphoinositide 3-kinase, p110delta, autoimmunity, GS-1101, CAL-101, IC87114, non-Hodgkin lymphoma, leukemia

THE ROLE OF PI3K SIGNALING IN B-CELL DEVELOPMENT AND ACTIVATION

B cells play a critical role in immune system function and dysfunction (e.g., autoimmunity) by producing antibodies and by acting as antigen-presenting cells (APCs) for T cells. Signaling via phosphoinositide 3-kinase (PI3K) controls many essential B cell functions and is therefore a promising target for preventing aberrant B cell activation. The class I PI3K enzymes consist of a regulatory subunit that allows receptors to recruit PI3K to the plasma membrane, and a catalytic subunit, which can then phosphorylate phosphoinositide lipids on the 3′ position of their inositol head group. The lipid second messengers generated by PI3K, primarily phosphatidylinositol 3,4,5-trisphosphate (PIP_3), can recruit cytosolic signaling enzymes that contain pleckstrin homology (PH) domains to the plasma membrane (Lemmon, 2008). The resulting formation of protein complexes facilitates the activation of these signaling enzymes and brings them in close proximity to their substrates. The PI3K "effectors" that are recruited to the plasma membrane and activated in this manner include signaling enzymes that control cell survival, activation growth, proliferation, and differentiation, as well as cell motility, cell adhesion, and specialized processes such as phagocytosis (Okkenhaug and Vanhaesebroeck, 2003; Fruman, 2004). The structure, enzymatic activity, and functions of the class I PI3Ks have been described in detail in many excellent reviews (Okkenhaug and Fruman, 2010; Vanhaesebroeck et al., 2010; So and Fruman, 2012).

PI3K signaling is important for B-cell development (Fruman et al., 1999; Suzuki et al., 1999; Clayton et al., 2002; Okkenhaug et al., 2002) due its role in mediating the survival and differentiation signals that are initiated by the pre-B-cell receptor (Ramadani et al., 2010). In mature B cells, PI3K transduces signals from a wide variety of receptors that control nearly all aspects of B-cell function. The survival of naïve B cells depends

on constitutive low-level antigen-independent activation of PI3K by the B-cell receptor (BCR) (Srinivasan et al., 2009) and by the receptor for the cytokine B-cell activating factor (BAFF) (Henley et al., 2008). These survival signals may be mediated, at least in part, by the Akt protein kinase, a major downstream target of PI3K signaling. Akt activates multiple pro-survival pathways while inhibiting pro-apoptotic pathways.

The detection of foreign antigens by circulating B cells depends on the ability of B cells to traffic into lymphoid organs and migrate into the lymphoid follicles, where antigens are captured and retained via multiple mechanisms (Batista and Harwood, 2009). The *in vivo* trafficking of B cells is directed by chemokines such as CXCL13 and CCL21 as well as the lipid chemoattractant sphingosine 1-phosphate (S1P) (Stein and Nombela-Arrieta, 2005). The G protein-coupled receptors that bind chemoattractants activate PI3K, and this is critical for B cells to migrate towards these stimuli. Once B cells encounter a foreign antigen, which in the case of an infection will occur in the presence of microbially-derived ligands for Toll-like receptors (TLRs), PI3K signaling is essential for B-cell activation and proliferation as well as the subsequent differentiation of B cells into antibody-producing cells and the survival of memory B cells. As described in detail elsewhere (Fruman, 2004), PI3K plays a central role in the activation of many BCR signaling pathways. In particular, PIP_3-dependent activation of Bruton's tyrosine kinase (Btk) is crucial for the activation of phospholipase C-γ, an enzyme that splits phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, second messengers that lead to increases in intracellular Ca^{2+}, activation of multiple protein kinase C isoforms, activation of the NF-κB and NF-AT transcription factors, and activation of the Ras and Rap1 GTPases, the latter of which is a master regulator of cytoskeletal reorganization and integrin-mediated adhesion. B-cell activation also requires critical input from co-stimulatory receptors such as CD40 and the IL-4 receptor, which transduce signals from helper T cells, as well as TLRs, which are now thought of as a third signal for B-cell activation. CD40, the IL-4 receptor, and the main TLRs expressed by B cells, TLR4 and TLR9, all signal via PI3K (**Figure 1**). Not only does PI3K signaling act downstream of multiple receptors that drive different steps in the B-cell activation process (Donahue and Fruman, 2004), it is also important for B cells to bind to T cells so that they can act as APCs and elicit T cell help in the form of CD40 ligand and IL-4 (Al-Alwan et al., 2007).

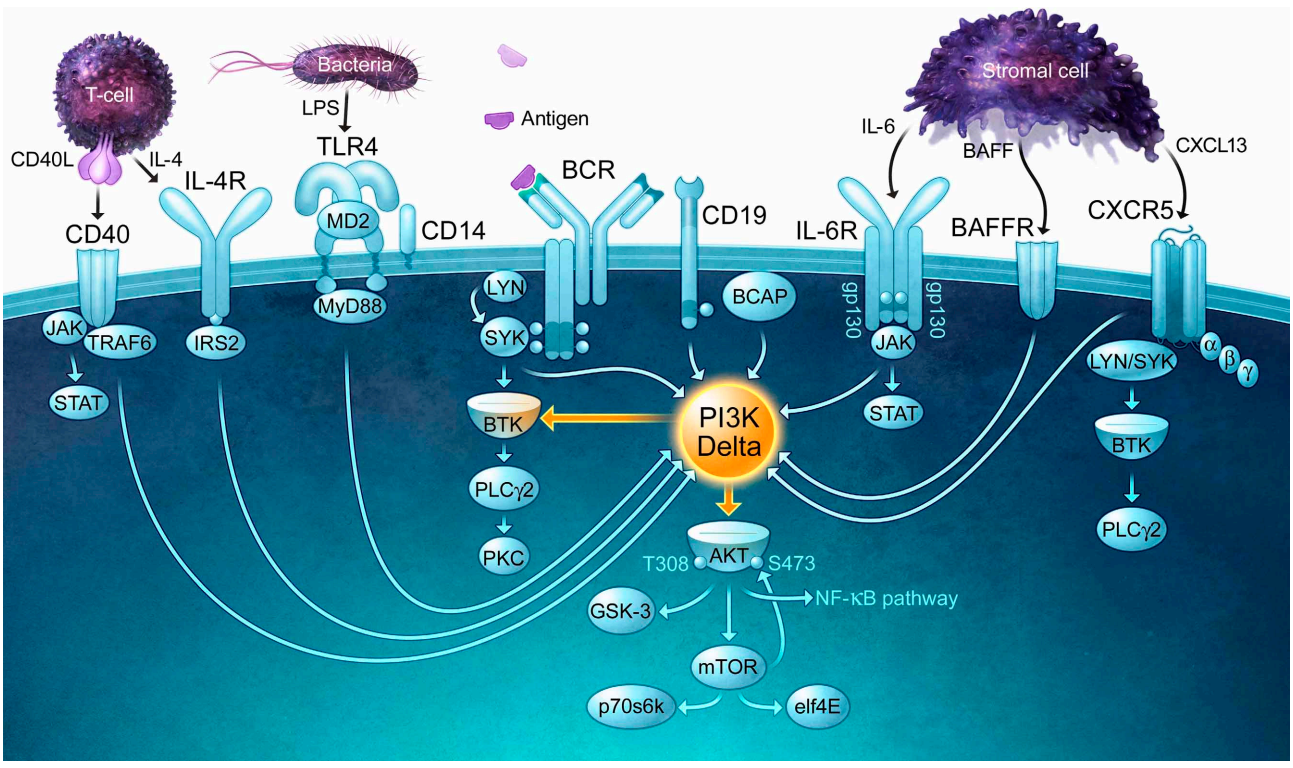

FIGURE 1 | PI3Kδ is a central signaling enzyme that mediates the effects of multiple receptors on B cells. PI3Kδ signaling is important for B-cell survival, migration, and activation, functioning downstream of the B-cell antigen receptor (BCR) and its co-receptor CD19, chemokine receptors (CXCR5), and activating/co-stimulatory receptors such as CD40 and Toll-like receptors (TLRs). Cytokines derived from lymphoid stromal cells (BAFF, IL-6) and T cells (IL-4) that are essential for the expansion and survival of B cells also require PI3Kδ for their actions and bind receptors that activate PI3Kδ. The receptors depicted in this figure use a variety of kinases and adaptor proteins to recruit PI3Kδ to the plasma membrane, where it can produce the lipid second messenger PIP_3. By binding to PH domain-containing proteins, PIP_3 promotes the activation of multiple signaling enzymes including PLCγ2 and Akt, both of which control key signaling networks. Note that the connecting arrows may represent multiple intermediate signaling reactions.

ISOFORM-SPECIFIC FUNCTIONS: A CRITICAL ROLE FOR PI3Kδ IN B CELLS

There are four isoforms of class I PI3K catalytic subunits: p110α, p110β, p110γ, and p110δ. Although these enzymes appear to have identical substrate specificity, they clearly have distinct functions *in vivo*, as revealed by the generation of mouse strains in which the genes encoding these different catalytic subunits have either been disrupted or replaced by a catalytically inactive version (Rommel et al., 2007; Vanhaesebroeck et al., 2010; So and Fruman, 2012). This could be explained in part by differential expression or abundance of the various isoforms in specific tissues. In particular, p110γ and p110δ are expressed primarily in hematopoietic cells and have important roles in the functions of both innate and adaptive immune cells (Rommel et al., 2007). It is more difficult to explain how different PI3K isoforms can play distinct roles within the same cell. Proposed models usually invoke preferential recruitment of specific PI3K isoforms by different receptors or to different membrane domains, which results in the formation of distinct signaling complexes. This would presumably depend on unique structural features of the different catalytic subunits mediating specific interactions.

An intriguing finding is that p110α is important for the tonic antigen-independent survival signals generated by the pre-BCR and BCR whereas p110δ (but not p110α) is required for antigen-dependent B-cell activation driven by the BCR (Ramadani et al., 2010). These distinct roles of p110α and p110δ in homeostatic versus antigen-induced BCR signaling could reflect different mechanisms by which PI3K complexes are recruited to the plasma membrane in resting versus activated B cells. The TC21 GTPase can directly bridge PI3K catalytic subunits to the non-phosphorylated BCR immunoreceptor tyrosine-based activation motifs (ITAMs) in resting B cells, thereby promoting homeostatic PI3K signaling (Delgado et al., 2009). In contrast, antigen-induced BCR clustering, and the resulting tyrosine kinase activation and ITAM phosphorylation, could abrogate the binding of TC21 to the BCR and instead promote SH2-mediated binding of PI3K to BCAP and CD19, scaffolding proteins that are tyrosine phosphorylated after BCR engagement. This model would require that TC21 preferentially recruit p110α-containing PI3K complexes whereas BCAP and CD19 preferentially recruit p110δ-containing PI3K complexes. The finding that TC21 recruits p110δ to the BCR in resting B cells (Delgado et al., 2009) does not support this idea. However, this group did not assess whether p110α was also recruited to the BCR by TC21. Thus, the basis for the differential role of p110α and p110δ in homeostatic versus antigen-initiated BCR signaling remains to be determined.

Although B cells express all four isoforms of the class I PI3K p110 catalytic subunit (Bilancio et al., 2006), the p110δ isoform has an essential and non-redundant role in many aspects of B cell development and activation. In mice lacking a functional p110δ protein, fewer mature circulating B cells (i.e., follicular or B-2-B cells) are generated and the B cells that do develop exhibit impaired chemokine-induced migration, BCR signaling, and BCR-induced proliferation, which correlates with reduced differentiation into antibody-producing cells and reduced serum Ig levels both before and after immunization (Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002; Reif et al., 2004). This is in contrast to knockout mice that lack the leukocyte-specific p110γ catalytic subunit, which is often linked to signaling by G protein-coupled receptors. Even though p110γ is normally expressed in B cells, mice lacking p110γ have no apparent defects in B-cell development or function (Sasaki et al., 2000). PI3Kδ also appears to have a unique role in B-cell development and function in humans. Several genetic polymorphisms in the p110δ gene have been identified and a patient with a primary B-cell immunodeficiency was found to have a missense mutation that resulted in an aspartic acid to lysine replacement within the catalytic domain of p110δ (Jou et al., 2006).

Highly selective small molecule inhibitors of p110δ such as IC87114 (Sadhu et al., 2003a,b) and GS-1101, which is also known as CAL-101 (Lannutti et al., 2011), allow inhibition of this PI3K isoform both *in vivo* and in cells derived from normal animals. The inhibitory effects of IC87114 phenocopy the effects of disrupting the p110δ gene or replacing it with a mutant version that encodes a catalytically inactive form of p110δ (Okkenhaug et al., 2002; Bilancio et al., 2006), supporting the idea that this drug is highly selective for p110δ and can therefore be used as a probe for p110δ function. The use of IC87114 has provided significant insights into the role of p110δ in mature B cells. *In vitro*, treating murine splenic B cells with IC87114 significantly reduces IL-4-dependent B-cell survival, CXCL13-induced B-cell migration, the ability of B cells to present antigens to T cells, TLR-induced cytokine production, and both BCR- and TLR-mediated B-cell activation, proliferation, differentiation, and antibody secretion (Bilancio et al., 2006; Dil and Marshall, 2009; Durand et al., 2009). IC87114 is also highly active *in vivo* and we have shown that it can reduce antigen-specific antibody responses in rodents (Durand et al., 2009).

THE ROLE OF PI3Kδ IN THE DEVELOPMENT, LOCALIZATION, AND FUNCTION OF INNATE-LIKE B CELLS

The majority of B cells are conventional circulating B-2 cells. However the marginal zone (MZ) B cells and B-1 subsets also play important roles in host defense (Pillai et al., 2005; Baumgarth, 2011). B-1 cells are found in the peritoneal cavity and the spleen, whereas MZ B cells reside near the marginal sinus of the spleen where they are positioned to rapidly detect blood-borne microbial infections. The Ig repertoires of B-1 and MZ B cells are skewed toward the recognition of both microbial antigens and self-antigens. So-called natural antibodies that are produced by these B-cell subsets in the absence of any antigen stimulation (as well as in germ-free mice) recognize cross-reactive epitopes on Gram-positive bacteria, viruses, apoptotic cells, and oxidized low-density lipoproteins (Ochsenbein et al., 1999; Zhang and Carroll, 2007; Binder et al., 2008). This provides protection against infection and also prevents inflammation by clearing oxidized lipids, oxidized proteins, and apoptotic cells (Binder et al., 2008; Chou et al., 2008; Baumgarth, 2011). In addition, exposure to TLR ligands leads to a rapid increase in antibody production by B-1 and MZ B cells. Hence, these B-cell subsets have been termed "innate-like" B cells because of their constitutive production of protective antibodies, their ability to mount rapid responses to infection, and their localization to specific sites where they act as sentinels of infection.

A striking observation is that mice in which the gene encoding the p110δ subunit has been disrupted or replaced with a catalytically inactive version have very few B-1 or MZ B cells (Clayton et al., 2002; Okkenhaug et al., 2002). The reasons why the development of these B-cell subsets is strongly dependent on p110δ are not fully understood although PI3K-dependent Akt activation appears to be more important for the development of B-1 and MZ B cells than conventional B cells. Irradiated mice reconstituted with hematopoietic progenitors that lack both the Akt1 and Akt2 isoforms develop normal numbers of circulating B-2 cells but lack MZ B cells and have greatly reduced numbers of B-1 cells (Calamito et al., 2010). The self-reactive nature of B-1 and MZ B cells has led to the idea that B-cell progenitors are directed into these lineages by weak BCR signals initiated by the binding of self-antigens, which could induce PI3K signaling. Where and when this self-antigen encounter occurs is not clear. B-1 cells are thought to arise from a unique population of committed B-1 progenitor cells that retain self-renewal capacity (Montecino-Rodriguez et al., 2006). MZ B cells arise from transitional cells in the spleen that can give rise to either conventional "follicular" B-2 cells or to MZ cells, a cell fate decision in which Notch signaling promotes the MZ B cell fate (Pillai and Cariappa, 2009). The role of PI3Kδ signaling in these cell fate decisions is not known, although it has been proposed that FOXO transcription factors, which are inhibited by PI3K-Akt signaling, may oppose the effects of Notch signaling (Chen et al., 2010).

Because B-1 and MZ B cells are nearly absent in mice lacking a functional p110δ protein, we have used the p110δ-selective inhibitor IC87114 to assess the role of this PI3K isoform in B-1 and MZ B cells that had developed in normal wild-type mice. These studies revealed a number of critical roles for p110δ activity in innate-like B cells (Durand et al., 2009). The localization of innate-like B cells is critical for their ability to mount rapid T cell-independent antibody responses against pathogens. For MZ B cells, their location near the marginal sinus of the spleen positions them to rapidly detect blood-borne pathogens. The retention of MZ B cells in this region surrounding the B-cell follicles is maintained by integrin-mediated adhesion and by opposing chemoattractant gradients established by the chemokine CXCL13, which is produced by follicular dendritic cells within the B-cell follicle and the lipid chemoattractant S1P, which is present at high concentrations in the blood (Cinamon et al., 2004). In response to TLR ligands as an indicator of infection or tissue damage, MZ B cells can move into the B-cell follicles and may eventually exit the spleen and migrate to other tissues in response to CXCL13 (Marino et al., 2008; Rubtsov et al., 2008). Similarly, the mobilization of peritoneal B-1 cells to mucosal sites of infection is stimulated by TLR ligands and depends on a robust migratory response to the chemokine CXCL13 (Ha et al., 2006). Our *in vitro* studies showed that p110δ activity is important for B-1 cells to migrate toward CXCL13 and for MZ B cells to exhibit chemotactic responses to both CXCL13 and S1P (Durand et al., 2009). In addition, CXCL13-stimulated adhesion of MZ B cells to the integrin ligand ICAM-1 was reduced to below basal level when these cells were treated with IC87114 (Durand et al., 2009). Consistent with the finding that *in vitro* treatment of MZ B cells with IC87114 inhibited both chemoattractant-induced migration and adhesion, oral administration of IC87114 to mice dramatically reduced the number of MZ B cells surrounding the B-cell follicles of the spleen, similar to what is seen in mice lacking a functional p110δ protein (Durand et al., 2009).

What are the molecular mechanisms by which PI3Kδ signaling promotes the cell motility and cell adhesion that control the localization of MZ B cells? CXCL13- and S1P-induced B-cell migration and adhesion are strongly dependent on activation of the Rap1 GTPase (Durand et al., 2006), a master regulator of cytoskeletal organization, cell polarity, cell motility, and integrin activation (Bos, 2005). Our finding that p110δ activity is required for CXCL13 and S1P to activate Rap1 in B cells (Durand et al., 2009), combined with the observation that Rap1b-deficient mice have greatly reduced numbers of MZ B cells (Chen et al., 2008), suggests that p110δ-dependent activation of Rap1b is essential for the *in vivo* localization of MZ B cells.

PI3Kδ signaling also plays an essential role in the activation of innate-like B cells. Treating murine splenic MZ B cells or B-1 cells with either IC87114 or the pan-PI3K inhibitor LY294002 cause nearly complete inhibition of Akt phosphorylation that is stimulated by CXCL13, by the TLR9 ligand CpG DNA, or anti-Ig antibodies that cluster the BCR. IC87114 also inhibits S1P-induced Akt phosphorylation in MZ B cells (neither B-1 nor B-2 cells respond well to S1P). These findings suggest that p110δ is the main PI3K isoform linking the BCR, TLR9, and the receptors for CXCL13 and S1P to activation of the Akt pro-survival kinase in innate-like B cells. This is somewhat surprising with regard to chemoattractant signaling since G protein-coupled receptors such as those that bind CXCL13 and S1P are often coupled to PI3Kγ. PI3Kγ is expressed in B cells (Bilancio et al., 2006), although this analysis has not been carried out on isolated B-1 or MZ B cell populations. However, mice lacking p110γ have no apparent defects in B-cell development or function (Sasaki et al., 2000), suggesting that p110δ substitutes for p110γ in linking G protein-coupled receptors to the PI3K/Akt pathway in B cells. Befitting their role as sentinels that provide early detection and protection from infection, innate-like B cells are most frequently activated in a polyclonal manner by TLR ligands. The ability of the TLR ligands lipopolysaccharide (LPS) and CpG DNA to induce the proliferation of B-1 and MZ B cells and to increase antibody secretion by purified splenic MZ cells or peritoneal B-1 cells is substantially reduced in the presence of the p110δ inhibitor IC87114 (Durand et al., 2009). Recent work has suggested the TLRs may activate p110γ in myeloid cells (Schmid et al., 2011) but its contribution to TLR-induced activation of innate-like B cells has not been assessed. Nevertheless, it is clear that p110δ activity is critical for the activation of innate-like B cell populations by chemokines, antigens, and TLR ligands.

Taken together, these data suggest that p110δ inhibitors such as IC87114 may be useful for inhibiting the *in vivo* trafficking, localization, and activation of MZ B cells and B-1 cells, innate-like B-cell populations that have been strongly implicated in a variety of autoimmune diseases.

PI3Kδ AS A TARGET FOR TREATING B CELL-MEDIATED AUTOIMMUNE DISEASES

Autoimmune inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), and type 1 diabetes affect millions of people worldwide. The aging population, especially in the developed world, as well as complex environmental factors, has contributed to a significant increase in the number of patients requiring treatment. Although there have been dramatic advances in the development of biological treatments in the form of antibodies and recombinant proteins, not all patients experience effective disease management. Hence there continues to be a need for new treatment approaches that provide effective long-term protection from disease progression with minimal side effects, simple delivery, and reasonable cost.

THE ROLE OF B CELLS IN CHRONIC AUTOIMMUNE INFLAMMATORY DISEASES

B cells play a major role in a number of autoimmune diseases via their production of autoantibodies, their ability to act as APCs that present self-antigens to autoreactive T cells, and their production of pro-inflammatory cytokines (Murakami and Honjo, 1997; Fields and Erikson, 2003; Martin and Chan, 2006; Shlomchik, 2008; Yanaba et al., 2008). The role of B cells in the pathogenesis of rheumatoid arthritis, type 1 diabetes, multiple sclerosis, SLE, and several other autoimmune inflammatory diseases has been described in detail elsewhere (Yanaba et al., 2008).

Aberrant B-cell activation and polyclonal antibody production can occur when there are defects in negative regulators of BCR signaling such as the Shp1 tyrosine phosphatase. In mice, the loss of Shp1 only in B cells is sufficient to cause an SLE-like disease (Pao et al., 2007). Viral and bacterial infections may also trigger autoimmunity by causing polyclonal activation of B cells via TLRs. In contrast to polyclonal B-cell activation, the loss of B-cell tolerance due either to intrinsic defects or to aberrant T-cell activation can result in the production of characteristic autoantibodies, for example the "rheumatoid factor" anti-IgG antibodies that are associated with rheumatoid arthritis. Autoantibodies can greatly amplify inflammatory responses and thereby play an important role in promoting tissue damage. Immune complexes containing IgM or IgG autoantibodies can initiate complement-mediated inflammation and can activate inflammatory cells by binding to Fc receptors. The formation of large immune complexes can also cause blockages that lead to glomerulonephritis or thrombosis (Lipsky, 2001). Finally, autoantibodies that bind to host cells may promote antibody-dependent cellular cytotoxicity carried out by NK cells or macrophages.

More recent data have shown that B cells can also initiate and amplify autoimmune disease via their ability to present antigens to T cells. This has been demonstrated convincingly in the non-obese diabetic (NOD) mouse model of spontaneous autoimmune type 1 diabetes. NOD mice that lack B cells (NOD μMT mice) do not develop diabetes (Serreze et al., 1996). However, NOD mice in which B cells are present but cannot secrete antibodies develop diabetes to the same extent as wild-type NOD mice (Wong et al., 2004). This suggests that the major role for B cells in autoimmune diabetes is as APCs. Consistent with this conclusion, B cells are required for the proliferation of diabetogenic T cells in NOD mice (Katz et al., 1993; Serreze et al., 1996; Falcone et al., 1998; Noorchashm et al., 1999a; Tian et al., 2006; Marino and Grey, 2008) and for the activation and survival of $CD8^+$ T cells that infiltrate the pancreas and attack the insulin-producing beta cells (Brodie et al., 2008). The hyperactivated B cells present in autoimmune animals and patients are highly effective APCs because they express elevated levels of MHC proteins and the T cell co-stimulatory molecules CD80 and CD86 on their surface.

Cytokine production by B cells may also contribute to the progressive amplification of autoimmune processes. Activated B cells can produce pro-inflammatory cytokines such as IL-6 (Shlomchik, 2008; Yanaba et al., 2008). Moreover, in chronic inflammatory autoimmune diseases such as rheumatoid arthritis, lymphotoxin-β produced by B cells promotes the formation of ectopic GCs within the inflamed tissue (Takemura et al., 2001; Bugatti et al., 2007). Activated B cells that enter these germinal centers undergo clonal expansion, somatic hypermutation, and affinity maturation, leading to increased production of higher affinity self-reactive antibodies (Kim and Berek, 2000).

THE ROLE OF INNATE-LIKE B CELLS IN AUTOIMMUNITY

In a number of mouse models of autoimmune inflammatory diseases such as rheumatoid arthritis and SLE, disease progression is strongly associated with an expansion of B-1 and MZ B cell populations and a corresponding increase in the production of self-reactive antibodies that may contribute to the autoimmune pathology (Batten et al., 2000; Duan and Morel, 2006; Ishida et al., 2006; Bugatti et al., 2007; Pao et al., 2007; Marino et al., 2008).

The role of innate-like B cells in autoimmunity has been characterized extensively in mouse models of autoimmune diabetes that closely resemble autoimmune type 1 diabetes in humans. In type 1 diabetes, T cells specific for pancreatic beta cells become activated and infiltrate the islets of Langerhans. $CD8^+$ cytotoxic T cells directly kill beta cells while pathogenic $CD4^+$ T_H1 cells recruit macrophages that produce inflammatory mediators such as IL-1β, TNF-α, and nitric oxide, which combine to cause beta cell dysfunction and death. The net result is progressive destruction of the beta cells, leading to insulin deficiency and hyperglycemia. At least in the NOD mouse model of spontaneous autoimmune diabetes, innate-like B cells appear to play an important role in disease progression. NOD mice have increased numbers of MZ B cells (Marino et al., 2008), a phenotype that maps to the Idd9/11 diabetes susceptibility locus (Rolf et al., 2005). Prior to the onset of diabetes in NOD mice, activated MZ B cells accumulate in the pancreatic lymph node (Marino et al., 2008), the main site of activation of diabetogenic T cells. MZ B cells are efficient APCs (Attanavanich and Kearney, 2004), express T cell co-stimulatory molecules when activated (Oliver et al., 1999; Wither et al., 2000; Marino et al., 2008), and can effectively present beta cell antigens to self-reactive T cells from NOD mice and induce their proliferation (Marino et al., 2008). Consistent with a role for MZ B cells in the development of autoimmune diabetes, the somewhat selective depletion of MZ B cells with antibodies to CD21/CD35 reduces the disease incidence in a mouse model (Noorchashm et al., 1999b). B-1 cells may also promote the autoimmune destruction of islet cells. B-1 cells are

found primarily in the peritoneal cavity but have been shown to traffic to the pancreas prior to the onset of type 1 diabetes in a mouse model of the disease (Ryan et al., 2010). In the pancreas, B-1 cells promote the infiltration of diabetogenic T cells by inducing the pancreatic vasculature to express VCAM-1 (Ryan et al., 2010), an integrin ligand that supports T cell extravasation. Thus the combined actions of MZ B cells and B-1 cells may be a key factor in the activation of diabetogenic T cells and their ability to infiltrate the pancreas, where they orchestrate the destruction of insulin-producing islet cells.

The role of innate-like B cells in autoimmune diseases in humans has yet to be fully explored because the equivalent cell populations have been more difficult to identify. A $CD27^+$ human B-cell subset has been likened to murine MZ B cells in that these cells can generate rapid T-independent antibody responses against the carbohydrate capsules of pathogenic Gram-positive bacteria. However, unlike murine MZ B cells that are localized mainly to the spleen and express an "innate" germ-line encoded Ig repertoire, $CD27^+$ human B cells circulate and their Ig repertoire appears to have undergone antigen-independent somatic hypermutation that may be similar to the way that the B cell Ig repertoire is generated in sheep and other species (Weill et al., 2009). These cells may therefore represent an innate-like B-cell population that does not have an equivalent in the mouse. It is not known whether their Ig repertoire is skewed towards self-reactivity or if they contribute to autoimmunity. In contrast, two distinct subpopulations of human B-1 cells can be distinguished by surface markers and transcriptome profile and a recent report has shown that the $CD11b^+$ human B-1 subset can activate T cells and is dramatically increased in SLE patients (Griffin and Rothstein, 2011). The identification of these subsets will undoubtedly lead to further studies of their functions and their potential roles in autoimmunity.

TARGETING B CELLS TO TREAT AUTOIMMUNITY

The multiple mechanisms by which B cells contribute to chronic inflammatory autoimmune diseases suggested that B cell depletion could be an effective approach for treating these diseases. Indeed, rituximab, a B cell-depleting anti-CD20 monoclonal antibody that was first developed for the treatment of B-cell malignancies, is now a standard therapy for rheumatoid arthritis (Edwards and Cambridge, 2006). B-cell depletion with monoclonal antibodies against B cell-specific membrane proteins such as CD20 and CD22 is also being considered as a treatment for a variety of other B cell-mediated autoimmune diseases (Kazkaz and Isenberg, 2004; Fiorina et al., 2008; Chan and Carter, 2010). The neutralization of BAFF, a cytokine that promotes the survival of B-2 and MZ B cells may also be a useful approach for depleting autoreactive B cells. Patients with rheumatoid arthritis (Baker, 2004) and SLE (Lebien and Tedder, 2008) exhibit elevated serum levels of BAFF and mice overexpressing BAFF develop SLE-like syndrome with expanded B-cell populations, elevated levels of anti-DNA antibodies, and immune complex deposition in the kidney (Mackay et al., 1999; Khare et al., 2000). In NOD mice, *in vivo* neutralization of BAFF reduces the level of autoantibodies against insulin, decreases the severity of islet inflammation, and reduces the incidence of spontaneous type 1 diabetes (Zekavat et al., 2008). Although global B-cell depletion holds promise as a treatment for autoimmunity, the strong association between autoimmunity and the expansion of innate-like B cells with inherent self-reactivity suggests that inhibiting the activation and function of the human equivalents of MZ B cells and B-1 cells may be an important consideration for effectively treating B cell-mediated autoimmune diseases. In mouse models of autoimmune disease, there is evidence suggesting that selectively targeting innate-like B cell populations can reduce disease incidence and progression. For example, in $(NZB \times NZW)F_1$ mice, which spontaneously develop autoimmune disease that is very similar to SLE in humans, B-1 cell depletion delays disease onset and reduces disease severity (Murakami et al., 1995), as does depletion of B-1 and MZ B cells using B-cell super antigens (Viau and Zouali, 2005). Similarly, as mentioned above, the somewhat-selective depletion of MZ B cells reduces disease incidence in a mouse model of type 1 diabetes (Noorchashm et al., 1999b). Thus, targeting MZ and B-1 B cells could be a useful approach for treating B cell-mediated autoimmune inflammatory diseases.

TARGETING PI3Kδ TO TREAT AUTOIMMUNITY

Although a number of different approaches have been used to treat autoimmune diseases, there are currently no curative therapies and it appears that a combination of approaches may be needed to provide effective and long-term protection from disease progression. In the context of treating type 1 diabetes, Luo et al. (2010) have provided a critical analysis of past and current strategies for treating autoimmunity by targeting immune cells. In particular, agents that deplete B cells (e.g., the anti-CD20 monoclonal antibody rituximab) are now being investigated as therapies for B cell-mediated autoimmune diseases. Mouse models of disease first showed the potential of this approach. The incidence of spontaneous autoimmune diabetes in NOD mice can be reduced by depleting B cells with anti-IgM-specific antibodies (Noorchashm et al., 1997), antibodies to CD20 (Hu et al., 2007; Xiu et al., 2008), anti-CD22 antibodies conjugated to a cytotoxic agent (Fiorina et al., 2008), or a fusion protein that sequesters the B-cell survival cytokine BAFF (Marino and Grey, 2008; Zekavat et al., 2008). Similar results have been obtained in other mouse models of autoimmunity (Yu et al., 2008). B-cell depletion with rituximab has now been tested in patients with a variety of B cell-mediated autoimmune diseases, with some success (Kazkaz and Isenberg, 2004; Martin and Chan, 2006; Sanz et al., 2007; Pescovitz et al., 2009; Hegedus et al., 2011). In a phase 2 clinical trial, rituximab treatment partially preserved beta cell function in patients with type 1 diabetes (Pescovitz et al., 2009). This incomplete efficacy could be due the variable penetration of monoclonal antibodies into tissues, resulting in poor depletion on innate-like B cell subsets (Gong et al., 2005; Yu et al., 2008). Moreover, the use of antibodies for treating chronic autoimmune diseases has been associated with infusion site reactions and other adverse reactions caused by immune complex formation (Vogel, 2010; El Fassi et al., 2011). A more serious issue associated with B-cell depletion as a long-term treatment approach for treating autoimmune diseases is chronic immunosuppression (Luo et al., 2010), as well as the potential

for eliminating beneficial B cells such as the recently identified "Bregs" that produce IL-10 and limit autoimmune reactions (Mizoguchi and Bhan, 2006; Matsushita et al., 2008). Indeed, in two mouse models of experimental autoimmune encephalomyelitis, anti-CD20-induced B-cell depletion increased the severity of the disease (Weber et al., 2010). The caveats associated with antibody-mediated B-cell depletion strategies suggest that small molecule inhibitors of B-cell function that are orally available, have good tissue penetration, and whose effects are reversible over a short period of time (in contrast to causing long-term immunosuppression) may allow for "tunable" moderate levels of B cell inhibition that can preserve beneficial B-cell functions while having limited side effects over the prolonged treatment course for chronic autoimmune diseases. Such B-cell inhibitors could be useful for managing disease, but may also be useful as an adjunct to rituximab, perhaps by reducing the frequency at which B-cell depletion would need to be done.

Based on the findings described earlier, there are a number of reasons why PI3Kδ is a very promising target for the treatment of autoimmune inflammatory diseases, particularly those in which B cells play a major role. By blocking B-cell migration, adhesion, survival, activation, and proliferation, inhibition of p110δ activity would impair the ability of B cells to act as APCs that activate autoreactive T cells, prevent their activation, and reduces their secretion of autoantibodies and pro-inflammatory cytokines. Moreover, innate-like B cells, which have been strongly linked to autoimmunity, appear to be the most sensitive to loss of PI3Kδ activity as MZ and B-1 cells are nearly absent in mice lacking a functional p110δ gene. We have directly shown that PI3Kδ inhibitors reduce the trafficking and activation of MZ and B-1 cells *in vitro*, as well as the localization of MZ B cells in mice (Durand et al., 2009). Importantly, the restricted expression of p110δ mainly in hematopoietic cells, the observation that B cells are the cell type most impaired in mice lacking a functional version of p110δ, and phase I clinical trial data showing that the PI3Kδ inhibitor GS-1101 is safe and well-tolerated by patients (Kahl et al., 2010) indicate that these compounds can be used to modulate B-cell function *in vivo* without deleterious effects on other cell types or significant side effects. As a proof-of-principle that PI3Kδ inhibitors can inhibit B cell-mediated autoimmune reactions *in vivo*, we showed that administering IC87114 to rats reduces autoantibody production in a model of collagen-induced arthritis (Durand et al., 2009).

PI3Kδ inhibitors such as IC87114 have now been shown to reduce the incidence and severity of autoimmune arthritis (Randis et al., 2008), asthma (Lee et al., 2006; Park et al., 2010), experimental autoimmune encephalomyelitis (Haylock-Jacobs et al., 2011), and SLE (Maxwell et al., 2012) in mouse models. Our current work suggests that oral administration of IC87114 opposes the progression of autoimmune diabetes in NOD mice. The ability of PI3Kδ inhibitors to reduce the severity of these inflammatory diseases may be due not only to their actions on innate-like B cells, but also to inhibitory effects on other immune cells that contribute to autoimmune disease (Fung-Leung, 2011). IC87114 inhibits the trafficking of neutrophils into inflamed tissues (Puri et al., 2004) and prevents the activation of mast cells (Ali et al., 2004, 2008). PI3Kδ also plays an important role in TCR-induced T-cell activation (Okkenhaug et al., 2002, 2006) and IC87114 treatment inhibits TCR-induced cytokine production by both naïve and memory human T cells *in vitro* (Soond et al., 2010). In particular, p110δ signaling is important for IL-17 production by both mouse and human T cells (Park et al., 2010; Soond et al., 2010; Haylock-Jacobs et al., 2011). IL-17-producing T_H17 cells have been implicated in a number of autoimmune diseases (Jain et al., 2008; Emamaullee et al., 2009; Crome et al., 2010). Finally, PI3Kδ mediates the TCR-induced downregulation of CD62L (Sinclair et al., 2008), which is required for the trafficking of activated T cells into tissues. The broad spectrum of action against cells that contribute to autoimmune processes, combined with the limited expression of p110δ in non-hematopoietic tissues, suggests that PI3Kδ inhibitors could have an excellent therapeutic index for the treatment of autoimmune diseases.

Providing a further rationale for the use of PI3Kδ inhibitors to treat autoimmune disease is the recent finding that enhanced PI3Kδ activity is associated with autoimmunity in humans (Suarez-Fueyo et al., 2011). PI3Kδ activity is significantly increased compared to normal individuals in T cells from ∼70% of SLE patients, and this difference is greatest in patients with active disease. Consistent with a role for PI3K in promoting cell survival, SLE patients exhibit an expanded population of CD4+ memory T cells, as well as defective activation-induced cell death (AICD) of T cells, a defect that can be corrected *in vitro* by pharmacological inhibition of p110δ with IC87114. AICD normally limits the expansion of activated T cells and terminates immune responses. Defects in AICD, for example in mice or humans with loss-of-function mutations in Fas or FasL, are strongly correlated with autoimmunity. This suggests that excessive activation of PI3Kδ could contribute to the T cell dysregulation associated with SLE. In this case, PI3Kδ inhibitors may be useful for treating SLE, for which there is currently no effective cure or treatment. The basis for the excessive PI3Kδ activity in SLE patients is not known nor is it known whether aberrant PI3Kδ activity is associated with other autoimmune diseases.

For treating autoimmune diseases, the therapeutic effects of inhibiting PI3Kδ could potentially be enhanced by simultaneously inhibiting PI3Kγ (Rommel et al., 2007). In addition to participating in the activation of T cells through the TCR, PI3Kγ signaling is essential for neutrophils and macrophages to invade tissues and produce inflammatory mediators (Hirsch et al., 2000; Sasaki et al., 2000; Alcazar et al., 2007). Like p110δ, p110γ is expressed mainly in hematopoietic cells, such that selective inhibition of PI3K isoforms containing these catalytic subunits would have limited toxicity on other cell types. Treating mice with the PI3Kγ-selective inhibitor AS605240 can reduce disease incidence and severity in mouse models of rheumatoid arthritis, SLE, and type 1 diabetes (Barber et al., 2005; Camps et al., 2005; Azzi et al., 2012), implicating PI3Kγ in these disease processes and suggesting that PI3Kγ, like PI3Kδ, is a good therapeutic target for treating autoimmune diseases. Abdi and colleagues (Azzi et al., 2012) provided several mechanistic insights into how inhibition of PI3Kγ may prevent the progression of autoimmune diabetes in NOD mice. Both *in vitro* and *in vivo* studies showed that AS605240 inhibited the expansion and effector functions (e.g., cytokine production) of

autoreactive CD4+ T cells while expanding the number of regulatory T cells (Tregs), consistent with previous findings that PI3K inhibition causes T cells to become Tregs instead of T-effector cells (Haxhinasto et al., 2008; Sauer et al., 2008) This shift in the T_{eff}/Treg balance toward a more immunoregulatory or immunosuppressive state correlated with a delayed onset of diabetes in NOD mice. Importantly, similar to our preliminary findings with the PI3Kδ inhibitor IC87114, treating NOD mice with the PI3Kγ inhibitor when they first exhibited elevated blood glucose levels provided sustained protection from progression to overt diabetes in a number of animals (Azzi et al., 2012). These findings suggest that combined inhibition of PI3Kγ and PI3Kδ could have even greater efficacy in preventing diabetes progression. Recently described dual inhibitors of PI3Kγ and PI3Kδ (Williams et al., 2010) could therefore hold substantial promise as a therapeutic approach for preventing disease progression in patients with early-stage type 1 diabetes and perhaps other chronic autoimmune inflammatory diseases. However dual inhibition of PI3Kγ and PI3Kδ may need to be carefully titrated, as loss of both PI3Kγ and PI3Kδ activity in mice is associated with a shift in the T_{h2}/T_{h1} balance that leads to eosinophil-mediated multi-organ inflammation (Ji et al., 2007).

The successful use of PI3Kγ/δ inhibitors to treat inflammatory autoimmune diseases must take into account the need to balance suppression of autoimmune responses with maintenance of protective immunity as well as the potential for unexpected pro-inflammatory consequences of PI3Kγ/δ inhibition, as has been observed in mice. As mentioned above, eosinophil-mediated inflammation is associated with the loss of both PI3Kγ and PI3Kδ activity in mice (Ji et al., 2007). Moreover, loss of PI3Kδ activity in mice greatly enhances isotype switching to IgE (Zhang et al., 2008, 2012), which could lead to atopic reactions. It remains to be seen whether these pro-inflammatory consequences of PI3Kγ/δ inhibition occur in humans. The paradoxical effects of PI3Kδ inhibition on Tregs, cells that play a critical role in limiting autoimmune reactions, may also be an important factor to consider. Inhibition of PI3Kδ activity has been reported to impair Treg function, even though it promotes Treg development (Patton et al., 2006; Sauer et al., 2008; Okkenhaug and Fruman, 2010). Despite these caveats, phase I clinical trial data have shown that the PI3Kδ inhibitor GS-1101 is safe and well-tolerated by patients (Kahl et al., 2010). Thus optimized dosing regimens that cause partial inhibition of PI3Kγ/δ may have the ability to substantially inhibit autoimmune reactions without rendering protective immune responses ineffective or causing significant unwanted side effects.

PI3Kδ AS A TARGET FOR TREATING B-CELL MALIGNANCIES

In addition to a key role for PI3Kδ in autoimmune inflammation, this pathway has emerged as a central mechanism underlying the survival and expansion of various malignant B-cells. BCR signaling is a central pathologic mechanism in B-cell malignancies, including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL) (Davis et al., 2010), and mantle cell lymphoma (MCL). Comparative gene expression profiling data demonstrate that BCR signaling is the most prominent pathway activated in CLL cells isolated from lymphatic tissues (Herishanu et al., 2011). Similarly, MCL cells display signs of constitutive activation of BCR (Rinaldi et al., 2006; Cecconi et al., 2008; Pighi et al., 2011) and PI3K signaling (Martinez et al., 2003; Rizzatti et al., 2005) in the absence of activating mutations. The role of BCR-induced PI3K signaling in promoting the survival of malignant B cells, combined with the fact that PI3Kδ is the predominant PI3K isoform involved in BCR signaling, make this PI3K isoform a very promising target for the treatment of B-cell cancers. Substantial progress, both preclinical and clinical, has been made with GS-1101 (CAL-101), a selective inhibitor of the PI3Kδ pathway. The importance of the PI3Kδ pathway in B cells, as well as the initial development of GS-1101 as a treatment for B-cell malignancies, has been reviewed recently by Fruman and Rommel (2011).

ROLE OF PI3Kδ SIGNALING IN B-CELL MALIGNANCIES

The potential role of excessive PI3K signaling in the development of B-cell malignancies was initially reported by Borlado et al. (2000), who showed that mice expressing a constitutively active form of PI3K develop infiltrating lymphoproliferative disorders as well as autoimmune disease. There is now mounting evidence for the importance of PI3K signaling in B-cell malignancies. Uddin et al. (2006) demonstrated the constitutive activation of the PI3K pathway in several DLBCL cell lines, importantly, in primary cells from the majority of patients with DLBCL. Constitutive activation of the PI3K pathway, as measured by elevated levels of phospho-Akt, has also been demonstrated in MCL cell lines and in primary cells from patients with MCL (Rudelius et al., 2006). PI3K activation has also been observed in follicular lymphoma (FL), mediastinal DLBCL, and Hodgkin's lymphoma (Renne et al., 2007; Garcia-Martinez et al., 2011). More recently, a role for the PI3Kδ isoform in the pathophysiology of B-cell malignancies has emerged. Several experiments have shown that there is excessive p110δ activity in malignant lymphoid cells. Herman et al. (2010) showed that there were significantly higher levels of PI3K p110δ activity in primary cells from patients with CLL than in normal hematopoietic cells. Similarly, PI3K p110δ is hyperactivated in plasma cell myeloma (PCM) cell lines and malignant cells from patients with PCM (Ikeda et al., 2010). Excessive PI3Kδ activity has also been observed in cell lines and primary cells from patients with Hodgkin's lymphoma (Meadows et al., 2012).

The development of GS-1101, a potent and selective inhibitor of PI3K p110δ, has enhanced our understanding of the role of p110δ in B-cell malignancies. GS-1101 (5-Fluoro-3-phenyl-2-[(S)-1-(9H-purin-6-ylamino)-propyl]-3H-quinazolin-4-one) inhibits PI3K p110δ with an IC_{50} value of 2.5 nM *in vitro* whereas its IC_{50} values for the p110α, p110β and p110γ subunits are 820, 565 and 89 nM, respectively (Lannutti et al., 2011). GS-1101 also shows a greater selectivity for inhibition of p110δ than for mTOR and other related kinases, and demonstrates high selectivity for p110δ when tested against a panel of more than 400 diverse kinases (Lannutti et al., 2011). Additionally, in isoform-specific cell-based assays, GS-1101 blocks PI3Kδ-dependent responses at concentrations that are >240-fold lower than those required to inhibit responses that are dependent on other PI3K isoforms (Lannutti et al., 2011). In addition to GS-1101, several other

PI3Kδ inhibitors are now in clinical trials and many more are in preclinical development, as described in a recent review (Norman, 2011).

The mechanism of action of GS-1101 has been studied in cell lines and primary patient samples representing diverse B-cell malignancies including CLL, DLBCL, and multiple myeloma (MM) (Herman et al., 2010; Ikeda et al., 2010; Hoellenriegel et al., 2011; Lannutti et al., 2011). Primary cells derived from CLL patients have high levels of p110δ and treating these cells with low micromolar concentrations of GS-1101 promotes apoptosis through caspase activation (Herman et al., 2010). This cytotoxic effect of GS-1101 is independent of the IgV_H mutational status of the cells as well as interphase cytogenetic abnormalities, the common prognostic factors associated with poor response to therapy in CLL. Interestingly, GS-1101 inhibits not only constitutive PI3K signaling in these cells but also antagonizes CLL cell survival by blocking the protective effect of CD40-ligand (CD40L) and microenvironmental stimuli (Herman et al., 2010). In particular, the cytokines BAFF and TNFα enhance the survival of primary CLL cells *in vitro* but this effect is attenuated by submicromolar concentrations of GS-1101 (Herman et al., 2010). Similarly, the survival advantage conferred to CLL cells by growth on fibronectin or stromal cell layers is reversed by GS-1101 (Herman et al., 2010).

More recent work demonstrated that GS-1101 completely blocks survival signals mediated by BCR engagement and significantly reduces survival of CLL cells cultured on specialized nurse-like cells (NLC) derived from peripheral blood monocytes (Hoellenriegel et al., 2011). Furthermore, GS-1101 decreases the secretion of survival-associated chemokines by both the CLL cells (CCL2, CCL3) and stromal cells (CXCL13) in a co-culture model. The co-culture of CLL and NLC triggers the release of additional survival factors (CCL7, CCL17, CCL22, soluble CD40L, IL-6, and TNFα) whose secretion is also reduced by GS-1101. Additionally, GS-1101 inhibits CLL cell chemotaxis toward CXCL12 and CXCL13. These chemokines are commonly present in the B-cell follicles of lymph nodes and chemoattract CLL cells to this protective microenvironment. Together these observations suggest that PI3Kδ inhibition with GS-1101 can antagonize intrinsic survival signals and also block survival signals provided by extrinsic factors in the tumor microenvironment.

PI3Kδ plays an essential role in PCM cell function (Ikeda et al., 2010). All PCM cell lines express p110δ and treatment with micromolar concentrations of GS-1101 inhibits the constitutive phosphorylation of Akt observed in these cells. This correlates with caspase-dependent apoptosis in p110δ-positive PCM cells, with minimal cytotoxicity in p110δ-negative cells (Ikeda et al., 2010). IL-6 and IGF-1, which are present in the bone marrow (BM) microenvironment, promote MM cell proliferation and survival. Importantly, the cytotoxic effect of GS-1101 on PCM cells was not diminished in the presence of IL-6 and IGF-1, suggesting that GS-1101 can overcome the protective effects of these cytokines within the BM milieu. GS-1101 also exhibits an inhibitory effect on PCM cells in the presence of BM stromal cells (BMSCs), and inhibited the growth, cytokine production, and Akt phosphorylation that are induced in PCM cells by their association with BMSCs (Ikeda et al., 2010).

PI3K p110δ INHIBITION WITH GS-1101 IN PATIENTS WITH B-CELL MALIGNANCIES

GS-1101 was the first selective inhibitor of PI3Kδ to enter clinical testing for various B-cell malignancies including CLL, non-Hodgkin lymphoma (NHL), acute myeloid leukemia (AML), DLBCL, and MM. Remarkably, treatment with GS-1101 as a single agent provided durable remissions to a significant percentage of patients with CLL and certain subtypes of NHL (Sharman et al., 2011). Most of these patients had relapsed from multiple other treatment regimens yet responded to the p110δ inhibitor. GS-1101 also showed impressive efficacy when combined with standard-of-care agents (e.g., rituximab, bendamustine, and fludarabine) for indolent NHL (iNHL) and CLL. Currently GS-1101 has advanced to phase 3 clinical testing in CLL as a single agent or in combination with rituximab and bendamustine (clinical trial identifiers: NCT01569295, NCT01539291, and NCT01539512). In addition, an ongoing phase 2 clinical study is evaluating the efficacy and safety of GS-1101 in patients with relapsed or refractory Hodgkin lymphoma (clinical trial identifier: NCT01393106).

Although most of the data have not yet been published in peer-reviewed journals, some of the clinical data for trials involving GS-1101 have been presented at meetings over the past few years and described in abstracts of the conference proceedings (Flinn et al., 2009a,b,c; Brown et al., 2010; Flinn et al., 2010; Furman et al., 2010a; Kahl et al., 2010; de Vos et al., 2011; Kahl et al., 2011; Leonard et al., 2011; Sharman et al., 2011). Most recently, Miller and colleagues (Sharman et al., 2011) reported on a phase I study of GS-1101 as single agent and in combination with rituximab and/or bendamustine in patients with relapsed/refractory CLL (clinical trial identifiers: NCT00710528 and NCT01088048). Data were presented from 55 patients with CLL who were administered GS-1101 monotherapy and 54 patients who received GS-1101-based combination therapies. GS-1101 as single agent or as part of a combination therapy caused lymph node shrinkage in a large majority of CLL patients (>79%) across all dose levels. Nodal size changes occurred in two phases: a steep initial reduction (>50% reduction by week 8) that was followed by a persistent continuing decline over several months. The extent and kinetics of the nodal responses were similar in patients treated with single-agent GS-1101 or with combination therapies. A significant percentage of patients showed durable responses (approximately 25% overall response rate) with single-agent GS-1101 and remained on study for many cycles of treatment. GS-1101-based combinations substantially increased the overall response rate to 81% compared to single-agent therapy. The median progression-free survival in patients on GS-1101 monotherapy was >12 months and the median progression-free survival for any combination therapy has not yet been reached but has exceeded 12 months.

The initial lymph node reduction observed in the majority of CLL patients treated with single-agent GS-1101 was accompanied by a transient elevation in circulating lymphocyte counts (Sharman et al., 2011), a phenomenon known as lymphocytosis. This lymphocytosis resolved over several months of continued GS-1101 treatment. This redistribution of CLL cells from the tissues into the blood is likely the result of lymphocytes being released from lymphoid tissue microenvironments or failing to home from the blood into lymph nodes, resulting in the

eventual death of tumor cells that are prevented by GS-1101 from accessing the supportive microenvironment of the lymph nodes. Importantly, the pattern and extent of lymphocytosis in CLL patients were altered by combination therapy. The combination of GS-1101 with rituximab or fludarabine led to a shorter duration of lymphocytosis while the combination with bendamustine largely eliminated the increase in circulating lymphocyte counts (Sharman et al., 2011).

The CLL patients treated with GS-1101 monotherapy and combination therapy also showed significantly reduced plasma levels of the chemokines CXCL13, CCL3, CCL4 and cytokine TNFα after 28 days of treatment compared with pretreatment measurements, further suggesting disruption of the CLL microenvironment by GS-1101 (Hoellenriegel et al., 2011; Sharman et al., 2011). As described earlier, previous studies demonstrated an important role for PI3Kδ in B-cell adhesion, migration, and homing to lymphatic tissues (Reif et al., 2004; Durand et al., 2009). B cells from PI3K p110δ knockout mice respond poorly to CXCL13, and exhibit reduced homing to lymphatic tissues in adoptive transfer experiments (Reif et al., 2004). PI3Kδ activity is also required for chemotactic and adhesive signals that mediate the localization of B cells in lymphoid tissue, as indicated by the ability of *in vitro* IC87114 treatment to inhibit B cell chemotaxis toward CXCL13 and S1P as well as CXCL13-stimulated adhesion to ICAM-1, which is mediated by the LFA-1-integrin (Durand et al., 2009). Consistent with this, treating mice with IC87114, a selective PI3Kδ inhibitor (Sadhu et al., 2003b), disrupted the *in vivo* localization of MZ B cells in mice (Durand et al., 2009). Indeed, the notable clinical activity of GS-1101 in CLL is associated with mobilization of CLL cells from tissues into the blood, which results in pronounced lymph node shrinkage and transient lymphocytosis. Thus GS-1101 displays a dual mechanism of action against CLL, directly decreasing BCR-induced CLL cell survival (see above) and inhibiting chemokine-dependent interactions that retain CLL cells in survival-promoting tissue microenvironments. These combined actions may account for the ability of this PI3Kδ inhibitor to sensitize CLL cells to the current standard-of-care agents. Similar effects have been observed with inhibitors of the Syk, Btk, and mTOR kinases, which also function in both chemokine receptor and BCR signaling pathways (Friedberg et al., 2010; Zent et al., 2010; Burger, 2012; de Rooij et al., 2012). The common mechanism underlying the antitumor activity of these kinase inhibitors that are active against CLL may be their ability to inhibit multifunctional signaling pathways that control leukemia cell survival, adhesion, migration, and homing.

Similar promising results have been obtained in a phase 1 study (de Vos et al., 2011) of GS-1101 as a single agent and in combination with rituximab and/or bendamustine for treatment of patients with relapsing/remitting iNHL (clinical trial identifiers: NCT00710528 and NCT01088048). Data were presented from 63 patients with iNHL on GS-1101 monotherapy and 52 patients on GS-1101-based combination therapies representing all four subtypes of iNHL: follicular, small lymphocytic, lymphoplasmacytic, and MZ, with FL being the most common in all treatment arms. GS-1101 as single agent or as combination therapy caused substantial tumor regression in a large majority of patients with indolent NHL and across all dose levels. The overall response rate for GS-1101 monotherapy was 38% across all dose levels and 59% at doses $\geq$ 100 mg twice daily. GS-1101-based combinations substantially increased the overall response rate to 83% compared to single-agent therapy. Furthermore, complete remission was achieved in 15–25% of patients on GS-1101-based combination therapies. Similar to clinical data in CLL, both GS-1101 monotherapy and combination therapy were associated with durable tumor control with the median progression-free survival in patients receiving GS-1101 monotherapy at doses $\geq$ 100 mg twice daily, receiving any combination therapy, was >12 months.

Consistent with observations in CLL patients, treatment of iNHL patients with single-agent GS-1101 or combinations significantly decreased plasma levels of CCL17, CCL22, CXCL13, and TNFα. However, unlike the biphasic nodal response and transient lymphocytosis observed in CLL patients treated with GS-1101 monotherapy, iNHL patients show profound and rapid reductions in lymph nodes. The lymphocytosis has not been reported in patients with iNHL treated with single-agent GS-1101 or with combination therapies, suggesting that redistribution of cells to the periphery may not be a critical mechanism of antitumor activity of GS-1101 in iNHL. This is also consistent with the observation that iNHL cells are not generally found in circulation; hence, they are likely being cleared in the tissue sites rather than in the periphery.

GS-1101 has also been tested as a single agent treatment in a small number of patients (9–11 each) with MM, AML, and DLBCL malignancies. However, as reported in conference proceeding abstracts, it has not shown the same level of efficacy as for CLL and NHL (Flinn et al., 2009a; Furman et al., 2010b; Kahl et al., 2010). Because the effects of GS-1101 on the redistribution of malignant cells and on plasma chemokine and cytokine levels in these patients has not been reported, it is not known if the lack of efficacy was due to insufficient inhibition of the PI3K pathway or to protective microenvironmental signals in these malignancies. Whether GS-1101 treatment would make these malignant cells more susceptible to other standard-of-care treatments remains to be determined.

An important consideration for therapeutics that target signaling pathways that are essential for the survival and dissemination of malignant lymphocytes is whether they will also suppress beneficial anti-tumor immunity. Several studies have reported that PI3Kδ is required for the function of immune cells that could influence the immune response to tumor cells. GS-1101 strongly suppresses cytokine production by human T cells (Herman et al., 2010). Moreover, inhibition of PI3Kδ may also have detrimental effects on tumor immunosurveillance by NK cells and cytotoxic T lymphocytes (CTL). PI3Kδ activity is important for NK cell activation and function (Kim et al., 2007; Saudemont et al., 2007) and for induction of both the perforin-granzyme and death-receptor pathways of CTL-mediated tumor cell killing (Putz et al., 2012). In addition, Tregs play a key role in restraining immune responses including anti-tumor responses but the net effect of PI3Kδ inhibition on Tregs is still unclear. Loss of PI3Kδ activity promotes the differentiation of T cells into Tregs but can also inhibit Treg function (Patton et al., 2006; Sauer et al., 2008;

Okkenhaug and Fruman, 2010). Thus determining how to balance inhibition of malignant cell survival with maintenance of anti-tumor immunity will be the key to treatment success.

CONCLUSIONS

Recent data has demonstrated that the PI3Kδ pathway is a critical signaling circuit in B cells and plays a key role in autoimmune inflammation as well as some lymphoid malignancies. This pathway is hyperactivated in various B-cell malignancies and its inhibition with PI3Kδ-targeted compound has achieved promising clinical responses in patients with CLL, iNHL, and other B-cell neoplasia. The PI3Kδ inhibitor GS-1101 appears to antagonize both intrinsic and extrinsic cell survival signals, decreases the survival of CLL cells directly, and abrogates cellular interactions between CLL cells and components of the tissue microenvironment that normally sustain leukemia and lymphoma cells in a protective niche. Ongoing clinical studies are further characterizing the risks and benefits of long-term PI3Kδ inhibition. Nevertheless, the initial trials suggest that GS-1101 has significant potential both as a single agent and in combination with bendamustine and rituximab for the treatment of CLL and iNHL (clinical trial identifiers: NCT01569295, NCT01539512, and NCT01539291). Ongoing clinical studies are also evaluating GS-1101 to potentially expand its utility in additional B-cell malignancies such as Hodgkin's lymphoma (clinical trial identifier: NCT01393106).

The PI3Kδ pathway is also constitutively activated in various inflammatory conditions and pharmacological inhibition or genetic inactivation of this pathway has demonstrated efficacy in several preclinical models of inflammation. Clinical studies are now evaluating the potential of PI3Kδ inhibitors for the treatment of a variety of inflammatory diseases (clinical trial identifiers: NCT00836914 and NCT01462617). In addition to B-cell inhibition, PI3Kδ inhibitors are also likely to significantly impact differentiation, migration, and cytokine production by T cells as well as the degranulation of mast cells, suggesting that these agents may be effective beyond autoimmune diseases. As is the case for all immunosuppressive agents, a balance between suppressing autoimmunity and maintaining protective immunity needs to be established. Orally available small molecule inhibitors that have a short *in vivo* half-life would allow this balance to be adjusted in a continual manner.

In conclusion, PI3Kδ plays a major role in both B-cell-mediated inflammation and B-cell malignancies, and PI3Kδ inhibitors therefore represent a promising therapeutic approach to treating these diseases.

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AKTivation of PI3K/AKT/mTOR signaling pathway by KSHV

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As an obligate intracellular parasite, Kaposi sarcoma-associated herpesvirus (KSHV) relies on the host cell machinery to meet its needs for survival, viral replication, production, and dissemination of progeny virions. KSHV is a gammaherpesvirus that is associated with three different malignancies: Kaposi sarcoma (KS), and two B cell lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease. KSHV viral proteins modulate the cellular phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway, which is a ubiquitous pathway that also controls B lymphocyte proliferation and development. We review the mechanisms by which KSHV manipulates the PI3K/AKT/mTOR pathway, with a specific focus on B cells.

Keywords: Akt, KSHV, mTOR, PI3K, B cells

INTRODUCTION

Kaposi sarcoma-associated herpesvirus (KSHV; also known as human herpesvirus 8) is a human gammaherpesvirus that was discovered in Kaposi's sarcoma (KS) biopsies in 1994 (Chang et al., 1994). Following this seminal discovery, KSHV has been found in all forms of KS, including KS associated with AIDS patients, as well as HIV-negative and transplant-associated KS. In addition to KS, which is a vascular endotheliosarcoma, KSHV is also tightly associated with two lymphoproliferative disorders: primary effusion lymphoma (PEL; Cesarman et al., 1995) and the plasmablastic variant of multicentric Castleman's disease (MCD; Gessain et al., 1996), both arising from infection of B cells. Owing to the association with these three cancers, KSHV has been extensively studied, and the results of these studies have revealed fascinating mechanisms by which this oncogenic herpesvirus alters the infected cell in order to promote transformation and tumorigenesis.

B LYMPHOCYTE DEVELOPMENT

B and T cells descend from a common lymphoid progenitor cell, itself derived from a hematopoietic stem cell precursor. In humans, B cell development occurs in the bone marrow, where the earliest progenitor (or pre-pro) B cell expresses germline heavy- and light-chain immunoglobulin genes (Murphy et al., 2008). As the B cell matures, movement along the bone marrow and interaction with stromal cells leads to maturation. D-J gene rearrangement occurs in early pro-B cells, and continues to V-DJ rearrangement in the late pro-B cell. These gene rearrangements create a unique variable domain in the immunoglobulin. Allelic exclusion is enforced by the pre-B cell receptor, whereby only one allele encoding the rearranged heavy chain is expressed, thereby ensuring that each B cell has specificity for a single antigen (Murphy et al., 2008). Many rounds of cell division occur during the transition of pro-B cells to the pre-B cell stage, leading to the formation of numerous small pre-B cells with a specific rearranged μ heavy-chain gene. Pre-B cells undergo light-chain gene rearrangement, which is also accompanied by allelic exclusion. Since these pre-B cells now produce both heavy- and light-chain proteins, they are classified as immature B cells, and bear intact IgM molecules on their cell surface (Murphy et al., 2008). For a review describing normal B cell development, please see Montecino-Rodriguez and Dorshkind (2012).

In addition to allelic exclusion, isotype exclusion also occurs in immature B cells, wherein the immature B cell expresses only one light chain (either λ or κ; Murphy et al., 2008). In humans, because the κ gene rearranges prior to the λ gene, many more mature B cells express the κ light chain rather than λ. The average distribution of κ to λ-bearing B cells in humans is approximately 65:35%, and aberration from this ratio is indicative of lymphoproliferative disorders, reflecting dominance of one clone (Murphy et al., 2008).

PATHOPHYSIOLOGY OF KSHV-ASSOCIATED B CELL MALIGNANCIES

PRIMARY EFFUSION LYMPHOMA

Primary effusion lymphoma mainly afflicts HIV-infected patients, and occurs in body cavities such as the peritoneal, pleural, and pericardial cavities (Green et al., 1995; Nador et al., 1996). Some KSHV-positive lymphomas can also present as extranodal solid masses, which may subsequently develop into an effusion. Cells have an immunoblastic appearance with a high mitotic index. KSHV-positive solid lymphomas represent an extracavitary variant of PEL (Arvanitakis et al., 1996). In PEL, every tumor cell expresses between 50 and 150 copies of the KSHV genome. The genome is found as an episome tethered to the host cell chromosome by the virus-encoded latency-associated nuclear antigen (LANA) protein (Ballestas et al., 1999; Cotter and Robertson, 1999; Schwam et al., 2000; Garber et al., 2001). Some PEL are co-infected with Epstein–Barr virus (EBV), another lymphotropic gammaherpesvirus (Cesarman et al., 1996; Nador et al., 1996).

Patients with PEL present with lymphomatous effusions within body cavities, in the absence of a solid tumor mass (Nador et al., 1996; Ambinder and Cesarman, 2007). Cells contained within the effusions are large, with abundant cytoplasm, and display morphological aspects common to both large-cell immunoblastic and anaplastic large cell lymphoma (Nador et al., 1996). Analysis of *Ig* rearrangements suggests that PEL arises from clonal expansion of an infected B cell (Green et al., 1995). PEL express syndecan 1/CD138, which is a plasma cell surface marker, in addition to CD45 (Gaidano et al., 1997).

Although most PEL cell lines do not have translocations and mutations e.g., *c-myc* and *p53*, many PEL possess numerous genetic aberrations (Luan et al., 2010). Sophisticated comparative genome hybridizations (CGH) studies reveal extensive copy number aberrations comprising predominantly of gains and amplifications. Two genes, *SELPLG* and *CORO1C* were found to be the targets of amplification at chromosome 12q24.11. *SELPLG* encodes a membrane-bound glycoprotein that binds to P, E, and L-selectins, and is important for leukocyte recruitment to sites of inflammation (Laszik et al., 1996). *CORO1C* is a member of the coronin gene family that regulates actin-dependent processes such as motility and vesicle trafficking, and whose expression is associated with enhanced invasion and metastatic capability (Roadcap et al., 2008). Deletions of the two fragile site tumor suppressors, *WWOX* and *FHIT*, were also recently reported in PEL (Roy et al., 2011).

MULTICENTRIC CASTLEMAN'S DISEASE

Multicentric Castleman's disease, an atypical lymphoproliferative disorder, is divided into the hyaline vascular type, and the plasmablastic type. KSHV is associated with the plasma cell variant of MCD, which is multicentric in that several lymph nodes and the spleen are involved in disease. In the context of HIV infection, MCD is systemic, aggressive, and is associated with high fatality. KSHV genomes are detected in almost all HIV+ MCD cases, and ∼50% of non-HIV+ cases of MCD (Soulier et al., 1995; Dupin et al., 2000). AIDS patients diagnosed with MCD suffer sustained fevers, weight loss, lymphadenopathy, and hepatosplenomegaly (Du et al., 2001). MCD frequently progresses to lymphoma or KS.

Multicentric Castleman's disease is localized to the marginal zone of lymph nodes and the spleen. The germinal centers resemble follicular hyperplasia, and the mantle zone is generally intact and surrounded by mature KSHV-infected plasmablasts (Dupin et al., 1999; Katano et al., 2000; Parravicini et al., 2000). MCD cells resemble plasmablast or pre-plasma cells (Jenner et al., 2003). All KSHV-infected plasmablasts within the lesion exclusively express the λ light chain of IgM (Du et al., 2001), and the presence of λ light chains and absence of CD138 on MCD cells further suggests they originate from the infection of a less differentiated B cell (Hassman et al., 2011). Lymph nodes involved in MCD are characterized by germinal center expansion and vascular endothelial proliferation. MCD is characterized by elevated serum interleukin (IL)-6 levels in the patient (Yoshizaki et al., 1989). These elevated IL-6 levels, partially augmented by virally encoded IL-6 (vIL-6), create an inflammatory microenvironment which significantly contributes to the pathophysiology of MCD.

KSHV ALTERS NORMAL B CELL PROLIFERATION AND DIFFERENTIATION, LEADING TO LYMPHOPROLIFERATIVE DISORDERS

Both MCD and PEL are associated with infection of a preterminally differentiated plasma cell. Gene expression arrays indicate that PEL have a plasma cell expression profile, and enhanced expression of genes involved in inflammation, adhesion, and invasion (Jenner et al., 2003), likely contributing to their malignant phenotype. MCD is characterized by the polyclonal expansion of KSHV-infected plasmablasts that exclusively express the λ light chain. No functional significance exists in whether a plasmablast bears either λ or κ, as the isotype exclusion is purely a function of order of light chain gene rearrangement.

It was unknown whether KSHV preferentially infected λ light-chain bearing B cells due to an inherent, yet unknown survival advantage to the virus/infected cell, or whether KSHV infection of a more undifferentiated cell (prior to light chain rearrangement) drove the expansion of λ-expressing B cells.

Hassman et al. (2011) attempted to address this question in a recent study in which they infected *ex vivo* suspensions of human tonsillar cells with purified KSHV. Despite the presence of various cell types, KSHV infection was shown to preferentially occur in B cells, as evidenced by LANA+ staining. The tonsillar cultures contained both κ- and λ-expressing B cells, however, all LANA+ staining was observed within the λ subset. Furthermore, infection with KSHV enhanced the proliferation of this IgMλ subset, which was further augmented by treatment with IL-6. KSHV-positive cells mirrored phenotypic characteristics of MCD cells, such as blasting morphology and increased expression of IgMλ, CD27, Ki67, and IL-6R.

This study also suggested that rather than naïve B cells, KSHV preferentially infects IgM memory B cells, resident within subepithelial regions of the tonsil and spleen. During plasmablast differentiation, IgM memory B cells acquire phenotypic markers such as Ki67+, IgM+, and CD27+, similar to MCD. This model is concordant with molecular and seroepidemiological data suggesting that the primary mode of KSHV transmission is via saliva (Vieira et al., 1997).

Plasmablast differentiation, including that of IgM memory B cells, can result from NF-κB activation, a signaling event that occurs during KSHV infection, and also during latency. The viral FADD-like IL-1β-converting enzyme (FLICE/caspase-8) inhibitory protein (vFLIP) activates NF-κB signaling in latently infected cells by associating with the IκB kinase (IKK) complex, and driving degradation of IκBα, and thus, NF-κB translocation into the nucleus. A consequence of NF-κB signaling is the promotion of cell survival and proliferation pathways. Ballon et al. (2011) constructed an inducible CD19- or Cγ1-driven conditional vFLIP knock-in mouse, which targeted vFLIP expression to various stages of B cell development. vFLIP expression in B cells was found to prevent germinal center development, Ig class switching, and affinity maturation; vFLIP expression resulted in splenomegaly in the mice. Although transgenic mice did not recapitulate phenotypes of PEL, B cells mimicked an MCD phenotype, with expansion of IgM λ plasmablasts, concordant with the findings of Hassman et al. (2011), indicating that vFLIP leads to expansion of the IgMλ subset upon KSHV infection. Moreover, impaired GC formation

and class switch recombination resulting from vFLIP expression suggests that inhibition of the adaptive response is a means of escaping immune surveillance.

Interestingly, 20-month old vFLIP-expressing mice developed B cell-derived histiocytic/dendritic cell (DC) sarcoma. This finding is important as it underscores the ability of vFLIP to either reprogram or transdifferentiate the infected lymphocytes or the bystander cells in a paracrine manner, into other cell types. These findings also demonstrate the inherent plasticity of B lymphocytes (Cobaleda and Busslinger, 2008).

The collective body of research suggests that KSHV viral oncogenesis is mediated by expression of both latent and lytic viral gene products. Viral latent proteins are expressed in every tumor cell, whereas lytic proteins are expressed in a small percentage of tumor cells undergoing reactivation, and are thought to promote cell proliferation in an autocrine or paracrine manner. KSHV can infect a wide range of cell types *in vitro* and *in vivo* including monocytes, plasmacytoid DCs, fibroblasts, keratinocytes, B lymphocytes, endothelial, and epithelial cells (Kliche et al., 1998; Renne et al., 1998; Lagunoff et al., 2002; Akula et al., 2003; Inoue et al., 2003; Krishnan et al., 2004; Naranatt et al., 2004; Kaleeba and Berger, 2006a,b; Rappocciolo et al., 2006, 2008; Jarousse et al., 2008; Greene and Gao, 2009; Hassman et al., 2011; West et al., 2011; West and Damania, 2008). To guarantee successful replication within these distinct cell types, KSHV encodes an arsenal of viral proteins that are capable of modifying the host cell environment, either directly or indirectly, with the outcome being beneficial for the virus. Modulation of host cell pathways includes evasion of both immunity as well as apoptosis, induction of cell proliferation, and the promotion of cellular metabolism, macromolecular synthesis, and protein translation. One way KSHV accomplishes these alterations is by targeting the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway. Similar to KSHV, many DNA viruses encode one or more viral proteins that either activate or inactivate various nodes of this pathway (Buchkovich et al., 2008).

KSHV LIFE CYCLE

Kaposi sarcoma-associated herpesvirus establishes lifelong latency within the host, punctuated with sporadic bouts of reactivation and lytic replication. During latency, the KSHV genome exists as a circular, extra-chromosomal viral genome (episome), with minimal expression of a subset of latent viral proteins: K12/Kaposin, K13/vFLIP, ORF72/vCyclin, ORF73/LANA, vIL-6, and K1 (Wen and Damania, 2010b). In contrast, during the lytic replication phase, most of the viral genome is expressed and viral replication is followed by viral assembly, egress, and dissemination. The lytic switch protein replication and transcription activator (RTA) governs KSHV reactivation (Sun et al., 1998; Lukac et al., 1999). Chemicals such as phorbol esters, sodium butyrate, and histone deacetylase inhibitors reactivate the virus by activating the RTA promoter (Yu et al., 1999), and thus are useful for studying the viral life cycle. Hormones (norepinephrine and epinephrine), cytokines such as interferon-γ, oncostatin M, and hepatocyte growth factor reactivate KSHV, as does the hypoxic microenvironment typical of solid tumors and serous cavities, thus stimulating the expression of viral proteins that are beneficial to the host cell (Chang et al., 2000; Mercader et al., 2000). Terminal differentiation of B cells, resulting from expression of X-box binding protein 1 (XBP-1) can also activate the RTA promoter, inextricably linking virion production to the host cell life cycle (Wilson et al., 2007; Yu et al., 2007). Furthermore, stimulation of Toll-like receptors (TLRs) 7 and 8 by microbes can also reactivate KSHV from latently infected cells (Gregory et al., 2009); stimulation of these pattern recognition receptors generates an anti-viral state, and expressed lytic proteins have many functions which antagonize the host immune system.

Kaposi sarcoma-associated herpesvirus reactivation from latency significantly alters the physiology of the infected cell. Viral replication can reveal viral nucleic acid or peptide motifs that can activate host immune surveillance pathways. Viral replication also increases the demand for macromolecules such as nucleotides and amino acids to synthesize progeny virions. Cellular energy pools are substantially depleted in order to fuel the increased biosynthetic rates associated with viral replication. The combined effect of enhanced biosynthetic rates and unfulfilled energy demands is the activation of cellular stress responses, resulting in cell cycle arrest or apoptosis, in an attempt to resolve these deficits. To circumvent activation of stress responses, lytic proteins efficiently block cell death pathways, and maintain the host cell in a constant state of proliferation. Moreover, many lytic and latent proteins co-opt cellular signaling pathways, which sustain proliferation, block cell death, and enhance cellular metabolism, in order to maintain latent virus or facilitate lytic replication and dissemination of KSHV. One such pathway is the pleiotropic PI3K/AKT/mTOR pathway, which governs many cellular processes.

THE PI3K/AKT/mTOR SIGNALING PATHWAY

The following brief introduction describes the various effectors of PI3K/AKT/mTOR signaling, and relates their activation to distinct physiological outcomes for the cell. While no means exhaustive, this description provides a primer for subsequent sections, which describe KSHV's interaction with this pathway. For recent reviews describing up-to-date targets of PI3K/AKT/mTOR signaling, their regulation, and relevance to malignancies, please refer to Bunney and Katan (2010), Hsieh et al. (2011), Zoncu et al. (2011), and De Luca et al. (2012).

The PI3K are lipid kinases that phosphorylate the 3′-hydroxyl of the inositol ring of phosphoinositide, a component of the interior side of eukaryotic cell membranes (Engelman et al., 2006). Phosphoinositides help form membrane cell signaling complexes and are essential for intracellular trafficking. PI3K are divided into four classes, IA, IB, II, and III, all of which have differing substrate specificities and modes of regulation. Class IA and IB PI3K catalyze the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3′ carbon on the inositol ring into phosphatidylinositol-3,4,5-triphosphate (PIP_3). $PI(3,4,5)P_3$ production by PI3K allows for pleckstrin homology (PH)-domain containing proteins to localize to the plasma membrane (Engelman et al., 2006). PIP_3 also functions as a cellular second messenger capable of controlling cell shape, survival, proliferation, growth, and motility. Class I PI3Ks are heterodimeric proteins comprising a regulatory subunit and a catalytic subunit. The regulatory subunits are p85α, p85β, p55α, p55γ and p50α (Engelman et al., 2006). The catalytic

subunits are comprised of one of four isoforms: p110α, p110β, p110δ and p110γ. Most mammalian tissues widely express p110α, β, and γ catalytic subunits, whereas p110δ is restricted to lymphocytes (Donahue and Fruman, 2004). Phosphatase and tensin homology (PTEN) is a phosphatase that catalyzes dephosphorylation of the 3′ carbon on the inositol of $PI(3,4,5)P_3$ back to PIP_2 (Cantley and Neel, 1999). PTEN is one of the most frequently lost tumor suppressors in various cancers. Mutations or deletions in *PTEN* cause hyperactivation of PI3K signaling, leading to increased cell proliferation as well as evasion of apoptosis. AKT is a PH-domain-containing protein that plays a central role in varied cellular processes such as glucose metabolism, evasion of apoptosis, and promotion of cell proliferation, transcription and cell migration (Manning and Cantley, 2007). AKT can also stimulate protein synthesis via activation of mTOR (discussed below; Hsieh et al., 2011). Once AKT is localized to the cell membrane through its PH-domain, it is phosphorylated on Threonine^{308} by phosphoinositide-dependent kinase 1 (PDK1), and serine^{473} through the mTORC2 complex (Lawlor and Alessi, 2001), both of which are activating modifications. AKT has several different effectors, which control distinct biological processes, thus, AKT activation has pleiotropic effects upon the cell. AKT inhibits cell death by phosphorylation-mediated inactivation of pro-apoptotic factors Bad, Caspase-9, and the FOXO group of transcription factors (Cross et al., 1995; Datta et al., 1997; del Peso et al., 1997; Cardone et al., 1998). Phosphorylation of the FOXO transcription factors sequesters them within the cytoplasm, thus preventing them from transcriptionally activating target pro-apoptotic genes such as Fas ligand and Bim.

AKT regulates the cell cycle by phosphorylating and inactivating key regulators of cell cycle progression. For instance, AKT promotes the transcriptional activation of *c-Myc* and *cyclin D1* genes (Gera et al., 2004), and interacts with two regulators of the cell cycle, p27 and p21 (Zhou et al., 2001; Shin et al., 2002). Maintaining a quiescent state requires high intracellular p27 levels, and PI3K activation was shown to reduce the cellular reserves of p27 (Sun et al., 1999). AKT phosphorylates the p53-induced protein p21, a negative regulator of cell cycle progression (Zhou et al., 2001). The net result of these inhibitory phosphorylation events is the maintenance of cell cycle progression and the de-regulation of cellular checkpoint signaling.

AKT also activates the non-canonical branch of the NF-κB family of transcription factors. NF-κB regulates many aspects of innate and adaptive immunity and cell survival. NF-κB is normally sequestered in the cytoplasm by inhibitory proteins such as inhibitor of κB (IκBα), which is degraded following phosphorylation by the upstream IKK complex, comprised of IKKα and IKKβ. IκBα is a direct target of IKKβ, that when degraded, releases NF-κB p65 thus activating genes involved in innate immune responses IKKα activates non-canonical NF-κB, leading to formation of p52 which activates genes involved in adaptive immunity (Verma et al., 1995; Ghosh et al., 1998; Karin and Ben-Neriah, 2000), and AKT regulates non-canonical NF-κB p52 processing by increasing the activity of IKKα (Gustin et al., 2006). Studies using constitutively active AKT demonstrated elevated p52 transcriptional activity. Non-canonical NF-κB activity was severely inhibited by using PI3K inhibitors, kinase-dead AKT or cells lacking AKT isoforms 1 or 2 (Gustin et al., 2006; Comb et al., 2012). Thus, AKT modulates both cell survival and regulation of adaptive immune responses. de Oliveira et al. (2010) provide an excellent review of the role of NF-κB signaling with regard to KSHV infection.

The serine/threonine kinase mTOR is a downstream target of PI3K/AKT signaling. In addition to being activated by essential signaling pathways such as PI3K and MAPK, mTOR is activated by a wide range of cellular stimuli such as growth factors, stress signals, and nutrient, energy, and amino acid abundance. mTOR activity is negatively regulated by the tuberous sclerosis complex (TSC), comprised of TSC1 and TSC2. TSC2 is an AKT target, and when phosphorylated, inhibits Rheb, also a negative regulator of mTOR (Manning and Cantley, 2007). mTOR exists in two distinct multiprotein signaling complexes, mTORC1 and mTORC2, which have differing sensitivities to the macrolide rapamycin; mTORC1 is sensitive, whereas mTORC2 is insensitive. mTORC1 regulates protein translation, cell size regulation, intracellular transport, metabolism, and lipid biogenesis. mTORC1 phosphorylates S6K1 and 4EBP1, which are two proteins critical for translation of eukaryotic capped mRNAs. S6K1 phosphorylates the S6 ribosome, thus stimulating protein synthesis. Unphosphorylated 4EBP1 tightly binds and represses the eukaryotic initiation factor 4E (eIF4E); hyperphosphorylated 4EBP1 releases eIF4E, thereby enabling cap-dependent translation (Gingras et al., 1999). mTOR has a wide plethora of other targets, for example ULK1 which regulates autophagy (Zoncu et al., 2011). Because protein translation is central to both cancer growth and viral persistence, mTOR is a very important signaling protein. The rapamycin-insensitive mTORC2 complex regulates cell survival and cytoskeleton dynamics. mTORC2 activates AKT, thereby paradoxically activating AKT/mTOR signaling even upon rapamycin treatment, demonstrating a feedback activation loop (Sarbassov et al., 2005).

Solid tumors are characterized by hypoxic microenvironments, therefore, *de novo* angiogenesis as well as remodeling existing blood vessels is essential to provide the rapidly growing cells with nutrients and oxygen. The viscosity and shear forces of blood against the walls of blood vessels govern the enzymatic activity of endothelial cell-expressed NOS (eNOS), and consequently, the continual synthesis and release of nitric oxide (NO). In endothelial cells, AKT is activated in a PI3K-dependent manner, both by shear forces and VEGF (vascular endothelial growth factor), which collectively activate eNOS (Dimmeler et al., 1999). NO has pleiotropic functions ranging from angiogenesis, remodeling of the vasculature, and the control of blood vessel tone (Thomas et al., 2008).

The AKT/mTOR axis is a critical regulator of cellular metabolism. Activated AKT stimulates glucose uptake by relocalizing the GLUT1 glucose transporter, thus bringing glucose into the cell for fueling various cellular processes (Wieman et al., 2007). Activation of the mTORC1 complex promotes glycolytic flux, up-regulates the pentose phosphate pathway, and stimulates *de novo* lipogenesis, all of which contribute to metabolic reprogramming essential for rapidly dividing cancer cells (Duvel et al., 2010).

As mentioned above, hyperactivation of PI3K/AKT/mTOR signaling is a characteristic of many malignancies (Kodaki et al., 1994;

Samuels et al., 2004). Deregulated signaling may result from inactivation of negative regulator phosphatases, e.g., TSC2 or PTEN, or from mutations in catalytic domains of kinases, e.g., PIK3CA. Although KSHV-infected PEL cells are not known to have activating mutations in any of these three kinases, PI3K/AKT/mTOR signaling is highly up-regulated in these cells (Sin et al., 2007; Bhatt et al., 2010). As we will discuss further, a variety of viral proteins can activate this signaling pathway.

Proper PI3K/AKT/mTOR signaling is essential for the differentiation and developmental program of normal T and B lymphocytes, as well as other immune cells. PI3K is downstream of numerous cytokine receptors – CD40, TLRs, and the BCR itself. The current body of research suggests that PI3K signaling regulates the development of bone marrow B cell precursors, as well as the differentiation and development of B cell subsets (Hodson and Turner, 2009; Srinivasan et al., 2009; Beer-Hammer et al., 2010). Moreover, PI3K signaling also governs many aspects of activation and proliferation of mature B cells. For an excellent review describing the PI3K pathway with regard to B cell development, please see Donahue and Fruman (2004).

KSHV ACTIVATES PI3K SIGNALING DURING *DE NOVO* INFECTION

Kaposi sarcoma-associated herpesvirus activates the PI3K signaling pathway during viral infection. The widely expressed proteoglycan heparan sulfate, α3β1 integrins, DC-SIGN, and xCT are the primary receptors for KSHV, and their differential distribution in various cell types contributes to the wide tropism of KSHV (reviewed in Chandran, 2010). KSHV enters target cells by endocytosis. Viral entry activates many cellular signaling pathways, which does not require active viral replication, as demonstrated by studies using UV-inactivated KSHV (Chandran, 2010). Viral ligation of cell-surface integrins triggers the phosphorylation and activation of focal adhesion kinase (FAK) in fibroblasts (Krishnan et al., 2006). FAK further activates downstream signaling molecules including Src, Rho GTPases, Diaphanous 2, and PI3K (Naranatt et al., 2003; Sharma-Walia et al., 2004, 2005; Veettil et al., 2006; Raghu et al., 2007). In turn, these molecules further activate their own downstream effectors such as Ezrin, protein kinase C (PKC), MEK, NF-κB, ERK-1/2 and p38 MAPKs, and AKT.

Integrin-mediated tyrosine phosphorylation of FAK occurs minutes after KSHV infection of fibroblasts (Chandran, 2010). Reduced viral genomes and gene expression are observed in cells lacking either FAK or Pyk2, a FAK family member, illustrating their essential role in viral infection of target cells (Naranatt et al., 2003; Krishnan et al., 2006). FAK and Pyk2 signaling converge onto the Src kinase family, whose downstream effectors are PI3K and Rho GTPases. FAK, Src, and PI3K are also phosphorylated following infection of THP-1 monocytes, as are NF-κB and ERK-1/2 (Kerur et al., 2010). PI3K activation is crucial for *de novo* infection due to its activation of various GTPases involved in actin cytoskeleton remodeling, endosome formation, and vesicle trafficking. These intracellular processes allow for viral entry and delivery into the nuclear compartment. PI3K and Rho GTPase activation collectively induces other Rho GTPase family members, which precipitate the formation of subcellular structures such as lamellipodia (through Rac), stress fibers (through RhoA), and filopodia (through Cdc42; Krishnan et al., 2006). Further, activation of the PI3K target, AKT, leads to the inhibition of pro-apoptotic factors, thus protecting KSHV-infected cells from cell death.

Activation of cellular signaling is imperative for successful viral infection. The signaling nodes described above activate the processes of vesicle formation, intracellular motility, and evasion of cell death. Furthermore, transcription factors activated by these signaling pathways also play a role in activation of the viral transcription program, as well as induction of cellular proteins that facilitate viral replication. Thus, the collective activation of these intracellular signaling pathways creates an environment benefiting the KSHV life cycle.

KSHV VIRAL PROTEINS THAT ACTIVATE PI3K/AKT/mTOR SIGNALING

Currently, four of the approximately 100 genes and microRNAs encoded by KSHV are known to impinge upon the PI3K/AKT/mTOR signaling pathway. They are K1, viral G protein-coupled receptor (vGPCR), vIL-6, and ORF45. We will discuss the mechanisms, extent, context and physiological relevance of each of these proteins below.

K1

K1 is the first ORF encoded by KSHV, and is located at the extreme left end of the viral genome. K1 is a transmembrane glycoprotein whose expression in rodent fibroblasts induces morphological changes and the ability to grow in foci, indicating K1's transformation capacity (Lee et al., 1998). Further, infection of T lymphocytes with a recombinant herpesvirus Saimiri expressing K1 instead of the oncoprotein, Saimiri transforming protein (STP), conferred IL-2 independent growth, suggesting that K1 is also an oncoprotein (Lee et al., 1998). All KSHV-associated tumors express low levels of K1 transcript, and K1 expression is highly up-regulated early during lytic replication (Lagunoff and Ganem, 1997; Jenner et al., 2001; Lee et al., 2003; Wang et al., 2006; Chandriani et al., 2010). K1 transgenic mice display constitutively active NF-κB and Oct-2 transcription factors, increase in expression of basic fibroblast growth factor (bFGF), as well as up-regulated expression and activity of the Lyn tyrosine kinase (Prakash et al., 2002). A physiological consequence of K1-mediated alteration of the cellular transcription program is the development of tumors similar to spindle-cell sarcomatoid tumor and malignant plasmablastic lymphoma (Prakash et al., 2002).

Various studies describe the extent to which K1 also deregulates normal cellular signaling (**Figure 1**). The regulatory p85 subunit modulates PI3K activity, and tyrosine-phosphorylation of p85 results in activation of PI3K (Cuevas et al., 2001). K1 expression leads to increased tyrosine phosphorylation of p85, in addition to phosphorylation of Vav and Syk, thus activating signaling networks downstream of these kinases, which have pleiotropic effects on the cell (Lagunoff et al., 1999; Lee et al., 2003; Tomlinson and Damania, 2004). Further, activation of transcription factors downstream of these kinases, for example, NFAT, downstream of Syk signaling, further augments deregulation of cellular signaling and promotes cell survival.

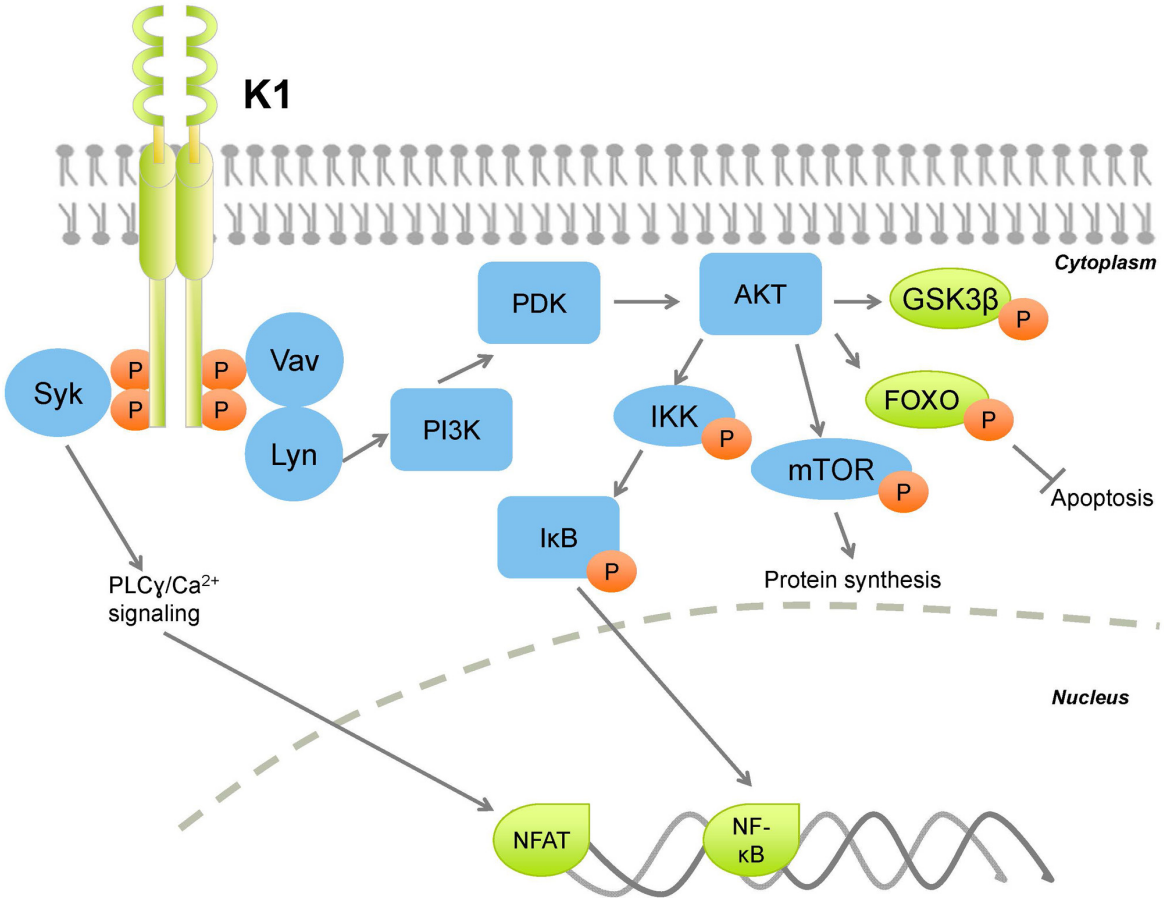

FIGURE 1 | Kaposi sarcoma-associated herpesvirus K1 activates PI3K/AKT/mTOR signaling thereby activating protein synthesis and survival pathways, while inhibiting apoptotic pathways. Orange circles denote phosphorylation.

In B lymphocytes, ectopic K1 expression was found to activate AKT signaling in two simultaneous ways: K1 expression induced AKT phosphorylation on Thr^{308} and Ser^{473}, and also inactivation of the negative regulator PTEN (Tomlinson and Damania, 2004). K1-mediated AKT activation induced the cytoplasmic sequestration of the FOXO family of transcription factors, and subsequent reduction of Fas ligand expression, thus conferring a cell survival advantage to K1-expressing cells (Tomlinson and Damania, 2004). K1 also stabilizes AKT through interaction with the cellular chaperones heat shock protein 90 β (Hsp90β) and the endoplasmic reticulum-associated Hsp40 (Erdj3/DnaJB11), (Wen and Damania, 2010a), both of which are important for enhancing the signaling function of AKT (Sato et al., 2000; Gao et al., 2003). Chaperone-mediated stabilization of AKT by K1 is essential for sustained signaling, as their inhibition induced caspase-3-dependent apoptosis in FasL-treated, K1-expressing cells (Wen and Damania, 2010a).

K1's cytoplasmic tail contains an immunoreceptor tyrosine-based activation motif (ITAM; Lagunoff and Ganem, 1997; Lee et al., 2003). ITAMs are essential for signal transduction in immune cells, therefore are found on immunoreceptors, for example, CD79α and β, subunits of the B cell receptor complex. Upon ligand binding, the tyrosine residues on ITAMs are phosphorylated, which allow for docking of SH2 domain-containing molecules (**Figure 1**). Downstream transduction of the extracellular signal induces calcium mobilization from the endoplasmic reticulum, and activates the lymphocyte. K1 does not require ligand binding to induce signaling, and functions as a constitutively active receptor (Asmuth et al., 2003). The K1 ITAM is closely conserved across KSHV strains, indicating the importance of this motif for K1 function (Zong et al., 1999, 2002). The constitutive activity of the K1 ITAM activates a variety of downstream signaling pathways that not only protect the infected cell, but also neighboring cells in a paracrine fashion. Notably, K1 also activates PI3K/AKT/mTOR signaling in endothelial cells (Wang et al., 2004, 2006). Components of the K1 signalosome have been identified, and indicate that the K1 ITAM interacts with a diverse set of cellular signaling proteins (Lee et al., 2005). Overall, K1 interactions with cellular proteins augments global cellular signal transduction, activation of transcription facts such as NF-κB and AP-1, and induction of inflammatory cytokines (Lee et al., 2005).

Interactions of the K1 N-terminal domain with the BCR complex induces BCR sequestration within the endoplasmic reticulum (Lee et al., 2000). Because normal BCR signaling can potentially

induce apoptosis, BCR sequestration preempts this possibility, thus conferring a long-term survival advantage to the infected cell.

K1 expression is up-regulated during viral reactivation from latency. Lytic replication may induce pro-apoptotic signals resulting from immune detection of replicating KSHV. Viral replication also places increased demands for energy and nutrients on the cell (Munger et al., 2006), and induces a stress response that can activate apoptosis. These collective pro-apoptotic signals can be subverted by K1 expression (Tomlinson and Damania, 2004; Wen and Damania, 2010a), thereby supporting productive lytic replication and further dissemination of KSHV. Moreover, PI3K activation can also re-start protein translation and metabolic programs, halted as a result of apoptotic signals, ensuring that raw materials for production of progeny virions are plentiful.

KSHV vGPCR

Kaposi sarcoma-associated herpesvirus ORF74 encodes a vGPCR, a seven-pass transmembrane protein homologous to the cellular IL-8 chemokine receptor (Cesarman et al., 1996). vGPCR is a ligand-independent receptor expressed during the lytic cycle. Genes expressed during lytic replication are potently transforming, as they exert strong survival signals to prolong the life of the host cell, which ultimately dies as a result of viral replication and associated cellular stress. vGPCR has transforming properties; it promotes focus formation in mouse NIH3T3 cells, and vGPCR-expressing cells form tumors in nude mice (Bais et al., 1998). Human umbilical vein endothelial cells (HUVECs) are immortalized by vGPCR expression, and also protected from apoptosis induced by serum-starvation (Montaner et al., 2001). In various mouse models, vGPCR expression leads to formation of vascular tumors and KS-like angioproliferative lesions, with cell surface markers and circulating cytokine profiles resembling KS (Yang et al., 2000; Guo et al., 2003; Montaner et al., 2003).

Being a constitutively active receptor protein, vGPCR can function without the need for ligand binding (Rosenkilde et al., 1999). However, vGPCR is capable of binding members of both CXC and CC chemokine families (Sodhi et al., 2004). Some CXC chemokines, such as GRO-α and IL-8, enhance vGPCR signaling (Rosenkilde et al., 2000), whereas interferon γ-inducible 10-kDa protein (IP-10/CXCL10) and stromal cell-derived factor 1 α (SDF-1α) inhibit vGPCR signaling (Gershengorn et al., 1998). IL-8 is a major mediator of inflammation, and recruits neutrophils, basophils, and T cells. IL-8 released during the immune response to lytic KSHV replication may enhance the function of vGPCR in lytically infected cells, thereby inducing an anti-apoptotic signal to delay the death of the infected cell. More puzzling is why SDF-1/CXCL12, a stimulator of B cell progenitor proliferation, inhibits vGPCR activity (Gershengorn et al., 1998). However, since vGPCR is primarily expressed during the lytic cycle, this might not have a consequence for latently infected B cells.

KSHV vGPCR activates a plethora of cellular signaling molecules as well as transcription factors, by means of which it promotes transformation in endothelial, epithelial, and fibroblast cells (**Figure 2**). vGPCR activates pathways such as PLC/PKC, Pyk2/Lyn, ERK, p38, and JNK, downstream of which are transcription factors that control many growth- and angiogenesis-promoting genes. For example, HIF-1α activation resulting from vGPCR-dependent p38 and MAPK signaling activates the VEGF promoter (Sodhi et al., 2000). vGPCR also activates AP-1, NF-AT, and NF-κB transcription factors, which in turn promote expression of a panoply of pro-inflammatory cytokines, growth factors, and adhesion molecules (Couty et al., 2001; Montaner et al., 2001; Schwarz and Murphy, 2001; Pati et al., 2003). The vGPCR-mediated secretion of such a wide array of factors may enhance proliferation and survival in neighboring, uninfected cells in a paracrine manner (**Figure 3**). Indeed, a recent report demonstrates that cytokines secreted by a small number of vGPCR-positive tumor cells activate signaling pathways in neighboring cells, converging on mTOR-dependent VEGF up-regulation (Jham et al., 2011). Inhibiting paracrine mTOR activity in non-expressing cells abrogated the tumor-promoting activities of vGPCR-expressing cells *in vivo*. On the other hand, vGPCR expression may directly induce transformation of the cell, due to up-regulated signaling resulting from the same secreted factors. These two methods of transformation may act in concert, rather than in isolation, leading to transformation of both the infected and bystander cells.

PI3K/AKT/mTOR signaling is a common pathway downstream of many growth factor and cytokine receptors. In particular, the tissue-restricted γ isoform of PI3K is crucial for relaying vGPCR signaling downstream to AKT/mTOR in endothelial cells (Martin et al., 2011). Paracrine secretions resulting from vGPCR expression activate PI3K/AKT/mTOR signaling (e.g., VEGF), and moreover, vGPCR directly activates AKT in a PI3K-dependent manner (Montaner et al., 2001).

vGPCR expression in the B cell neoplasms, PEL and MCD, exhibits a distinct gene expression profile compared to endothelial cells (Polson et al., 2002), and is also characterized by elevated PI3K/AKT and ERK/p38 MAPK signaling. Ectopic vGPCR expression in B cells activates several transcription factors: AP-1, CREB, NF-AT, and NF-κB, thereby promoting cell survival, although the mechanisms of activation of these transcription factors differ (Cannon et al., 2003).

As mentioned above, although capable of constitutive activity, vGPCR can also signal by coupling with cellular $G\alpha_q$ and $G\alpha_i$ subunits (Cannon and Cesarman, 2004), further amplifying PI3K/AKT signaling, as both $G\alpha_q$ and $G\alpha_i$ subunits signal through this pathway (Murga et al., 1998). Additionally, vGPCR-mediated activation of AP-1 and CREB (but not NF-κB and NFAT) in B cells was found to be dependent on PI3K/AKT (Cannon and Cesarman, 2004). vGPCR also activates endogenous Lyn tyrosine kinase in a $G\alpha_i$- and PI3K-dependent manner. Pharmacologic and genetic ablation of Src family kinases abolished AP-1 and CREB transcriptional activity, confirming that these transcription factors are activated by vGPCR through a $G\alpha_i$-PI3K/AKT-Src signaling axis. Further, this study showed that Src inhibitors decreased AKT phosphorylation in PEL, indicating that in B cells, Src may be upstream rather than downstream of PI3K/AKT signaling, suggestive of a positive feedback loop. Importantly, this study revealed that NF-κB and NF-AT are not activated in a PI3K/AKT-dependent manner in B cells (Cannon and Cesarman, 2004). Subsequent studies indicated that the Ras-related small G protein Rac1 may be required for NF-κB activation *via* vGPCR (Montaner et al., 2004).

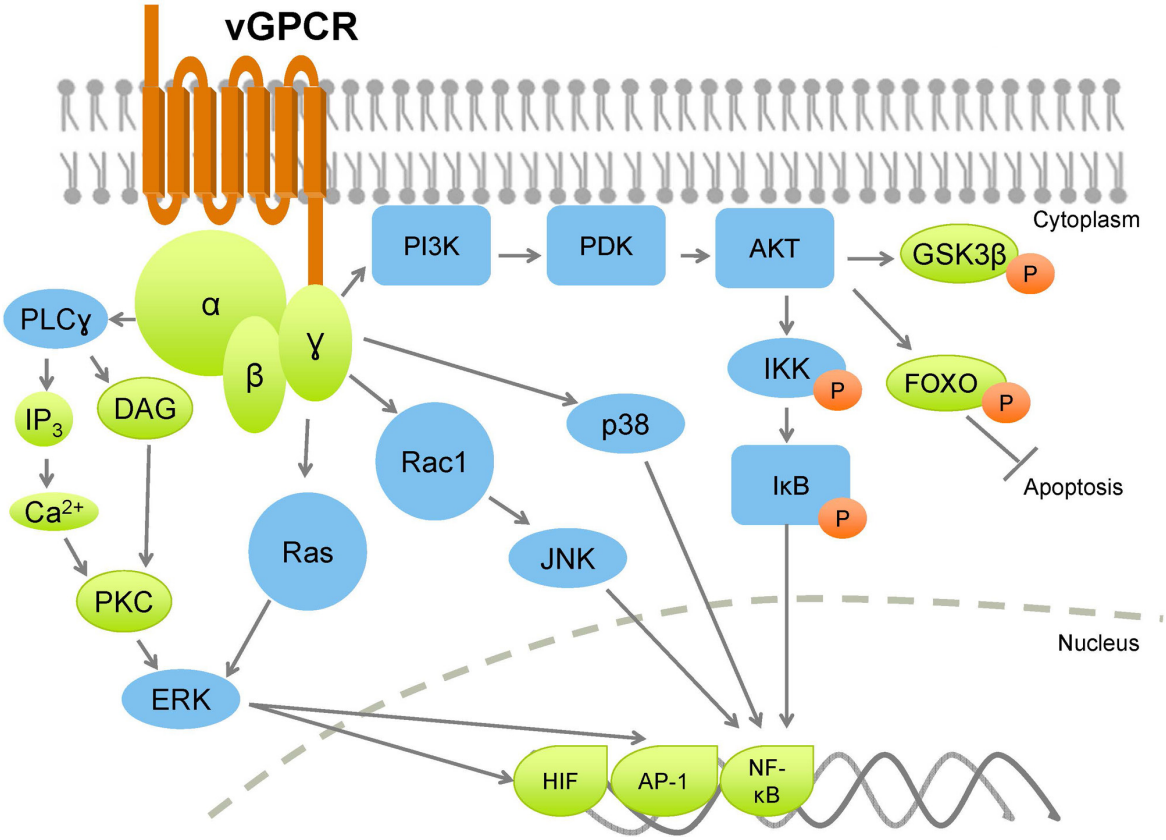

FIGURE 2 | Kaposi sarcoma-associated herpesvirus vGPCR broadly activates PI3K and MAPK pathways, leading to increased production of cytokines and growth factors, with a concurrent increase in cell proliferation, and inhibition of apoptotic pathways. Orange circles denote phosphorylation.

The previous paragraphs describe the extent to which vGPCR activates cellular signaling. The lytically replicating, vGPCR-expressing cell activates transcription factors and signaling entities within the infected cell; induction of secreted factors further amplifies signaling in neighboring cells, with the collective outcome of enhanced proliferation and sustained survival (**Figure 3**).

VIRAL IL-6

Viral IL-6, encoded by ORF-K2, is a homolog of the human IL-6 (hIL-6) cytokine, with 24.8% amino acid sequence similarity and 49.7% sequence identity (Moore et al., 1996). Functionally, vIL-6 is a faithful mimic of hIL-6, as vIL-6 secreted by KSHV-infected B lymphocytes supports proliferation of B lymphocytes and also hIL-6-dependent mouse myeloma cell lines, demonstrating its functional similarity (Moore et al., 1996; Neipel et al., 1997; Nicholas et al., 1997). Most latently infected cells express low levels of vIL-6, with up-regulated expression during lytic replication (Cannon et al., 1999; Staskus et al., 1999; Parravicini et al., 2000; Chandriani et al., 2010). Similar to its cellular counterpart, vIL-6 signaling activates the JAK/STAT, MAPK, and H7-sensitive pathways (Osborne et al., 1999). Although vIL-6 and hIL-6 have similar sequence and function, their receptor usage is substantially different. Cellular IL-6 requires two IL-6 receptor subunits: IL-6Rα and gp130. However, vIL-6 is capable of signaling via only the gp130 subunit (Molden et al., 1997). Thus, KSHV vIL-6 circumvents the requirement for a second receptor, thereby subverting cellular checks against uncontrolled, exuberant signaling.

vIL-6 is implicated as a linchpin in the pathology of all KSHV-associated malignancies, due to its angiogenic properties, as well as potent proliferative and survival effects. vIL-6 is detected, in increasing order, in KS, PEL, and MCD patients (Aoki et al., 2000, 2001). However, only a subset of cells express vIL-6 in KS tissue (Cannon et al., 1999; Staskus et al., 1999; Parravicini et al., 2000), suggesting that like vGPCR, vIL-6 effects are primarily paracrine, activating proliferative signaling pathways in bystander cells. Similarly, in MCD, vIL-6 is expressed in lymphoid cells in mantle zones, and hIL-6 was detected in germinal centers (Cannon et al., 1999; Staskus et al., 1999; Parravicini et al., 2000).

Injection of vIL-6-expressing NIH3T3 cells into athymic mice led to tumor formation, hematopoiesis, and plasmacytosis, all of which were absent in control mice. Tumors derived from vIL-6-expressing cells were highly vascularized, and correlated with elevated secreted VEGF (Aoki and Tosato, 1999). A recent report of transgenic mice constitutively expressing vIL-6 describes elevated serum vIL-6, increased levels of phospho-STAT3 levels in the spleen and lymph nodes, and a manifestation of human MCD-like symptoms (Suthaus et al., 2012). Importantly, when vIL-6 was constitutively expressed in a mouse lacking endogenous hIL-6, no

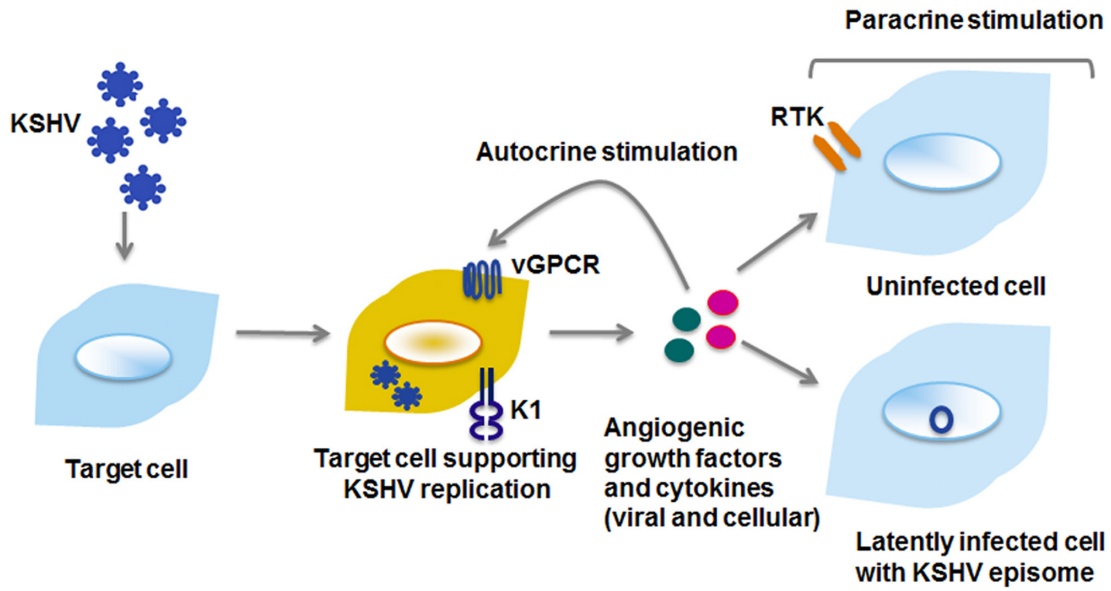

FIGURE 3 | Viral proteins enhance cell proliferation by autocrine and paracrine mechanisms. Viral and cellular cytokines and growth factors can activate signaling pathways within the cell they are secreted from (autocrine), or on distant cells that may either be uninfected or latently infected with KSHV (paracrine). RTK, receptor tyrosine kinase.

MCD-like symptoms were observed. These studies indicate that vIL-6 and hIL-6 cooperate in the pathogenesis of MCD.

Lymphatic reprogramming resulting from KSHV infection of endothelial cells also occurs via engagement of the gp130 receptor (Morris et al., 2008). Specifically, gp130 receptor activation leads to the activation of JAK2/STAT3 and PI3K/AKT pathways. Activated AKT promotes the expression of Prox1, a lymphatic transcription factor necessary for VEGFR-3 induction, and Prox1 itself is known to potentiate the lymphatic reprogramming of endothelial cells upon *de novo* KSHV infection (Hong et al., 2004). Furthermore, STAT3 amplifies this signaling cascade by activating gp130 receptor expression. Podoplanin, another marker of lymphatic reprogramming, is also expressed following KSHV activation of gp130. While not found to be necessary, vIL-6 is sufficient to induce lymphatic reprogramming in endothelial cells infected with KSHV (Morris et al., 2012). Thus, through gp130, vIL-6 can enhance lymphangiogenesis and lymphatic reprogramming in both a paracrine and autocrine manner.

ORF45

ORF45 is an immediate early gene product that plays a crucial role in lytic replication. ORF45 expression is induced upon entry into the lytic cycle and subsequently increases as the life cycle advances, with expression restricted to the cytoplasm. ORF45 is incorporated into the KSHV virion (Zhu and Yuan, 2003), suggesting that it may immediately influence the environment of the *de novo* infected host cell, exemplified by the observation that ORF45 acutely inhibits type 1 IFN induction upon infection. ORF45 mediates the inhibition of innate immune responses by sequestering the cellular transcription factor, interferon regulatory factor-7 (IRF-7), to the cytoplasm (Zhu et al., 2002).

In addition to the modulation of cellular anti-viral responses, ORF45 also exerts an effect upon the cellular signaling *milieu*. Cellular MAPKs are activated during KSHV infection (Sharma-Walia et al., 2005; Xie et al., 2005), and ORF45 interacts with two important MAPK substrates RSK1 and RSK2, which are both p90 ribosomal S6 kinase (RSK) family members. RSK1 and 2 not only phosphorylate ORF45, but their association further augments the kinase activities of these two proteins (Kuang et al., 2008, 2009).

Another consequence of formation of the RSK/ERK/ORF45 complexes is phosphorylation of ribosomal protein S6, and eIF4B, an important member of the complex that recruits ribosomes to 5′ capped mRNAs. Phosphorylated eIF4B complexes with eIF4A, eIF4G, 4E-BP1, and 5′ capped mRNAs to recruit the 40S ribosome, thereby initiating protein translation. Normally, S6 and eIF4B are activated by p70 S6 kinase, itself regulated by upstream mTOR signaling; eIF4B is also a target of the p90 S6 kinase, regulated by MAPK signaling. However, ORF45 allows for eIF4B phosphorylation in an mTOR- and MAPK-independent manner. These observations indicate that protein translation may occur upon KSHV infection in an mTOR-independent manner. They also demonstrate the existence of unique viral strategies to directly enhance protein translation despite a situation within the host cell that may potentially be inhibitory to protein translation.

KSHV-MEDIATED TRANSFORMATION AND THE HALLMARKS OF CANCER

The hallmarks of cancer are a conceptual framework to understand the multistep progression of cancer (Hanahan and Weinberg, 2000, 2011). The hallmarks of cancer take into account that neoplasms, rather than being a singular, isolated entity, are a collection of distinct cell types, comprised of tumor cells, tumor-associated

stroma as well as normal, non-cancerous cells. These diverse cell types cooperate to collectively confer hallmark capabilities, which further enhance the development of a tumor microenvironment. Acquisition of one hallmark by a normal cell facilitates the development of others, thus increasing the likelihood of cellular transformation. The classical hallmarks of cancer are sustained proliferation signaling, evasion of growth suppression, replicative immortality, induction of angiogenesis, evasion of cell death, and invasion and metastasis. Three additional hallmarks have also been recently proposed: deregulation of cellular energetics, tumor-promoting inflammation, and avoidance of immune recognition.

The endpoint of the productive KSHV life cycle is the production of new virions that can subsequently infect new cells, and begin another round of the viral life cycle. To successfully replicate and generate progeny virions, KSHV must evade immune recognition during latency, and also counter antiviral and death-inducing signaling during lytic replication. A plethora of viral proteins successfully grant invisibility from immune recognition, and prolong the life of the infected cell by altering cellular signaling, as described above.

SUSTAINED PROLIFERATIVE SIGNALING

Proliferation is a carefully controlled process in normal cells. Uncontrolled growth would lead to nutrient scarcity as well as overgrowth in the physical niche. KSHV encodes genes that activate cellular proliferative pathways both in a paracrine and autocrine fashion (**Figure 3**; Lagunoff et al., 1999; Lee et al., 2003; Tomlinson and Damania, 2004). This hallmark is particularly evident in KS, as not all cells within the lesion are KSHV-positive; expression of viral and cellular factors bestows neighboring cells with enhanced proliferative capabilities. Sustained proliferation results from growth factors binding to their cognate cell surface receptors, and subsequent signaling that further promotes expression of the same growth factors and receptors, in a positive feedback loop.

EVASION OF GROWTH SUPPRESSION AND CELL DEATH

Powerful growth inhibitory programs are intrinsic to the host cell to prevent uncontrolled proliferation. Hyperproliferation can trigger cell senescence via activation of checkpoint signaling. Alternatively, apoptosis may also result from aberrant oncogene activation. Immune cells that detect virally infected cells may also transduce apoptotic signals to facilitate viral clearance.

KSHV encodes several proteins to protect the infected cell from these growth suppressive and cell death-inducing signals, during latency and the lytic cycle. For example, vCyclin, a homolog of cellular cyclin D, forces quiescent rodent cells to enter the S-phase to overcome RB-mediated growth arrest induced by CDK inhibitors (Swanton et al., 1997). In the same vein, cells supporting lytic replication do not die prematurely despite increased cellular stress, due to the presence of viral proteins that inhibit apoptotic signaling. As described above, K1-dependent, AKT-mediated sequestration of FOXO transcription factors and inactivation of Bad can protect B cells from apoptosis (Tomlinson and Damania, 2004). Similarly, vFLIP expression also protects cells from apoptosis by up-regulating NF-κB transcription and pro-survival factors (Sun et al., 2003; Matta and Chaudhary, 2004; Thurau et al., 2009). In the context of the whole virus, KSHV-infected primary HUVEC cells are more resistant to apoptotic stimuli such as etoposide, staurosporine, and serum-starvation, compared to uninfected cells (Wang and Damania, 2008). Thus, evasion of both cell death and growth suppression by viral proteins expressed during latency and the lytic cycle contribute to the development of KSHV-associated cancers.

REPLICATIVE IMMORTALITY

Somatic cells within the body divide a finite number of times, i.e., they have limited replicative potential. Upon reaching their limit, normal cells senesce and cannot proliferate any further. The number of cell divisions is governed in part by telomeres, which are stretches of repetitive DNA at the ends of chromosomes that shorten after every cell division. However, cancer cells can replicate indefinitely owing to activation of one of two pathways: activation of human telomerase (hTERT), or the activation of an alternative (ALT) pathway, both of which lead to lengthening of telomeres. KSHV LANA has been shown to increase the expression of the catalytic subunit of telomerase, hTERT by up-regulating its promoter, thereby contributing to replicative immortality (Knight et al., 2001). Additionally, K1 expression in primary HUVECs endows replicative immortality, primarily through the ALT pathway (Wang et al., 2006).

INDUCTION OF ANGIOGENESIS

All cells, whether normal or cancerous, require a reliable blood supply to provide nutrients and oxygen, and to eliminate carbon dioxide and metabolic waste products. Tumor cells promote formation of vasculature by activating angiogenesis, as well as remodeling of existing vasculature and sprouting new vessel growth. Often, tumor-associated vessels are erratically branched, aberrantly sized, excessively convoluted and structurally unsound, all resulting from hyperactivated induction of angiogenic factors (Baluk et al., 2005; Nagy et al., 2010). VEGF is a key mediator of angiogenesis, and its expression is governed by upstream signaling pathways such as PI3K/AKT (Jiang and Liu, 2009). Other inflammatory cytokines can also drive angiogenesis. KSHV vGPCR induces VEGF and VEGF receptor 2 secretion in endothelial cells (Bais et al., 2003). Moreover, K1 expression in epithelial and endothelial cells also induces secretion of VEGF and the invasion factor matrix metalloprotease-9 (MMP-9; discussed below), as does KSHV infection of endothelial cells (Wang et al., 2004; Wang and Damania, 2008).

INVASION AND METASTASIS

Invasion and metastasis is a multistep process that begins with local invasion due to cancer cells exceeding their occupied niche, resulting from hyperproliferation. Cancer cells that weakly adhere to neighboring cells easily escape and intravasate nearby blood vessels and lymph nodes and travel to distal sites through vasculature and lymphatics (Langley and Fidler, 2011). Some cells escape these vessels and enter a new environment, often substantially different than the first, forming micrometastases; in the final stage, these grow into larger masses that colonize the new niche, generating metastases (Langley and Fidler, 2011). Activation of a developmental

regulatory program termed "epithelial–mesenchymal transition" (EMT) bestows onto epithelial cells the ability to invade and metastasize (Thiery, 2002; Mani et al., 2008). EMT includes a transcriptional and signaling program, and a similar, "endothelial to mesenchymal" (EndMT) transition occurs in the context of KSHV infection (Cheng et al., 2011). vFLIP and vGPCR activate the Notch signaling pathway, resulting in secretion of the mesenchymal marker membrane type-1 MMP (MT1-MMP), granting invasive properties to KSHV-positive cells. Significantly, MT1-MMP was found co-localized with LANA in KS biopsies. These data suggest that the presence of heterogeneous cell types within KS lesions can result from viral proteins driving EndMT within infected cells, bestowing them with invasion capabilities, and the creation of a microenvironment that benefits viral dissemination.

DEREGULATION OF CELLULAR ENERGETICS

Recent evidence suggests that the reprogramming of cellular energetics and metabolism is an emerging hallmark of cancer (reviewed in Hanahan and Weinberg, 2011). Fueling uncontrolled proliferation and cell division of tumor cells requires rewiring of normal cellular energetics. Normal cells, in aerobic conditions, utilize glucose to first generate pyruvate and ATP by glycolysis, and subsequent mitochondrial oxidative phosphorylation (OXPHOS). Anaerobic conditions result in a switch to glycolysis, which is relatively inefficient and generates smaller quantities of ATP, which may or may not be accompanied by reduced OXPHOS. Warburg observed that cancer cells preferentially oxidize glucose by glycolysis even in aerobic conditions, limiting their energy production; this phenomenon is termed the Warburg effect, or aerobic glycolysis (Warburg et al., 1924). The Warburg effect is an adaptation of tumors growing in hypoxic conditions to generate ATP. KSHV infection of endothelial cells induces the Warburg effect, and glycolysis inhibition of latently infected cells leads to apoptosis (Delgado et al., 2010). Moreover, we reported that in KSHV-infected PEL, aerobic glycolysis fuels *de novo* lipid synthesis to generate precursors for daughter cells, explaining the significance of up-regulating an energetically unfavorable biochemical process (Bhatt et al., 2012). This study also demonstrated that glycolysis and fatty acid synthesis (FAS) occur in a PI3K/AKT-dependent manner, providing a mechanism for metabolic reprogramming in PEL. Further, PEL viability was found to be susceptible to FAS inhibitors, revealing a new molecular therapeutic target.

IMMUNE EVASION

An ever-watchful immune system surveys the body for signs of nascent neoplasms, and eliminates such cells. The ability to escape immune surveillance is a frequent consequence of genetic instability and aberrant signaling in tumors. KSHV-associated tumors are even more adept at hiding from the immune system as viral protein expression can subvert various aspects of the innate and adaptive immune response. Viral proteins, e.g., KSHV vIRFs, K3, K5, etc. inhibit immune signaling, protecting the infected cell from host detection. For example, the K3 and K5 viral proteins can down-regulate both class I and II major histocompatibility complexes (MHC), enhancing the immunoevasion capabilities of infected cells (Coscoy and Ganem, 2000; Ishido et al., 2000). The KSHV vIRFs also contribute to immune evasion (reviewed in Jacobs and Damania, 2011). As discussed in previous sections, apoptotic signaling resulting from immune detection is also potently inhibited by viral protein expression.

TUMOR-PROMOTING INFLAMMATORY MICROENVIRONMENT

Similar to non-viral tumors, KSHV-associated lesions are infiltrated by a large number of immune cells. KSHV-associated neoplasms are also characterized by elevated local and systemic levels of inflammatory cytokines and chemokines, further augmented by virally encoded cytokines such as vIL-6, vMIPs/vCCls, and vOX2. KSHV infection up-regulates cyclooxygenase-2 (COX-2), an enzyme that converts arachidonic acid into prostaglandins, which are inflammation mediators (Sharma-Walia et al., 2010). COX-2 is essential for survival of KSHV-infected cells, and viral genome maintenance, both of which are susceptible to COX-2 pharmacological inhibitors. Creation of an inflammatory environment is functionally significant, since it activates signaling in surrounding tissues, and recruits readily infectable cell types to facilitate viral dissemination.

EXPLOITING THE PI3K/AKT/mTOR PATHWAY TO TREAT KSHV-ASSOCIATED MALIGNANCIES

Individual KSHV proteins can activate PI3K/AKT/mTOR signaling in B cells and endothelial cells, and this pathway is important for both lytic and latent phases of the KSHV life cycle. Additionally, both KS and PEL display highly activated AKT and mTOR kinases (Montaner et al., 2001; Uddin et al., 2005; Sin et al., 2007). Because aberrant PI3K/AKT/mTOR signaling is a characteristic of almost all human cancers, a plethora of small molecule inhibitors exist that target various nodes of this pathway. These inhibitors include allosteric inhibitors such as rapamycin and FK506, and also ATP-competitive small molecule kinase inhibitors that usually target the kinase activity of specific proteins.

Rapamycin is a macrolide that binds to FKBP12, a component of the mTOR signaling complex (mTORC), thus making it an allosteric inhibitor (Sawyers, 2003). Rapamycin is commonly used as an oral immunosuppressant for solid organ transplant recipients, as it inhibits the production and secretion of IL-2 in T cells, thus blocking T cell proliferation. Moreover, rapamycin blocks protein translation. Therefore, rapamycin and its derivative compounds called "rapalogs" are extensively studied for their therapeutic benefit in a variety of human cancers, including those associated with viral infection (Dittmer et al., 2012). Rapamycin treatment resolved transplant-associated KS (Stallone et al., 2005), a seminal finding that has prompted many other studies which confirm that rapamycin is an effective anti-cancer drug for PEL (Sin et al., 2007). Specifically, rapamycin is effective at halting the proliferation of PEL in cell culture, and in a xenograft model of PEL, rapamycin inhibits tumor formation and induces tumor regression (Sin et al., 2007). One drawback of rapamycin therapy is that it slows tumor growth (tumorstatic), rather than killing tumor cells (tumortoxic). Therefore, single agent therapy with rapamycin alone has limited benefit in a majority of cancers.

A class of AKT inhibitors called alkyl-lysophospholipids (e.g., miltefosine and perifosine) also inhibited PEL cell proliferation both *in vitro* and *in vivo* (Bhatt et al., 2010). Moreover, NVP-BEZ235, a dual inhibitor of both PI3K and mTOR kinases, is a

potent inhibitor of PEL cell proliferation and tumor formation in xenograft mouse models. NVP-BEZ235 treatment induced high levels of apoptosis in PEL (Bhatt et al., 2010). Thus, it appears that the PI3K/AKT/mTOR signaling pathway is essential for the survival of both PEL and KS tumors. It is of critical importance to evaluate whether long-term treatment with small molecule inhibitors breeds resistance to pathway-focused inhibitors. Selective pressure resulting from these inhibitors could drive expression of viral proteins that may contribute to resistance. Therefore in the future, it will be important to investigate whether as yet uncharacterized KSHV proteins influence PI3K/AKT/mTOR signaling, both in the context of latency and lytic viral replication.

CONCLUSION

Kaposi sarcoma-associated herpesvirus is an obligate intracellular parasite, and is a salient example of a successful pathogen. Viral manipulation of cellular pathways enhances the synthesis and secretion of growth factors and cytokines of both viral and cellular origin, which in turn support angiogenesis and proliferation. These secreted growth factors and cytokines can also activate pro-survival, proliferative, and angiogenic processes in uninfected or latently infected cells (**Figure 3**). Thus, by manipulating cellular signaling, KSHV viral proteins create an environment beneficial for both lytic and latent phases of the viral life cycle. Given the central role it plays in cell survival and proliferation, it comes as no surprise that KSHV targets the PI3K/AKT/mTOR signaling pathway at multiple nodes, in order to induce and sustain a survival and proliferative signal that is advantageous for the virus.

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The role of the PI3K signaling pathway in CD4$^+$ T cell differentiation and function

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The relative activity of regulatory versus conventional CD4$^+$ T cells ultimately maintains the delicate balance between immune tolerance and inflammation. At the molecular level, the activity of phosphatidylinositol 3-kinase (PI3K) and its downstream positive and negative regulators has a major role in controlling the balance between immune regulation and activation of different subsets of effector CD4$^+$ T cells. In contrast to effector T cells which require activation of the PI3K to differentiate and mediate their effector function, regulatory T cells rely on minimal activation of this pathway to develop and maintain their characteristic phenotype, function, and metabolic state. In this review, we discuss the role of the PI3K signaling pathway in CD4$^+$ T cell differentiation and function, and focus on how modulation of this pathway in T cells can alter the outcome of an immune response, ultimately tipping the balance between tolerance and inflammation.

Keywords: CD4$^+$ T cells, regulatory T cells, PI3K, AKT, FOXP3, mTOR, rapamycin

INTRODUCTION

Immune tolerance is a state where the immune system is unable to mount an inflammatory response toward a particular substance, thus ensuring inappropriate immunity to self or non-harmful foreign antigens is kept in check. Tolerance is controlled by two major mechanisms: central tolerance in the thymus that results in deletion of the majority of self-reactive T cells; and peripheral tolerance, which is mediated by a variety of pathways and processes including anergy, deletion, and immune regulation. Since the breakdown of immune tolerance can lead to a variety of diseases such as type 1 diabetes, allergy, and inflammatory bowel disease, there is intense research into the cellular and molecular mechanisms which control this process.

In the past 10 years, immune regulation mediated by specific types of T cells has emerged as one of the most important mechanisms of peripheral tolerance. Specifically, specialized T cells known as regulatory T cells (or T_{regs}) emerge in the thymus and periphery and are dedicated to turning off immune responses (Sakaguchi et al., 2010; Rudensky, 2011). There are several different types T_{regs}, including specialized subsets of CD4$^+$, CD8$^+$, double negative CD3$^+$CD4$^-$CD8$^-$, γδ T cells, and NKT cells (Allan et al., 2008). While it is likely that these different types of T_{regs} work together in a network to maintain immune homeostasis, the majority of current research is focused on CD4$^+$ T_{regs} since these cells are known to mediate dominant, long lasting, and transferable tolerance in experimental models (McMurchy et al., 2011).

Recently, the phosphatidylinositol 3-kinase (PI3K) signaling pathway has emerged as a key molecular regulator of immune tolerance. Modulating this pathway using drugs or genetic manipulation has revealed the importance of PI3K and its downstream signaling components in regulating T_{reg} development and function, maintaining the balance between T_{regs} and conventional CD4$^+$ T cells, and controlling the distinct metabolic requirements of different CD4$^+$ T cell subsets. In this review, we will discuss the current state of knowledge on how PI3K, its downstream signaling pathways, and its negative regulators, control the development and function of CD4$^+$ T cells, with a specific focus on T_{regs} and immune tolerance.

OVERVIEW OF THE PI3K SIGNALING PATHWAY IN T CELLS

Although there are four classes of PI3K, only class IA and class IB PI3K have been comprehensively studied in T cells. Most research is focused on the p110δ class IA catalytic subunit and the p110γ class IB catalytic subunit since these proteins are preferentially expressed in leukocytes (Huang and Sauer, 2010). Class IA PI3Ks are activated by receptor tyrosine kinases such as cytokine receptors and the T cell receptor (TCR), while class IB PI3Ks are primarily activated by G protein-coupled receptors (GPCRs) such as chemokine receptors. Class I PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to form phosphatidylinositol-3,4,5-triphosphate [PIP3] on the inner membrane of the cell, thus initiating the recruitment and activation of downstream signaling components such as PDK1 and its substrate AKT (**Figure 1**). AKT activation requires phosphorylation by PDK1 at Thr308, and for full activation, and a subsequent second phosphorylation by mTORC2 or DNA-PK at Ser473 (Bhaskar and Hay, 2007; Fayard et al., 2010). In the nucleus, activated AKT phosphorylates and consequently promotes nuclear exclusion and inhibition of FOXO transcription factors, which consist of four family members (FOXO1, FOXO3a, FOXO4, and FOXO6) (Hay, 2011; Ouyang and Li, 2011). Another consequence of AKT kinase activity is activation of mTORC1 via Rheb-GTPase (Huang and Manning, 2009). Several phosphatases negatively regulate the PI3K pathway, including the lipid phosphatases PTEN

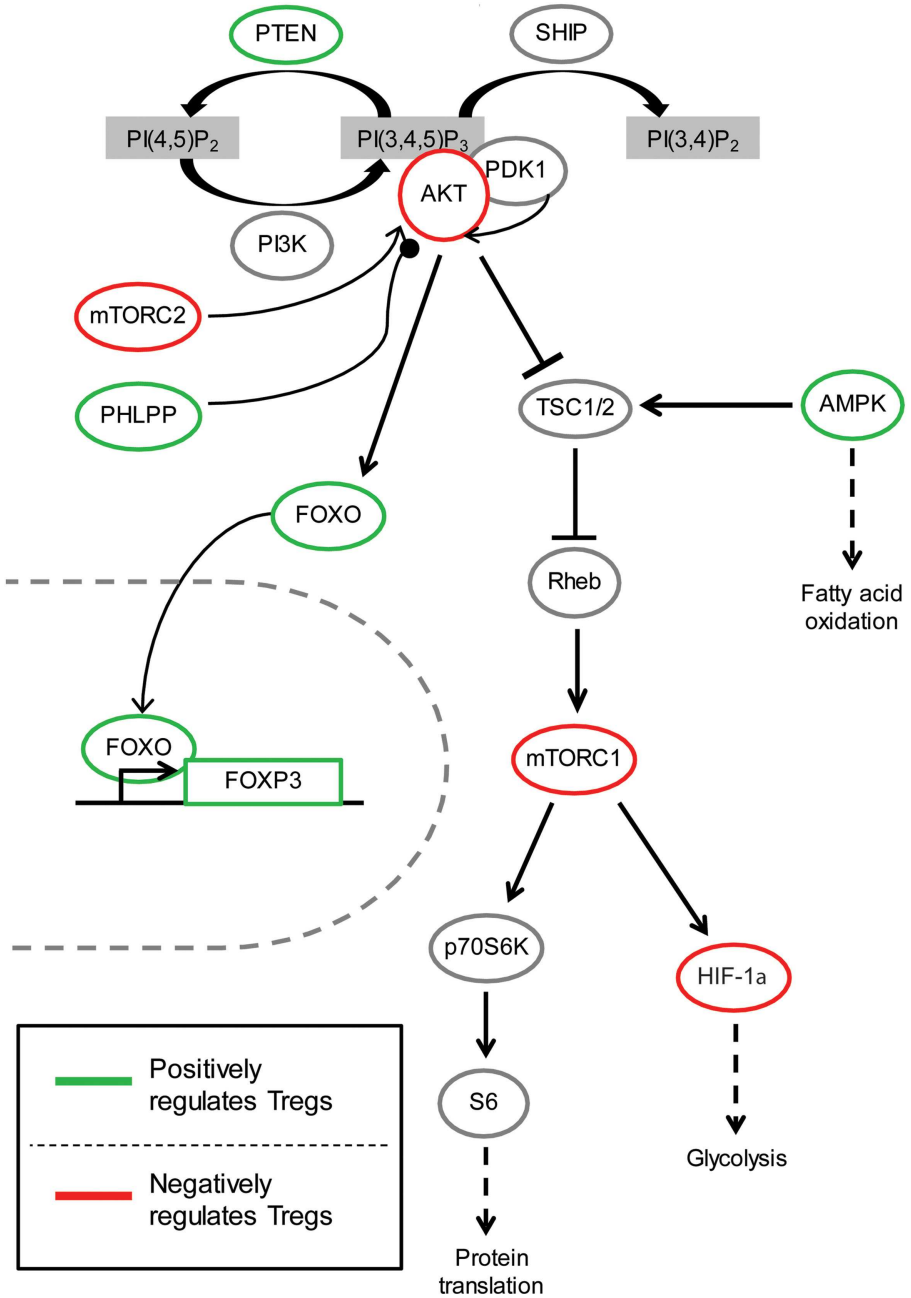

FIGURE 1 | The role of PI3K/AKT signaling pathway in T_{regs}. Arrow indicates activating phosphorylation; line with a perpendicular line at the end indicates inhibitory phosphorylation; line with a circle at the end indicates dephosphorylation; dashed line indicates resulting outcome of signaling. Green represents components of the PI3K/AKT pathway which have been shown to be beneficial for T_{reg} function and/or development. On the contrary, red indicates molecules with activity thought to be inhibitory for T_{reg} function and/or development.

and SHIP that dephosphorylate PIP3 (Harris et al., 2008), and the protein phosphatase PHLPP that dephosphorylates AKT (Brognard and Newton, 2008). The generation of PIP3 by PI3K also plays a role in the recruitment and activation of other signaling proteins in T cells such as Tec family of kinases (Huang and Sauer, 2010), which have not been extensively studied in T_{regs} and will not be discussed.

THE ROLE OF THE PI3K PATHWAY IN T_{reg} DEVELOPMENT AND FUNCTION

The best-defined type of T_{regs} is $CD4^+$ and characterized by high and constitutive expression of a transcription factor known as FOXP3. Genetic mutations in FOXP3, which cause defects in the development and function of T_{regs}, result in a severe and often fatal multi-organ autoimmune disease called Scurfy in mice and

Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked (IPEX) in humans, illustrating the essential role of Tregs in immune tolerance (Bacchetta et al., 2006; Gambineri et al., 2008; McMurchy et al., 2009, 2010). FOXP3-expressing T_{regs} can be divided into two distinct subsets: natural T_{regs} that develop in the thymus via central tolerance mechanisms, and peripherally induced T_{regs}, which differentiate from naïve T cells when self or non-self antigen is encountered in the periphery under tolerogenic conditions. T_{regs} utilize a variety of mechanisms to suppress conventional T cells as well as other immune cells such as macrophages, dendritic cells, and NK cells (Ghiringhelli et al., 2005; Tiemessen et al., 2007; Onishi et al., 2008). Some of the mechanisms used by T_{regs} to suppress immunity include expression of surface inhibitory molecules such as CTLA-4 and CD39, and secretion of anti-inflammatory cytokines such as TGF-β, IL-10, and IL-35 (Vignali et al., 2008; Sakaguchi et al., 2009). This section of the review will discuss the role of PI3K signaling in the development and function of thymically derived natural T_{regs}.

KINASES IN THE PI3K SIGNALING PATHWAY THAT AFFECT NATURAL T_{regs}

In order to define the function of PI3K in natural T_{regs}, most studies have focused on the p110δ catalytic subunit and used mice with a kinase-inactive knocked-in form of p110δ ($p110\delta^{D910A}$). The role of p110γ in T_{reg} development and function has not been clearly defined, although chemical inhibition of p110γ can induce peripheral T_{reg} differentiation *in vivo* (Dutra et al., 2011; discussed further below). $p110\delta^{D910A}$ mice have an increased proportion of T_{regs} in the thymus, but reduced in the spleen and lymph nodes (Patton et al., 2006). In addition, these T_{regs} are less suppressive and cannot produce the anti-inflammatory cytokine IL-10, as a result, $p110\delta^{D910A}$ mice develop spontaneous colitis (Patton et al., 2006) and enhanced resistance to *Leishmania major* infections (Liu et al., 2009). These data suggest that p110δ activity is not required for the development of T_{regs}, but rather for their function and maintenance in the periphery. The effect of p110δ inactivation is not specific to T_{regs} since $CD4^+$ T cells in these mice are less proliferative and have reduced IL-2, IL-4, and IFN-γ production, suggesting a general impairment in both Th1 and Th2 responses. Despite the defects in T_{regs} and resistance to primary *L. major* infections, $p110\delta^{D910A}$ mice are more susceptible to secondary *L. major* infections, due to insufficient generation of Th1-polarized memory cells (Liu and Uzonna, 2010). A subsequent study reported that the $p110\delta^{D910A}$ mice have a specific reduction in T_{regs} expressing high levels of CD38, a marker thought to define a highly suppressive population of T_{regs} (Patton et al., 2011). Together these studies suggest that reduced activity of the p110δ form of PI3K is detrimental to the effector and suppressive functions of Th cells and T_{regs}, respectively. On the other hand, as discussed below, there is also evidence that excessive activity of PI3K signaling is inhibitory to T_{regs}. Thus maintaining the correct threshold of PI3K activity is critical for the normal function of these cells.

Although there is clearly a requirement for a certain level of PI3K activity to maintain T_{regs} in the periphery, T_{regs} have a significantly diminished ability to activate the PI3K pathway downstream of the TCR (Crellin et al., 2007). Diminished signaling is evident not only in terms of reduced AKT phosphorylation, but also at the level of downstream effectors including reduced phosphorylation of p70 S6K and of FOXO1 and FOXO3a at Ser256 (Crellin et al., 2007). Notably, diminished AKT phosphorylation is most evident at Ser473, with normal phosphorylation of Thr308, suggesting that activation of PDK1 is normal. This low activity of AKT is essential for the normal function of T_{regs} since over-expression of an inducibly active form of AKT abolishes their suppressive function (Crellin et al., 2007). Mechanistically, it remains unknown why high activity of AKT block suppression in mature T_{regs} since it does not result in a change in expression of FOXP3, IL-2, CTLA-4, or granzyme B; although trans-differentiation into effector cells may play a role since enforced AKT activation causes T_{regs} to produce high amounts of IFN-γ and IL-4 (Crellin et al., 2007). Constitutive activation of AKT also represses thymic T_{reg} development (Haxhinasto et al., 2008) suggesting that high PI3K activity is detrimental to both the development and function of natural T_{regs}.

Many of the studies investigating the role of mTOR in T_{regs} have relied on the use of rapamycin (also known as sirolimus), which selectively inhibits mTORC1 at low doses but can also inhibit mTORC2 at higher doses (Delgoffe et al., 2011). Unlike conventional T cells, T_{regs} are resistant to rapamycin-induced apoptosis (Strauss et al., 2009) and hence this drug can selectively block pro-inflammatory T cells while preserving T_{regs} (Battaglia et al., 2006; Qu et al., 2007; Lu et al., 2010; Zuber et al., 2011) and their suppressive function (Singh et al., 2012). These data support the conclusion that activation of T_{regs} does not require strong activity of the PI3K pathway. Because of this distinct molecular property, the PI3K signaling pathway represents an ideal target for pharmacological immunomodulation. Indeed in mouse models, rapamycin induces T_{reg}-mediated tolerance and protects mice against graft rejection (Eng et al., 1991; Zheng et al., 2003; Gagliani et al., 2011), and acute graft versus host disease (Shin et al., 2011). Clinically, use of rapamycin is associated with increased frequency of T_{regs} following lung transplantation (Lange et al., 2010), and increased suppressive activity of T_{regs} in islet transplantation (Monti et al., 2008). On the other hand, some clinical data show an association between rapamycin and an increased incidence of acute rejection (Zuber et al., 2011), possibly due to the parallel ability of rapamycin to expand memory T cells and enhance cytokine production by antigen presenting cells (Saemann et al., 2009; Li et al., 2011). Moreover, rapamycin has many deleterious side effects such as inhibition of islet survival and function (Tanemura et al., 2012), and induction of glucose intolerance and hyperlipidemia (Morrisett et al., 2002; Houde et al., 2010). Thus the favorable effects of rapamycin on immune tolerance must be weighed against the adverse effects of this drug.

THE ROLE FOXO PROTEINS IN NATURAL T_{regs}

Since natural T_{regs} have diminished AKT activity it was predicted that continued activity of FOXO may be important for their development and function. Indeed, when both FOXO1 and FOXO3a are deleted specifically in T cells, there is reduced development and function of natural T_{regs}, resulting in a multi-organ inflammatory disorder (Ouyang et al., 2010). By corollary, enforced FOXO activity results in impaired proliferation and survival of conventional T

cells (Fabre et al., 2005), illustrating that the relative activity of this transcription factor is key for maintaining the balance between tolerance and immunity. Mechanistically, FOXO1 and FOXO3a are likely required for T_{reg} development and function because they bind and transactivate the FOXP3 promoter, the essential lineage defining transcription factor for T_{regs} (Ouyang et al., 2010). Interestingly, the FOXO-deficient T_{regs} that do develop produce large amounts of IFN-γ and IL-17, and only weakly express FOXP3, CD25, and CTLA-4 (Ouyang et al., 2010), suggesting that beyond developmental control, FOXO (and the PI3K pathway) can also control the stability of the T_{reg} lineage. Further investigation is required to study how different environments affect the activity of the PI3K in T_{regs} and hence their stability and function.

THE ROLE OF PHOSPHATASES IN THE PI3K PATHWAY IN NATURAL T_{regs}

One reason that could explain why natural T_{regs} have diminished activity of the PI3K pathway could be that they have high activity of one or more of the phosphatases that negatively regulate the pathway. SHIP is a lipid phosphatase that dephosphorylates PIP3 into phosphatidylinositol-3,4-bisphosphate [PI(3,4)P2]. It is now clear that SHIP does not terminate PI3K signaling, but rather modulates it as some proteins, such as TAPP1 and TAPP2, are preferentially recruited to PI(3,4)P2 and initiate distinct signaling pathways (Zhang et al., 2009). SHIP-$1^{-/-}$ mice have an elevated percentage of natural T_{regs} which are suppressive *in vitro* and *in vivo* (Locke et al., 2009), but this apparent enhanced T_{reg} development is likely due to a T cell extrinsic effect of SHIP, since mice with a SHIP-1 deletion only in $CD4^+$ T cells do not display this phenotype (Tarasenko et al., 2007). Moreover, T_{regs} do not express high levels of SHIP-1 (our unpublished data), supporting the overall conclusion that there is no intrinsic role for SHIP-1 in T_{reg} development or function.

PTEN is another lipid phosphatase that directly counteracts and terminates the activity of PI3K. T_{regs} from mice with a $CD4^+$ T cell specific PTEN deficiency develop and function normally, but they are hyper-proliferative in response to stimulation with IL-2, even in the absence of TCR activation (Walsh et al., 2006; discussed further below). PTEN may thus have an important role in maintaining peripheral T_{reg} expansion by regulating IL-2-induced PI3K signaling in the context of continual expression of the high affinity IL-2R.

Since the reduction of AKT activity in T_{regs} is consistently found at the level of phosphorylation of Ser473 but not Thr308, when a novel Ser473-specific protein phosphatase, known as PHLPP (Gao et al., 2005; Brognard et al., 2007; Liu et al., 2011), was identified in 2005 it was an attractive candidate for a negative regulator of the PI3K pathway in T_{regs}. There are two genes in this family: *PHLLP1* and *PHLPP2*. *PHLPP1* is expressed as two isoforms, resulting in a total of three isozymes that differentially control the phosphorylation of the three different isoforms of AKT. We found that both mouse and human natural T_{regs} express significantly more *PHLPP1* mRNA compared to conventional T cells, and moreover, that expression of the protein was critical for their function (Patterson et al., 2011). Although natural T_{regs} in *PHLPP1*$^{-/-}$ mice developed normally, they were dysfunctional both *in vitro* and *in vivo*. In addition, *PHLPP1*$^{-/-}$ T_{regs} had completely restored phosphorylation of AKT at Ser473, suggesting that high expression of PHLPP1 in T_{regs} is the molecular mechanism controlling low activity of the PI3K pathway in these cells. More recent work indicates that PHLPP can also dephosphorylate conventional PKCs, and it will be critical to determine whether T_{regs} also have altered activity of this pathway and how this may impinge on their function (Gao et al., 2005; Brognard et al., 2007; Liu et al., 2011).

REGULATION OF PI3K SIGNALING PATHWAY BY CO-STIMULATORY AND CO-INHIBITORY MOLECULES IN T_{regs}

Much of the biochemistry of the PI3K pathway in T_{regs} has been studied in the context of TCR activation, but it is important consider that this pathway is activated by many different receptors and that the net result will be the integrated signaling that is stimulated by all the factors in the local environment (**Figure 2**). Co-stimulatory molecules are essential for full T cell activation and have long been known to modulate the level of PI3K signaling induced by the TCR. Indeed CD28-stimulated activation of AKT is a prototypic signaling mechanism that is required for full activation of conventional T cells.

CD28 co-stimulation is an integral part of the development (Tai et al., 2005) and function of T_{regs} (Golovina et al., 2008). Somewhat unexpectedly, we found that even co-stimulation via CD28 in combination with the TCR was not sufficient to restore AKT phosphorylation in T_{regs}, illustrating the strength of negative regulation of this pathway in these cells (Crellin et al., 2007; Patterson et al., 2011). In addition to PI3K, many of the effects of CD28 on thymic T_{reg} development are mediated via the NF-κB

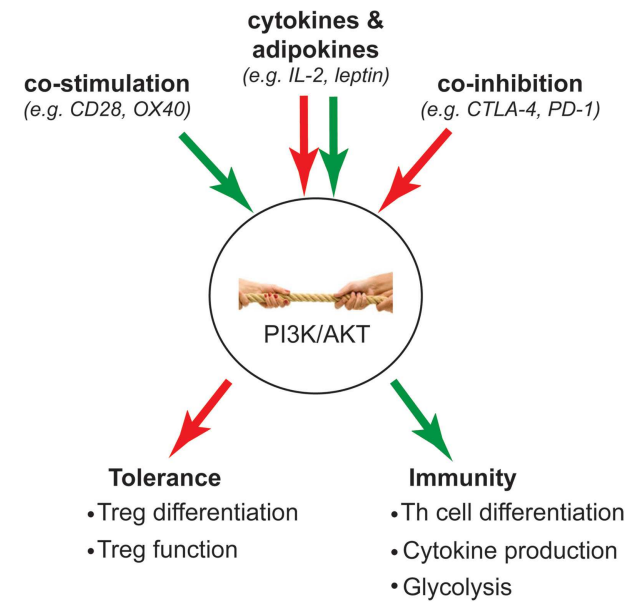

FIGURE 2 | Intracellular "tug-of-war" determines T cell fate. Green arrow indicates activation of PI3K signaling; red arrow indicates inhibition of PI3K signaling. The strength of PI3K signaling is influenced by opposing activation and inhibitory signals that are integrated from extracellular stimuli such as co-stimulation, co-inhibition, and cytokines/adipokines. Ultimately, the outcome of this "tug-of-war" determines whether a T cell becomes a T_{reg} to mediate tolerance, or inflammatory Th subsets to mediate immunity.

pathway, and specifically the c-Rel family member (Deenick et al., 2010; Vang et al., 2010). The role of CD28-mediated activation of the NF-κB pathways in fully developed T_{regs} remains to be investigated. In conventional T cells, CD28-mediated activation of the PI3K pathway is necessary for the induction of anti-apoptotic proteins (Okkenhaug et al., 2001), and the induction of glucose uptake via surface expression of GLUT1 glucose transporter (Frauwirth et al., 2002; Jacobs et al., 2008), suggesting that T_{regs}, which have diminished CD28-induced PI3K signaling, may use distinct signaling mechanisms to survive and fulfill their metabolic demands (discussed further below). There is evidence that excessive CD28 signaling inhibits immune tolerance, for example, CD28 blockade promotes T_{regs} in organ transplantation (Poirier et al., 2010), but whether the underlying mechanism of CD28 blockade involves modulation of PI3K activity remains to be investigated.

In addition to CD28, the function and biochemical activity of other co-stimulatory and co-inhibitory pathways, such as OX40, CLTA-4, ICOS, and PD-1, have recently been studied in T_{regs}. Whereas CLTA-4 and PD-1 suppress PI3K activation, OX40L, and ICOS strongly activate this pathway, leading to the prediction that ligation of the former molecules should promote T_{reg} development and function whereas the latter should block these processes. Curiously, T_{regs} express high levels of all these molecules, suggesting they are poised to have their PI3K pathway turned on or off in response to different environments.

OX40 is expressed on T_{regs} in the absence of immune activation (Sakaguchi, 2004), and, as in activated effector T cells (So et al., 2011), OX40 engagement in T_{regs} activates AKT (Xiao et al., 2012). Studies to investigate whether OX40 engagement positively or negatively affects T_{regs} have produced conflicting data. Some studies suggest that T_{regs} lacking OX40 lose suppressive function *in vivo* (Griseri et al., 2010), while others report that OX40 activation interferes with T_{reg} function (Vu et al., 2007; Piconese et al., 2008). A recent study suggests that the effect of OX40 on T_{regs} may depend on the abundance of IL-2 (Xiao et al., 2012), which activates STAT5 but not the PI3K pathway in T_{regs} (Bensinger et al., 2004). Specifically, OX40 stimulation renders T_{regs} non-suppressive unless IL-2 is abundant. Thus an optimal balance between the PI3K pathway activated by OX40 and the STAT5 pathway activated by IL-2 may be important for regulating both T_{reg} proliferation and function.

ICOS expression defines a subset of effector T_{regs} that are highly suppressive and selectively produce high amounts of IL-10 (Ito et al., 2008) and IL-35 (Whitehead et al., 2012), a phenotype which is likely related to the fact that ICOS expression is induced upon antigen specific activation of T_{regs} *in vivo* (Vocanson et al., 2010). ICOS ligation potently stimulates PI3K activation in conventional T cells (Fos et al., 2008; Simpson et al., 2010), but it is not known whether ICOS stimulation can similarly induce strong PI3K signaling in T_{regs}. Thus it remains to be investigated whether the reduced numbers of peripheral T_{regs} in the absence of ICOS (Burmeister et al., 2008) is related to activation of the PI3K pathway in T_{regs}.

In contrast to CD28 and other positive co-stimulatory receptors, co-inhibitory receptors such as CTLA-4 and PD-1 typically inhibit TCR-induced PI3K signaling (Parry et al., 2005), and both proteins are highly expressed in T_{regs} (Takahashi et al., 2000; Raimondi et al., 2006). Although CTLA-4 engagement does not inhibit PI3K directly, it is thought that CTLA-4 utilizes the serine/threonine protein phosphatase PP2A to dephosphorylate and inactivate AKT in $CD4^+$ T cells (Parry et al., 2005). However, others claim that the inhibitory property of CTLA-4 on T cells is separate from the PI3K/AKT pathway, and that CTLA-4 can signal and activate the PI3K/AKT pathway to promote T cell survival (Schneider et al., 2008). A recent study supports the concept that T_{reg} suppression mediated via CTLA-4 inhibits intracellular signaling in T_{regs} (Tai et al., 2012).

PD-1 stimulation disrupts the accumulation of PIP3 in $CD4^+$ T cells by recruiting SHP-2, which subsequently blocks the recruitment and activation of PI3K (Parry et al., 2005; Saunders et al., 2005). PD-L1 and PD-L2 expression on antigen presenting cells, such as tolerogenic dendritic cells, is crucial for efficient differentiation of induced T_{regs} from conventional T cells (Francisco et al., 2009). Mechanistically this role in T_{reg} differentiation is mediated by PD-1-induced down-regulation of AKT and mTOR activity and parallel up-regulation of PTEN (Wang et al., 2007; Francisco et al., 2009; Maldonado and von Andrian, 2010).

Clearly, the effects of these co-receptors on conventional T cells versus T_{regs}, and the consequent balance of PI3K signaling are crucial in dictating the state of immune tolerance. As biological agents blocking, or in some cases stimulating, the function of these molecules enter clinical trials (Vincenti, 2007; Weber, 2007; Ford and Larsen, 2009), further research is needed to explore the functional consequences on the activity of the PI3K pathway and the resulting biological effects of T_{regs} versus conventional T cells.

REGULATION OF PI3K SIGNALING PATHWAY BY CYTOKINES IN T_{regs}

Cytokines have a major role in directing and sustaining T cell responses, and these molecules also directly regulate the PI3K pathway. Although mature, fully developed T_{regs} respond to many cytokines, to date only the biochemical effects of IL-2 and leptin, an adipo-cytokine, have been intensively studied in these cells. IL-2R signaling is essential for T_{reg} development and survival (Almeida et al., 2002), but the signaling pathway triggered by the receptor is different compared to conventional T cells. Although STAT5 signaling downstream of IL-2R remains intact, as for the TCR, IL-2-stimulated PI3K signaling is selectively inhibited in T_{regs} (Bensinger et al., 2004). This defect in PI3K signaling downstream of the IL-2R has been attributed to the expression of PTEN as $PTEN^{-/-}$ T_{regs} are hyper-proliferative to IL-2 stimulation, even in the absence of TCR stimulation. These data suggest that PTEN is responsible for keeping IL-2-stimulated proliferation of T_{regs} in check despite their continuous expression of the high affinity IL-2R (Walsh et al., 2006). It would be of interest study whether T_{regs} also have defective PI3K pathway activation upon stimulation with other common gamma chain cytokines such as IL-7, which has recently been shown to be required for T_{reg} maturation and homeostasis (Di Caro et al., 2011; Kim et al., 2012), and IL-15, which, much like IL-2, also stimulates expansion of T_{regs} *ex vivo* (Levings et al., 2002). In addition, since polarizing cytokines such as IL-6 and IL-12 have been suggested to affect the stability of the T_{reg} lineage, their downstream receptor signaling pathways should be explored in T_{regs}. Finally the neuropeptide hormone vasoactive intestinal peptide inhibits PI3K signaling in T cells and promotes T_{reg} differentiation, indicating that the effects of cytokines which

are not normally considered part of the immune response should also be considered (Anderson and Gonzalez-Rey, 2010).

Recent studies have shown that adipocyte-derived cytokines, or adipokines, modulate T cell responses via the PI3K signaling pathway, and that this process affects the function of T_{regs}. Most research has focused on leptin, an adipokine induced by food intake and glucose metabolism to control appetite. Specifically, leptin is thought to negatively regulate T_{reg} proliferation by activating mTOR. In parallel, leptin promotes T cell mediated inflammation by enhancing Th1 and Th17 responses, and the survival of autoreactive T cells (Galgani et al., 2010; Matarese et al., 2010). Surprisingly, T_{regs} themselves secrete leptin, and the autocrine effects of this adipokine are thought to induce activation of mTOR (De Rosa et al., 2007; Procaccini et al., 2010). Leptin-induced mTOR activity in T_{regs} causes them to be anergic *in vitro*, and by corollary leptin blockade restores T_{reg} activation and proliferation. Thus oscillatory changes in mTOR activity, controlled partially by leptin, could be necessary for the ability of T_{regs} to vigorously proliferate *in vivo* (Procaccini et al., 2010).

In support of a major role for adipokines in controlling immune tolerance, leptin receptor deficient T_{regs} maintain their suppressive function but have an increased proliferative potential (De Rosa et al., 2007). Similarly, leptin deficient (ob/ob) mice have increased numbers of peripheral T_{regs} and are resistant to experimental autoimmune encephalomyelitis (Matarese et al., 2001). These data contrast to a recent observation that the inflamed adipose tissue in ob/ob mice has a decreased proportion of adipose-resident T_{regs} (Feuerer et al., 2009; Winer et al., 2009), suggesting there may be tissue specific effects of adipokines. Overall, the data from the above studies are consistent with the widely accepted notion that chronic activation of mTOR inhibits T_{regs}. With growing evidence that T_{regs} have a role in metabolic disorders, it is important to understand how signals from metabolic and classical immune stimuli are integrated.

T_{regs} MODULATE PI3K SIGNALING ACTIVITY IN TARGET CELLS AS PART OF THEIR SUPPRESSIVE MECHANISM

Since damping of PI3K signaling is strongly associated with depressed T cell activation, it can be hypothesized that T_{regs} may modulate this pathway in order to suppress their targets. In support of this concept, effector T cells with hyperactive PI3K/AKT activity become resistant to suppression by T_{regs} (King et al., 2006; Ben Ahmed et al., 2009) and T_{regs} attenuate the activation of AKT in $CD8^+$ T cells (Kojima et al., 2005). Via CTLA-4 expression, T_{regs} also compete with CD28 expressed on conventional T cells for access to CD80/86 on antigen presenting cells (Greenwald et al., 2005), and can physically remove these co-stimulatory ligands from APCs (Qureshi et al., 2011). As a result, T_{regs} can indirectly limit CD28-induced PI3K activation in their targets. Furthermore, by producing high levels of IL-10, T_{regs} can cause phosphorylation and activation of SHP-1, a tyrosine phosphatase that inhibits the recruitment of PI3K, thus hindering T cell activation (Taylor et al., 2007). In addition, IL-10 can stabilize the expression of SHIP-1 via blocking miR-155, a micro RNA that targets SHIP-1 for degradation, in macrophages (McCoy et al., 2010). Lastly, T_{regs} also express PD-L1 (Francisco et al., 2010), which upon ligation to PD-1 on effector T cells, can inhibit PI3K activity via induction of SHP-2 (Parry et al., 2005). It can be speculated that the ability of T_{regs} to limit PI3K signal strength in conventional T cells would create a condition favorable for peripheral T_{reg} differentiation, hence contributing to infectious tolerance (Kendal et al., 2011).

THE ROLE OF PI3K SIGNALING PATHWAY IN PERIPHERAL $CD4^+$ T CELL POLARIZATION

Depending on the context of stimulation upon activation, naive T cells differentiate into distinct subsets, which are characterized by lineage defining transcription factors and profiles of cytokine production. One arm of T cell differentiation includes the peripheral development of induced T_{regs} which are important for tolerance to harmless commensals and prevention of over-active immune responses against pathogens (Chen and Konkel, 2010). The other arms include Th1, Th2, and Th17 cells, as well as a variety of other newly described Th cell subsets (Zhu and Paul, 2010). Since the relative activity of PI3K plays a key role in regulating Th cell polarization, this in an additional way that the activity of this pathway modulates the balance between tolerance and immunity.

KINASES IN THE PI3K SIGNALING PATHWAY WHICH AFFECT $CD4^+$ T CELL DIFFERENTIATION

Studies involving inhibition of PI3K activity have revealed separate roles for p110δ and p110γ in peripheral $CD4^+$ Th polarization. Specific inhibition of p110δ using IC87114 blocks the release of multiple cytokines by human T cells, including IFN-γ, TNF-α, IL-5, and IL-17 (Soond et al., 2010). Similarly, genetic manipulations to inactivate p110δ results in reduced production of IL-4, IL-17, IFN-γ, and IL-10 by different T cell subsets (Okkenhaug et al., 2006; Patton et al., 2006), hence disrupting Th1, Th2, Th17, and T_{reg} associated cytokines. These data suggest that p110δ plays an indispensable role in multiple $CD4^+$ Th cell subsets. On the other hand, p110γ does not seem to have a major role in T cell activation (Gruen et al., 2010), and its expression is dispensable for Th1 and Th17 differentiation (Berod et al., 2011). Interestingly, blockade of p110γ by administration of its inhibitor AS605240 in mice can induce T_{regs} *in vivo* and consequently ameliorate colitis (Dutra et al., 2011). Together, these studies suggest that inhibition of p110δ may be beneficial for treating inflammatory disorders where cytokines are over-produced; however, since p110δ activity is essential for T_{regs}, immune tolerance would likely not be achieved in parallel. On the contrary, inhibition of p110γ may be beneficial in achieving long lasting tolerance by inducing T_{regs}, but may be relatively ineffective at controlling ongoing Th1 and Th17 responses.

There are contradicting results regarding the role of AKT in peripheral differentiation of induced T_{regs}. Constitutive AKT activation impairs FOXP3 induction during *in vitro* TGF-β driven T_{reg} differentiation (Haxhinasto et al., 2008), suggesting a requirement for reduced AKT activity in peripheral T_{reg} differentiation similar to that in natural T_{reg} development. In contrast, another study found that in the absence of CD28 co-stimulation, AKT transgenic $CD4^+$ T cells have an enhanced capacity to differentiate into T_{regs} (Pierau et al., 2009). In addition, CD28 signaling is required for the survival of induced T_{regs} (Liu et al., 2006), suggesting that in the former study constitutive AKT activity may substitute for the requirement of co-stimulation. On the other

hand, CD28 co-stimulation may influence peripheral T_{reg} differentiation via other signaling pathways such as activation of c-Rel, which has been shown to play a role in thymic T_{reg} development (Deenick et al., 2010; Vang et al., 2010). Since AKT is central to various cellular processes including cell survival pathways, it is possible that peripheral T_{reg} development requires some level of AKT activation, provided by CD28 co-stimulation, but which must then be maintained at a relatively low level for the cells to stabilize FOXP3 expression and retain suppressive function.

The activity of mTOR, which forms part of the mTORC1 or mTORC2 kinase complexes when bound to the scaffold proteins Raptor or Rictor, respectively (Laplante and Sabatini, 2009), tightly regulates Th cell differentiation. Deletion of Rictor, which disrupts mTORC2, impairs both Th1 and Th2 differentiation (Lee et al., 2010). The effect on Th1 cells is due to the fact that expression of TBET, the defining transcription factor for Th1 cells, is repressed by FOXO1. In the absence of mTORC2, AKT activity is diminished, FOXO1 is not repressed and hence TBET expression is prevented. In contrast, the effect of mTORC2 deletion on Th2 cells does not seem to be related to AKT or FOXO1. On the contrary, another study reported that while *Rictor*$^{-/-}$ T cells fail to differentiate into Th2 cells, they can still differentiate into Th1 cells (Delgoffe et al., 2011). *Rheb*$^{-/-}$ T cells, which lack the GTPase required for mTORC1 activity, cannot successfully differentiate into Th1 or Th17 cells, but maintain the capacity for Th2 differentiation.

Both mTORC1 and mTORC2 antagonize the peripheral differentiation of T_{regs}. While *Rictor*$^{-/-}$ T cells have enhanced TGF-β mediated T_{reg} differentiation (Lee et al., 2010), mTOR-deficient T cells that lack both mTORC1 and mTORC2 readily differentiate into T_{regs} in the absence of TGF-β (Delgoffe et al., 2011). Furthermore, the lack of both mTOR complexes renders T cells unable to skew into Th1, Th2, and Th17 cells (Delgoffe et al., 2009). In agreement with these genetic data, inhibition of mTOR by rapamycin, promotes FOXP3 expression and T_{reg} generation (Kopf et al., 2007; Kang et al., 2008).

In summary, studies of mTOR have shown that mTORC1 is required for differentiation of Th1 and Th17 cells, but not Th2 cells. mTORC2 is most important for Th2 differentiation, but also plays a role in Th1 differentiation, and both mTORC1 and mTORC2 negatively regulate the peripheral differentiation of T_{regs}. Hence differential targeting of mTORC1 versus mTORC2 could be used to alter the balance of effector T cell subsets and promote immune suppression.

THE ROLE OF FOXO PROTEINS IN CD4+ T CELL DIFFERENTIATION

As discussed above, one of the main ways that the PI3K pathway blocks the differentiation of T_{regs} is via inactivation of FOXO1 and FOXO3a, transcription factors which are necessary for induction of FOXP3 expression (Ouyang et al., 2010). In agreement, ablation of Cbl-b, which results in FOXO3a inactivation in a PI3K dependent manner, also impairs T_{reg} differentiation *in vitro* and *in vivo* (Harada et al., 2010). Impaired T_{reg} differentiation can be rescued by over-expression of FOXO3a, and mice lacking FOXO3a have increased Th1 and Th2 cells (Lin et al., 2004). Together these data indicate that regulation of FOXO activity is the critical arm of the PI3K pathway controlling the balance between immune tolerance and inflammation.

PHOSPHATASES IN THE PI3K SIGNALING PATHWAY THAT AFFECT CD4+ T CELL DIFFERENTIATION

As negative regulators of the PI3K pathway, phosphatases such as SHIP also have crucial roles in Th cell differentiation. Systemic *SHIP-1* deletion results in reduced numbers of Th17 but not Th1 cells. Furthermore, when naive T cells from *SHIP-1*$^{-/-}$ mice are transferred into immunodeficient mice, they are less able to induce colitis, possibly due to their reduced IL-17 production and parallel tendency to differentiate into induced T_{regs} (Locke et al., 2009). In studies of mice with a T cell specific *SHIP-1* deletion, *SHIP-1*$^{-/-}$ T cells themselves have a reduced capacity to differentiate into Th2 cells (Tarasenko et al., 2007). Furthermore, *SHIP-1*$^{-/-}$ Th2 cells produce less IL-4, suggesting that SHIP-1 is an intrinsic positive regulator of Th2 responses (Tarasenko et al., 2007; Roongapinun et al., 2010). Interpretations on effects of the PI3K pathway from these studies of *SHIP*$^{-/-}$ T cells have to be taken with caution as SHIP does not simply reverse PI3K activity, but rather modulates the downstream signaling effects through a modified lipid second messenger PI(3,4)P2, which can also act by recruiting adaptor proteins (Zhang et al., 2009).

T cell deficiency of *PTEN* results in enhanced AKT activation and resistance to TGF-β driven differentiation of induced T_{regs} (Sauer et al., 2008). On the other hand, T cell specific *PTEN* deficiency also causes uncontrolled proliferation and cytokine production by both Th1 and Th2 cells, ultimately leading to the development of lymphoma (Suzuki et al., 2001). Collectively, PTEN is necessary to keep T cell proliferation in check and maintain tolerance. We have shown that expression of PHLPP is crucial for the induction of FOXP3 expression in T cells (Patterson et al., 2011). When *PHLPP1* is deleted, conventional T cells lose the ability to convert into induced T_{regs} in the presence of TGF-β. Furthermore, PHLPP expression is up-regulated in response to TGF-β, consistent with high PHLPP expression found in natural T_{reg}.

Overall, the differentiation of Th cells into distinct subsets is clearly modulated by the PI3K pathway. Since these different Th cell subsets have distinct roles in different immune responses, modulating the pathway could be used in different therapeutic approaches. For example, in the case of infectious diseases, it may be advantageous to enhance PI3K activity and block T_{regs} and Th2 cells. On the other hand, since inhibitors of p110α, p110γ, AKT, or mTOR all favor the conversion of conventional T cells into T_{regs} (Fruman and Bismuth, 2009; Dutra et al., 2011), these agents have promise in strategies to induce tolerance.

THE ROLE OF PI3K SIGNALING PATHWAY IN REGULATING T CELL METABOLISM

Cellular metabolism is a previously under-studied aspect of T cell biology that has recently gained much attention. As with all cells, T cells have energy requirements and must generate ATP to survive and function. In their naive quiescent state, T cells rely on oxidative metabolism to survive. Upon activation, however, T cells increase their energy requirements to support proliferation and effector functions such as cytokine production. Activated effector T cells must meet this increase of demand for energy and building blocks for cellular macromolecules by switching to the catabolic process of glycolysis (Maciver et al., 2008).

Upon TCR activation in conjunction with CD28 co-stimulation, T cells increase their ability to uptake glucose by promoting surface trafficking of the glucose transporter GLUT1 and glycolysis via a process that depends on the PI3K signaling pathway (Frauwirth et al., 2002; Jacobs et al., 2008). If co-stimulation is lacking, T cells have a reduced ability to proliferate due to failure to activate PI3K and increase glycolysis. Furthermore, T cells with constitutive AKT activation have increased glycolytic activity, and lose their dependence on CD28 co-stimulation to proliferate and secrete cytokines. Since ICOS and OX40 co-stimulatory molecules induce strong PI3K activity on activated T cells, it is possible that their stimulation promotes even stronger glycolytic activity on antigen experienced T cells. In line with this observation, activation of co-inhibitory receptors CTLA-4 and PD-1, and the use of inhibitors of the PI3K pathway, prevents the up-regulation of glucose uptake in T cells (Frauwirth et al., 2002; Parry et al., 2005; Wieman et al., 2007). In this section, we will review the differential cellular metabolic requirements between T_{reg} and conventional T cells as they relate to the PI3K signaling pathway.

The distinct lineages of $CD4^+$ Th cells differ in their metabolic requirements. Even though Th1, Th2, and Th17 cells all express GLUT1 and require glycolysis (Michalek et al., 2011), Th17 cells uniquely require a protein known as HIF-1α for their glycolytic activity (Shi et al., 2011). Expression of HIF-1α in Th17 cells requires mTOR activation, and thus inhibition of mTOR by rapamycin blocks HIF-1α induction and expression of glycolytic enzymes in Th17 cells. HIF-1α is a transcription factor which responds to changes in oxygen tension and directs cells to switch from oxidative phosphorylation to aerobic glycolysis (Semenza, 2007). Indeed hypoxia, which activates HIF-1α, promotes skewing toward Th17 cells and away from T_{regs} (Dang et al., 2011). Similarly, *HIF-1α*$^{-/-}$ T cells have defective Th17 differentiation, and are more prone to express FOXP3 and become T_{regs}. Interestingly, HIF-1α has been reported to bind and target FOXP3 for ubiquitination and proteasomal degradation (Dang et al., 2011), providing a possible mechanism for the observed effects on T_{regs}. Along with the role of FOXO on FOXP3 expression and T_{reg} function, these recent findings on HIF-1α provide an additional mechanism for how activation of the PI3K pathway can negatively regulate T_{regs}.

Unlike Th1, Th2, and Th17 cell subsets, T_{regs} and memory T cells are relatively quiescent, expressing low amounts of GLUT1 and not requiring high glycolytic activity (Michalek et al., 2011). Instead of glycolysis, T_{regs} depend on AMPK, an enzyme which antagonizes mTOR activation, to perform lipid oxidation and meet their energetic demands. Metformin, a drug commonly used as to treat type 2 diabetes, activates AMP, and increases lipid oxidation and T_{reg} numbers *in vivo* (Michalek et al., 2011). Since enhancing T_{reg} numbers *in vivo* ameliorates insulin resistance in mice (Feuerer et al., 2009; Winer et al., 2009), further investigation into whether part of the mechanism of action of metformin in type 2 diabetes is related to enhanced T_{reg} function is warranted.

Since AMPK inhibits Rheb-GTPase mediated mTORC1 activation (Laplante and Sabatini, 2009), modulating the balance between mTOR and AMPK can be used to alter T cell metabolism and hence lineage differentiation. For example, rapamycin-mediated inhibition of mTOR favors AMPK activity and the lipid oxidation of T_{regs} (Michalek et al., 2011). Rapamycin can also reverse the effect of *AMPK* or *LKB1* (upstream kinase for AMPK activation) deletion, resulting in increased mTORC1 activity, glycolysis, and over-production of IFN-γ (Maclver et al., 2011). Since T_{regs} and memory T cells are metabolically similar, it is no surprise that rapamycin can promote the generation of both of these cell types (Araki et al., 2009; Golovina et al., 2011). Interestingly, TCR stimulation can activate both mTOR and AMPK (Tamas et al., 2006), and therefore, the relative strength of the PI3K pathway activation may be crucial in determining whether a T cell passes the threshold of mTOR activity to proceed to glycolysis.

Notably, one of the mechanisms that T_{regs} use to suppress conventional T cells is through metabolic disruption via CD39, an ectonucleotidase that hydrolyzes extracellular ATP (Borsellino et al., 2007). AMPK is preferentially activated in conditions of high AMP:ATP ratio (Carling et al., 2011). Thus via CD39, T_{regs} may be able to promote AMPK activity in their target cells, ultimately antagonizing mTOR activity. AICAR, a drug that promotes the activation of AMPK, has been shown to promote T cell anergy (Zheng et al., 2009), supporting the notion that AMPK activity is beneficial for immune tolerance.

CONCLUSION

Collectively, the above studies reveal the complexity and intricacies of signaling requirements for T_{regs} and different Th cell subsets. The studies of mice expressing $p110\delta^{D910A}$ reveal that too little activity of the PI3K/AKT pathway is detrimental for T_{regs}. On the other hand, many studies show that strong PI3K/AKT signaling activity negatively affects T_{regs}. These differential effects suggest that there is likely a certain range of PI3K/AKT signal strength that is permissive for T_{regs}. This signal strength is likely determined by the collective outcome of various extracellular stimuli that can activate or inhibit PI3K/Akt signaling, thus regulating cellular changes (**Figure 2**). Because the PI3K/Akt pathway serves as a critical signaling hub, which directs the balance between inflammation and immune tolerance, it is an ideal target for therapeutic manipulation.

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Does the PI3K pathway promote or antagonize regulatory T cell development and function?

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Regulatory T cells (Tregs) prevent autoimmunity and inflammation by suppressing the activation of other T cells and antigen presenting cells. The role of phosphoinositide 3-kinase (PI3K) signaling in Treg is controversial. Some studies suggest that inhibition of the PI3K pathway is essential for the development of Tregs whereas other studies have shown reduced Treg numbers and function when PI3K activity is suppressed. Here we attempt to reconcile the different studies that have explored PI3K and the downstream effectors Akt, Foxo, and mTOR in regulatory T cell development and function and discuss the implications for health and therapeutic intervention.

Keywords: Akt, autoimmunity, Foxo, inflammation, mTOR, PI3K, T cell, Treg

INTRODUCTION

The class I phosphoinositide 3-kinases (PI3Ks) consist of heterodimers between one regulatory and one catalytic subunit. The Class IA catalytic subunit isoforms (p110α, p110β, and p110δ) can be activated by tyrosine kinase-associated and sometimes G protein-coupled receptors while the Class IB isoform (p110γ) can only be activated by G protein-coupled receptors. Each class I PI3K isoform uses PtdIns(4,5)P_2 as its preferred substrate to generate the second messenger PtdIns(3,4,5)P_3, which helps activate PH domain-containing signaling proteins. Key downstream targets include Akt, PDK-1, and Tec family kinases (Okkenhaug and Fruman, 2010; So and Fruman, 2012).

PtdIns(3,4,5)P_3 generation recruits and co-localizes Akt and PDK1 to the plasma membrane. PDK1 can then phosphorylate Akt on Thr^{308}. A second phosphorylation at Ser^{473} is required for optimal Akt activity. This residue is phosphorylated by the rapamycin-insensitive mTOR/Rictor complex (mTORC2) and de-phosphorylated by PHLPP. Akt, together with numerous other upstream regulators, can then indirectly contribute to activation of the rapamycin-sensitive mTOR/Raptor complex (mTORC1). Akt also phosphorylates the transcription factor FOXO, which leads to its exclusion from the nucleus, thus altering T cell homeostasis and trafficking (Kerdiles et al., 2009; Finlay and Cantrell, 2010).

PtdIns(3,4,5)P_3 signaling is terminated by two classes of phosphatases. Pten dephosphorylates PtdIns(3,4,5)P_3 on the D3 position to maintain resting levels of PtdIns(4,5)P_2. SHIP phosphatases dephosphorylate PtdIns(3,4,5)P_3 on the D5 position to generate PtdIns(3,4)P_2, which has signaling properties of its own (Okkenhaug and Fruman, 2010; So and Fruman, 2012).

The PI3K isoform p110δ was shown to be the dominant isoform downstream of the T cell receptor (TCR), the co-stimulatory receptor ICOS, and the IL-2 receptor (Okkenhaug et al., 2002, 2006; Rolf et al., 2010; Soond et al., 2010; Macintyre et al., 2011). Consequently, p110δ controlled proliferation, cytokine production, differentiation into helper T cells (Th) subsets, and trafficking (Okkenhaug et al., 2002, 2006; Nashed et al., 2007; Garcon et al., 2008; Jarmin et al., 2008; Sinclair et al., 2008; Liu et al., 2009; Rolf et al., 2010; Soond et al., 2010; Macintyre et al., 2011). The p110γ isoform of PI3K is required for migration toward inflammatory chemokines and memory T cell survival (Barber et al., 2006; Martin et al., 2008; Thomas et al., 2008). Conversely, T cells lacking Pten are hypersensitive to TCR and IL-2 signaling leading to augmented Th cell functions, autoimmunity, and leukemia (Suzuki et al., 2001; Buckler et al., 2006; Liu et al., 2010; Guo et al., 2011; Soond et al., 2012).

The role of the PI3K pathway in T cells has been addressed experimentally by either inhibiting PI3K signaling (e.g. by inactivating PI3K or downstream proteins such as PDK1 or mTOR) or by increasing PI3K signaling (e.g. by deleting Pten, SHIP or Foxo, or by overexpressing membrane-targeted Akt). Although there seems to be a clear positive role for the PI3K pathway in inducing the activation, differentiation, and maintenance of Th cells, data regarding its precise effect in regulatory T cells (Tregs) appears contradictory. In this review, we will briefly summarize what is known about the development and function of Tregs, describe how Tregs are regulated by the PI3K pathway, and propose how conflicting data can be reconciled.

Tregs SUPPRESS IMMUNE RESPONSES

Tregs are defined as the 5–10% of $CD4^+$ T cells that express the transcription factor Foxp3. Foxp3 expression is both necessary and sufficient to confer suppressive ability to Tregs. Tregs prevent autoimmunity, restrain the responses to infectious agents, aid maternal tolerance toward fetuses and block tumor immunity. Their importance has been shown in cases where Foxp3 is lost or attenuated such as IPEX syndrome in humans or *scurfy* mice, where by multi-organ autoimmunity and inflammation rapidly develops, leading to death of the organism (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001; Yamaguchi et al., 2011; Josefowicz et al., 2012a). Depletion of $Foxp3^+$ cells in adult mice also leads to fatal disease, highlighting their role in preventing responses throughout the life of the organism (Kim et al., 2007).

Treg DEVELOP IN THE THYMUS AND PERIPHERY

The majority of Treg cells are generated in the thymus and are termed "natural Tregs" (nTregs). Commitment to this lineage occurs in two steps. First, TCR signaling in $CD4^+CD8^+$ double positive T cells poises them to express Foxp3, which then occurs in a second IL-2-dependent but TCR-independent step (Lio and Hsieh, 2008). The amount of TCR signaling required for the positive selection of Tregs is higher than for conventional T cells, but less than is required for negative selection. Hence, the TCRs expressed by Tregs tend to have higher affinity for self-peptide/MHC complexes than those expressed by Th cells (Hsieh et al., 2012).

Tregs can also be generated outside the thymus from naïve $CD4^+$ T cells. These "induced Tregs" (iTregs) develop when the TCR is activated under immunosuppressive conditions in the presence of TGFβ1 (Chen et al., 2003), indoleamine 2,3-dioxygenase, or other amino acid metabolizing enzymes (Chen et al., 2008; Chung et al., 2009; Cobbold et al., 2009), or when T cells are activated by Ag at low doses or low affinity antigen (Daniel et al., 2010; Gottschalk et al., 2010). Although there is a lack of reliable markers to unequivocally track the survival of iTreg, it is estimated that iTreg represent only a small proportion of the total Treg population under homeostatic conditions (Zheng et al., 2010; Josefowicz et al., 2012b).

The Foxp3 gene locus contains a promoter and three additional conserved non-coding DNA sequences (CNS1-3) which include binding sites for diverse transcription factors such as NFAT, NF-κB, AP1, STAT5, Cbf, Runx, Foxo, Foxp3, SMAD, and other factors (Merkenschlager and Von Boehmer, 2010; Zheng et al., 2010). The number of elements involved in *Foxp3* regulation suggests this locus is tightly controlled and highly responsive to context-dependent cues. Not all of these transcription factors or promoter regions are required for *Foxp3* transcription at all times. CNS3, which binds c-Rel but not other members of the NF-κB transcription factor family, is considered to be a pioneer element accessible in Treg precursors. Consistent with this, Treg development in the thymus is blocked in the absence of CNS3 or c-Rel (Isomura et al., 2009; Visekruna et al., 2010; Zheng et al., 2010). The CNS1 element binds Smad3 and—along with CNS3—is required for TGF-β-induced conversion to iTreg. CNS1 deficiency primarily affects Treg numbers at environmentally exposed tissues such as the intestine and lung where iTregs are most frequently found, but is dispensable for nTreg development (Zheng et al., 2010; Josefowicz et al., 2012b). CNS2, but not CNS3, is required for maintenance of Treg in the periphery (Zheng et al., 2010). This is of interest as CNS2 binds Foxp3 protein and may hence stabilize the lineage as part of a positive feedback loop. Multiple regulatory inputs mean that a genetic lesion may alter Treg numbers by affecting the development and/or maintenance of Foxp3.

Fate-mapping studies have shown somewhat conflicting results with regards to Treg plasticity. While some studies had suggested that some Treg can be re-differentiated to other Th lineages (Tsuji et al., 2009; Zhou et al., 2009), further studies suggest that the expression of Foxp3 is highly stable and irreversible (Rubtsov et al., 2010; Miyao et al., 2012). However, it is possible that a certain percentage of Th cells express Foxp3 transiently, but subsequently are diverted to other lineages (Komatsu et al., 2009). Consistent with this notion, when expression of Foxp3 was intentionally destabilized, the $Foxp3^{low}$ T cells were subverted into Th2 cells that caused disease (Wan and Flavell, 2007). It should be noted however, that fully committed $Foxp3^+$ Treg can co-express transcription factors associated with other T cells lineages, such as Tbet, Gata3, IRF4, or Bcl6. This may help adapt the $Foxp3^+$ Treg to limit particular types of immune responses, for instance by targeting them to the correct anatomical location (Josefowicz et al., 2012a).

PI3K ACTIVITY SUPRESSES THE DEVELOPMENT OF nTreg

How does the PI3K pathway affect development of nTregs in the thymus? The $p110\delta^{D910A}$ mouse, in which p110δ is inactivated by point mutation, showed increased proportions of Tregs in the thymus (Patton et al., 2006). There were more immature as well as mature thymic Tregs, suggesting that the increased $Foxp3^+$ population reflects enhanced development of $Foxp3^+$ T cells rather than accumulation of mature Treg that fail to emigrate to the periphery (Patton et al., 2006). Consistent with a negative role for PI3K in nTreg development, retroviral expression of oncogenic Akt reduced the number of nTregs (Haxhinasto et al., 2008). Treg numbers were also dramatically decreased in the thymi of mice lacking Foxo1 and Foxo3 expression in T cells, although this defect resolved as the mice aged (Kerdiles et al., 2010; Ouyang et al., 2010). Foxo transcription factors have been found to directly bind CNS1 and CNS3 regions of the *Foxp3* locus, providing a direct mechanism for their role in nTreg and iTreg development (Harada et al., 2010; Ouyang et al., 2010). By contrast, in mice lacking mTOR in T cells there was no difference in nTreg (Delgoffe et al., 2009). The simplest conclusion from these experiments is that the PI3K p110δ antagonizes nTreg development by activating Akt, leading to the exclusion of Foxo from the nucleus.

In apparent contradiction to these results, mice lacking PDK1 in T cells had reduced Treg numbers in the thymus (Park et al., 2010). It is important to appreciate, however, that PDK1 regulates multiple protein kinase C isoforms independently of PI3K and Akt (Mcmanus et al., 2004; Waugh et al., 2009); hence the reduced

numbers of thymic Treg in these mice might be a consequence of impaired c-Rel activation (which depends on PKC activity) rather than interrupted PI3K signaling.

PI3K SIGNALS CAN ENHANCE OR BLOCK PERIPHERAL CONVERSION OF NAÏVE CD4$^+$ T CELLS

We found that TGF-β1-stimulated iTreg conversion was reduced when we used the pan-PI3K inhibitor PI-103, the p110δ-selective inhibitor IC87114 or rapamycin (Patton et al., 2011); however, others have observed enhanced iTreg conversion upon addition of PI3K or mTOR inhibitors (Harada et al., 2010; Patterson et al., 2011). We do not have an explanation for these differences other than it may depend on the amount of costimulation provided in the conversion cultures, as in some cases CD28 signals can compensate for the lack of PI3K activity in T cells (Okkenhaug et al., 2002; Garcon et al., 2008; Gogishvili et al., 2008). PDK1$^{-/-}$ T cells showed reduced conversion to iTreg *in vitro* and *in vivo* (Park et al., 2010) while more iTregs developed upon stable transgenic expression of active Akt (Pierau et al., 2009). These latter studies suggest that PI3K pathway activation is required for iTreg differentiation, possibly at the level of initial activation, stabilization of Foxp3 expression, or survival of Foxp3$^+$ cells. However, TGF-β-dependent conversion was reduced upon deletion of Pten, PHLPP or Foxo transcription factors, or by retroviral expression of Akt (Haxhinasto et al., 2008; Sauer et al., 2008; Patterson et al., 2011). Therefore, very high PI3K-Akt activity may be incompatible with iTreg conversion, presumably because it would eliminate Foxo from the nucleus.

Foxp3 expression also can be induced in naïve CD4$^+$ T cells independently of TGF-β1 by removing cells from TCR stimuli 18 h after initial activation *in vitro* (Sauer et al., 2008). Interestingly, this effect could be enhanced by the addition of PI3K and mTOR inhibitors also added 18 h after activation (Sauer et al., 2008). TCR deprivation could not induce Foxp3 expression in Foxo1$^{-/-}$Foxo3$^{-/-}$ T cells, suggesting that the effect of the PI3K inhibitors depends on nuclear expression of Foxo (Ouyang et al., 2010).

PI3K AND mTOR INHIBITION HAVE OPPOSING EFFECTS ON PERIPHERAL Treg HOMEOSTASIS AND EXPANSION

Once Foxp3 is expressed, Tregs must process external cues in order to be maintained. One indicator of intact maintenance is the preservation of normal levels of Tregs under homeostatic conditions. There were 2-fold fewer peripheral Tregs in p110δ^{D910A} mice despite increased nTreg generation (Patton et al., 2006). This implies that PI3K signals are important for maintenance of Tregs. Consistent with this, Pten$^{-/-}$ Treg show enhanced proliferation in response to IL-2 and mice in which Pten was deleted in Treg (as well as activated CD4$^+$ T cells) have increased numbers of peripheral Treg (Walsh et al., 2006; Soond et al., 2012). Although Tregs with inactive p110δ proliferated normally in responses to IL-2 *in vitro* (Patton et al., 2006), it is possible that integrated signals from the TCR and IL-2R fail to support normal Treg numbers in p110δ^{D910A} mice *in vivo*. Mice with Foxo1 and Foxo3 deleted in T cells show a gradual recovery of Treg numbers with age, suggesting that Foxo may play a more important role in initial development of Tregs than in their maintenance in the periphery (Kerdiles et al., 2010).

Deprivation of mTOR signals by rapamycin or by deleting the gene encoding the mTOR catalytic subunit in T cells favors the expansion of Tregs (Battaglia et al., 2005; Delgoffe et al., 2009). Immunization with very low peptide concentrations promotes Treg differentiation and this is enhanced by rapamycin (Daniel et al., 2010). Whether mTOR inhibition actually enhances Treg expansion or gives Treg a selective growth advantage over other Th cell lineages remains a subject of debate. In a further twist, a recent study has also shown reduced expansion of Foxp3$^-$ T cells after transfer of Foxp3$^+$ T cells into lymphopenic hosts (Yurchenko et al., 2012). Whether this represented true reprogramming or these apparently converted Foxp3$^-$ T cells were derived from contaminating Foxp3$^-$ progenitors or partially differentiated Foxp3$^+$ cells could not be established conclusively.

There are a number of potential mechanisms that render Treg insensitive, or even activated, by mTOR inhibition. Tregs express high levels of the serine-threonine kinase Pim2, which shares many targets in common with mTOR (Basu et al., 2008). Prolonged treatment with rapamycin can partially inhibit phosphorylation of Akt and hence enhance nuclear retention of Foxo (Sarbassov et al., 2006). Rapamycin can also mimic the effect of amino acid deprivation which favors Treg expansion (Cobbold et al., 2009). Furthermore, in contrast to Th, Tregs depend highly on lipid oxidation rather than glycolysis. By inhibiting mTOR-dependent glycolysis in favor of lipid oxidation, rapamycin may favor the expansion of Tregs (Michalek et al., 2011; Shi et al., 2011).

Tregs HAVE MANY MECHANISMS TO SUPPRESS IMMUNE RESPONSES

Perhaps more important than the role in the development and maintenance is the question of whether PI3K signaling controls Treg-mediated suppression. Tregs provide a dominant mechanism of peripheral tolerance and hence moderate variations in their absolute numbers do not necessarily have significant impacts on their ability to prevent disease. Rather, it is the amount of Foxp3 expressed per Treg that is essential (Wan and Flavell, 2007).

Tregs employ a variety of mechanisms to suppress the immune system, and these differ whether the Tregs are suppressing immune responses elicited by self-antigens or commensal bacteria (Yamaguchi et al., 2011). Tregs can secrete suppressive cytokines such as IL-10, TGF-β, and IL-35 (Read et al., 2000; Collison et al., 2007; Rubtsov et al., 2008) which directly inhibit T cells and accessory leukocytes. IL-10 is critical in the gut, as mice with a Treg-specific deficiency in IL-10 develop inflammatory bowel disease, but not systemic autoimmunity (Rubtsov et al., 2008). Tregs constitutively express high levels of the IL-2R, and they can deprive conventional T cells of access to IL-2 (Pandiyan et al., 2007, 2011). CTLA-4 is also essential for Treg suppression, as Treg-specific CTLA-4 knockout mice succumb to a severe autoimmune syndrome (Wing et al., 2008). CTLA-4 blocks T cell activation by physically removing CD80 and CD86

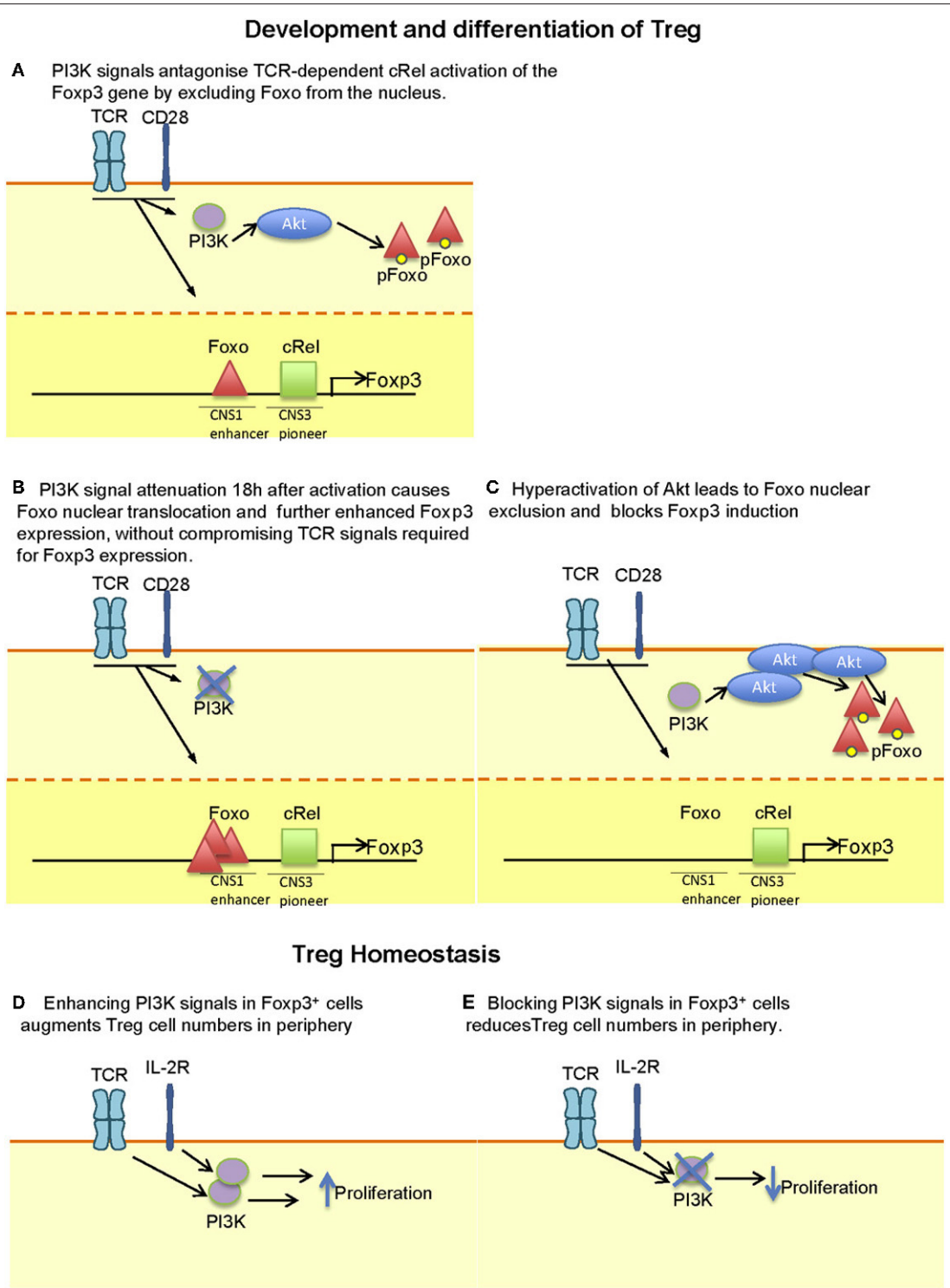

FIGURE 1 | Context-dependent effect of PI3K signaling on Treg development, differentiation and maintenance. (A) During thymic development, attenuation of PI3K signaling may be required to enhance the translocation of Foxo proteins to the nucleus where they enhance the expression of Foxp3. **(B)** The capacity of PI3K signaling to antagonize is illustrated during the *in vitro* differentiation of Foxp3$^-$ CD4$^+$ T cells to Foxp3$^+$ T cells where signal attenuation after 18 h facilitates Foxo nuclear translocation. It is worth noting that earlier inhibition may interfere with TCR-dependent signals that favor Foxp3 expression. **(C)** Hyperactivation of Akt (e.g. by expression of a membrane targeted Akt transgene) leads to Foxo nuclear exclusion and reduced Foxp3 expression. Whether such extent of Akt activation can be achieved by physiological receptor activation has yet to be determined. **(D)** Treg numbers in the periphery is controlled by both TCR and IL-2 signals. Inhibition of PI3K is likely to reduce the ability of these receptors to maintain Treg homeostasis, thus explaining the reduced number of Treg in p110δ-deficient mice. **(E)** Enhanced numbers of Treg are found upon deletion of Pten or Ship in T cells in which PI3K signaling is enhanced, probably reflecting enhanced IL-2-dependent homeostatic expansion.

from dendritic cells, thus depriving effector T cells of costimulation (Yokosuka et al., 2010; Qureshi et al., 2011). CTLA-4 binding can also instruct dendritic cells to release indoleamine 2,3-dioxygenase, which produces pro-apoptotic kyneurenines and deprives proliferating T cells of the tryptophan needed for growth (Grohmann et al., 2002). Tregs can transfer the inhibitory second messenger cAMP via gap junctions (Bopp et al., 2007), alter levels of extracellular nucleotides using CD38, CD39, and CD73 (Chen et al., 2006; Deaglio et al., 2007; Hubert et al., 2010), and kill activated leukocytes through the release of perforin or granzyme (Cao et al., 2007; Boissonnas et al., 2010). However, no single mechanism has been identified which either is unique to Tregs or which accounts for all aspects of Treg-mediated suppression (Yamaguchi et al., 2011; Josefowicz et al., 2012a).

PI3K PROMOTES Treg-MEDIATED SUPPRESSION

p110δ^{D910A} Tregs produce less IL-10 and show reduced suppression of $CD4^+$ T cell proliferation *in vitro* (Patton et al., 2006, 2011). The reduced ability of p110δ-deficient Treg to supress was correlated with lower expression of CD38, a marker of a highly suppressive Treg population in the gut, suggesting that "effector" Tregs may not develop normally in p110δ^{D910A} mice (Cretney et al., 2011; Patton et al., 2011). Tregs from p110δ^{D910A} cannot block the development of experimental colitis induced by transfer of naïve T cells in Rag knockout mice (Patton et al., 2006). Consistent with this, p110δ^{D910A} mice spontaneously develop colitis of similar severity to that observed in IL-10-deficient mice (Okkenhaug et al., 2002; Uno et al., 2010). IL-$10^{-/-}$p110δ^{D910A} double deficient mice develop a more severe form of colitis (Uno et al., 2010), suggesting that the disease caused by p110δ-deficiency is not only a consequence of impaired IL-10 production. $PDK1^{-/-}$ Tregs had multiple functional defects that resulted in the failure to suppress γδ T cell-dependent colitis, again suggesting a key role for PI3K signaling in suppression of gut-associated inflammation (Park et al., 2010). Mice lacking p85 regulatory subunits in T cells develop Sjogrens syndrome, perhaps also as a consequence of impaired Treg function, although this mechanism was not examined directly (Oak et al., 2006). In the context of infection, p110δ^{D910A} mice showed a surprising increased ability to eliminate *Leishmania* parasites despite mounting a reduced Th1 response. This was explained, at least in part, by reduced Treg expansion and/or function in p110δ^{D910A} mice as the transfer of Treg into p110δ^{D910A} mice reversed their resistance to *Leishmania* (Liu et al., 2009). It should be noted that so far, no evidence of autoimmunity has been described in PI3K-deficient mice, suggesting that defective Treg function in these mice primarily affect responses to commensal or pathogenic organisms.

Both $SHIP^{-/-}$ and $Pten^{-/-}$ Treg suppressed normally *in vitro* and *in vivo* suggesting that moderately increased levels of PI3K activity is compatible with normal Treg function (Kashiwada et al., 2006; Walsh et al., 2006; Collazo et al., 2009; Locke et al., 2009; Patterson et al., 2011; Soond et al., 2012). However, Tregs overexpressing active Akt or in which the Akt-phosphatase PHLPP were deleted showed reduced capacity to suppress $CD4^+$ T cell proliferation (Crellin et al., 2007; Patterson et al., 2011). Thus, some PI3K activity is required for optimal Treg-mediated suppression, but very high Akt activity can inhibit Treg suppression.

CONCLUSIONS

Does the PI3K pathway promote or antagonize regulatory T cell development and function? The answer seems to be: both.

The findings that the *Foxp3* gene contains Foxo-binding elements in its promoter region, along with the observation that PI3K inhibitors added 18 h after activation could be used to induce Treg differentiation raised the possibility that PI3K inhibitors could be used to enhance Treg induction *in vivo* (Bruno and Merkenschlager, 2008; Ohkura et al., 2011). However, inhibition of PI3K signaling in mice does not lead to increased numbers of peripheral Treg, even when deleted after activation using OX40-Cre (Rolf et al., 2010). Moreover, the evidence for enhanced Treg proportions or numbers in patients taking rapamycin or its analogs is lacking, despite the demonstrated property of rapamycin to favor the expansion of established Treg *in vitro* and *in vivo* (Zuber et al., 2011). **Figure 1** illustrates how PI3K signaling can both antagonize and augment Treg numbers, depending on the stage of differentiation, timing, and extent of activation/inactivation.

The development of colitis and Sjogren's syndrome in mice with chronic inhibition of the PI3K pathway suggests that, on balance, inhibition of PI3K is more likely to inhibit Treg function than enhance it. It is therefore unlikely that patients who are administered p110δ-selective inhibitors would have enhanced Treg function. Whether impairment in Treg function or homeostasis leading to a clinical manifestation will be a significant detrimental side effect in patients who are administered PI3K inhibitors is not yet clear; however, serious side effects have not been reported in initial clinical trials so far (Furman et al., 2010; Fruman and Rommel, 2011). Indeed, protective effects of p110δ inhibition in mouse models of asthma, multiple sclerosis, arthritis, and lupus suggest that blockade of effector T cells dominates (Nashed et al., 2007; Durand et al., 2009; Haylock-Jacobs et al., 2011). A number of pharmaceutical companies are also developing p110δ or p110γ inhibitors to treat leukemia or autoimmune diseases (Fruman and Rommel, 2011; Norman, 2011; So and Fruman, 2012). The first publically available results from clinical trials using p110δ inhibitors suggest a remarkable response rate in patients with chronic lymphocytic leukemia, nearly all of whom showed reduced lymph node size after treatments in phase I trials (Furman et al., 2010). Could inhibition of Treg by p110δ inhibitors be exploited therapeutically? One positive outcome of reduced Treg function was shown in p110δ^{D910A} mice which were resistant to infection with *Leishmania* (Liu et al., 2009). A recent study suggested a beneficial effect of PI3K inhibitors as adjuvants to cancer vaccines (Marshall et al., 2012). We are currently exploring whether p110δ-inhibition of Treg function could be used to enhance anti-tumor responses. In summary, inhibiting PI3K can facilitate the differentiation of Treg *in vitro*, but *in vivo*, the net results of PI3K inhibition is fewer Tregs with reduced, but not abolished, suppressive capacity.

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Regulation of T cell homeostasis and responses by *Pten*

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The generation of lipid products catalyzed by PI3K is critical for normal T cell homeostasis and a productive immune response. PI3K can be activated in response to antigen receptor, co-stimulatory, cytokine, and chemokine signals. Moreover, dysregulation of this pathway frequently occurs in T cell lymphomas and is implicated in lymphoproliferative autoimmune disease. Akt acts as a central mediator of PI3K signals, downstream of which is the mTOR pathway, controlling cell growth and metabolism. Members of the Foxo family of transcription factors are also regulated by Akt, thus linking control over homing and migration of T cells, as well cell cycle entry, apoptosis, and DNA damage and oxidative stress responses, to PI3K signaling. PTEN, first identified as a tumor suppressor gene, encodes a lipid phosphatase that, by catalyzing the reverse of the PI3K "reaction," directly opposes PI3K signaling. However, PTEN may have other functions as well, and recent reports have suggested roles for PTEN as a tumor suppressor independent of its effects on PI3K signaling. Through the use of models in which *Pten* is deleted specifically in T cells, it is becoming increasingly clear that control over autoimmunity and lymphomagenesis by PTEN involves multi-faceted functions of this molecule at multiple stages within the T cell compartment.

Keywords: Pten, T cells, autoimmunity, lymphoma

INTRODUCTION

Class 1A PI3Ks are directly linked to lymphocyte activation mainly through receptor tyrosine kinases, such as the antigen and cytokine receptors (Engelman et al., 2006; Huang and Sauer, 2010; So and Fruman, 2012). Class 1A PI3Ks, hereafter referred to as PI3K, are comprised of a catalytic subunit of 110 kDa (of which there are three isoforms) that generates phosphatidylinositol 3,4,5 phosphate (PIP3) from its main substrate phosphatidylinositol 4,5 phosphate (PIP2), and a regulatory subunit (of which there are five isoforms). *Pten* encodes a protein with a lipid phosphatase function that directly opposes PI3K signaling by dephosphorylating PIP3 at the 3′ position to generate PIP2. Cells lacking PTEN have elevated levels of PIP3 and constitutive activation of PI3K signaling pathways (Stambolic et al., 1998; Cantley and Neel, 1999). With increasing age, mice heterozygous for *Pten* develop T cell lymphomas and cancers in multiple tissues, and develop a lethal polyclonal autoimmune disorder, similar to that seen in Fas-deficient mice (Di Cristofano et al., 1998, 1999; Podsypanina et al., 1999; Suzuki et al., 2001). Germline mutations in *PTEN* occur in a group of autosomal dominant syndromes known as the PTEN hamartoma tumor syndromes, which include Cowden syndrome, Proteus syndrome, Proteus-like syndrome, and Bannayan–Riley–Ruvalcaba syndrome, demonstrating the importance of PTEN as a tumor suppressor. Consistent with this, genomic amplification and mutation of either PI3K or Akt has been reported in a large number of cancers as well (Samuels et al., 2004; Lee et al., 2005; Carpten et al., 2007; Jaiswal et al., 2009), although the role of PTEN as a tumor suppressor is now believed to involve more than its ability to oppose PI3K signaling. While disruption of regulation by PTEN has the overt phenotype of cancer progression, PTEN also plays an important role in maintaining T cell tolerance at multiple stages within the T cell compartment.

ACTIVATION AND REGULATION OF PI3K CLASS 1A IN T LYMPHOCYTES

Upon ligation of the T cell receptor (TCR) in the presence of co-stimulatory molecules, PI3K recruitment and activation leads to the production of lipid products which in turn recruit downstream PH-domain containing targets such as PDK1 and Akt (see **Figure 1**). Ultimately, these events lead to activation of the mTOR pathway and inactivation of members of the Foxo family, inducing growth and proliferation of T cells and acquisition of effector function (for review, see Engelman et al., 2006; Finlay and Cantrell, 2010; So and Fruman, 2012). In the absence of PTEN, TCR stimulation alone results in hyperactivation of the PI3K pathway, resulting in effective cytokine production and proliferation independent of co-stimulation (Buckler et al., 2006). Thus, put another way, negative regulation of PI3K signaling by PTEN enforces the requirement for co-stimulation in naïve T cells. However, the notion that PI3K signaling is required for co-stimulation to mediate its effects has been challenged by the generation of mice which lack all isoforms of the regulatory subunit of class 1A PI3K in T cells (Deane et al., 2007). T cells from these mice are able to proliferate under co-stimulatory conditions in the absence of detectable Akt signaling. These mice also maintained a normal anti-viral response upon MHV infection, although *in vivo* T helper function to B cell antibody responses was impaired. Whether the relative lack of defects is due to the fact that lack of PI3K was genetic, rather than acquired, is not known. However, these studies indicate that potential therapies targeting PI3K for inflammatory diseases and cancer may not compromise all aspects of cellular immunity, and further suggest that PI3K signaling has specialized functions in the context of T cell activation.

PI3K activation is critical for optimal responses of T cells to cytokines which utilize the common gamma chain. For example,

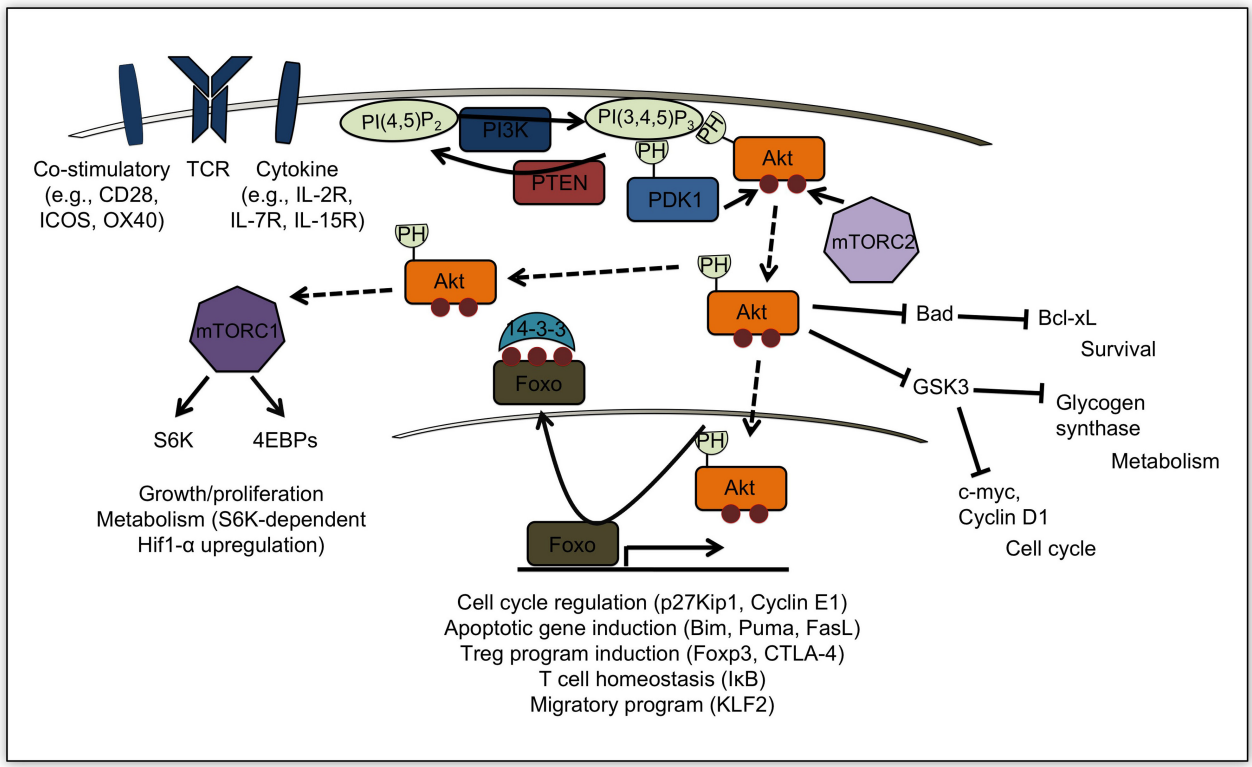

FIGURE 1 | PTEN/PI3K signaling in mature T cells. Class 1A PI3Ks are activated by receptor tyrosine kinases that drive co-stimulatory, T cell receptor (TCR), and cytokine signaling pathways. PTEN directly opposes PI3K signaling by converting PI(3,4,5)P3 to PI(4,5)P2. Through their PH-domains, PDK1 and Akt bind PIP3, enabling Akt to be phosphorylated by PDK1 on Ser308. Full activation of Akt requires phosphorylation on Ser473 by mTORC2. Akt can then target downstream substrates such as Foxos in the nucleus, leading to their inactivation and export from the nucleus. This results in attenuation of a Foxo-dependent gene program normally acting to promote pathways involved in, but not limited to, T cell homeostasis (in resting T cells), apoptosis (upon, for example, cytokine withdrawal), Treg induction, and homing to secondary lymphoid organs (in circulating, naïve T cells). Akt also targets TSC2 and Pras40 (not shown), negative regulators of mTORC1, allowing for activation of mTORC1. Activation of S6K and 4EBPs by mTORC1 results in increased protein translation, allowing growth and proliferation, and upregulation of Hif-1α, important for glycolytic metabolism. Inhibitory targeting of Bad and GSK3 also contribute to Akt's effect on cell cycle, survival and metabolism.

engagement of the IL-2R on activated T cells results in activation of the Jak/Stat pathway, as well as the PI3K and MAPK pathways, and the combination of these signals is required for the observed proliferation and cell survival in response to IL-2. Thus, appropriate responses to TCR ligation and cytokines require downregulation of PTEN, which is constitutively expressed in naïve T cells. This normally occurs as a consequence of TCR stimulation itself, which terminates detectable PTEN expression within 24–48 h. The importance of this is demonstrated by two findings. First, retroviral mediated "enforced" expression of PTEN renders activated IL-2R$^+$ T cells unable to fully respond to IL-2 stimulation. Second, regulatory T cells, which normally do not divide in response to IL-2 alone, have complete responses and signaling to IL-2 restored solely by genetic ablation of *Pten* (Bensinger et al., 2004; Walsh et al., 2006; Locke et al., 2009). This demonstrates that control of PI3K through PTEN plays an important role not only in modulating the degree of activation signals within lymphocytes, but in maintaining traits of a specific lineage.

PI3K-related signals can modulate ongoing responses as well. Deletion of *Pten*, or expression of constitutively active myristoylated-Akt, within the CD8$^+$ T cell compartment inhibits the development and survival of memory CD8$^+$ T cells (Hand et al., 2010). On the contrary, Akt is required for a transcriptional program leading to upregulation of cytolytic effector, chemokine, and adhesion molecules (Macintyre et al., 2011). Conditional deletion of *Pten* using OX40-Cre has demonstrated that PTEN plays a critical role in limiting the expansion of Tfh cells and in maintaining control over GC reactions, indicating that T cell intrinsic roles of PTEN are crucial for maintaining global levels of tolerance (Rolf et al., 2010). Lastly, the promotion of iTreg generation and maintenance by PD-L1 is associated with downregulation of Akt signaling and concomitant upregulation of PTEN expression (Francisco et al., 2009). These examples all provide evidence that the balance of Akt signaling within multiple stages of development and differentiation states helps determine cellular fate.

PTEN also has been shown to play an important role in central tolerance and in regulating proliferation of developing cells in the thymus (Suzuki et al., 2001; Hagenbeek et al., 2004). In the absence of IL-7R and pre-TCR signaling, loss of Pten allows cells to bypass the β-selection checkpoint, indicating that PI3K signaling is crucial

for the ongoing development of early T cell precursors. Interestingly, thymic cellularity and subset percentages are relatively unperturbed prior to tumor development, which likely occurs at the DP stage during thymic maturation, suggesting that secondary events emerge in this context of Pten loss to promote transformation (discussed below; Hagenbeek et al., 2004; Hagenbeek and Spits, 2008; Xue et al., 2008; Guo et al., 2011).

SIGNALING DOWNSTREAM OF Pten-PI3K-Akt: A CRITICAL ROLE FOR Foxos

Members of the Foxo family of transcription factors play a critical role in DNA damage and oxidative stress responses (Tran et al., 2002; Miyamoto et al., 2007; Tothova et al., 2007; Choi et al., 2009) and in preventing entry into cell cycle (Medema et al., 2000), acting as bona fide tumor suppressors as demonstrated in hematopoietic and epithelial tissues (Coffer, 2003; Accili and Arden, 2004; Paik et al., 2007). It is becoming increasingly appreciated that Foxos play important roles within the T cell compartment as well, regulating homing (Fabre et al., 2008; Sinclair et al., 2008; Finlay et al., 2009; Kerdiles et al., 2009; Finlay and Cantrell, 2010), survival (Hedrick, 2009; Kerdiles et al., 2009; Dejean et al., 2011), and the development and function of effector and memory subsets (Kerdiles et al., 2010; Ouyang et al., 2010; Rao et al., 2012). In quiescent cells, Foxos are active within the nucleus, where they maintain the pattern of chemokine and adhesion molecule expression, and expression of the IL-7R, needed for the migration and survival of circulating T cells (Fabre et al., 2008; Finlay et al., 2009; Kerdiles et al., 2010). Akt phosphorylation on Ser473, mediated by mTORC2, is critical for Foxo inactivation, implicating mTOR in control over Foxos, and placing PTEN upstream in this pathway. Importantly, mTORC2 has been shown to be critical for the phenotype in models of PTEN loss. Prostate cancer caused by conditional *Pten* deletion in prostate epithelium requires mTORC2, and deletion of one copy of *Rictor*, required for mTORC2 complex assembly and activity, was sufficient to protect *Pten* heterozygous mice from prostate cancer (Guertin et al., 2009).

The role of Foxos in T cells was first described using mice in which retroviral gene-trap targeting of embryonic stem cells was used to generate a null *Foxo3* allele, although the authors could not rule out the possibility that undetectable levels of a truncated form of Foxo3 was produced (Lin et al., 2004). These mice exhibited spontaneous lymphoproliferation and multi-organ lymphocyte infiltration, indicating that Foxo3 is important for the control of T cell tolerance and homeostasis. This hyper-activated phenotype was correlated with decreased expression of IκBε and IκBβ, leading to increased NF-κB activation. As IKKβ has been shown to phosphorylate and inactivate Foxos (Hu et al., 2004), this implicates Foxos in a critical negative feedback loop that may serve to limit inflammatory responses in certain settings, and is potentially important in the context of tumors which maintain low levels of Akt activation. Other studies using *Foxo3*-deficient strains did not find the same immunological defects (Hosaka et al., 2004; Dejean et al., 2009), attesting first to the redundancy of members of this family and, second, to the possibility of dominant-negative effects of undetected Foxo3 gene products in the aforementioned mice. T cell-specific loss of Foxo1 was shown to severely disrupt T cell homeostasis, resulting in multi-organ lymphocyte infiltration as well as exocrine pancreatitis and hind limb paralysis (Kerdiles et al., 2010). This was attributed to a defect in both the development and function of $Foxp3^+$ regulatory T cells, and this phenotype was exacerbated by combined deletion of *Foxo1* and *Foxo3* (Kerdiles et al., 2010; Ouyang et al., 2010). More recently, Foxo3a has been mechanistically linked to anergy induction in T cells through upregulation of Sirt1 (Gao et al., 2012). IL-2 was shown to reverse T cell anergy through the PI3K pathway by inactivation of Foxos, which prevented transcriptional upregulation of Sirt1.

In mouse embryonic fibroblasts and lymphoid cells, growth factor and cytokine withdrawal upregulates BH3-only pro-apoptotic mediators Puma and Bim in a Foxo3a-dependent manner that requires downregulation of PI3K/Akt signaling (You et al., 2006). These findings strongly support the idea that impaired downregulation of PI3K activity, as seen in T cells lacking PTEN, results in survival effects and apoptotic resistance in response to cytokine deprivation through inactivation of Foxo family members. FasL has been shown to be another important target of Foxo (Brunet et al., 1999). Given that *Pten* heterozygous mice display decreased sensitivity to Fas-mediated AICD, similar to *lpr* and *gld* mice (Van Parijs and Abbas, 1996), constitutive inactivation of Foxos due to loss of PTEN is likely a partial mechanism underlying the phenotype observed in these mice. Additionally, the finding that a deficiency in Foxos is sufficient to drive the development of hemangiomas, similar to what is seen in patients with Cowden disease and Bannayan–Zonana syndrome, and thymic lymphomas, similar to mice with a T cell-specific deletion of *Pten*, as well suggests that inactivation of Foxos may be critical for the phenotype brought on by loss of *Pten* (Paik et al., 2007).

LYMPHOMA AND DYSREGULATED IMMUNE FUNCTION IN THE ABSENCE OF PTEN

Genetic studies in mice expressing conditional alleles of *Pten* have been crucial for studying the tissue specific role of PTEN in tumorigenesis. Deletion of *Pten* during hematopoiesis using Mx-1-Cre results in myeloproliferative disease and transplantable leukemia, and has shown that PTEN is required for maintaining the hematopoietic stem cell (HSC) compartment (Yilmaz et al., 2006; Lee et al., 2010). Similar to the Mx-1-Cre model of PTEN loss, Vec-Cre-mediated PTEN loss, in which nearly 40% of fetal liver HSCs were subject to deletion of *Pten*, led to impaired HSC self-renewal and the development of a myeloproliferative disorder followed by leukemia (Guo et al., 2008). It is notable that this phenotype shares striking similarity to mice in which all six alleles of *Foxo1/3a/4* were deleted by Mx-1-Cre (Tothova et al., 2007), and to $Foxo3a^{-/-}$ mice (Miyamoto et al., 2007). Importantly, this study reported the same t(14;15) chromosomal translocation (seen within a subset of human T-ALL), in all blast-crisis samples analyzed from these mice, suggesting a critical genomic destabilizing event potentially independent of PTEN's role in controlling PI3K/Akt signaling. Similarly, this translocation, involving the *c-myc* and *TCRα/δ* loci and resulting in constitutively high levels of c-myc, was found to recur with 100% incidence in T cell lymphomas from mice in which *Pten* was deleted specifically

in T cells (CD4-Cre × $Pten^{fl/fl}$ mice, hereafter referred to as PTEN-ΔT mice; Liu et al., 2010). Thus, well after T lineage commitment in mice subject to lymphoma and lymphoproliferative autoimmune disease, PTEN is critical to prevent the emergence of the same genetic abnormality present in HSCs that correlates with the selective pressure of these cells for the development of leukemia.

A deficiency in *Rag1* or *TCRα* prevented the t(14;15) translocation event in PTEN-ΔT mice, but did not inhibit malignant transformation, although lymphomagenesis developed with delayed onset and was primarily restricted to the thymus (Liu et al., 2010). Interestingly, mature T cells from 3-week-old PTEN-ΔT mice that had not undergone transformation, when transferred into either immunocompetent or immunoincompetent recipients, did not develop a malignant phenotype throughout the duration of the host's life, suggesting that malignancy arises within the thymus after a period of latency in these mice, consistent with an earlier report demonstrating that transplanted $Pten^{-/-}$ thymocytes gave rise to T cell lymphomas in immunodeficient recipients (Hagenbeek and Spits, 2008). Supporting this, PTEN-ΔT mice thymectomized at 3 weeks of age did not develop lymphoma, although later in life these mice exhibited signs of systemic autoimmunity. Thus, within distinct developmental stages, PTEN is required for the prevention of lymphoma and autoimmunity.

In the context of DNA damage, loss of PTEN enables cells to bypass the normal G2/M checkpoint enforced by CHK1 as a result of Akt-mediated CHK1 sequestration in the cytoplasm (Puc et al., 2005). Akt has also been shown to promote prosurvival responses following DNA double-strand breaks (DSBs; Bozulic et al., 2008). It is possible that secondary mutations or other genetic alterations occurred in $Rag1^{-/-}$ or $TCR\alpha^{-/-}$ × PTEN-ΔT mice as a result of genetic instability due to PTEN loss, and that hyper-active Akt maintains the survival of these cells that would normally undergo apoptosis. Spectral karyotyping analyses, however, failed to detect any chromosomal translocations in these mice to support this idea. Induction of the Notch pathway in malignant cells from these mice, however, was shown to increase cellular levels of c-myc, suggesting that PI3K and c-myc cooperate in multiple models of PTEN loss to promote lymphomagenesis and lymphoproliferative autoimmune disorder.

Disruption of Foxo function has been shown to accelerate c-myc-driven lymphomagenesis (Bouchard et al., 2007), and constitutively active mutants of Foxo3a resistant to inactivation by Akt directly repress multiple target genes of c-myc and block c-myc-dependent proliferation and transformation (Bouchard et al., 2004; Jensen et al., 2011). Together, this offers the possibility that c-myc and PI3K cooperate in tumorigenesis through inactivation of Foxos. Whether or not restoration of Foxo signaling in mice lacking PTEN in T cells has an effect on metabolism, survival, infiltrative capacity, lymphomagenesis, or the prevention of autoimmunity remains to be seen.

A recent study in which $Pten^{fl/fl}$ × CD4-Cre mice were crossed to $PDK1^{fl/fl}$ mice has shown that while lymphoma did not develop in this model of T cell-specific *Pten* deletion, PDK1/Akt signaling was dispensable for the survival, proliferation and differentiation of T cell progenitors, and *in vivo* expansion of peripheral T cells (Finlay et al., 2009). The mechanism for control of cell metabolism independent of PDK1 is unclear in these mice, although thymocyte proliferation in the absence of PTEN was shown to require RhoA-dependent pathways, and a dependence on c-myc, which was recently shown to play a pivotal role in T cell metabolism following activation (Wang et al., 2011), was not ruled out. Mice which express a single hypomorphic *PDK1* allele similarly prevented a wide range of tumors when crossed to *Pten* heterozygous mice, indicating that the requirement for PDK1 in the context of tumor formation brought on by loss of PTEN is not limited to its role in controlling migratory capacity of T cells (Bayascas et al., 2005). Additionally, deletion of S6K1 in the Mx-1-Cre model of Pten loss resulted in delayed development of leukemia, indicating that an mTORC1-mediated pathway, in this context shown to involve induction of a Hif-1α-dependent glycolytic program, contributes to leukemogenesis in Pten-deficient cells (Tandon et al., 2011).

Akt-INDEPENDENT ROLES OF PTEN

PTEN nuclear function, independent of its lipid phosphatase activity, has been shown to contribute to its tumor-suppressive effects and, in particular, in maintaining genomic stability. Control of DNA stability and DSB repair has in part been attributed to interaction of PTEN with an integral kinetochore protein, CENP-C. Mutants originally identified in Cowden disease patients demonstrated that the C-terminus of PTEN, but not the phosphatase domain, was required for interaction with centromeres and with CENP-C, and that DNA instability and DSBs could be prevented by a phosphatase-dead PTEN mutant which retained its ability to bind to CENP-C (Shen et al., 2007). Additionally, nuclear exclusion of PTEN, but not a phosphatase-inactive mutant, was shown to impair the tumor-suppressive APC-CDH1 nuclear complex, again demonstrating tumor-suppressive capability of catalytically inactive PTEN (Song et al., 2011). Additional protein–protein interactions within the nucleus, independent of PTEN catalytic activity, have been shown to increase the activity and stability of p53 (Li et al., 2006; Salmena et al., 2008), and upregulate expression of Rad51, an essential component of the DSB repair machinery (Baker, 2007; Shen et al., 2007). A lysine to glutamate mutation in PTEN (K289E) identified in a Cowden syndrome family, which lead to a dramatic decrease in nuclear import without disrupting phosphatase activity or membrane localization of PTEN, as well strongly supports the idea that nuclear PTEN can be critical for tumor suppression independent of its role in opposing PI3K signaling in certain contexts (Trotman et al., 2007; See **Figure 2**).

Inactivation of PTEN phosphatase activity is sufficient to abrogate its tumor-suppressive effects, emphasizing the importance of PTEN's role in downregulation of PI3K signaling. This has been demonstrated by a subset of Cowden disease patients that harbor a missense mutation at a cysteine residue (C124) critical for phosphatase activity. A C124S mutant has been shown to form a stable complex with PIP3, potentially protecting it from dephosphorylation by other lipid phosphatases to account for higher cellular levels of PIP3 compared to conditions in which PTEN is completely absent (Myers et al., 1998). As well, the importance of Akt activity in tumor development induced by loss of PTEN has been reported in a number of studies (Stiles et al., 2002;

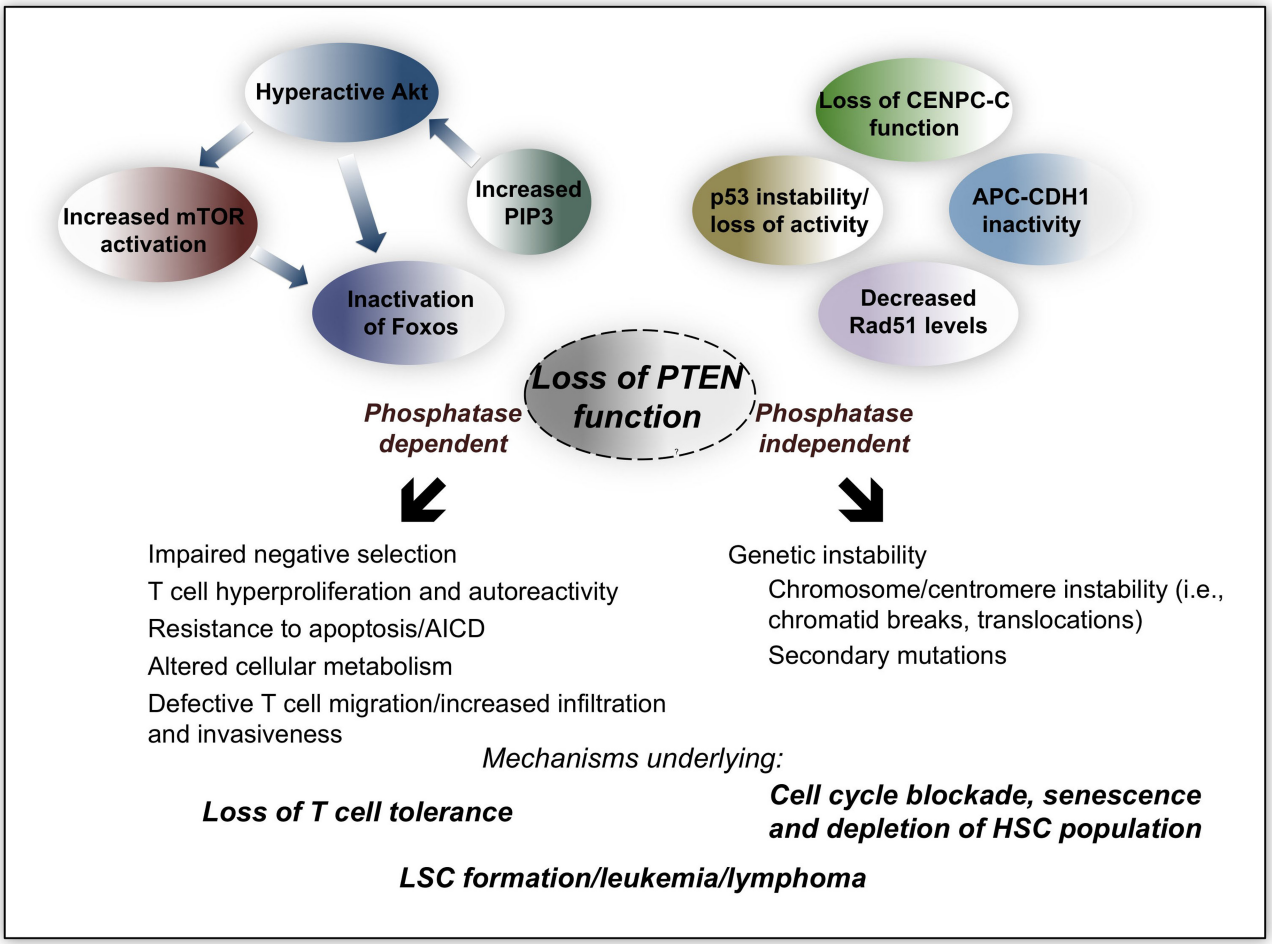

FIGURE 2 | Molecular and phenotypic consequences of loss of PTEN phosphatase-dependent and -independent functions during hematopoiesis and within the T cell compartment. Loss of PTEN phosphatase activity results in increased levels of PIP3, causing hyperactivation of the Akt pathway and subsequent activation of the mTOR pathway, and inactivation of the Foxo family of transcription factors. Disruption of PTEN phosphatase-independent function accounts for loss of much of its nuclear activity, compromising genetic stability and DSB repair, leading to chromatid breaks and translocations. That mature T cells do not undergo transformation, and that lymphoma in T cell-specific PTEN knockout mice is of thymic origin, suggests that PTEN phosphatase-independent function contributes less to the phenotype at the mature T cell stage, and that loss of T cell tolerance can likely be attributed to loss of PTEN phosphatase activity. Whether depletion of hematopoietic stem cells (HSCs) and development of leukemia stem cells (LSCs), as well lymphomagenesis, depends on the absence of PTEN phosphatase activity (which likely maintains survival of genetically aberrant cells), PTEN phosphatase-independent activity (promoting genetic instability), or both, remains an open question.

Bayascas et al., 2005; Chen et al., 2006). Constitutive Akt activation in T cells and thymocytes has been shown to be sufficient to drive autoimmunity and lymphoma (Rathmell et al., 2003) and thymomas (Malstrom et al., 2001), respectively, and bone marrow chimera experiments have demonstrated that enforced expression of constitutively active Akt in HSCs was sufficient for the development of myeloproliferative disease, T cell lymphoma, or AML (Kharas et al., 2010).

PTEN-ΔT mice crossed onto the *Rag1*$^{-/-}$ background, which do not harbor the t(14;15) translocation, potentially serve as an example in which Akt signaling is not sufficient to drive cancer progression (Liu et al., 2010). *In vitro* studies using malignant cells from these mice demonstrated a dependence on Notch, suggesting that Akt at the very least must cooperate with other oncogenic pathways to allow tumor growth in this model, similar to what has been suggested in an MMTV-myrAkt breast cancer model with respect to tumorigenesis induced by loss of PTEN (Blanco-Aparicio et al., 2007). In contrast to this, in a model of Notch-induced tumorigenesis, genetic loss of *Pten* induced an oncogene addiction switch that rendered T-ALL cells resistant to Notch inhibition through GSIs and dependent on PI3K/Akt signaling to maintain tumor growth (Palomero et al., 2007). Thus, the reliance on Akt signaling differs among models of PTEN loss. This idea is perhaps emphasized by the observation that PTEN-ΔT mice, regardless of whether the t(14;15) translocation event occurs, maintain constitutively high levels of c-myc.

The reliance on c-myc in the context of PTEN loss remains an important question. In tumors driven by inducible c-myc activation in a zebrafish model of T-ALL, constitutive activation of the Akt pathway through genetic disruption of *Pten* or transgenic

expression of Akt2 rendered cells in this model independent of c-myc for tumor progression (Gutierrez et al., 2011). Conversely, conditional genetic disruption of both *Pten* and *c-myc* in mice, while still subject to myeloproliferative and lymphoproliferative disorders, prevented the development of hematopoietic malignancies, highlighting an important dependence on c-myc in this context of PTEN loss (Zhang et al., 2011). Whether loss of PTEN allows the survival of cells that, through other mechanisms, have acquired genetic aberrations, such as translocations resulting in constitutive c-myc, or whether PTEN, independent of its role in PI3K downregulation, prevents genetic instability that collaborates with dysregulation of PI3K to allow cancer progression, remains an open question that needs to be addressed in a context-dependent manner, with consideration of what function of PTEN is lost, changes in levels of expression, and in which tissues abnormal growth originates.

FUTURE DIRECTIONS

Pten is the one of the most frequently mutated or lost genes in human cancer. Given the emerging roles of PI3K in immune system regulation, it is possible that PTEN is also playing a prominent role in the prevention of autoimmune disease and inflammatory/lymphoproliferative syndromes. The finding that PTEN did not play a role in lymphomagenesis in mature T cells from mice which lack PTEN specifically in T cells suggested that PTEN has a specific function in preventing lymphoma within a distinct time frame in this compartment. That these non-malignant T cells were able to provoke autoimmunity demonstrates that well beyond the stage at which Pten is required for prevention of lymphomagenesis in this model, PTEN plays a critical role in the maintenance of T cell tolerance. It will be important to determine which biochemical requirements PTEN fulfills in each stage of protection, and whether these requirements are distinct within different stages from cells of the same lineage, or whether lack of particular features leads to different outcomes depending on developmental stage. Given the importance of PI3K signaling in both normal immunity and the development of cancer, it will be interesting to see how closely PTEN's role as a lipid phosphatase is tied to prevention of disease, and how integral PI3K signaling remains throughout disease progression. Uncovering the specific biochemical functions of PTEN within these contexts will be key to the development of targeted therapies for the prevention and treatment of T cell malignancies and autoimmune disease.

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All PI3Kinase signaling is not mTOR: dissecting mTOR-dependent and independent signaling pathways in T cells

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The mechanistic target of rapamycin (mTOR) is emerging as playing a central role in regulating T cell activation, differentiation, and function. mTOR integrates diverse signals from the immune microenvironment to shape the outcome of T cell receptor (TCR) antigen recognition. Phosphatidylinositol 3-kinase (PI3K) enzymes are critical mediators of T cell activation through their generation of the second messenger phosphatidylinositol (3,4,5) triphosphate (PIP3). Indeed, PIP3 generation results in the activation of Protein Kinase B (PKB, also known as AKT), a key activator of mTOR. However, recent genetic studies have demonstrated inconsistencies between PI3K disruption and loss of mTOR expression with regard to the regulation of effector and regulatory T cell homeostasis and function. In this review, we focus on how PI3K activation directs mature CD4 T cell activation and effector function by pathways dependent on and independent of mTOR signaling. Importantly, what has become clear is that targeting both mTOR-dependent and mTOR-independent PI3K-induced signaling distally affords the opportunity for more selective regulation of T cell differentiation and function.

Keywords: mTOR pathway, PI3K, CD4 T cells, effector function, tolerance

INTRODUCTION

The Class IA phosphatidylinositol 3-kinase (PI3K) family consists of a heterodimeric complex of one of the 110-kDa catalytic subunits (p110α, β, δ) with a regulatory subunit (p85α, p55α, p50α, p85β, and p55γ), reviewed in Kane and Weiss (2003); Okkenhaug and Vanhaesebroeck (2003). Catalytic subunits are each encoded by separate genes, while the *pik3r1* gene generates p85α, p55α, and p50α from alternative promoters and the *pik3r2* and *pik3r3* genes encode p85β and p55γ, respectively. Details of receptor interactions with PI3Ks have recently been reviewed elsewhere (Okkenhaug and Fruman, 2010). Co-localization of catalytic subunits with regulatory subunits permits juxtaposition of catalytic subunits to the cell membrane in response to receptor ligation, increasing the local synthesis of phosphatidylinositol (3,4,5) triphosphate (PIP3). The p110α and β subunits are ubiquitously expressed while p110δ expression is restricted to hematopoietic cells. The Class IB PI3 Kinase family consists of a complex of the p110γ catalytic subunit and either the p101 or p84 regulatory subunits. This complex interacts with G-protein coupled receptors including chemokine receptors via binding to Gβ and γ regulatory proteins. Like p110δ, p110γ expression is restricted to hematopoietic cells. Accumulation of the PIP3 signaling intermediate is opposed by phosphatase and tensin homolog (PTEN) that converts PIP3 back to phosphatidylinositol (4,5) bisphosphate and Src homology 2 domain-containing inositol phosphatase (SHIP)1 and SHIP2 that hydrolyze PIP3 to phosphatidylinositol (3,4) bisphosphate (Okkenhaug and Fruman, 2010).

PIP3 acts as a second messenger to mediate downstream signaling by recruitment of pleckstrin homology (PH) domain containing proteins that bind to the high local concentrations of PIP3 generated by PI3Ks at the inner leaflet of the plasma membrane. Examples include the Tec family of tyrosine kinases that mediate signals to phospholipase C-γ (PLCγ), the 3-phosphoinositide-dependent protein kinase 1 (PDK1), and Protein Kinase B (PKB), also known as AKT [reviewed in Kane and Weiss (2003)]. Members of the Vav family of guanine nucleotide exchange factors that regulate cellular motility may also be recruited via PIP3 binding, although some data suggest recruitment may be indirect via other protein interactions. AKT binding to PIP3 induces a conformational change that renders it accessible to phosphorylation at residue T308 by PDK1 co-localized at the plasma membrane, resulting in activation of AKT serine/threonine kinase activity (Stokoe et al., 1997; Currie et al., 1999; Milburn et al., 2003). One critical substrate for activated AKT is tuberous sclerosis complex (TSC)-2. TSC2 functions with TSC1 as a GTPase activating complex for Ras homolog enriched in brain (Rheb). TSC2 is inactivated by phosphorylation, resulting in accumulation of GTP-bound Rheb that activates the mechanistic target of rapamycin (mTOR) that is in a complex with regulatory-associated protein of mTOR (Raptor) termed mTORC1. Activated mTORC1 phosphorylates and inhibits the eukaryotic initiation factor 4E-binding proteins (4E-BP1, 2, 3) and activates the p70 ribosomal S6 kinases (S6K1, 2), resulting in increased protein translation and upregulation of glycolysis, promoting cell growth and division [reviewed in Laplante and Sabatini (2012)]. In addition,

mTORC1 activation is required for several key elements of T cell effector differentiation that are discussed in further detail below.

A second mTOR complex containing the rapamycin insensitive companion of TOR (Rictor) and the mammalian homolog of the yeast *SIN1* gene (mSIN1), termed mTORC2, is also critical to mediating PI3K signaling via AKT. Although the precise mechanisms underlying activation of mTORC2 remain incompletely understood, it was recently observed that physical association with ribosomes stimulates mTORC2 activity (Zinzalla et al., 2011) and that Rictor acetylation by p300 stimulates mTORC2 activity (Glidden et al., 2012). mTORC2 is strongly activated in T cells by costimulation and cytokines [reviewed in Cantrell (2002)]. mTORC2 has several substrates including serum- and glucocorticoid-induced protein kinase 1 (SGK1), protein kinase C-α (PKC-α), and importantly, AKT itself. Phosphorylation at residue S473 in the AKT hydrophobic motif modifies substrate specificity of AKT and enhances its kinase activity (Sarbassov et al., 2005; Facchinetti et al., 2008; Garcia-Martinez and Alessi, 2008). In particular, S473 phosphorylation is required to permit AKT to phosphorylate members of the Forkhead box family of transcription factors Foxo1 and Foxo3a (Jacinto et al., 2006). Foxo phosphorylation leads to the cytoplasmic retention of these transcription factors with resulting downregulation of target gene expression (Brownawell et al., 2001). In T cells, a key Foxo target is KLF2 that regulates expression of CD62L, the c–c chemokine receptor 7 (CCR7), and the sphingosine-1-phosphate receptor (S1P1R) which each control homing of lymphocytes into and out of secondary lymphoid tissues [reviewed in Finlay and Cantrell (2010)].

The signaling cascade from receptors through PI3K to mTOR described above appears to imply a linear relationship, and older studies utilizing small molecule inhibitors of either PI3Ks such as wortmannin or LY294002 or mTOR using rapamycin demonstrated similar profound inhibition of T cell function *in vitro* (Kay et al., 1991; Ward et al., 1995). However, recent analysis of the pathways using more specific inhibitors and genetic manipulation has identified a more complex signaling cascade with both overlapping and unique functions. Phenotypes of mice discussed in this review with targeted disruption of components of the PI3K or mTOR pathways are summarized in **Table 1** below.

INTERRUPTION OF THE PI3K/AKT/mTOR PATHWAY AT DIFFERENT LEVELS HAS DIFFERENTIAL EFFECTS ON T CELL ACTIVATION

A critical aspect to TCR signaling is its ability to activate PLCγ and generate the second messenger inositol triphosphate (IP3) that triggers Ca^{++} entry following activation. This Ca^{++} binds calmodulin and activates calcineurin, which dephosphorylates NF-AT transcription factors that translocate to the nucleus to activate transcription of genes that regulate both T cell activation/effector function and tolerance (Macian et al., 2002). Cyclosporine acts as a potent immunosuppressant by binding to cyclophilin and blocking activation of calcineurin and thus NF-AT. Rapamycin inhibits T cell activation by a different mechanism, by binding to FKBP12 and preventing association of Raptor and mTOR to form the mTORC1 complex. The importance of this difference is highlighted by the fact that activation of Th1 cells in the presence of rapamycin renders them subsequently anergic and incapable of proliferation and IL-2 secretion upon secondary stimulation after rapamycin is washed away, in a manner similar to Th1 cells stimulated through the T cell receptor (Signal 1) in the absence of costimulation via CD28 (Signal 2) (Powell et al., 1999). This occurs despite the fact that while rapamycin acutely inhibits T cell proliferation, it does not block initial IL-2 secretion. Cyclosporine, in contrast, blocks both proliferation and IL-2 secretion acutely but does not impair secondary stimulation after drug is removed. Rapamycin appears to promote anergy and tolerance by permitting normal TCR-mediated activation of *NF-AT* gene targets including the transcription factors Egr-2 and Egr-3 and their targets such as Cbl-b, DGKα, and Fas-L that limit T cell activation and promote apoptosis (Sanjuan et al., 2001; Macian et al., 2002; Safford et al., 2005; Zha et al., 2006). As these signaling pathways would predict, co-incubation of T cells with cyclosporine and rapamycin blocks rapamycin-induced anergy (Powell et al., 1999). That is, blocking proximal TCR-mediated signaling with cyclosporine mitigates the effects of blocking mTOR with rapamycin. We emphasize the consequences of blocking proximal signaling with cyclosporine and more distal signaling with rapamycin because a parallel can be drawn comparing genetic blockade of mTOR that promotes generation of regulatory T cells (Treg) and genetic blockade of proximal PI3K signaling that results in decreased Treg function *in vivo* (to be discussed in depth below).

The generation of mice with T cell conditional deletion of mTOR using a floxed allele crossed with a CD4-Cre transgenic mouse confirms the specificity of rapamycin for blocking mTOR-mediated signaling (Delgoffe et al., 2009). Stimulation of mTOR deficient T cells with anti CD3/CD28 induces robust, even slightly increased, proximal AKT T308 phosphorylation but it is unable to elicit S6K1 and AKT S473 phosphorylation. Like rapamycin treated T cells, mTOR deficient T cells show normal IL-2 secretion during primary stimulation but reduced proliferation. However, the consequence of activation of naïve T cells lacking mTOR is the inhibition of effector differentiation and the generation of Tregs.

Since PI3K is upstream of mTOR, the effect of a PI3K inhibitor would be predicted to mimic rapamycin. In fact, stimulation of T cells in the presence of wortmannin does acutely block proliferation, but IL-2 secretion is also inhibited, similar to cyclosporine (Ward et al., 1995). Despite this difference, the presence of wortmannin during T cell priming does result in anergy upon restimulation similar in magnitude to that achieved by costimulatory blockade with CTLA4-Ig which blocks B7/CD28 interactions (Taub et al., 1997). Importantly, subsequent work has demonstrated that wortmannin is not selective for PI3K as originally thought, but it can inhibit mTOR as well (Brunn et al., 1996), therefore it is not clear if the findings reported by Taub et al. merely mimic the effect of rapamycin-induced mTOR blockade. Clarification of distinctions between PI3K and mTOR-dependent signals has subsequently been provided by genetic analysis.

Initial work deleting individual PI3K regulatory subunits demonstrated significant functional redundancy in T cells, with no overt defects in T cell development and signaling after disruption of p85α and paradoxically enhanced TCR and IL-2-induced proliferation in p85β knockouts (Fruman et al., 1999; Suzuki

Table 1 | T cell phenotype and function in PI3K and mTOR pathway gene-targeted mice.

Gene	Phenotype	References
pik3r1 ($p85\alpha^{-/-}$, $p55\alpha^{-/-}$, $p50\alpha^{-/-}$)	Normal T cell development and proliferation	Fruman et al., 1999
pik3r1 ($p85\alpha^{-/-}$)	Normal T cell development and proliferation	Suzuki et al., 1999
pik3r2 ($p85\beta^{-/-}$)	Normal T cell development Enhanced proliferation to TCR stimulation or TCR + IL-2	Deane et al., 2004
r1ΔT/r2n ($p85\alpha^{-/-}$, $p55\alpha^{-/-}$, $p50\alpha^{-/-}$, $p85\beta^{-/-}$)	Normal T cell development Normal Th1 but reduced Th2 differentiation Impaired T-dependent antibody response Reduced Treg numbers, spontaneous autoimmunity	Oak et al., 2006 Deane et al., 2007
pik3cd ($p110\delta^{-/-}$, CD4-Cre;$p110\delta^{flox/flox}$, or OX40-Cre;$p110\delta^{flox/flox}$)	Normal T cell development and proliferation Impaired T-dependent antibody response	Clayton et al., 2002 Rolf et al., 2010
pik3cd ($p110\delta^{D910A/D910A}$)	Decreased peripheral T cell numbers CD3/CD28-induced proliferation and IL-2 normal Antigen-induced proliferation and IL-2 reduced Impaired Th1 and Th2 differentiation *in vitro* Reduced Th1 contact hypersensitivity Inappropriate Th1 differentiation to Th2 stimulus *in vivo* Reduced Th17 differentiation with attenuated EAE Reduced IL-10 production Impaired Treg function with autoimmune colitis Impaired T-dependent antibody response	Okkenhaug et al., 2002 Okkenhaug et al., 2006 Patton et al., 2006 Nashed et al., 2007 Liu et al., 2009 Rolf et al., 2010 Soond et al., 2010 Haylock-Jacobs et al., 2011
pik3cg ($p110\gamma^{-/-}$)	Normal T cell development Variably decreased or normal proliferation Variably decreased or normal cytokine production Abnormal trafficking to sites of inflammation Impaired trafficking to chemokines	Sasaki et al., 2000 Alcazar et al., 2007 Garcon et al., 2008 Martin et al., 2008 Thomas et al., 2008
pten (Lck-Cre;$PTEN^{flox/-}$ or OX40-Cre;$PTEN^{flox/flox}$)	Lymphoproliferative disorder (Lck-Cre) Enhanced T cell proliferation and cytokine production Spontaneous autoimmunity (Lck-Cre) Enhanced T cell help (OX40-Cre) Enhanced tumor rejection (OX40-Cre)	Suzuki et al., 2001 Soond et al., 2012
frap1 (CD4-Cre;$mTOR^{flox/flox}$)	Normal T cell development Reduced proliferation Normal initial IL-2 secretion Impaired Th1, Th2, and Th17 differentiation Spontaneous iTreg differentiation	Delgoffe et al., 2009
rheb (CD4-cre;$rheb^{flox/flox}$)	Normal T cell development Impaired Th1 and Th17 differentiation Normal Th2 and iTreg differentiation	Delgoffe et al., 2011
raptor (Lck-Cre;$raptor^{flox/flox}$)	Reduced peripheral T cell numbers Impaired Th17 differentiation	Kurebayashi et al., 2012
rictor (CD4-Cre;$rictor^{flox/flox}$ or proximal Lck-Cre;$rictor^{flox/flox}$)	Normal T cell development Variably decreased Th1 and Th17 differentiation Impaired Th2 differentiation Normal iTreg differentiation Impaired T-dependent antibody response	Lee et al., 2010 Delgoffe et al., 2011

et al., 1999; Deane et al., 2004); therefore the p85α and p85β knockouts were crossed to disrupt all Class IA PI3K activity. This was accomplished by crossing *pik3r1*$^{2loxP/2loxP}$ mice with *pik3r2*$^{-/-}$ mice and breeding these onto an Lck-Cre background to cause T cell specific deletion of both regulatory subunits (Deane et al., 2007). Resulting mice, termed r1ΔT/r2n, have normal generation of T cells, but such cells have profound decreases in expression of multiple PI3K catalytic subunits with greatly reduced p110β and undetectable p110α and p110δ. Such T cells demonstrate expected loss of CD3/CD28-induced AKT S473 phosphorylation. This may be due to impaired mTORC2 signaling but it is important to comment that recruitment of AKT to the cell membrane to be phosphorylated by PDK1 at T308 is predicted to be interrupted as well. Since T308 phosphorylation

was not directly measured, it cannot formally be determined whether the loss of AKT S473 phosphorylation represents a lack of singly phosphorylated AKT substrate for mTORC2 or is a consequence of reduced mTORC2 activity. Also, phosphorylation of other mTORC2 substrates such as SGK1 and PKCα was not reported in this study. Similar data on AKT S473 phosphorylation in isolation without other measures of mTORC2 activity has been extensively reported in other systems described in this review. Thus, interpretation of relative levels of mTORC2 activity in some of these systems remains speculative but is helpful to consider when comparing phenotypes with systems where mTORC2 is selectively disrupted. Deane et al. unexpectedly provide evidence for preservation of some CD3/CD28-induced phosphorylation of ribosomal S6 at S235/236. While some data have suggested that this particular S6 phosphorylation site may be more dependent on RSK activity than mTOR activity upstream of S6K (Salmond et al., 2009), Deane et al. demonstrate that this S6 phosphorylation in r1ΔT/r2n T cells is blocked by rapamycin, supporting that in r1ΔT/r2n T cells some mTORC1 activity is preserved. TCR-induced calcium flux was modestly reduced in r1ΔT/r2n T cells and TCR-induced NF-κB activation as measured by IκB phosphorylation was significantly reduced, consistent with reduced activation of the Tec kinase/PLCγ pathway due to inefficient PIP3 generation. However, IκB phosphorylation could be normalized with CD28 costimulation. This partial preservation of CD3/CD28-induced signals including some mTOR activation may explain why proliferation is only slightly reduced while IL-2 and IFN-γ are more profoundly suppressed, which would not be predicted by the PI3K and rapamycin inhibitor studies described above and differs from the phenotype following T cell specific deletion of mTOR.

Since disruption of the regulatory subunits indirectly affects expression of multiple PI3K catalytic subunits, targeted deletion of individual catalytic subunits has been used to dissect how each one contributes to T cell activation with the rationale that a selective inhibitor of a T cell dominant PI3K isoform might have less off target effects in non-immune cells and be a more useful immunosuppressant. Two parallel approaches have been used to determine the role of the PI3K p110δ isoform in lymphocytes, simple gene deletion and targeted knock-in of a kinase inactive mutant p110δ D910A allele (Clayton et al., 2002; Okkenhaug et al., 2002). The latter mice were generated due to concern that deletion of individual regulatory or catalytic subunits appears to result in compensatory changes in expression of remaining *PI3K* genes. Also, the catalytically inactive protein could still function in a scaffolding capacity during assembly of receptor-induced signaling complexes (Okkenhaug et al., 2002). p110$\delta^{-/-}$ mice (completely deleted for p110δ) have normal peripheral T cell numbers but impaired T-dependent antibody production (Clayton et al., 2002). Subsequent work relying on T and B cell conditional deletion of a p110δ floxed allele demonstrated that loss of p110δ expression in T cells due to conditional deletion with Lck-Cre is sufficient to disrupt T-dependent antibody responses despite preserving essentially normal T cell numbers and normal T cell proliferation in response to CD3/CD28 stimulation. This is correlated with an absence of AKT S473 phosphorylation in response to stimulation with anti CD3 plus ICOS-L and significantly decreased expression of CD40L by activated p110$\delta^{-/--}$ T cells (Rolf et al., 2010). The p110$\delta^{D910A/D910A}$ mice demonstrate normal cellularity of the thymus but have about a 50% reduction in mature T cell numbers and a decreased frequency of CD44^{high} effector T cells in the periphery. Phosphorylation of AKT on S473 is severely reduced after CD3 crosslinking, however T cell proliferation and IL-2 secretion when stimulated with anti CD3/CD28 coated beads was the same as WT mice. This stands in contrast to the diminished proliferation and IL-2 secretion that was seen when p110$\delta^{D910A/D910A}$ were crossed to a TCR Tg and stimulated with cognate peptide/APC, indicating that strength of stimulation may compensate in some manner for loss of efficient PIP3 generation in such cells, perhaps via activation of p110γ as discussed below (Alcazar et al., 2007). Similar to r1ΔT/r2n mice, mTOR-independent TCR signaling to induce NF-κB activation was reduced in p110$\delta^{D910A/D910A}$ T cells but NF-κB activation normalized when CD28 costimulation was provided (Okkenhaug et al., 2006). In summary, loss of the individual p110δ subunit or all class IA PI3Ks results in consistent inhibition of AKT S473 phosphorylation implying decreased mTORC2 activity, but TCR-induced proliferation may be relatively spared, depending on the strength of the TCR stimulation. Loss of IL-2 production also appeared to be more severe in the absence of all Class IA PI3K expression. These effects differ from the effects of rapamycin treatment or deletion of mTOR where proliferation is more severely inhibited, initial IL-2 production is preserved, but recall cytokine responses are diminished. Mechanisms underlying these differences may relate to CD4 T cell effector differentiation and are discussed in the next section.

There are conflicting data on the coupling of p110γ to TCR signaling. In one report, T cells from p110$\gamma^{-/-}$ mice have reduced anti CD3 and anti CD3/CD28-induced proliferation, IL-2 production, and IFN-γ production (Sasaki et al., 2000), to some extent resembling T cell anergy. However, while the addition of exogenous IL-2 can rescue Th1 cell anergy induced by rapamycin (Powell et al., 1999), supplementation of IL-2 only partially corrects the defective proliferation of p110$\gamma^{-/-}$ T cells in response to anti CD3/CD28 (Alcazar et al., 2007). Furthermore, p110$\gamma^{-/-}$ T cells demonstrate reduced AKT S473 and mitogen activated protein kinase (MAPK) phosphorylation after stimulation with anti CD3 or anti CD3/CD28, correlating with decreased F-actin polymerization and impaired formation of stable conjugates between T cells and peptide loaded APCs (Alcazar et al., 2007). These data stand in contrast to studies which examined early signaling events in p110$\gamma^{-/-}$ T cells following TCR binding to cognate peptide on APC. Recruitment of an AKT PH domain-GFP fusion protein to the immunologic synapse following stimulation with peptide loaded APC is normal in T cells lacking p110γ while it is impaired in p110$\delta^{D910A/D910A}$ T cells and CD28$^{-/-}$ T cells (Garcon et al., 2008). Likewise, proliferation, downregulation of CD62L, and upregulation of CD44 in response to antigen *in vivo* is normal in CD4 T cells lacking p110γ (Thomas et al., 2008). Proliferation of CD8 T cells lacking p110γ was also normal in response to antigen pulsed APC *in vitro* and vaccinia virus expressing a model antigen *in vivo* (Martin et al., 2008). The mechanism underlying differences between the normal proliferation to antigen but reduced proliferation to anti CD3/CD28 crosslinking in p110$\gamma^{-/-}$ T cells

as compared to the reduced proliferation to antigen but near normal proliferation to anti CD3/CD28 in T cells lacking normal p110δ remains incompletely understood, but it may be related to increased susceptibility of p110$\gamma^{-/-}$ T cells to activation induced cell death as p110$\gamma^{-/-}$ thymocytes showed enhanced death compared to WT when exposed to anti CD3 in the presence of adenosine receptor agonists (Sasaki et al., 2000). It will be interesting to see if preservation of antigen induced mTOR activation contributes to the proliferation seen from p110$\gamma^{-/-}$ T cells.

Deletion of PTEN in tumors is associated with constitutive accumulation of PIP3 and appears to promote proliferation and tumor survival, thus it was not surprising when mice with T cell conditional deletion of PTEN were found to develop a lymphoproliferative disorder and die prematurely of CD4 T cell lymphomas (Suzuki et al., 2001). Prior to the onset of lymphoma, peripheral T cells from such mice demonstrate enhanced proliferation, IL-2 secretion, IFN-γ secretion, and AKT S473 phosphorylation after CD3/CD28 stimulation. T cells demonstrate a spontaneous activated, CD69 high phenotype and are autoreactive. Furthermore, such mice develop autoimmunity and hypergammaglobulinemia due to aberrant self-tolerance. This immunopathology appears strongly related to the timing of loss of PTEN expression because conditional deletion of PTEN after completion of thymic negative selection using OX40-Cre, which results in deletion in mature peripheral T cells after initial activation, does not lead to development of autoreactive cells or systemic autoimmunity despite increased lymph node cellularity (Soond et al., 2012). OX40-Cre PTEN null T cells do exhibit enhanced CD3-induced proliferation and IFN-γ production compare to WT. Furthermore, consistent with an exaggerated AKT/mTOR activation state, such mice develop an increased secondary contact hypersensitivity recall response.

NAïVE CD4 T CELLS REQUIRE PI3K-INDUCED mTOR-DEPENDENT AND mTOR-INDEPENDENT SIGNALS

Naïve CD4 T cells acquire restricted patterns of cytokine expression or regulatory function as a consequence of cytokine exposure or strength of antigenic stimulation during the period of initial activation. Specific patterns of cytokine expression are associated with master transcriptional regulators in the different lineages: Th1 cells express Tbet and IFN-γ; Th2 cells express GATA3 and IL-4, IL-5, and IL-13; Th17 cells express ROR-γT and IL-17A/F; Treg express FoxP3 and suppress other T cell responses [reviewed in Weaver et al. (2006)]. More recently an additional subset termed T follicular helper (Tfh) cell was identified that supports generation of T-dependent antibody production and is dependent on expression of the transcription factor Bcl6 and marked by surface ICOS and CXCR5 and secretion of IL-21 [reviewed in Crotty (2011)]. A requirement for mTOR activation in helper T cell differentiation was initially suggested by the observation that rapamycin inhibition of mTOR promotes *de novo* expression of FoxP3 by naïve T cells and promotes expansion of Tregs *in vitro* (Battaglia et al., 2005). Such rapamycin-induced FoxP3+ cells are functional Tregs capable of suppressing pancreatic allograft rejection *in vivo*. Additional evidence of a critical role for mTOR signals in promoting effector function and inhibiting Treg generation comes from the mice with conditional deletion of mTOR in T cells. Following initial activation, a high proportion of mTOR deficient T cells spontaneously become inducible FoxP3+ Tregs and Th effector differentiation to Th1, Th2, and Th17 fates *in vitro* is severely impaired (Delgoffe et al., 2009).

Mice with individual T cell conditional deletion of either mTORC1 or mTORC2 have provided surprising insight into non-overlapping roles for each of these complexes in normal T cell effector differentiation. Selective loss of mTORC1 following Rheb deletion results in CD4 T cells incapable of Th1 and Th17 differentiation but preserves the ability to make Th2 cells in response to IL-4 stimulation (Delgoffe et al., 2011). More recently, T cell conditional deletion of Raptor was shown to impair Th17 differentiation but Th1 differentiation was preserved (Kurebayashi et al., 2012). Conversely, deletion of Rictor with resulting loss of mTORC2 causes loss of Th2 differentiation, with variable effects on Th1 and Th17 differentiation depending on the timing of Cre expression in the thymus based on the particular Cre transgenic line that the floxed Rictor allele is crossed to (Lee et al., 2010; Delgoffe et al., 2011). T cell-dependent antibody production in mice with mTORC1 deficient T cells is normal but mice with mTORC2 deficient T cells have decreased antibody titers in both mouse lines, although enumeration of Tfh was not performed. As Th17 cells contribute significantly to the pathology of a murine model for multiple sclerosis, experimental autoimmune encephalitis (EAE), the Rheb deficient T cell mice were compared to WT in the EAE system. Rheb deficiency in T cells results in lower EAE severity scores than WT despite all mice developing some symptoms. This correlates with fewer T cells infiltrating the spinal cord and a lower frequency of IL-17 and IFN-γ secretion by T cells extracted from spinal cords of the Rheb deficient T cell mice. Strikingly, 60% of the Rheb deficient T cell mice immunized to induce EAE develop ataxia consistent with "non-classical EAE" that has been observed with induction of Th2 cells in other reports (Wensky et al., 2005). Rheb deficient mice with ataxia have T cell infiltrates in their cerebellum that produce Th2 cytokines *in vitro* in response to antigen (Delgoffe et al., 2011). Treg number and function are normal following T cell conditional deletion of Rheb and Rictor, but Rictor deficient T cells that only have mTORC1 demonstrate enhanced sensitivity to rapamycin in promoting expression of FoxP3 *in vitro* (Delgoffe et al., 2011). The observation that prolonged incubation of cells with higher doses of rapamycin blocks mTORC2 assembly serves to explain both this enhanced sensitivity of Rictor deficient T cells to rapamycin and the fact that loss of both mTORC1 and mTORC2 by deletion of mTOR is necessary to mimic the effect of rapamycin on promoting Tregs (Sarbassov et al., 2006).

Based on the regulation of mTOR by PI3K, the PI3K knockout models should recapitulate the effects of mTOR deletion on effector differentiation. However, just as the mTORC1 and mTORC2 deficient T cells demonstrated unexpected effector phenotypes, disruption of PI3K proximal signaling has also provided surprises. T cells from the r1ΔT/r2n mice lacking all p85 regulatory subunits and having low or absent p110α, β, and δ catalytic subunit expression supported normal Th1 polarization but Th2 cultures had decreased IL-4 and increased IFN-γ. This is consistent with the report above from Delgoffe et al. noting selective loss of IL-4 expression from T cells deficient in mTORC2 and the

relative preservation of mTORC1-dependent S6 phosphorylation over mTORC2-dependent AKT S473 phosphorylation that Deane et al. report in the r1ΔT/r2n mice. However, the frequency of $CD4^+CD25^+FoxP3^+$ natural Tregs in the spleen was significantly decreased in r1ΔT/r2n mice despite normal thymic Treg numbers. This is in contrast to normal Treg numbers in the periphery of mice with T cells lacking Rictor (Lee et al., 2010; Delgoffe et al., 2011). T-dependent B cell responses to NP-Ova were impaired and germinal centers following vaccination were decreased but antiviral responses to mouse hepatitis virus were normal (Deane et al., 2007). In addition, the mice were noted to develop spontaneous autoimmunity with corneal opacities and eye lesions found to resemble Sjögren's syndrome. On necropsy the r1ΔT/r2n mice were noted to have a predominantly CD4 T cell infiltrate into lacrimal and salivary lands with lymphadenopathy, splenomegaly, and anti-nuclear and anti SSA autoantibodies (Oak et al., 2006). A mechanistic link between decreased Tregs and autoimmunity was speculated upon but not formally tested in this report. These clinical findings arise in the mice despite the biochemical profile with loss of AKT S473 phosphorylation that resembles the Rictor deficient T cell mice that lack autoimmunity. This serves to highlight the importance of additional PI3K signaling pathways distinct from the AKT/mTOR axis in the maintenance and function of Tregs.

Further characterization of the $p110\delta^{D910A/D910A}$ mice demonstrated that Th1 and Th2 differentiation *in vitro* is markedly reduced (Okkenhaug et al., 2006). Interestingly, when *in vivo* Th2 differentiation was examined following immunization with Ova/Alum, $p110\delta^{D910A/D910A}$ mice exhibit decreased Th2 responses and inappropriate Th1 responses. That is, splenocytes have decreased Ova specific IL-4, IL-5, and IL-13 secretion but enhanced Ova specific IFN-γ and CXCL10 secretion, and the mice demonstrate a reduction in Th2-dependent allergic airway inflammation *in vivo*. This appears to be secondary to defective IL-10 production by $p110\delta^{D910A/D910A}$ T cells because neutralization of IL-10 *in vitro* during Ova stimulation enhances WT splenocyte IFN-γ production and addition of IL-10 suppresses $p110\delta^{D910A/D910A}$ splenocyte IFN-γ production (Nashed et al., 2007). Phosphorylation of mTORC1 substrates is not described in these manuscripts to see if the phenotype is related to imbalance of mTORC1 versus mTORC2, as is ostensibly predicted by the Rictor conditional deletion data above.

Th17 differentiation is also partially p110δ-dependent. IL-17A production from Th17 culture *in vitro* is strongly inhibited by the p110δ isoform specific inhibitor IC87114 while IFN-γ production in Th1 culture is only modestly decreased. Likewise, induction of Th17 cells in $p110\delta^{D910A/D910A}$ mice *in vivo* in EAE is decreased while Th1 responses are spared, resulting in less severe clinical symptoms (Haylock-Jacobs et al., 2011). This preservation of Th1 cytokine expression resembles the phenotype noted for the r1ΔT/r2n mice and to some degree the Rictor T cell deficient mice noted above (Deane et al., 2007; Delgoffe et al., 2011).

Despite the preferential Th1 phenotype under a typical Th2 stimulus and in the EAE model above, Th1-driven contact hypersensitivity responses in $p110\delta^{D910A/D910A}$ mice are reduced (Soond et al., 2010). This would lead to a prediction that such mice would have increased susceptibly to the Th1-sensitive pathogen *Leishmania major*. However, while $p110\delta^{D910A/D910A}$ mice have diminished T cell proliferation, IFN-γ, and TNF-α production to *Leishmania* antigens after infection, consistent with impaired Th1 function, the clinical outcome of infectious challenge is actually enhanced resistance, smaller lesion size, and more rapid parasite clearance. This was found to correlate with impaired generation of IL-10 producing Treg and recruitment of such Tregs to sites of infection in $p110\delta^{D910A/D910A}$ mice (Liu et al., 2009). Adoptive transfer of WT Tregs to $p110\delta^{D910A/D910A}$ mice reverses their *Leishmania* resistance, supporting the hypothesis that Treg dysfunction permits the inefficient $p110\delta^{D910A/D910A}$ Th1 response to be sufficient to clear infection. Additional evidence for functional Treg deficiency in the $p110\delta^{D910A/D910A}$ mice comes from their spontaneous development of autoimmune colitis. Such mice have 50% reduction in Treg frequency in the periphery compared to WT and no detectable IL-10 secretion by cultured Tregs. This is associated with reduced *in vitro* suppression compared to WT Tregs and an inability of $p110\delta^{D910A/D910A}$ Tregs to suppress colitis induced by adoptive transfer of naïve T cells into $RAG1^{-/-}$ mice (Patton et al., 2006). In contrast to these autoimmune findings in mice with proximal PI3K defects, to date, no spontaneous autoimmune phenotype has been reported in mice with T cell conditional disruption of mTORC1, mTORC2, or both (Delgoffe et al., 2009, 2011; Lee et al., 2010).

Reminiscent of the mice with T cells lacking mTORC2 complexes and the r1ΔT/r2n mice, $p110\delta^{D910A/D910A}$ mice demonstrate abnormal T-dependent antibody responses. Such mice are largely devoid of germinal centers in spleen and lymph nodes (Okkenhaug et al., 2002). This appears to be due to a requirement for p110δ expression in Tfh, rather than B cells, based on subsequent conditional deletion experiments. Mice bearing $p110\delta^{2loxP/2loxP}$ alleles were crossed with CD4-Cre to delete p110δ during the double positive thymocyte stage or Ox40-Cre to delete p110δ in peripheral T cells after their initial activation. In both types of mice, PD-1+ CXCR5+ Tfh are decreased 10-fold and 5-fold, respectively with corresponding decreases in the number of germinal center B cells and the number of germinal centers per follicle within lymph nodes. Mechanistically, this correlates with decreased ICOS-induced AKT S473 phosphorylation and reduced expression of factors critical for T cell-dependent B cell help including CD40L, IL-21, IL-4, and c-Maf in T cells lacking p110δ expression (Rolf et al., 2010). A detailed Tfh phenotype has not been reported following T cell conditional deletion of Rictor, however the data from the PI3K deficient mice would predict that Tfh effector cytokines might be reduced.

Despite the controversy surrounding whether p110γ is coupled directly to TCR or CD28, it is clear that loss of p110γ impacts T cell effector function. Both CD4 and CD8 $p110\gamma^{-/-}$ T cells have defective effector T cell migration *in vivo* that correlates with impaired migratory responses to chemokines *in vitro* (Martin et al., 2008; Thomas et al., 2008). Expression of granzyme B and IFN-γ appears normal in $p110\gamma^{-/-}$ CD8 effector T cells generated *in vivo* by antigen exposure (Martin et al., 2008). Effector differentiation of $p110\gamma^{-/-}$ CD4 T cells *in vivo* has not been fully described and impaired footpad swelling induced by LCMV and

impaired T-dependent antibody responses in p110$\gamma^{-/-}$ mice may be related to loss of p110γ expression in cells other that CD4 T cells (Sasaki et al., 2000). Anti CD3/CD28 induced phosphorylation of AKT S473 is decreased in p110$\gamma^{-/-}$ T cells, suggesting that CD4 effector differentiation may be abnormal in a manner analogous to mTOR or mTORC2 deficient T cells but in light of partial functional redundancy of p110δ activity in such cells, direct measurement of mTOR substrate phosphorylation in response to antigen or anti CD3/CD28 will ultimately need to be performed to address whether the p110$\gamma^{-/-}$ phenotype is mTOR related.

In contrast to immune dysregulation associated with Treg dysfunction after reducing PI3K activity, enhancing PI3K function by PTEN deletion in the OX40-Cre T cell conditional model not only enhances IFN-γ production *in vitro*, but also results in enhanced Th1 effector function *in vivo* by several measures. PTEN deletion in T cells increases contact hypersensitivity reactions. Adoptively transferred OX40-Cre PTEN floxed OT2 TCR Tg T cells secrete more IL-2 and IFN-γ after activation by infection with attenuated *Listeria* expressing ovalbumin and support better expansion of endogenous ovalbumin-specific CD8 T cells than WT OT2 cells. In addition, adoptively transferred OX40-Cre PTEN floxed OT2 T cells are better at rejecting syngeneic tumor expressing ovalbumin antigen (Soond et al., 2012). These findings are consistent with a robust Th1 response in the presence of increased PI3K/AKT/mTOR activity, but experiments to dissect if any findings were exclusively due to enhanced mTOR activity await experiments culturing PTEN null T cells or treating Ox40-Cre PTEN floxed mice with rapamycin or mTOR kinase inhibitors.

A MODEL FOR PI3K/AKT/mTOR REGULATION OF T CELL EFFECTOR FUNCTION

In an effort to propose a unifying model to explain the varied phenotypes arising from blockade of the PI3K/AKT/mTOR axis at different points, it is useful to consider experiments that disrupt more proximal and distal signaling events in T cells. Premature interruption of CD3/CD28 signaling or activation by very low doses of cognate peptide in CD4 T cells promotes expression of FoxP3 that is further enhanced by exposure to PI3K inhibitors and rapamycin (Kang et al., 2008; Sauer et al., 2008). Through use of dose responses to selective PI3K inhibitors, Sauer et al. attribute FoxP3 induction to inhibition of PI3Kα and δ upstream of AKT and mTOR. These findings raise a paradox: small molecule inhibitors of PI3K, AKT, and mTOR all result in a similar phenotype, promoting induction of FoxP3 expression following activation of naïve CD4 T cells, but the phenotypes of mice with genetic lesions resulting in loss of T cell PI3K expression demonstrate inefficient Treg production and decreased Treg function. Furthermore, mice with T cell conditional deletion of mTOR phenocopy the small molecule inhibitors and generate Tregs at the expense of effector helper T cells. These observations serve to highlight that there are mTOR-dependent effects of PI3K, mediated by AKT, that are necessary to suppress FoxP3 expression and promote normal effector function and that there are mTOR-independent effects of PI3K, some of which are necessary for normal inducible Treg differentiation. This is shown schematically in **Figure 1**, and details of the potential mechanisms for this effect are discussed below.

There are several potential mechanisms, currently largely speculative, to explain how loss of PI3K enzymatic activity, with associated decreased mTOR activity, may result in fewer and less functional Tregs. First, PI3K-dependent accumulation of PIP3 results in localization of other families of signaling molecules near the immunologic synapse including Vav guanine nucleotide exchange factors for Ras related GTPases Rac and Rho that control cell motility and Tec family tyrosine kinases that mediate PLCγ activation [reviewed in Cantrell (2001)]. A role for such pathways in T cell activation and effector cytokine expression is well established, but a direct connection between PI3K, Tec, Vav, and Treg function is incompletely characterized. However, disruption of other molecules downstream, including the Bcl-10 and PKCθ components of the NF-κB signaling pathway distal to the Tec family kinases, impairs generation of Tregs (Schmidt-Supprian et al., 2004). Also, Tregs normally have elevated levels of PTEN expression. This is associated with defective phosphorylation of AKT S473 in response to IL-2 stimulation in Tregs as compared to effector T cells and with defective Treg proliferation in response to TCR stimulation in the presence of IL-2 without concurrent CD28 ligation (Bensinger et al., 2004). Conditional deletion of PTEN in T cells does not affect generation of Tregs, the amount of FoxP3 they express, or the suppressive capacity of Tregs, but it does result in enhanced Treg proliferation in response to IL-2 or TCR stimulation (Walsh et al., 2006). Taken together, these data suggest that the Treg defects following loss of PI3K expression might be due to differential sensitivity of Tregs to decreased levels of PIP3 compared to effector cells due to high Treg PTEN expression. Preferential inhibition of Treg proliferation and suppressor cytokine production would be predicted to result in an enhanced ratio of effector T cells to Tregs and autoimmunity in the PI3K deficient mice. Such hypotheses await further experimental evaluation.

Another paradox that arises comparing T cells lacking PI3K with those lacking mTOR is how do PI3K null cells promote some effector function if they are unable to activate mTOR? It is important to remember that other inputs in addition to TCR and CD28-induced PI3K/AKT are able to activate mTOR. In particular, chemokine receptors via p110γ, cellular energy stores by regulating the adenosine monophosphate-dependent kinase (AMPK) pathway, cytoplasmic amino acids via the Ras-related small GTPase Rag proteins, and hypoxia via the hypoxia-induced factor protein regulated in the development of DNA damage response 1 (REDD1)/hypoxia-induced factor 1α (HIF1α) pathway, ERK and RSK downstream of Ras, and phosphatidic acid produced by phospholipase D are potent regulators of mTOR activation [reviewed in Laplante and Sabatini (2012); Waickman and Powell (2012)]. The capacity of these inputs to regulate mTOR activation in T cells is reflected by the ability of 2-deoxyglucose (2-DG) mimicking glucose deprivation, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) mimicking cellular energy depletion, and n-acetyl leucine (NALA) mimicking amino acid depletion to result in anergy when present during CD3/CD28 stimulation of T cells, analogous to rapamycin treatment (Zheng et al., 2009). The

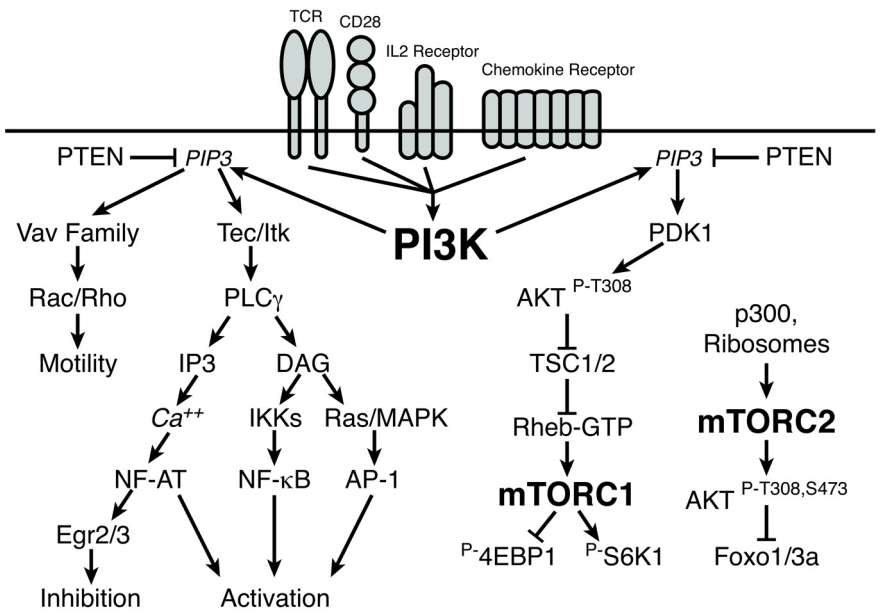

FIGURE 1 | PI3K signaling results in the activation of both mTOR-dependent and independent pathways in T cells. A schematic representation of PI3K signaling is shown demonstrating mTOR-independent and mTOR-dependent signaling cascades. Lines with arrows indicate activating signals and lines with bars indicate inhibitory signals. Importantly, this graphic does not include all of the nuanced inputs that are discussed in the text. Rather, these pathways represent connections and not absolute requirements for signaling. For example, while elimination of p110δ or all PI3K regulatory subunits leads to decreased NF-κB, such a deficit can be overcome by the addition of CD28 signaling. Overall, PI3K-induced mTOR-independent signaling can influence both the activation and inhibition of T cells as well as the generation and function of Tregs. The PI3K-induced mTOR-dependent pathways can influence CD4 effector differentiation and function as well as inhibit the generation of Tregs.

presence of normal energy, amino acids, and normoxia to promote mTOR activation is therefore consistent with the reported preservation of some mTORC1 activity in the r1ΔT/r2n mice (Deane et al., 2007). This relative increase in mTORC1 activity over mTORC2 activity in these mice supports the observed relative preservation of Th1 cytokine production over Th2 cytokines, analogous to the Rictor deficient T cell mice noted above (Lee et al., 2010; Delgoffe et al., 2011).

Finally, there are two mTOR-dependent pathways that can negatively regulate FoxP3 expression whose loss may explain the increased FoxP3 expression in mTOR null T cells. First, HIF1α is induced by mTORC1 and has been shown to directly bind to FoxP3 protein and promote its ubiquitination and degradation (Dang et al., 2011). Second, the Foxo transcription factors directly bind to and transactivate the FoxP3 promoter, and they are inactivated in an mTORC2-dependent manner following phosphorylation by doubly phosphorylated activated AKT, leading to nuclear export of the Foxos [reviewed in Coffer and Burgering (2004)]. The importance of the latter pathway to Tregs is seen following T cell conditional deletion of Foxo1 and Foxo3. Individually they have overlapping function in T cells to maintain naïve T cells in quiescence, but their simultaneous deletion in T cells results in functional Treg deficiency with early fatal autoimmunity that can be prevented by adoptive transfer of WT Treg (Ouyang et al., 2010). The fact that an increase in Tregs was observed following deletion of mTOR but not in the Rheb and Rictor T cell conditional knockouts suggests that loss of both of these pathways is necessary to result in enhanced generation of FoxP3+ Tregs (Lee et al., 2010; Delgoffe et al., 2011).

DISTAL INHIBITION OF THE PI3K/AKT/mTOR AXIS SHOULD YIELD MORE RELIABLE BLOCKADE OF T CELL MEDIATED PATHOLOGY

Based on the data and model above, treatment with mTOR inhibitors for immunologic diseases would be predicted to not only suppress immune responses but also potentially promote tolerance by fostering anergy and the induction of Treg. For example, in a mouse model of non-myeloablative peripheral blood stem cell transplantation, we found that treatment with cyclosporine led to eventual graft rejection while treatment with rapamycin led to stable mixed chimerism, even after the rapamycin was stopped. Such engrafted mice had negative mixed lymphocyte reactions to both donor and recipient target cells, indicating tolerance was achieved (Powell et al., 2005). This preclinical data served as the basis for a trial of human transplantation to treat sickle cell anemia with matched sibling donors that specifically avoided use of standard calcineurin inhibitors for graft versus host disease (GVHD) prophylaxis. Patients received alemtuzumab for T cell depletion, low dose total body irradiation, and peripheral blood stem cell transplant with rapamycin for GVHD

prophylaxis. Nine of ten patients engrafted with mixed donor chimerism sufficient to abrogate symptoms of sickle cell disease, and patients that have tapered off their immunosuppression have demonstrated stable engraftment, consistent with donor-specific tolerance (Hsieh et al., 2009).

In contrast to the effects of mTOR blockade, loss of PI3K expression in T cells leads to consequences on both Treg and effector T cell function, with the net effect of enhanced or suppressed immune responses *in vivo* strongly dependent on the context. A recent study examined PI3K inhibition as a means of suppressing cardiac allograft rejection (Ying et al., 2012). Transplanting male hearts into female mice results in HY antigen driven chronic allograft rejection that is mitigated if the hearts are transplanted into $p110\delta^{D910A/D910A}$ female recipients. Splenocytes from both rejecting WT and non-rejecting $p110\delta^{D910A/D910A}$ recipient mice proliferate equally well to HY peptide, however, indicating that the absence of rejection in $p110\delta^{D910A/D910A}$ recipients is not due to tolerance. Instead, defective trafficking appears to prevent rejection, as adoptive transfer of HY specific TCR Tg T cells demonstrates reduced localization of the $p110\delta^{D910A/D910A}$ HY-specific T cells into male allografts despite normal trafficking of adoptively transferred cells to other tissues. Based on this, the authors tested the selective PI3K p110δ inhibitor IC87114 and found it also prevents rejection by blocking localization of alloreactive cells to the graft but fails to induce tolerance. The inability of proximal PI3K blockade to induce transplant tolerance resembles the inability of cyclosporine to promote engraftment in the stem cell transplant model discussed above and stands in contrast to the tolerance induced by rapamycin in that model.

Interestingly, the capacity for proximal PI3K blockade to potentiate immune function in specific contexts may nicely synergize with the broader ability of PI3K blockade inhibit tumor cell proliferation to make PI3K inhibitors particularly well suited to cancer treatment, reviewed in So and Fruman (2012). To this end, mice were given subcutaneous implants of different syngeneic tumors and then treated with immunotherapy in the presence or absence of ZSTK474, a pan-class I PI3K inhibitor. It was found that this drug both inhibits tumor growth and has the ability to enhance the efficacy of immunotherapy (Marshall et al., 2012). The authors did not assess whether impaired Treg function contributed to tumor clearance, but they demonstrate that inhibition of PI3K is sufficient to suppress DC secretion of IL-10 while permitting secretion of IL-12. This appears to result in enhanced Th1 immunity since neutralization of IFN-γ *in vivo* by blocking antibody abrogated the protection afforded by immunotherapy plus ZSTK474. In this regard, we would predict that mTOR inhibition in the absence of proximal PI3K blockade might also have directs effects on inhibiting tumor growth but would serve to limit development of anti-tumor immunity.

SUMMARY

By employing genetic analysis it has become clear that in T cells what initially appeared to be a straightforward connection between components of the PI3K signaling cascade via AKT to mTOR is substantially more complex. We have only been able to show a small subset of data published on the topic to illustrate this point and apologize to authors for whom we were not able to include their work. While inhibiting PI3K and mTOR interferes with T cell effector function, what has become clear is that through the inhibition of distal targets in these signaling pathways, selective effects can be achieved. For example, a drug that blocks mTORC2 might be helpful to treat Th2-mediated asthma without blunting Th17 and Th1-mediated antifungal and antibacterial responses. Conversely, an mTORC1 selective drug, unlike rapamycin that can inhibit mTORC2 under prolonged exposure to higher doses, might be helpful in a disease like multiple sclerosis mediated by Th17 and Th1 cells but might permit effective vaccine responses to seasonal influenza by preserving Tfh function. As the PI3K-induced mTOR-independent pathways in T cells become more precisely defined, blockade of nodes distal in the pathway might avoid unintended effects that interfere with function of Treg. Finally, it may turn out that concomitant distal inhibition of both mTOR-dependent and independent pathways will lead to the most robust and precise clinical outcomes.

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Regulation of glucose metabolism in T cells: new insight into the role of phosphoinositide 3-kinases

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Naïve T cells are relatively quiescent cells that only require energy to prevent atrophy and for survival and migration. However, in response to developmental or extrinsic cues T cells can engage in rapid growth and robust proliferation, produce of a range of effector molecules and migrate through peripheral tissues. To meet the significantly increased metabolic demands of these activities, T cells switch from primarily metabolizing glucose to carbon dioxide through oxidative phosphorylation to utilizing glycolysis to convert glucose to lactate (termed aerobic glycolysis). This metabolic switch allows glucose to be used as a source of carbon to generate biosynthetic precursors for the production of protein, DNA, and phospholipids, and is crucial for T cells to meet metabolic demands. Phosphoinositide 3-kinases (PI3K) are a family of inositol lipid kinases linked with a broad range of cellular functions in T lymphocytes that include cell growth, proliferation, metabolism, differentiation, survival, and migration. Initial research described a critical role for PI3K signaling through Akt (also called protein kinase B) for the increased glucose uptake and glycolysis that accompanies T cell activation. This review article relates this original research with more recent data and discusses the evidence for and against a role for PI3K in regulating the metabolic switch to aerobic glycolysis in T cells.

Keywords: PI3K, Glucose metabolism, Akt, T lymphocyte, aerobic glycolysis, c-Myc, PDK1

PI3K IN T CELLS

Class 1 phosphoinositide 3-kinases (PI3K), lipid kinases that phosphorylate phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$] to generate the lipid signaling molecule phosphatidylinositol-(3,4,5)-trisphosphate [$PI(3,4,5)P_3$], play a crucial role in many aspects of T cell biology (Okkenhaug and Vanhaesebroeck, 2003). Class 1 PI3Ks consist of a catalytic subunit, responsible for the lipid kinase activity, and an adapter subunit which links the catalytic subunit to upstream activating signals. They are subdivided into Class 1A; p110α, β, or δ catalytic subunits coupled to p85 adapter subunits, and Class 1B; p110γ catalytic subunit coupled to the p101 adapter protein. The p85 subunit couples Class 1A PI3Ks to docking sites created by tyrosine-kinase signaling while the Class IB p101 links the p110γ kinase with G-protein-coupled receptors (Vanhaesebroeck et al., 2010). The levels of the lipid signaling molecule $PI(3,4,5)P_3$ are coordinately regulated by both class 1 PI3Ks and by the action of 3′ and 5′ phosphatases PTEN and SHIP1 that dephosphorylate $PI(3,4,5)P_3$ to generate $PI(4,5)P_2$ and $PI(3,4)P_2$ respectively (**Figure 1A**).

$PI(3,4,5)P_3$ is an important lipid signaling molecule in T cells, present at low levels in naïve T cells and elevated in response to signaling initiated by the T cell receptor (TCR) and various cytokine and chemokine receptors (Koyasu, 2003). Following TCR engagement by antigen presenting cells (APC) levels of $PI(3,4,5)P_3$ accumulate in the plasma membrane and are maintained for prolonged periods, requiring continual TCR engagement and activation of PI3K (Costello et al., 2002; Harriague and Bismuth, 2002; Huppa et al., 2003). Signals from co-stimulatory molecules such as CD28 are important for sustaining $PI(3,4,5)P_3$ levels following TCR engagement but are not in themselves sufficient to stimulate PI3K as $PI(3,4,5)P_3$ levels are only induced when the TCR engages APC presenting cognate peptide antigen (Costello et al., 2002; Garcon et al., 2007). Other physiological stimuli that can stimulate $PI(3,4,5)P_3$ levels in T cells include cytokines and chemokines (Koyasu, 2003). While $PI(3,4,5)P_3$ levels are induced by a number of cytokines it should be noted that the potency of these different cytokines can vary, e.g., IL2 maintains high levels of $PI(3,4,5)P_3$ while IL15 maintains comparatively low levels (Cornish et al., 2006; Sinclair et al., 2008).

In the thymus, PI3Kδ and PI3Kγ are the major isoforms required in developing thymocytes. While deletion of PI3Kδ or γ individually does not have a pronounced effect on thymopoiesis, deletion or inactivation of both isoforms in developing T cells results in a block early in thymocyte development at the CD4 CD8 double negative (DN) stage (Sasaki et al., 2000; Okkenhaug et al., 2002; Webb et al., 2005; Swat et al., 2006; Ji et al., 2007). Negative regulation of $PI(3,4,5)P_3$ signaling is also important in developing thymocytes. Thus, deletion of PTEN in early thymocyte progenitors results in constitutive $PI(3,4,5)P_3$ signaling that leads to the development of T cell leukemia or lymphoma (Suzuki et al., 2001; Hagenbeek and Spits, 2008; Finlay et al., 2009). In contrast to developing thymocytes, in mature T cells PI3Kδ appears to be the major PI3K isoform responsible for promoting $PI(3,4,5)P_3$ signaling in activated T cells. Thus, a point mutation that makes p110δ catalytically inactive (D910A) abolishes $PI(3,4,5)P_3$ signaling in activated T cell subsets (Okkenhaug et al., 2002, 2006; Macintyre et al., 2011).

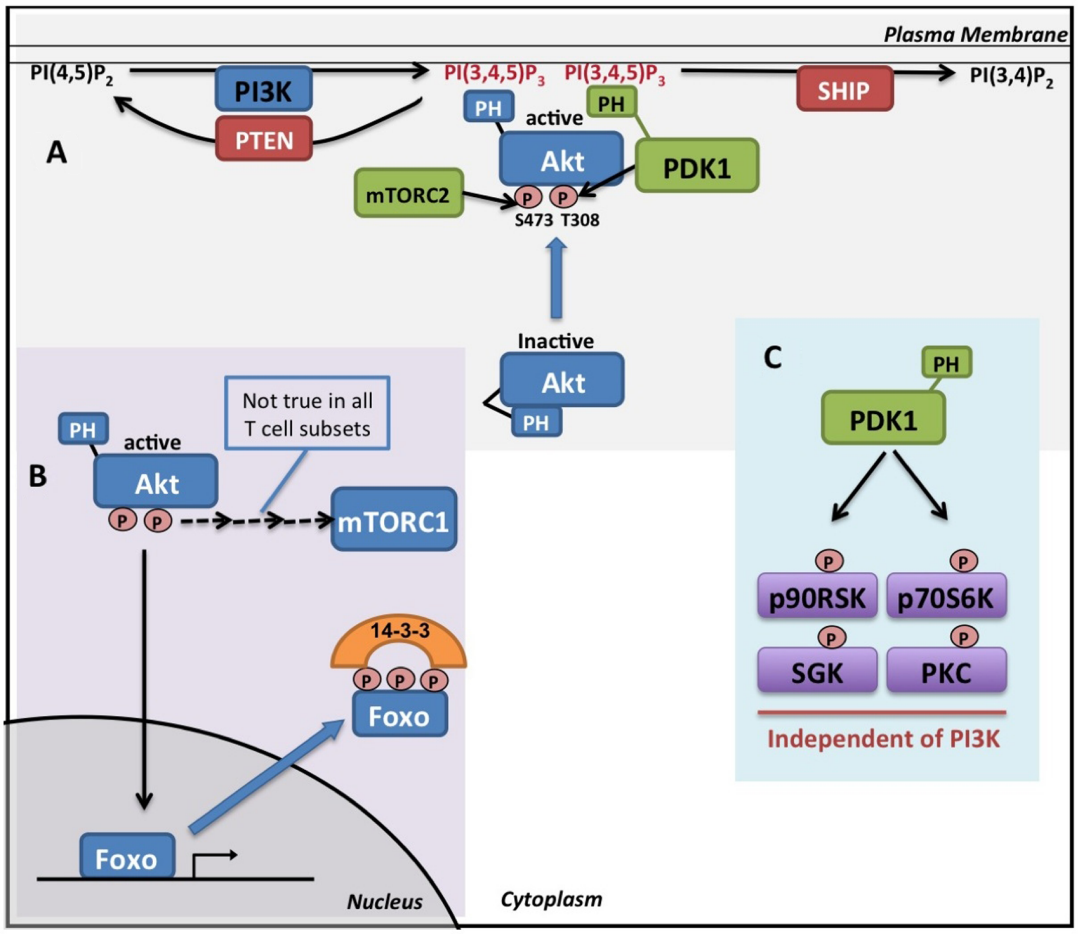

FIGURE 1 | PI3K and Akt signaling. (A) $PI(4,5)P_2$ is phosphorylated by Class 1 PI3K isoforms generating the second messenger molecule $PI(3,4,5)P_3$. Levels of $PI(3,4,5)P_3$ are negatively regulated by the lipid phosphatases PTEN and SHIP. Binding of the PH domain of Akt to $PI(3,4,5)P_3$ in the membrane results in a conformational change that allows for the phosphorylation of Akt on key residues(T308 and S473) by PDK1 and mTORC2. The recruitment of PDK1, via its PH domain, to the site of $PI(3,4,5)P_3$ is required for efficient Akt activation. **(B)** Active Akt phosphorylates Foxo transcription factors on multiple sites resulting in their translocation to the cytoplasm where they are retained in complex with 14-3-3 proteins. mTORC1 can be activated downstream of Akt, though this does not occur in all T cell types. **(C)** PDK1 also phosphorylates and activates a number of other members of the AGC kinase family in a PI3K independent manner.

PI3K SIGNALING

$PI(3,4,5)P_3$ acts as a signaling molecule through its interaction with the pleckstrin homology (PH) domains of a diverse array of signal transduction proteins. These proteins include Akt (also called PKB), Tec family kinases, and guanine-nucleotide-exchange proteins for Rho family GTPases. This interaction primarily controls the subcellular localization of PH domain containing proteins but can also control protein conformation and enzyme activity. Consider, for example, the activation of Akt, which is the best characterized $PI(3,4,5)P_3$ effector in T cells (for reviews Alessi and Cohen, 1998; Hanada et al., 2004; Cameron et al., 2007). The interaction of $PI(3,4,5)P_3$ with the PH domain of Akt stimulates its kinase activity by inducing a conformational change that allows Akt to be phosphorylated on threonine 308 and serine 473 by its upstream activating kinases phosphoinositide-dependent kinase 1 (PDK1) and mechanistic Target Of Rapamycin Complex 2 (mTORC2) respectively (**Figure 1A;** Calleja et al., 2007). PDK1 also contains a PH domain and co-localization of Akt and PDK1 to sites of $PI(3,4,5)P_3$ is required for efficient Akt activation (Bayascas et al., 2008; Waugh et al., 2009). Once activated, Akt phosphorylates a number of important signaling molecules including the Foxo transcription factors (Manning and Cantley, 2007). Foxo transcription factors localize to the nucleus where they promote the expression of target genes. Once phosphorylated by Akt, Foxos translocate into the cytoplasm where they are retained through their interaction with 14-3-3 proteins (**Figure 1B**; Coffer and Burgering, 2004; Burgering, 2008). PI3K/Akt signaling also activates the mTOR Complex 1 (mTORC1) in many cellular systems through multiple mechanisms (Laplante and Sabatini, 2009). mTORC1 is an important regulator of cellular metabolism that senses environmental cues such as nutrient availability and energy homeostasis (Delgoffe and Powell, 2009). However, it is now becoming apparent that mTORC1 activity is not universally dependent upon PI3K/Akt signaling in T cells. Thus, in activated

CD8 cells, mTORC1 activity is not blocked by the disruption of PI3K/Akt signaling by various pharmacological and genetic strategies (unpublished data; Macintyre et al., 2011).

MATCHING GLUCOSE METABOLISM TO METABOLIC DEMANDS

While naïve T cells only require energy to prevent atrophy and for survival and migration, activated T cell subsets have a greatly increased metabolic demand as they engage in rapid growth and proliferation, and the production of cytokines and other effector molecules. It is crucial that activated T cells increase their metabolism to meet the biosynthetic needs of the T cell as it responds either to developmental or pathogenic cues. To achieve this T cells respond to extrinsic signals from antigen receptors and cytokines to up-regulate the surface expression of key nutrient receptors: amino acid transporters, the transferrin receptor, and glucose transporters (Fox et al., 2005a; Kelly et al., 2007; Jacobs et al., 2008). Additionally, T cells switch their glucose metabolism from oxidative phosphorylation to aerobic glycolysis; i.e., glucose is metabolized to produce lactate even though oxygen is readily available (**Figure 2;** Greiner et al., 1994). Aerobic glycolysis is an inefficient route to generating ATP, producing two molecules ATP per molecule of glucose compared to >30 molecules ATP per glucose generated by oxidative phosphorylation. Therefore, cells must be able to sustain high levels of glucose uptake and an elevated glycolytic flux to generate sufficient ATP. This is achieved by increasing the expression of the GLUT1 glucose transporter and certain rate limiting enzymes within the glycolytic pathway (Vander Heiden et al., 2009; Marko et al., 2010). However, the real advantage of switching from oxidative phosphorylation to glycolysis is that it allows glucose to be used as a source of carbon to generate nucleic acid, amino acids and phospholipids (**Figure 2;** Vander Heiden et al., 2009). The generation of these biosynthetic precursors is critical for cells engaging in rapid growth, proliferation, and the synthesis of effector molecules. Therefore, to facilitate their differentiation and function, activated T cells up-regulate the expression of GLUT1, increase glucose uptake, and activate the switch to aerobic glycolysis

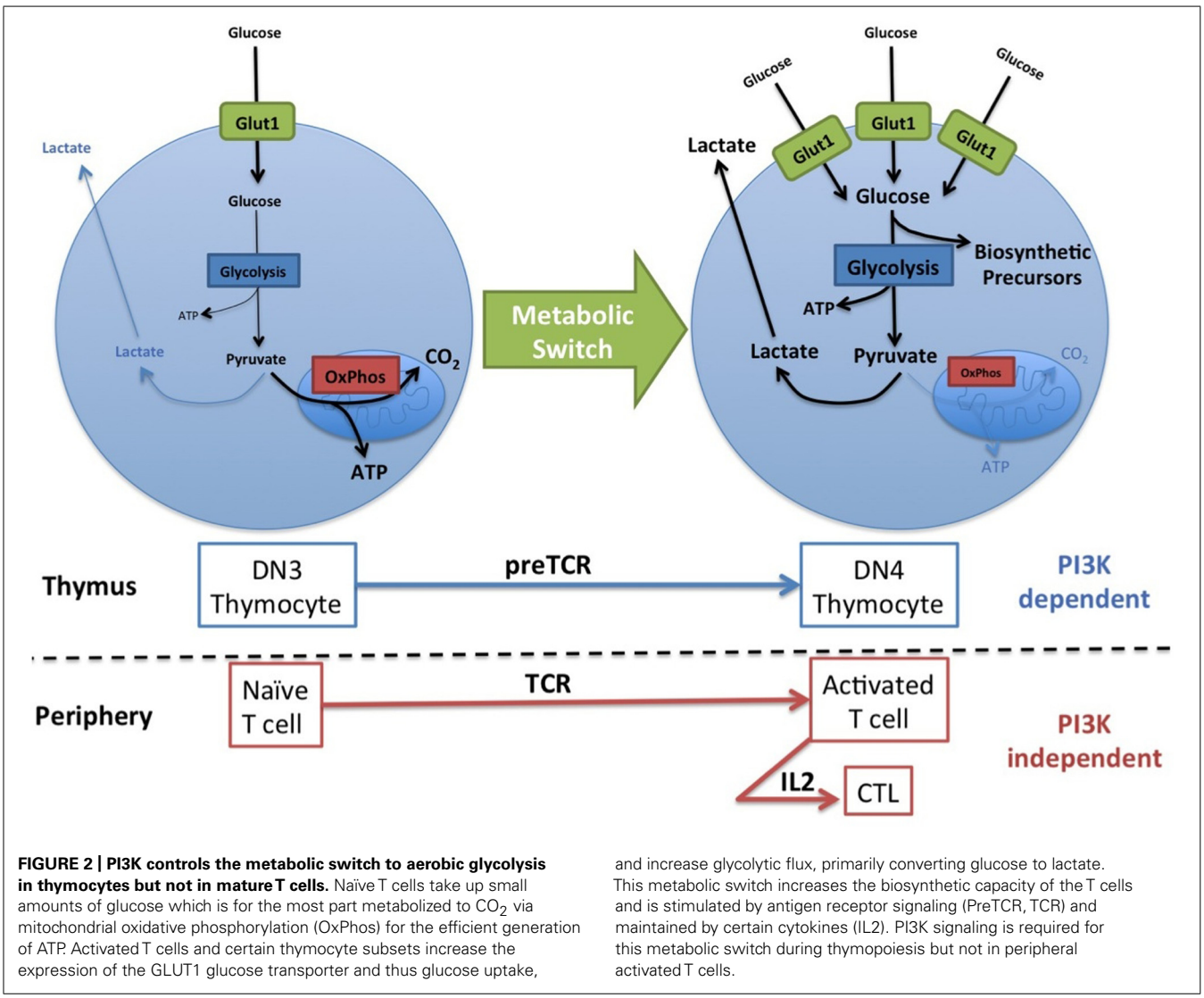

FIGURE 2 | PI3K controls the metabolic switch to aerobic glycolysis in thymocytes but not in mature T cells. Naïve T cells take up small amounts of glucose which is for the most part metabolized to CO_2 via mitochondrial oxidative phosphorylation (OxPhos) for the efficient generation of ATP. Activated T cells and certain thymocyte subsets increase the expression of the GLUT1 glucose transporter and thus glucose uptake, and increase glycolytic flux, primarily converting glucose to lactate. This metabolic switch increases the biosynthetic capacity of the T cells and is stimulated by antigen receptor signaling (PreTCR, TCR) and maintained by certain cytokines (IL2). PI3K signaling is required for this metabolic switch during thymopoiesis but not in peripheral activated T cells.

(Brand et al., 1984; Greiner et al., 1994; Frauwirth et al., 2002; Wofford et al., 2008).

PI3K AND GLUCOSE METABOLISM IN THE THYMUS

In the thymus discrete subpopulations, e.g., DN4 thymocytes, engage in rapid growth and robust proliferation. PI3K and Akt signaling is crucial in allowing these thymocyte subsets to match their metabolism with metabolic demands (Ciofani and Zuniga-Pflucker, 2005; Juntilla et al., 2007; Kelly et al., 2007; Finlay et al., 2010). Thus, mice lacking Akt or PDK1 or both PI3Kδ and γ isoforms during early thymopoiesis have a profound developmental block at the DN3/DN4 stage of T cell development(Hinton et al., 2004; Webb et al., 2005; Swat et al., 2006; Fayard et al., 2007; Juntilla et al., 2007; Mao et al., 2007). In the absence of PI3K/PDK1/Akt signaling DN4 thymocytes fail to up-regulate the expression of the glucose transporter, GLUT1, and also the expression of other key nutrient receptors for the uptake of amino acids (CD98, component of the L-amino acid transporter) and iron (transferrin receptor, CD71). Increased provision of these nutrients is a key requirement for these cells to meet the metabolic demands of rapid growth and proliferation and PI3K/PDK1/Akt deficient DN4 thymocytes that fail to do so atrophy and fail to develop (Juntilla et al., 2007; Kelly et al., 2007). Therefore, in T cells developing in the thymus, PI3K and Akt signaling is crucial to allow certain thymocyte subsets to match glucose metabolism with metabolic demands.

PI3K AND GLUCOSE METABOLISM IN ACTIVATED T CELLS

The metabolic switch to aerobic glycolysis is crucial during the activation and differentiation of T cells in the periphery. Thus, limiting glucose availability in activating T cells compromises TCR induced growth and proliferation and also the expression of certain effector molecules such as interferon γ (IFNγ; Cham et al., 2008; Jacobs et al., 2008). The transcription factor c-Myc is crucial for the metabolic switch in glucose metabolism that accompanies the activation of naïve T cells (Wang et al., 2011). Accordingly, deletion of c-Myc in naïve T cells prevents TCR induced glucose uptake and glycolysis, and activated c-Myc-null T cells completely fail to grow or proliferate (Trumpp et al., 2001; Iritani et al., 2002; Dose et al., 2006; Wang et al., 2011). Is PI3K and Akt signaling also required for the increase in glucose uptake and glycolysis in TCR activated T cells in the periphery? Certainly, antigen receptor induced c-Myc expression and glucose uptake have been attributed to PI3K signaling (Frauwirth et al., 2002; Grumont et al., 2002; Doughty et al., 2006; Jacobs et al., 2008; Wang et al., 2011). However, one criticism of these studies is that they rely on experiments involving the overexpression of Akt and the use of the PI3K inhibitor LY294002. Overexpression studies can be difficult to interrupt and while LY294002 was initially believed to be a highly specific PI3K inhibitor, and as such was used in good faith, it has since emerged that this inhibitor is rather non-specific. LY294002 potently inhibits a number kinases other than PI3K, including those with described roles in regulating T cell growth and proliferation, i.e., mTORC1 and Pim family kinases (Brunn et al., 1996; Davies et al., 2000; Fox et al., 2005b; Bain et al., 2007). The importance of these other LY204002 targets for T cell metabolism can be appreciated by a comparison of the cellular sizes of PI3K/Akt deficient CD8 cytotoxic T lymphocytes (CTL) and wild-type CTL cultured in the presence of LY294002. While PI3K/Akt deficient CTL are comparable in size to wild-type CTL, LY294002-treated CTL are substantially smaller (Cornish et al., 2006; Sinclair et al., 2008; Macintyre et al., 2011). Therefore, the question as to whether PI3K regulates TCR induced c-Myc expression has not been satisfactorily investigated to date. Nevertheless, a comparison of PI3K/Akt and c-Myc deficient T cells is extremely informative. In contrast to the failure of c-Myc-null T cells to engage in TCR stimulated growth and proliferation, PI3K deficient T cells show a relatively mild defect in growth and proliferation with activated T cells capable of completing numerous divisions, though at a reduced rate (Okkenhaug et al., 2002, 2006). Furthermore, T cells expressing a PDK1 K465E mutant which have defective TCR stimulated Akt activity, undergo normal TCR induced growth and proliferation (Waugh et al., 2009). These observations coupled to the fact that T cells activated in limiting concentrations of glucose do show a marked defect in proliferation argue that TCR induced c-Myc expression, glucose uptake and glycolysis is not compromised by disruption of PI3K and Akt signaling (Jacobs et al., 2008). More recently, PI3K/Akt independent glucose uptake and glycolysis in TCR activated T cells has been confirmed using pharmacological inhibitors of PI3Kδ (IC87114) and Akt (Akti1/2) with substantially greater selectivity than LY294002 (Macintyre et al., 2011). Akti1/2 is particularly selective toward Akt due to its unique allosteric mechanism of inhibition, binding to the PH domain of Akt and preventing the PH domain-PI(3,4,5)P_3 interaction and the resultant conformational change that is a prerequisite for Akt activation (Zhao et al., 2005; Bain et al., 2007). Both IC87114 and Akti1/2 prevent PI3K/Akt signaling in T cells while having no effect on TCR induced glucose uptake (Macintyre et al., 2011). Therefore, it seems clear that PI3K has differential roles in regulating glucose metabolism in developing thymocytes in the thymus and mature T cells in the periphery.

Once activated, T cells differentiate into various different effector T cell subsets depending on the local environment and cytokine availability. Many of these effector T cell subsets maintain an elevated glycolytic rate in response to cytokine signaling (Macintyre et al., 2011; Shi et al., 2011). For example, activated CD8 T cells undergo rapid growth and proliferation in response to interleukin 2 (IL2) as they differentiate into functional CTL. In response to IL2 signaling CTL maintain high levels of glucose uptake and lactate production indicative of elevated glycolysis (Macintyre et al., 2011). IL2 also promotes glucose uptake and glycolysis independently of PI3K and Akt but a key role has been revealed for PDK1. Thus, while IC87114 and Akti1/2 have no effect on CTL glucose uptake, a pronounced decrease is observed following the deletion of PDK1 using a Cre/loxP strategy (Macintyre et al., 2011). While PDK1 is responsible for the activation of Akt, it also activates a number of other members of the AGC kinase family including protein kinase C (PKC), 70-kDa ribosomal S6 kinase (p70S6K), 90-kDa ribosomal S6 kinase (p90RSK), and serum/glucocorticoid regulated kinase (SGK; Pearce et al., 2010). However, unlike the activation of Akt, PDK1 mediated activation of these other AGC family members are independent of PI3K signaling (**Figure 1C**). As members of this kinase family have overlapping substrate specificity it is likely that PDK1 dependent, Akt independent

regulation of glucose metabolism reflects functional redundancy within the AGC family of protein kinases (Brunet et al., 2001; Zhang et al., 2006; Sapkota et al., 2007).

mTORC1 has described roles in regulating various aspects of cellular metabolism and given that its activity is independent of PI3K and Akt in some activated T cell subsets, it remains likely that mTORC1 is involved in maintaining glucose uptake and glycolysis (unpublished data; Duvel et al., 2010; Macintyre et al., 2011). Indeed, inhibition of mTORC1 decreases glycolysis in T cells activated under Th17 polarizing conditions (Shi et al., 2011). However, as mTORC1 inhibition also disrupts Th17 differentiation, it is difficult to interpret whether the effect of rapamycin on glycolysis is direct or as a result of the differentiation of different T cells. Therefore, a role for mTORC1 in controlling glycolysis in T cells has still to be formally demonstrated.

T CELL MIGRATION AND METABOLISM

The expression of adhesion molecules and chemokine receptors orchestrate the peripheral trafficking of activated T cells. The p110γ catalytic subunit is the major PI3K isoform in T cells that promotes $PI(3,4,5)P_3$ signaling in response to chemokines. Thus, migration to a range of chemokines is deficient in $p110\gamma^{-/-}$ T cells and these T cells fail to traffic normally to sites of inflammation (Reif et al., 2004; Smith et al., 2007; Martin et al., 2008; Thomas et al., 2008). T cell migration and motility are energy demanding processes and it is tempting to speculate that chemokine receptor signaling might promote T cell glucose metabolism to meet these energy demands. However, while there is some tentative data linking chemokine receptor signaling to cell growth and metabolism in transformed T cells and developing thymocytes the relationship between chemokine receptor signaling and T cell metabolism has not been directly studied (Janas et al., 2010; Lo et al., 2010). Nonetheless, it is worth mentioning that factors that influence T cell migration and/or homing, and thus the peripheral tissue destination of T cells, will affect T cell metabolism, albeit indirectly, by determining the cytokine environment to which they are exposed. A comparison of activated T cells responding to related cytokines IL2 and IL15 illustrates the differential regulation of T cell metabolism by distinct cytokine environments. IL2 promotes elevated glucose metabolism and glycolysis while IL15 does not maintain this metabolic state and T cells responding to IL15 are smaller with reduced nutrient uptake and glycolysis (Cornish et al., 2006; Macintyre et al., 2011; unpublished data). While PI3Kγ controls T cell migration in response to chemokines, PI3Kδ regulates the repertoire of adhesion and chemokine receptors expressed by activated T cells. PI3Kδ, signaling through Akt and the Foxo transcription factors, regulates the expression of key molecules required for T cell homing between the blood and the lymphoid organs; the adhesion molecule CD62L (also called L-selectin) and the chemokine receptors CC-chemokine receptor 7 (CCR7) and sphingosine-1-phosphate receptor 1 ($S1P_1$). Disruption of PI3Kδ/Akt signaling in activated CD8 T cells prevents the down-regulation of CD62L, CCR7, and $S1P_1$ and these T cells retain a lymph node trafficking pattern rather than migrating to non-lymphoid tissues and the sites of inflammation (Sinclair et al., 2008; Waugh et al., 2009; Finlay and Cantrell, 2010; Macintyre et al., 2011). Thus, PI3Kδ deficient T cells activated *in vivo* or wild-type T cells activated in mice treated with a PI3Kδ inhibitor fail to traffic into the periphery to antigenic sites (Jarmin et al., 2008). Therefore, both PI3Kγ and δ isoforms coordinately regulate T cell peripheral tissue homing thereby dictating the cytokine environments encountered and indirectly impacting upon T cell metabolism.

FINAL REMARK

It has recently become clear that the PI3K/Akt signaling axis is not the important regulator of glucose uptake and glycolysis in mature T cells, as initially described. However, disrupting PI3K may in fact impact upon T cell metabolism through indirect mechanisms, i.e., through altering their *in vivo* trafficking pattern, which will dictate the cytokines these T cells encounter.

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RNA-binding proteins as a point of convergence of the PI3K and p38 MAPK pathways

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Understanding the mechanisms by which signal transduction pathways mediate changes in RNA abundance requires the examination of the fate of RNA from its transcription to its degradation. Evidence suggests that RNA abundance is partly regulated by post-transcriptional mechanisms affecting RNA decay and this in turn is modulated by some of the same signaling pathways that control transcription. Furthermore, the translation of mRNA is a key regulatory step that is influenced by signal transduction. These processes are regulated, in part, by RNA-binding proteins (RBPs) which bind to sequence-specific RNA elements. The function of RBPs is controlled and co-ordinated by phosphorylation. Based on the current literature we hypothesize that RBPs may be a point of convergence for the activity of different kinases such as phosphoinositide-3-kinase and mitogen-activated protein kinase which regulate RBP localization and function.

Keywords: RNA-binding proteins, PI3K, MAPK

INTRODUCTION

The response of cells to environmental stimuli frequently involves changes in gene expression. This may be controlled at multiple levels including the production of new RNA by transcription. Post-transcriptional regulation at the RNA level includes nuclear RNA processing (frequently a co-transcriptional process), as well as RNA export, decay, localization, and translation. These processes are integrated with changes in protein stability and function. Signaling pathways are a major mechanism for co-ordination of these distinct mechanisms (Schoenberg and Maquat, 2012).

Substantial evidence obtained over two decades has highlighted the importance of mRNA stability in gene regulation (Cheadle et al., 2005; Keene, 2007; Anderson, 2008; Hao and Baltimore, 2009). The half-life of different mRNAs can vary from 15 min to more than 24 h depending on the activation status of a cell, for example, the half-life for interleukin-2 (IL-2) mRNA is 17 min in non-stimulated T cells but upon activation with anti-CD3/CD28 the half-life for IL-2 mRNA is increased to 232 min (Raghavan et al., 2002, 2004; Yang et al., 2003). The mRNA half-lives in bacteria (Bernstein et al., 2002) and yeast (Wang et al., 2002) are mostly shorter in comparison to mammalian cells. The increased mRNA half-life correlates with increasing organismal complexity and a tendency for 3′ untranslated regions (UTR) within mRNA to become longer in more complex species (Mazumder et al., 2003; Dinger et al., 2011). Thus, post-transcriptional regulation of mRNA may be a more prevalent amongst complex multicellular organisms.

The difference in mRNA half-lives can lead to significant changes in the abundance of mRNA (Ross, 1995). This was illustrated in genome-wide studies which have shown that up to 50% of altered mRNA abundance in lymphocytes is due to the regulation of mRNA stability (Lam et al., 2001; Cheadle et al., 2005). The stability of mRNA is regulated by distinct sequences present in the coding and UTR of mRNA (Caput et al., 1986; Shaw and Kamen, 1986; Schoenberg and Maquat, 2012). Conservation of these sequences within the UTR region among different species further emphasizes their regulatory role.

The fate of RNA can be regulated by the interplay between sequences within the RNA (*cis*-acting) and *trans*-acting factors present in the nucleus and cytoplasm (Keene, 2007; Anderson, 2010; Elkon et al., 2010). *Trans*-acting factors such as non-coding RNA (Rinn and Chang, 2012), microRNA (Fabian and Sonenberg, 2012), and RNA-binding proteins (RBPs) have been reported to regulate mRNA stability and translation. Translation can also be regulated through control of the length of the poly(A) tail in the cytoplasm (Weill et al., 2012). The class of *trans*-acting factors we will focus on in this review are the RBPs. The function of RBPs can be controlled by different signaling pathways and several excellent reviews covering the detailed regulation of RBPs in response to stress pathways have been published (Eberhardt et al., 2007; Doller et al., 2008; Sandler and Stoecklin, 2008; Kim et al., 2010). Here we will discuss the function of selected RBPs at the molecular level and how they are being controlled by phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinases (MAPK) signaling.

RNA-BINDING PROTEINS

It has been estimated that approximately 1000 RBPs are encoded in the mammalian genome (Keene, 2007; Araujo et al., 2012; Baltz et al., 2012; Castello et al., 2012). These play important roles in splicing, nuclear export, mRNA stability, localization, and translation. RBPs exert their function by physically interacting with RNA and can do so in a sequence-specific manner. Amongst the well-characterized sequences that bind RBPs are the adenine- and uridine-rich elements (ARE). RBPs that bind to ARE include KSRP as well as TTP (TIS11) and its homologs TIS11b (also called BRF-1; butyrate response factor-1), and TIS11d (BRF-2). These

have been shown to promote ARE-dependent mRNA decay but may also affect translation. HuR and its close relatives have also been suggested to promote mRNA stability and to regulate translation (Srikantan and Gorospe, 2012; Yiakouvaki et al., 2012). The domain structures of these RBPs are depicted in **Figure 1**.

CONTROL OF RBP FUNCTION BY SIGNAL TRANSDUCTION PATHWAYS

Diverse stimuli (such as chemical and environmental mediators, reviewed in Eberhardt et al., 2007) induce signaling cascades which control RBP function by means of protein phosphorylation. However the details of how these signaling pathways control the abundance and RNA-binding properties of RBPs at molecular level are only beginning to be understood. Amongst the signaling kinases discussed below and summarized in **Table 1** are PI3K, PKB (protein kinase B), mTOR (mammalian target of rapamycin), and p38 MAPK. Experimentally determined phosphorylation sites on the RBPs we will focus on are depicted in **Figure 1**. A more extensive list derived from phospho-proteomics studies is given in **Table 2**.

THE PI3K PATHWAY

The PI3K pathway plays an important role in controlling cell growth, differentiation, survival, chemotaxis, and metabolism. The activation of PI3K stimulates the generation of phosphatidylinositol 3,4,5 trisphosphate at the cell membrane, which mediates the recruitment of PKB (also known as AKT) and phosphoinositide-dependent kinase 1 (PDK1). PKB is then activated following phosphorylation by PDK1 at Thr^{308}. Mammalian target of rapamycin complex 2 (mTORC2) phosphorylates PKB at hydrophobic motif (Ser^{473}; Jacinto et al., 2006; Sarbassov et al., 2006; Facchinetti et al., 2008). PKB, which has three isoforms encoded by different genes, is involved in regulating cell proliferation and survival (Pearce et al., 2010). Genetic alterations in the PI3K pathway, such as mutations in catalytic subunits and loss of the negative regulator PTEN have been found in cancers (Kok et al., 2009). PI3K also regulates immunity and inflammation by controlling the recruitment and activation of immune cells. Thus, PI3K is an important signal integrator in maintaining immune homeostasis.

An early report implicated PI3K in the regulation of the stability of IL-3 mRNA by TIS11/TTP but did not identify phosphorylation sites within TTP (Ming et al., 2001). Subsequent studies using mass spectroscopy identified over 30 phosphorylation sites on TTP (Cao et al., 2006). Both Ser^{60} and Ser^{113} in human TTP are predicted to be PKB phosphorylation sites (Cao et al., 2007) but this has not been experimentally confirmed.

Following activation of the PI3K pathway the phosphorylation of TIS11b by PKB at Ser^{90}, Ser^{92}, and Ser^{203} facilitates its binding with 14-3-3. This sequesters TIS11b in the cytoplasm and

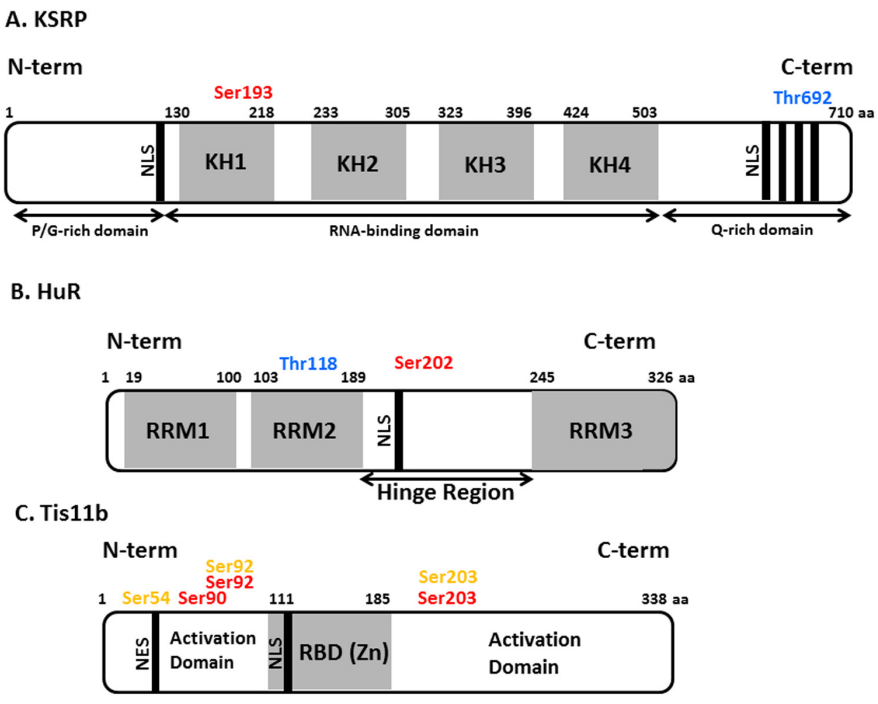

FIGURE 1 | Schematic view of domains and phosphorylation sites in KSRP, HuR and Tis11b proteins. (A) KH domains and nuclear localization signals are represented in light gray and black colors respectively. The amino acid numbers are depicted on top of protein domain structure. Proline/glycine-rich and glutamine-rich domains on N-terminal and C-teriminal end respectively are marked. **(B)** Hinge region (containing nucleocytoplasmic sequence and nuclear localization signal, NLS); RNA recognition motifs (RRM) and their corresponding amino acid positions are depicted. **(C)** Activation domains on N- and C-terminal ends, NLS, nuclear export sequence (NES), and RBD (RNA-binding domain) are shown. The experimentally determined phosphorylation sites on RBPs are given colors in following manner, red: PKB phosphorylation sites, blue: p38 phosphorylation sites, yellow: MK2 phosphorylation sites.

Table 1 | Representative list of signaling kinases regulating RBP function.

RBP	Kinase	Phosphosite/ inhibitor/siRNA	Type of cell	Target gene	Fate of RBP or its target gene function	Reference
TIS11	Protor-2	siRNA	Jurkat	TNF-α, COX-2, GM-CSF, IL-3	Regulate RBP function	Holmes et al. (2012)
TIS11	MK2	siRNA	HPMECs	ICAM-1 and IL-8	Target gene mRNA stability	Shi et al. (2012)
TIS11	MK2	Ser52, Ser178	3T3		Inhibition of mRNA decay property, RBP stability	Chrestensen et al. (2004)
TIS11b	PKB	Ser92, Ser203	HT1080 and MEF		Inhibition of mRNA decay property, RBP stability	Schmidlin et al. (2004)
TIS11b	MK2	Ser54, Ser92, Ser203	HT1080	GM-CSF	Inhibition of mRNA decay property	Maitra et al. (2008)
HuR	mTOR	siRNA	RIE-1	Ornithine decarboxylase	Target gene mRNA stability	Origanti et al. (2012)
HuR	MK2	Inhibitor	184B5/HER	COX-2	Target gene mRNA stability	Subbaramaiah et al. (2003)
TIA1	mTOR	Inhibitor	HeLa	5′ terminal oligopyrimidine tracts	Translation repressor	Damgaard and Lykke-Andersen (2011)
TIAR	mTOR	Inhibitor	HeLa	5′ terminal oligopyrimidine tracts (5′TOP)	Translation repressor	Damgaard and Lykke-Andersen (2011)
KSRP	PKB	Ser193	Alpha-T3-1	β-catenin	Inhibition of mRNA decay property	Gherzi et al. (2006)
NF90	PKB	Ser647	Jurkat	IL-2	Stabilizing mRNA	Pei et al. (2008)
hnRNP F	mTOR	Inhibitor	HEK293	controls proliferation		Goh et al. (2010)
CELF1	PKB	Ser28	Myoblasts	CcnD1		Salisbury et al. (2008)
Nucleolin	PI3K	Inhibitor	Endothelial cell line	KLF2	Up regulation of KLF2	Huddleson et al. (2006)
IMP2	mTOR	Ser162/164	Human RD	IGF2	Regulate translation of IGF2	Dai et al. (2011)
YB1	PKB	Ser99	CEF		Regulate translation capacity of YB1	Bader and Vogt (2008)

Table 1 shows type of kinase and/or its phosphorylation site on RBP function over its target gene in a specific cell. In studies where phosphorylation site is not reported, authors used either inhibitor or siRNA against studied kinase to analyse its role on RBP function.

inhibits its ability to promote mRNA decay (Schmidlin et al., 2004; Benjamin et al., 2006). Phosphorylation of these sites appears to increase the stability of the TIS11b protein which is consistent with a shorter half-life/increased degradation of TIS11b protein in PKBα knockout mouse embryo-derived fibroblasts (MEFs; Benjamin et al., 2006). Mutagenesis of Ser^{90}, Ser^{92}, and Ser^{203} in TIS11b uncoupled it from regulation by PKB and the mutated protein, which retained the ability to promote RNA decay, could no longer associate with 14-3-3 (Benjamin et al., 2006).

KSRP mediates mRNA destabilization by binding to AREs in target mRNAs. Phosphorylation of KSRP at Ser^{193} by PKB facilitates its binding to 14-3-3 which inhibits its interaction with the RNA decay machinery (in this case the exosome; Gherzi et al., 2006). In this way, KSRP is prevented from promoting the degradation of β-catenin mRNA (Gherzi et al., 2006). Phosphorylation at Ser^{193} creates a binding site for 14-3-3ζ in the N-terminal KH domain of KSRP (Diaz-Moreno et al., 2009) which, upon interaction with KSRP, promotes its nuclear localization. Thus, the availability of KSRP in cytoplasm and its ability to mediate mRNA decay is limited by phosphorylation. Interestingly, phosphorylation at Ser^{193} redirects the function of KSRP to become a regulator of the maturation of miRNA (Trabucchi et al., 2009). In C2C12 myoblasts PI3K-dependent phosphorylation of KSRP enhanced its ability to accelerate myogenic miRNA processing while attenuating its ability to promote myogenic mRNA decay. Thus, during myogenesis, KSRP appears to function as a dynamic switch controlling RNA regulated by PI3K (Briata et al., 2012).

The importance of the PI3K pathway in controlling mRNA decay was further illustrated by a recent study which demonstrated that approximately 20 out of 50 transcripts regulated by PI3K were affected at the level of mRNA stability (Graham et al., 2010). Using

Table 2 | Phosphorylation sites on RBP from phospho-proteomic studies.

RBP	Kinase	Phosphorylation site	Type of cell	Reference
TIS11	MK2	Ser52	3T3	Wang et al. (2008)
TIS11	CAMK2	Ser52	CD8 T	Navarro et al. (2011)
TIS11	PKB	Ser248	CD8 T	Navarro et al. (2011)
TIS11	PKA	Ser197	HEK-293	Cao et al. (2006)
TIS11	GSK3	Ser218	HEK-293	Cao et al. (2006)
TIS11	ERK	Ser228	HEK-293	Cao et al. (2006)
TIS11b	PKA/PKB	Ser54/92	CD8 T	Hsu et al. (2011); Navarro et al. (2011)
TIS11b	mTOR	Ser334	MEF	Hsu et al. (2011)
TIS11d	PKB	Ser98	3T3	Wang et al. (2008)
TIS11d	PKA/PKB	Ser28/98	CD8 T	Navarro et al. (2011)
TIS11d	mTOR	Ser57/73/416/464	MEF	Hsu et al. (2011)
KSRP	mTOR	Ser182, Ser185	MEF	Yu et al. (2011)
KSRP	PKA	Ser481	CD8 T	Navarro et al. (2011)
Nucleolin	mTOR	Ser28/34/40/41/145/157/616/189/403/212/460,Thr121	MEF	Yu et al. (2011)
Roquin	ERK/MAPK	Ser770	CD8 T	Navarro et al. (2011)
Roquin	mTOR	Ser531/535	MEF	Hsu et al. (2011)
hnRNPk	CDK2	Ser284	CD8 T	Navarro et al. (2011)
hnRNPu	CK1	Ser187	CD8 T	Navarro et al. (2011)
hnRNPu	CAMK2	Ser247	CD8 T	Navarro et al. (2011)
hnRNPF	mTOR	Ser63	MEF	Hsu et al. (2011)
hnRNPA3	mTOR	Ser356/359/367	MEF	Hsu et al. (2011)
hnRNPA3	CK1	Ser359	CD8 T	Navarro et al. (2011)
hnRNPA3	PKA	Ser357	CD8 T	Navarro et al. (2011)
hnRNPAb	PKA	Ser260	CD8 T	Navarro et al. (2011)
AUF1	mTOR	Ser82/83, Thr177	MEF	Hsu et al. (2011)
hnRNPA2B1	mTOR	Ser245/247/266/272, Tyr254	MEF	Hsu et al. (2011)
hnRNPK	mTOR	Ser284/379	MEF	Hsu et al. (2011)
hnRNPC	mTOR	Ser229/232/241/268/306/313	MEF	Hsu et al. (2011)
hnRNPUL1	mTOR	Ser513	MEF	Hsu et al. (2011)
hnRNPUL1	CAMK2	Ser195	CD8 T	Navarro et al. (2011)
hnRNPA1	mTOR	Ser6/257	MEF	Hsu et al. (2011)
hnRNPA1	CDK1	Ser6	CD8 T	Navarro et al. (2011)
hnRNPH2	mTOR	Ser104	MEF	Hsu et al. (2011)
HuR	mTOR	Ser202	MEF	Yu et al. (2011)
IMP2	mTOR	Ser102	MEF	Yu et al. (2011)
IMP2	mTOR	Ser160/161/163	MEF	Hsu et al. (2011)

The phosphorylation sites on RBPs for respective kinases in a specific cell are obtained from published phospho-proteomic studies. In Yu et al. (2011)), mTOR phosphorylation sites on RBP in MEFs are derived using inhibitors rapamycin and KU-0063794 against mTOR in a SILAC experiment. Similarly in Hsu et al. (2011), mTOR regulated phosphosites on RBPs in MEFs were verified using Torin1 (mTOR) inhibitor in an iTRAQ experiment. By employing SILAC technology, Navarro et al. (2011) identified phosphorylation sites on different RBPs for the respective kinases in TCR stimulated CD8 T cells. Wang et al. (2008) identified phosphorylation sites on RBPs in 3T3 cells using iTRAQ.

siRNA knockdown experiments TIS11b and KSRP were shown to be involved in stabilization of the mRNAs of down-regulated genes (Graham et al., 2010). Interestingly Graham et al., 2010) observed no effect on mRNA stability by RBPs such as AUF1 and HuR which are not known to be controlled by PI3K signaling, suggesting a key role of PI3K pathway in maintaining mRNA stability via TIS11b and KSRP.

MAMMALIAN TARGET OF RAPAMYCIN

The mTOR is a kinase component of signaling complexes which play very important roles in immune cell function (Powell et al., 2012). The mTORC1 complex can be activated in a PI3K-dependent or independent manner and is highly susceptible to inhibition by rapamycin. By contrast, mTORC2 which phosphorylates PKB Ser^{473} and contributes to its activation is much less sensitive to rapamycin (Jacinto et al., 2006; Sarbassov et al., 2006; Facchinetti et al., 2008). mTOR can also be activated in a PI3K-independent manner by spleen tyrosine kinase (SYK), as reported in follicular lymphoma cells (Leseux et al., 2006). In addition to SYK, the Erk pathway can also activate mTOR (Shaw and Cantley, 2006). Thus, mTOR activated in a PI3K-dependent or independent manner exerts effector functions via a number of targets including PKB (Powell et al., 2012).

In a phospho-proteomic study the Blenis Group reported Ser^{182} and Ser^{185} as phosphorylation sites on KSRP for mTOR. These findings were verified by treating MEFs with the mTOR inhibitors rapamycin and KU-0063794 (**Table 2**; Yu et al., 2011). The same phosphorylation sites in KSRP were predicted by PHOSIDA (www.phosida.com; the post-translational modification database which provides information on the sites of phosphorylation, *N*-glycosylation, and acetylation across nine different species; Gnad et al., 2007; Gnad et al., 2011). However Ser^{185} of KSRP has also been predicted to be a casein kinase 1 (CK1) phosphorylation site and the function of this phosphorylation is presently unknown.

The Blenis group also reported HuR Ser^{202} as a phosphorylation site for mTOR and this finding was substantiated using the mTOR inhibitors rapamycin and KU-0063794 in MEFs (**Table 2**; Yu et al., 2011). However, limited evidence exists for the functional regulation of HuR by mTOR as it has not yet been reported whether or not mTOR regulates HuR localization and function. Previously the Gorospe group had reported Ser^{202} as a target for cyclin-dependent kinase 1 (Cdk1; Kim et al., 2008). Phosphorylation of HuR by Cdk1 promoted its movement into the nucleus where HuR appeared to be associated with 14-3-3 proteins. A modified HuR protein with a non-phosphorylatable serine to alanine mutation resided predominantly in the cytoplasm. Unphosphorylated HuR bound poorly to 14-3-3, which increased the availability of HuR for stabilizing its target mRNAs (Kim et al., 2008). Thus, the function of HuR is modulated by Cdk1 during the cell cycle (Blethrow et al., 2008; Kim et al., 2008). In another very recent report CDK5 phosphorylation of HuR at Ser202 has been shown to regulate its function in cell cycle progression (Filippova et al., 2012). HuR regulates ornithine decarboxylase (ODC) mRNA stability (Nowotarski and Shantz, 2010) and the binding of HuR to the ODC transcript is decreased when mTORC1 signaling is inhibited using rapamycin, an mTORC1 inhibitor (Origanti et al., 2012). The mTOR mediated phosphorylation at Ser^{202} on HuR might be one mechanism through which mTOR regulates proliferation.

Protor2, a component of mTORC2 kinase has been shown to bind to TTP in Jurkat cells following treatment with carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, an uncoupler of mitochondrial oxidative phosphorylation. The interaction between TTP and Protor2 was suggested to be necessary for enhancing TTP-mediated turnover of mRNAs such as IL-3, GM-CSF, COX-2, and TNF (Holmes et al., 2012). The siRNA knockdown of protor2 inhibited the localization of TTP to mRNA processing bodies (P-bodies), the sites where mRNA decay enzymes are concentrated (Parker and Sheth, 2007). Very recently mTOR has been reported to regulate iron homeostasis by modulating transferrin receptor 1 (TfR1) stability via TTP (Bayeva et al., 2012).

THE P38 MAPK PATHWAY

Mitogen-activated protein kinases are major regulatory hubs where inflammation and stress responses are regulated. Three major MAPK pathways are p38, JNK, and ERK. We discuss below the findings implicating p38 and its substrate MAPK activated protein kinase 2 (MK2) in regulating RBP activity.

The p38 pathway via MK2 regulates the mRNA decay property and the mRNA and protein expression of TTP (Dean et al., 2001; Tchen et al., 2004; Brook et al., 2006; Hitti et al., 2006). MK2 phosphorylates mouse TTP at Ser^{52} and Ser^{178} which stabilizes TTP protein (Brook et al., 2006). Upon dephosphorylation of these sites, TTP moves from the cytoplasm to the nucleus and undergoes degradation (Brook et al., 2006). MK2-mediated phosphorylation of TTP does not affect its binding to target mRNA, but inhibits the ability of TTP to recruit deadenylases to target mRNA for their degradation in cytoplasm (Carballo et al., 2001; Stoecklin et al., 2004; Clement et al., 2011). Further investigation of this demonstrated that carbon catabolite repressor protein 4-associated factor-1 (CAF1) was the major source of deadenylase activity responsible for TTP-directed deadenylation (Marchese et al., 2010). MK2 phosphorylation reduced the ability of TTP to promote deadenylation by inhibiting the recruitment of CAF1 deadenylase independently of 14-3-3. The Stoecklin group demonstrated that Not1, a component of carbon catabolite repressor protein 4 (Ccr4)-negative on TATA (NOT) complex, associates with TTP and is required for the decay of ARE-mRNAs (Sandler et al., 2011). Subsequently it has been reported that TTP regulates the translation of TNF mRNA at the endoplasmic reticulum (Tiedje et al., 2012). Phosphorylation of TTP by MK2 weakens its ability to bind to TNF mRNA and allowed HuR-binding to TNF mRNA which promoted its translation. Conflicting data exists on the ability of phospho-TTP to bind its target mRNA (Clement et al., 2011; Tiedje et al., 2012) The former group found that phosphorylation did not alter TTP binding to its target mRNA but the latter group found that phosphorylation reduced the affinity of TTP binding to its target mRNA.

TIS11b is also regulated by MK2. The phosphorylation of TIS11b at Ser^{54}, Ser^{92}, and Ser^{203} by MK2 inhibits the ability of TIS11b to promote ARE-mediated mRNA decay (Maitra et al.,

2008). The phosphorylation-dependent inhibitory effects of MK2 on TIS11b do not seem to alter its ability to bind RNA or its association with mRNA decay enzymes. Furthermore, the MK2-mediated effects on TIS11b were independent of PKB (Maitra et al., 2008).

Several studies indicate that the function of HuR is regulated by the p38 pathway. The abundance of COX-2 mRNA is controlled by the p38-dependent regulation of the binding of HuR to the COX-2 3′ UTR (Subbaramaiah et al., 2003). In neuronal cell line p38 activation following treatment with anisomycin promotes the cytoplasmic accumulation of HuR where it interacts with and stabilizes the survival motor neuron (SMN) transcript (Farooq et al., 2009). In mouse splenic T cells, LFA-1 engagement activates p38 which promotes HuR translocation and stabilization of IFN-γ and TNF mRNA (Ramgolam et al., 2010). In none of these studies was it established whether HuR was directly phosphorylated by p38 or MK2. However, an independent study has reported that phosphorylation of HuR at Thr^{118} by p38 promotes its localization to the cytoplasm where it stabilizes p21 mRNA during the DNA damage response (Lafarga et al., 2009). HuR was found to be phosphorylated at Thr^{118} a site previously identified to be phosphorylated by Chk2 (Abdelmohsen et al., 2007). HuR regulated translation of TNF mRNA at the endoplasmic reticulum appeared to be mediated by the effects of the p38 pathway on TTP (Tiedje et al., 2012) and no evidence was found for p38-mediated phosphorylation of HuR. It was suggested that Thr^{118} was instead phosphorylated by Chk2 as a consequence of the over-expression system being used (Tiedje et al., 2012).

The p38 pathway also regulates KSRP. During C2C12 muscle cell differentiation the stability of p21, myogenin, and MyoD mRNA is regulated by p38-mediated phosphorylation of KSRP (Briata et al., 2005). p38 phosphorylates KSRP at Thr^{692} which renders KSRP unable to bind to ARE-containing transcripts thus promoting their stabilization. However, this phosphorylation event does not alter the ability of KSRP to interact with the mRNA degradation machinery (Briata et al., 2005). To our knowledge it has not yet been reported whether or not p38 regulates KSRP function in microRNA maturation.

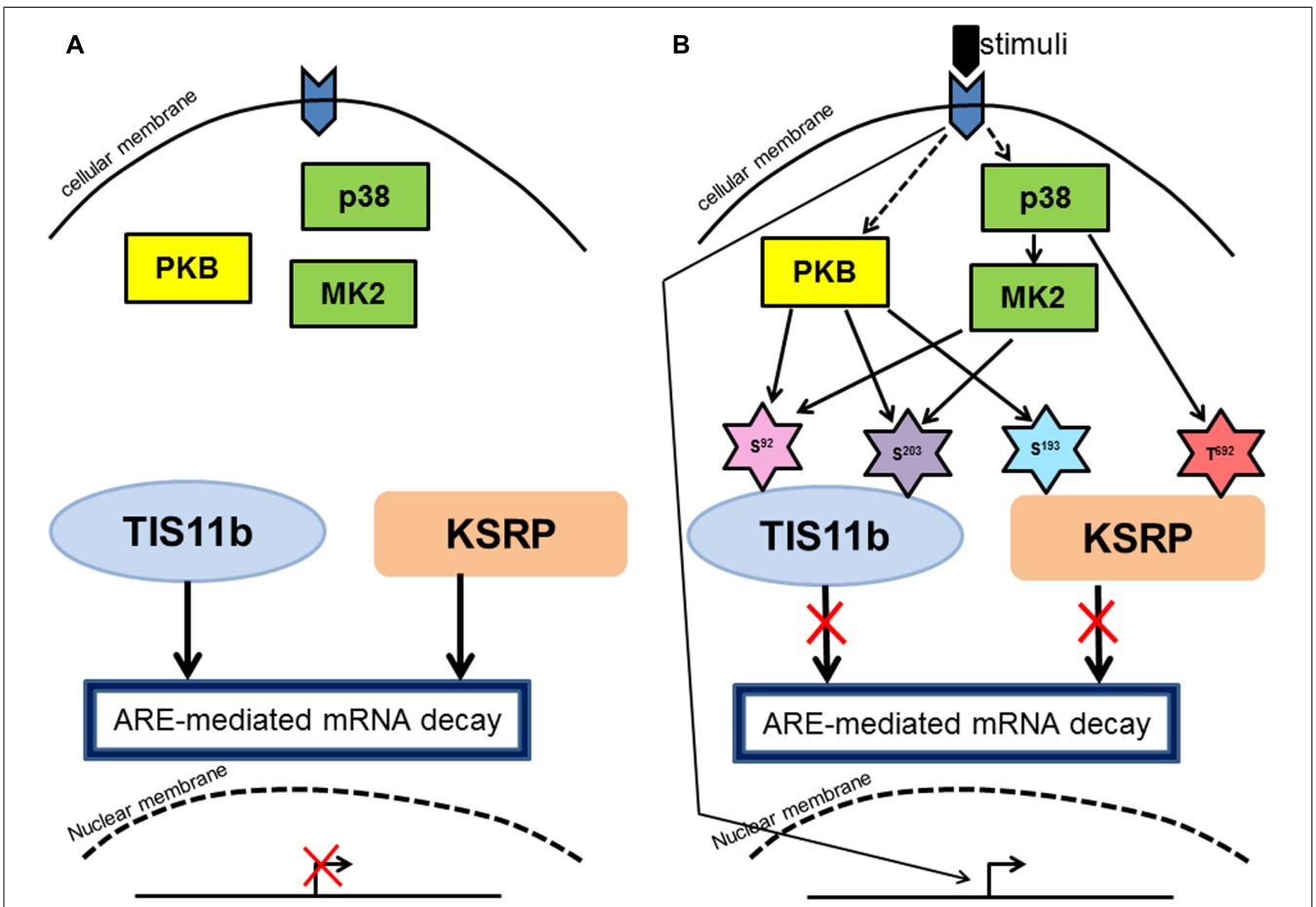

FIGURE 2 | Convergence of signaling on RBP and its function. (A) In non-activated cells, RBPs (TIS11b and KSRP) are active in promoting mRNA decay of ARE-containing transcripts. **(B)** Upon activation, PKB and MK2 phosphorylate TIS11b at Ser^{92} and Ser^{203} and subsequently TIS11b is prevented in mediating ARE-mediated mRNA decay. Similarly, but not at same phosphorylation sites, KSRP is phosphorylated at Ser^{193} and Thr^{692} by PKB and p38 respectively. These phosphorylation events inhibit KSRP in mediating ARE-mediated mRNA decay. Transcription factors upon activation by signals from kinases drive transcription which is illustrated in the **Figure 2**. The phosphorylation sites are shown in star shape cartoons.

ARE RBPs A POINT OF CONVERGENCE FOR PI3K AND p38 SIGNALING?

In NIH 3T3 fibroblasts, the stability of TPA induced IL-3 mRNA is regulated by the p38 and PI3K pathways (Ming et al., 2001). The latter mediates its effects independently of p38 suggesting that p38 and PI3K pathways control IL-3 mRNA turnover by parallel mechanisms. Stabilization of IL-3 mRNA mediated by either of these two pathways is antagonized by TTP and this effect can be overcome by HuR when it is in collaboration with p38 but not with PI3K (Ming et al., 2001). This suggests that signaling pathways activated upon stimulation lead either to activation of stabilizing RBP (HuR) or inactivation of destabilizing RBP (TTP) thus preventing the degradation of transcripts (Ming et al., 2001). Furthermore, another study reported that in U87 glioblastoma cells, the regulation of cyclin D1 and c-Myc mRNA stability by TTP is controlled by p38 in a PKB-dependent manner (Marderosian et al., 2006), implying interdependent roles for p38 and PKB. Data from phospho-proteomic (Cao et al., 2006, 2007; Navarro et al., 2011) and *in vitro* (Chrestensen et al., 2004) studies suggest that MK2 and PKB phosphorylate TTP at $Ser^{52/178}$ and Ser^{248} respectively (**Table 2**).

Both PKB and MK2 target the same phosphorylation sites on TIS11b (Ser^{92} and Ser^{203}) and inhibit its mRNA decay activity (Benjamin et al., 2006; Maitra et al., 2008). The mRNA decay property of KSRP is also regulated by PKB and p38 (Briata et al., 2005; Gherzi et al., 2006), but in this instance the kinases do not use the same phosphorylation sites.

HuR provides a further example of a point of convergence. In this case the circumstances under which the mTOR would affect HuR have not been studied but the phosphorylation site (Ser^{202}) has been shown to regulate the function of the protein. Similarly, p38-mediated phosphorylation of HuR at Thr^{118} targets a site previously shown to be targeted by Chk2 (Abdelmohsen et al., 2007). It is clear that multiple different kinases converge on HuR to regulate its function (**Figure 2**).

FEEDBACK BETWEEN RBPs AND PI3K mTOR PATHWAY

Current literature suggests that kinases control the function of RBPs, however RBPs have also been reported to regulate the expression of kinases suggesting a role for RBPs in feedback control over kinase expression. For example, the 68 kDa Src substrate associated during mitosis (Sam68) is an RBP reported to regulate alternative splicing of mTOR (Huot et al., 2012). In Sam68 knockout cells, intron five is retained in the mTOR transcript introducing a premature termination codon, which results in an unstable mRNA and subsequently reduced protein levels of mTOR. Consequently, the effector pathways of mTOR responsible for adipogenesis are deregulated in these mice, leading to a lean phenotype (Huot et al., 2012). Sam68 is also reported to associate with PI3K in insulin receptor signaling (Sanchez-Margalet and Najib, 2001) and this association may regulate the RNA-binding function of Sam68 (Taylor et al., 1995). Based on this information it is possible that PI3K is regulating RBP function via its downstream kinases and regulating the abundance of its downstream kinases (mTOR) via possible activation of Sam68. Conserved ARE elements in 3′ UTR of p38α, PKBα, PKBγ but not p38β, p38γ, and PKBβ, suggest that these proteins might be targets for regulation of ARE-mediated mRNA stability by RBPs (Gruber et al., 2011).

CONCLUSION

Gene expression is controlled at the post-transcriptional level by RBPs. However, regulation of the effector function of RBPs on RNA decay and translation is controlled by signals from protein kinases. These effects include inhibition of function by uncoupling from the RNA decay machinery and in some instances reassignment of function. Kinase-dependent relocation of RBP into different compartments of the cell seems to be a common theme amongst structurally diverse RBPs. RBPs represent a class of proteins upon which signaling by the PI3K and p38 pathways converge. Existing literature on this might indicate a potential redundancy of kinases phosphorylating the same serine or threonine amino acid in different cellular functions. A fuller understanding of the interplay between kinases, RBPs and target RNAs may provide important new insights into the dynamic regulation of gene expression.

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Pharmacological targeting of phosphoinositide lipid kinases and phosphatases in the immune system: success, disappointment, and new opportunities

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The predominant expression of the γ and δ isoforms of PI3K in cells of hematopoietic lineage prompted speculation that inhibitors of these isoforms could offer opportunities for selective targeting of PI3K in the immune system in a range of immune-related pathologies. While there has been some success in developing PI3Kδ inhibitors, progress in developing selective inhibitors of PI3Kγ has been rather disappointing. This has prompted the search for alternative targets with which to modulate PI3K signaling specifically in the immune system. One such target is the SH2 domain-containing inositol-5-phosphatase-1 (SHIP-1) which de-phosphorylates $PI(3,4,5)P_3$ at the D5 position of the inositol ring to create $PI(3,4)P_2$. In this article, we first describe the current state of PI3K isoform-selective inhibitor development. We then focus on the structure of SHIP-1 and its function in the immune system. Finally, we consider the current state of development of small molecule compounds that potently and selectively modulate SHIP activity and which offer novel opportunities to manipulate PI3K mediated signaling in the immune system.

Keywords: activators, inflammation, inhibitors, leukemia, lymphocytes, PI3K, SHIP-1, SHIP-2

INTRODUCTION

Studies using mice in which the genes encoding PI3Kδ or PI3Kγ have been either altered to encode kinase-inactive mutants (e.g., $PI3K\delta^{D910A}$ mice) or deleted, have revealed that PI3Kδ and PI3Kγ have non-redundant (but often co-ordinated), functions in B cells, T cells, NK cell, neutrophils, mast cells, and dendritic cells (Vanhaesebroeck et al., 2005; Crabbe et al., 2007; Randis et al., 2008; Saudemont et al., 2009; Ward and Marelli-Berg, 2009). Indeed, when the immune system of these mice is challenged they exhibit severely defective responses to infection (Vanhaesebroeck et al., 2005; Crabbe et al., 2007; Ward and Marelli-Berg, 2009). The predominant expression of the γ and δ isoforms of PI3K in cells of hematopoietic lineage prompted speculation that inhibitors of these isoforms could offer selective targeting of PI3K in the immune system in a range of inflammatory and autoimmune diseases as well as in transplantation and hematological malignancies. While there has been some success in developing PI3Kδ inhibitors, progress in developing selective inhibitors of PI3Kγ has been rather disappointing. This has prompted the search for alternative targets with which to modulate PI3K signaling specifically in the immune system. In this regard, attention has recently focused on the lipid phosphatase SH2 domain-containing inositol-5-phosphatase (SHIP), which de-phosphorylates $PI(3,4,5)P_3$ at the D5 position of the inositol ring to create $PI(3,4)P_2$. This review will focus predominantly on the role of SHIP as a potential therapeutic target in the immune system and consider progress in developing small molecule drugs that target this protein.

DEVELOPMENT OF INHIBITORS TARGETING PI3Kγ AND PI3Kδ – THE STORY SO FAR

There have been huge advances in the design of PI3K inhibitors which utilize the ATP-binding pocket of PI3K to achieve greater potency and selectivity as well as reduced toxicity (Walker et al., 2000; Knight et al., 2006; Berndt et al., 2010). The development of PI3K inhibitors with which to treat cancers has made substantial recent progress (for in depth reviews on this subject see Marone et al., 2008; Workman et al., 2010; Fruman and Rommel, 2011; Shuttleworth et al., 2011; So and Fruman, 2012). However, the development of PI3K inhibitors to treat inflammatory disorders has to date, been less successful.

The discovery of the quinazolinone purine series, exemplified by the ICOS compound IC-87114 (**Figure 1**) demonstrated that the design of isoform-selective PI3K inhibitors with at least 50-fold potency over other isoforms was possible to achieve (Sadhu et al., 2003). In 2006 several members of ICOS Corporation formed a spin-out company, Calistoga Pharmaceuticals. Calistoga developed CAL-101, a PI3Kδ specific inhibitor that exhibits 40–300-fold selectivity over other PI3K isoforms. CAL-101 which was acquired by Gilead in February 2011 and recently renamed GS-1101, has shown success in clinical trials for treatment of B cell malignancies where it causes rapid lymph node shrinkage and lymphocytosis (Fruman and Rommel, 2011; Hoellenriegel et al., 2011; Lannutti et al., 2011; So and Fruman, 2012). CAL-101 displays a dual mechanism of action whereby it both decreases cell survival and reduces chemokine-mediated interactions that retain CLL cells in protective tissue microenvironments (Hoellenriegel et al., 2011; Lannutti et al., 2011). These effects have been observed across a broad

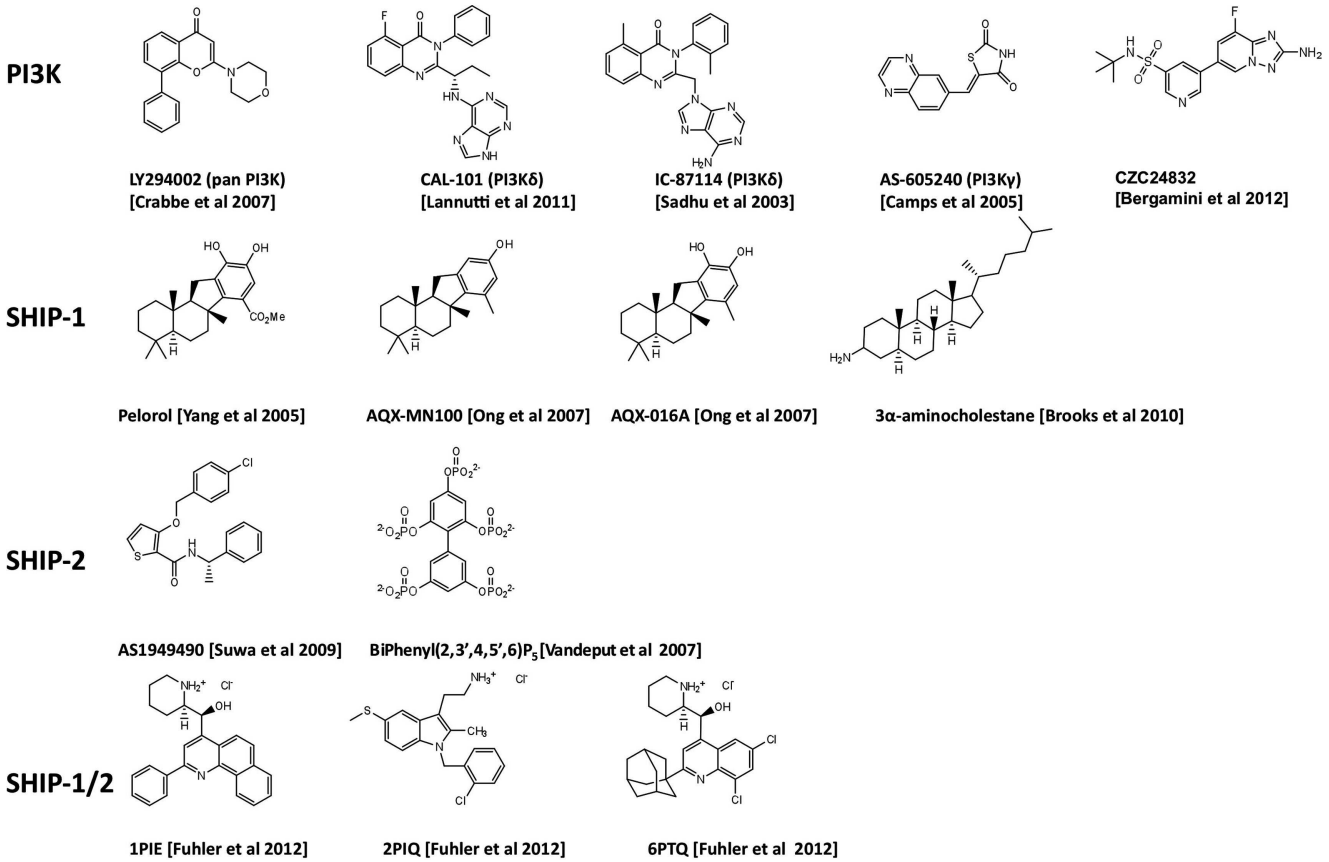

FIGURE 1 | Chemical structures of PI3K inhibitors and SHIP-targeting compounds.

range of other immature and mature B cell malignancies including $CD5^+$ mantle zone B cell lymphomas, follicular lymphomas, and multiple myeloma (Herman et al., 2010; Ikeda et al., 2010; Fruman and Rommel, 2011; Hoellenriegel et al., 2011; Lannutti et al., 2011). Hodgkin lymphoma (HL) is a malignant lymphoma of B-cell origin. The malignant cells, known as Reed-Sternberg (RS) cells, represent less than 2% of the tumor mass, the remainder composed of a mix of reactive inflammatory cells attracted by the RS cells. Recently Hodgkin Lymphoma (HL) cell lines and primary samples from patients with HL have been reported to express high level of PI3Kδ and constitutive PI3K pathway activation (Meadows et al., 2012). As with CLL, CAL-101 was able to reduce the positive interaction between stromal cells and malignant RS cells. This inhibitor has therefore, demonstrated an essential role for PI3Kδ in constitutive PI3K signaling that is required for the survival of malignant B cells. Oncogenic mutations of components of the PI3K signaling pathway are infrequent in B cell malignancies. A potential mechanism for PI3K activation in this setting is tonic antigen-independent B cell receptor (BCR) signaling that requires PI3Kδ for the transduction of proliferation and survival signals.

Inhibition of the PI3Kδ isoform for the treatment of inflammatory disorders is also being explored. Specifically, CAL-101 and CAL-263 have entered clinical trials for allergic rhinitis (**Table 1**). In addition, patents have been filed by several other companies (Amgen, Intellikine, and Incyte) describing PI3Kδ inhibitors and the majority are based on the same basic pharmacophore identified by ICOS (Norman, 2011). However, additional scaffolds have now been reported by several companies; almost all of these are with intended indications against B cell lymphomas (Norman, 2011).

Whilst there has been considerable success in designing PI3Kδ-selective inhibitors with promise against lymphoid malignancies, the progress in designing PI3K inhibitors for anti-inflammatory/autoimmune applications has been disappointing. Compounds that selectively inhibit PI3Kγ have been identified, with a series of compounds designed by Merck Serono SA based on the thiazolidinedione scaffold (Ruckle et al., 2006). One of these, AS-605240 (**Figure 1**), exhibited superior potency compared to related compounds, can be administered orally and has high cell membrane permeability (Barber et al., 2005). These class of compounds have been proven useful as experimental tools but do not have requisite drug-like properties and have limited selectivity over other class 1A PI3K isoforms. Possible reasons for the relatively slow progress in developing PI3Kγ inhibitors include the close structural conservation of class I PI3Ks and other lipid kinases in the ATP-binding pocket and the limited ability of the commonly used *in vitro* assays based on recombinant enzymes, to predict cellular and *in vivo* kinase selectivity. However, Cellzome recently

Table 1 | Clinical trials status of PI3K and SHIP-1 targeting compounds for the treatment of inflammatory disorders.

Compound	Inflammatory disorder	Protein target	Clinical trial phase	Status of clinical trial	Company	Reference
AQX-1125	Asthma	SHIP-1	IIa	Initiated	Aquinox Pharmaceuticals	http://www.aqxpharma.com/content/aquinox-pharmaceuticals-initiates-two-phase-iia-clinical-studies-airway-inflammation
CAL-101	Allergic rhinitis	PI3Kδ	I	Completed	Gilead Sciences	http://clinicaltrials.gov/ct2/show/NCT00836914
CAL-263	Allergic rhinitis	PI3Kδ	I	Completed	Gilead Sciences	http://clinicaltrials.gov/ct2/show/NCT01066611
IPI-145	Inflammatory disorders	PI3Kδ/γ	I	Initiated	Infinity and Intellikine	http://www.intellikine.com/pipeline/ipi145.html

described a chemoproteomics-based drug discovery platform that enables multiplexed high-throughput screening of native proteins in cell extracts. The chemoproteomic approach preserves post-translational modifications and protein interactions and hence allows targeting of PI3K proteins under close-to-physiological conditions in human primary cells (Bergamini et al., 2012). Using affinity enrichment of target kinases afforded by immobilized ATP-competitive lipid kinase inhibitors, the potency of small molecule test compounds was evaluated in competition binding assays. This revealed CZC24832 which exhibits superior selectivity for PI3Kγ than previously reported compounds (Camps et al., 2005; Bergamini et al., 2012). Interestingly, CZC24832 shows anti-inflammatory effects in a collagen-induced arthritis model that correlated with reduced Th17 differentiation, a pro-inflammatory helper T cell type characterized by expression of the cytokine IL-17 (Weaver and Murphy, 2007). Indeed, CZC24832 treatment also led to reduced IL-17 production (Bergamini et al., 2012). This confirms the long-held belief that pharmacological inactivation of PI3Kγ alone, can lead to amelioration of inflammatory disease. This recent breakthrough, may facilitate detailed mechanistic studies of PI3Kγ in human primary cells and allow human clinical studies in inflammation.

The non-redundant and often co-ordinated roles of PI3Kδ and PI3Kγ in immune cell function have been reported (Rommel et al., 2007) and provide a rationale for targeting both isoforms simultaneously with a single compound. Indeed, TargeGen described two diaminopteridine-diphenol-based compounds with good selectivity for PI3Kγ and PI3Kδ that showed early promise in animal models of myocardial ischemia as well as asthma and chronic obstructive pulmonary disease (Doukas et al., 2006, 2009). The TargeGen compounds did not progress beyond phase I/II clinical trails. However, Infinity and Intellikine are currently in pre-clinical trials with IPI-145 (**Table 1**), which is the only PI3Kγ/δ inhibitor currently in development for the treatment of inflammatory disease (Norman, 2011).

There is an increasing appreciation of a role for PI3Kβ in the immune system including cooperation with PI3Kδ in the generation of reactive oxygen species (ROS) in neutrophils in response to fungal infection or immune complexes (Boyle et al., 2011; Kulkarni et al., 2011). Signaling responses of several Gi-coupled receptors including those for the leukocyte chemoattractants C5a and fMLP has been demonstrated to occur at least in part via PI3Kβ (Guillermet-Guibert et al., 2008). Indeed, loss of PI3Kβ confers substantial protection in a mouse model of a human autoimmune blistering disease (Boyle et al., 2011; Kulkarni et al., 2011). Loss of PI3Kβ also partially (but significantly), protected against the development of clinical signs of arthritis in response to low does of arthritogenic serum in the K/BxN mouse model of rheumatoid arthritis. However, no protection was seen mice lacking either PI3Kβ or expressing kinase-dead PI3Kδ subjected to higher does of arthritogenic serum. Remarkably, mice lacking both PI3Kβ and PI3Kδ activity were highly protected at both high and low doses of K/BxN serum. Collectively, these data provide a rationale for targeting PI3Kβ as well as PI3Kδ in the treatment of inflammatory disorders. Such dual isoform inhibitors could offer some benefit in certain therapeutic settings, though it is important to recognize that the pathogenesis of human inflammatory diseases such as RA is complex and multi-factorial. As such, the precise contribution of each isoform to disease pathology is likely to be subtle and complex. Nevertheless, compounds with dual selectivity for PI3Kβ and PI3Kδ have been reported suggesting that this approach is feasible (Knight et al., 2006). However, caution should be applied to the use of PI3Kβ inhibitors in inflammatory disorders due to the described role of PI3Kβ in thrombus formation and circulatory homeostasis (Bird et al., 2011).

INCREASED UNDERSTANDING OF A ROLE FOR OTHER PI3KS IN THE IMMUNE SYSTEM

The difficulties of developing PI3Kγ inhibitors with sufficient selectivity over PI3K isoforms has led to the search for other targets that might offer opportunities to selectively disrupt PI3K signaling in immune cells. To this end, class II PI3KC2β, has been demonstrated to play an important and unexpected role in $CD4^+$ T-cell activation downstream of the TCR (Srivastava et al., 2009), while Vps34's role in autophagy (Backer, 2008; Simonsen and Tooze, 2009), suggests it may prove important for immune recognition of tumor antigens, regulation of T cell homeostasis, and immune tolerance (Li et al., 2008; Nedjic et al., 2008; Walsh and Edinger, 2010). There is considerable evidence that class III PI3K is important for phagocytosis (Fratti et al., 2001; Vieira et al., 2001; Ellson et al., 2006; Anderson et al., 2008). There may be opportunities to target Vps34 in destructive inflammatory/autoimmune diseases where there is dysregulated phagosomal activity and antigen presentation of self molecules, for example. The publication of the

Vps34 crystal structure in complex with PI3K inhibitors may allow the design of more potent and selective Vps34 inhibitors which are able to exploit differences between Vps34 and Class 1 PI3Ks (Miller et al., 2010). However, the largely ubiquitous expression of Class II and III PI3Ks makes selective targeting of the immune system problematic.

SHIP-1: AN ALTERNATIVE TARGET FOR MODULATION OF PI3K SIGNALING IN THE IMMUNE SYSTEM

The search for alternative targets with which to modulate PI3K signaling specifically has therefore, recently focused on the lipid phosphatase SH2 domain-containing inositol-5-phosphatase-1 (SHIP-1), which de-phosphorylates $PI(3,4,5)P_3$ at the D5 position of the inositol ring to create $PI(3,4)P_2$. The *INPP5D* gene located on chromosome 2 (2q37.1) encodes the 145-kDa SHIP-1 which was originally recognized as an important component of the inhibitory signaling pathway triggered by the IgG receptor FcγRIIB in mast cells and B cells (Ono et al., 1996). Once recruited to the plasma membrane by signaling complexes, its catalytic activity depletes $PI(3,4,5)P_3$ and prevents membrane localization of some PH domain-containing effectors, leading to inhibition of extracellular calcium influx and ultimately reducing transcription activation, and cytokine release. One would predict that activators of SHIP-1 would lead to a reduction of cellular $PI(3,4,5)P_3$ levels and hence, mimic the effect of PI3K inhibitors. Its hematopoietic-restricted expression should limit the impact of SHIP-1 targeted drugs to the immune system making SHIP-1 an attractive drug target for use in inflammatory and autoimmune diseases, hematological malignancies as well as in transplantation settings.

SHIP-1: A CROSSROADS IN PI3K-DEPENDENT SIGNALING

The classical view of SHIP-1 is that it acts to switch off PI3K-dependent signaling by degradation of $PI(3,4,5)P_3$. However, the metabolism of $PI(3,4,5)P_3$ by SHIP-1 yields $PI(3,4)P_2$ which retains the phosphate grouping on the third position of the inositol ring and thus, may retain some signaling ability (**Figure 2**). Pleckstrin homology (PH) domains encoded in many proteins (e.g., Grp-1, Gabs, and Btk) bind exclusively to $PI(3,4,5)P_3$, whereas others such as those found in dual adaptor of phosphotyrosine and 3-phosphoinositides-1 (DAPP1) and Src kinase-associated phosphoprotein (SKAP), can interact with both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (Lemmon and Ferguson, 2000; Zhang et al., 2009). In addition, the tandem PH domain-containing protein TAPP-1 encodes PH domains that show selectivity toward $PI(3,4)P_2$ (Dowler et al., 2000). The ability of PH domain-containing proteins to distinguish between different 3′-phosphoinositide lipids suggests that SHIP-1 can act as a switch to redirect PI3K-dependent signaling toward a set of distinct effectors that are temporally and functionally separate from $PI(3,4,5)P_3$-dependent events. Thus, SHIP-1 may function to fine-tune phosphoinositide signaling, rather than terminate it. In this regard, SHIP-1 promotes recruitment of the GTPase Irgm1 to sites of phagocytosis in macrophages

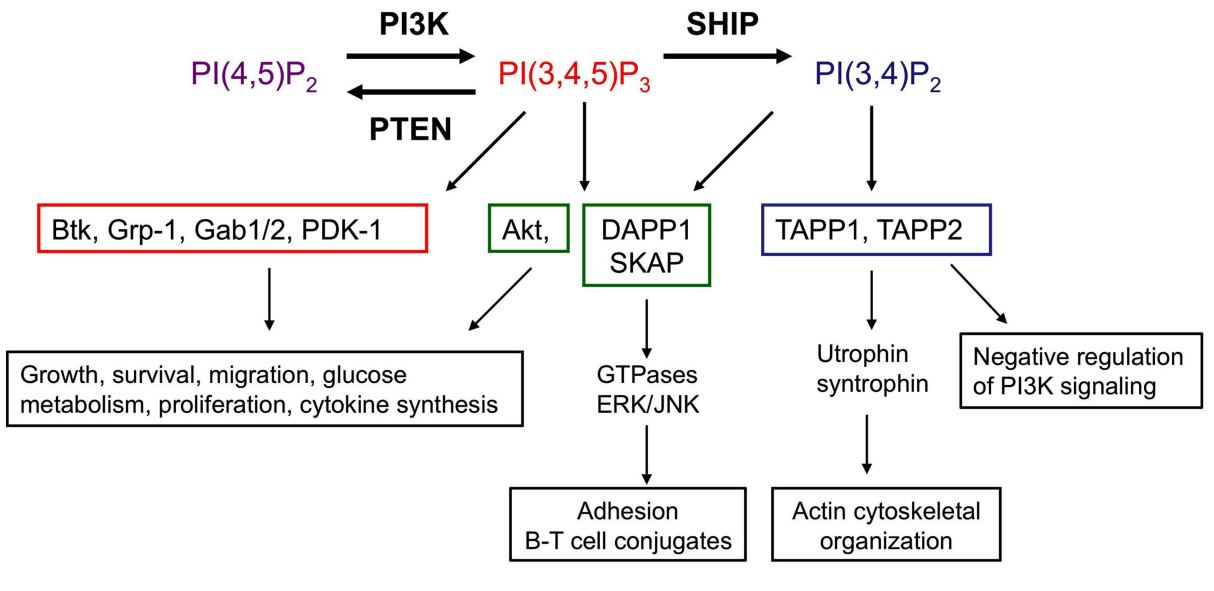

FIGURE 2 | SHIP acts as a molecular "switch." SHIP catalyzes the conversion of the PI3K lipid product $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Effector proteins which express PH domains are recruited and activated by these lipid second messengers at the cell surface membrane. PH domains of proteins are able to discriminate between $PI(3,4,5)P_3$ and $PI(3,4)P_2$. Examples of proteins which bind only $PI(3,4,5)P_3$ (red), both $PI(3,4,5)P_3$ and $PI(3,4)P_2$ (green), or only $PI(3,4)P_2$ (blue) as well as functional consequences are shown, though there are many other PH domain proteins present in immune cells and this is not an exhaustive list. Functional read-outs downstream of $PI(3,4,5)P_3$-interacting proteins and Akt are context dependent, have been extensively reviewed elsewhere (Manning and Cantley, 2007;Vanhaesebroeck et al., 2010, 2012) and are summarized in this figure. Lesser known interacting partners of PI(3,4)P2-dependent TAPP-1/2 and $PI(3,4)P2/PI(3,4,5)P_3$-dependent DAPP1 with roles in immune function are indicated (Costantini et al., 2009; Zhang et al., 2009; Vanhaesebroeck et al., 2010; Wullschleger et al., 2011; So and Fruman, 2012). Abbreviations: Btk, Bruton's tyrosine kinase; Gab1, GRB2-associated binding protein-1; Grp-1, general receptor for phosphoinositides 1; PDK-1, phosphoinositide lipid-dependent kinase-1; DAPP1, dual-adapter for phosphotyrosine and 3-phosphoinositides 1; SKAP, src kinase-associated phosphoprotein; TAPP, tandem pleckstrin homology domain protein.

via generation of PI(3,4)P_2, a critical step in maturation of the phagosome and engulfment of bacteria (Tiwari et al., 2009). PI(3,4,5)P_3 and PI(3,4)P_2 appear sequentially following agonist stimulation in many cell types including T lymphocytes, but show temporal overlap. Some cell types, notably B lymphocytes and platelets, exhibit sustained PI(3,4)P_2 production, lasting for up to 45–60 min post-stimulation (Sorisky et al., 1992; Brauweiler et al., 2001).

NON-ENZYMATIC ACTIVITIES OF SHIP-1

SH2 domain-containing inositol-5-phosphatase-1 protein possesses numerous structural domains in addition to its single catalytic domain (**Figure 3**). The catalytic domain is responsible for the hydrolysis of the 5-phosphate group on the PI3K product PI(3,4,5)P_3 to form PI(3,4)P_2. Under basal conditions, SHIP-1 is located in the cell cytosol and upon receptor ligation is recruited to the surface membrane, bringing SHIP-1 within close proximity to its lipid substrate. Numerous structural domains are required for SHIP-1 to successfully re-localize to the surface membrane. The SH2 domain within SHIP-1 interacts with proteins via the consensus amino acid sequence pY[Y/S][L/Y/M][L/M/I/V]. Through this SH2 domain, SHIP-1 binds to tyrosine phosphorylated proteins such as Shc, Doks, Gabs, CD150, platelet-endothelial cell adhesion molecule (PECAM), Cas, c-Cbl, certain immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and some immunoreceptor tyrosine-based activation motifs (ITAMs). Proline rich regions within the C-terminal enable SHIP-1 to bind proteins that contain a SH3 domain, for example phospholipase-Cγ and Grb-2. The phosphorylation of tyrosine residues within the NPXY motifs at the C-terminal tail of SHIP-1 provides sites of interaction for various proteins which express phosphotyrosine-binding (PTB) domains, such as Shc, Dok1, and Dok2. A newly identified structural domain has been recently identified whereby a segment of SHIP-1 adopts an independently folded structure predicted to have PH domain-like topology. This PH-related (PH-R) domain binds PI(3,4,5)P_3 and is required for localization of SHIP-1 to the

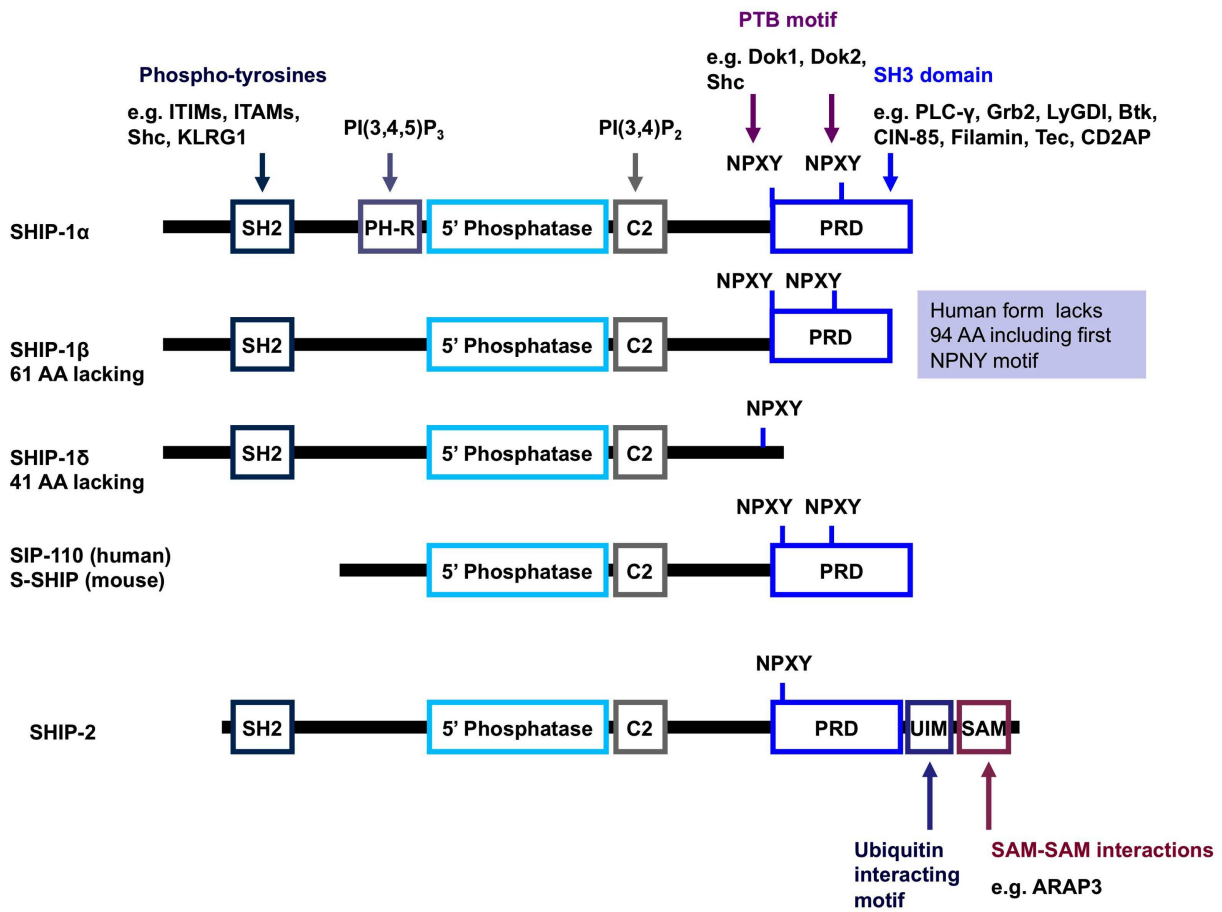

FIGURE 3 | Schematic representation of the structure of SHIP-1 and its isoforms. SHIP-1 possesses a centrally located 5′ phosphatase catalytic domain, an SH2 domain at the N-terminus as well as a proline rich domain and NPXY motifs at the C-terminus (Harris et al., 2008). SHIP-1 also has a C2 domain adjacent to the catalytic domain which, when bound to PI(3,4)P_2, acts to allosterically enhance the catalytic activity of SHIP (Ong et al., 2007). A pleckstrin homology-related domain that binds PI(3,4,5)P_3 has also been reported to exist adjacent to the catalytic domain in SHIP-1 (and most likely the other forms of SHIP-1). Structures depicted represent mouse protein; key differences in human protein structure are annotated on the right-hand side (shaded blue background). Abbreviations: PH-R, pleckstrin homology-related domain; PRD, proline rich domain; SH2, src homology domain; UIM, ubiquitin interacting motif; SAM, sterile alpha motif.

phagocytic cup and SHIP-1 mediated inhibition of FcγR-mediated phagocytosis by macrophages (Ming-Lum et al., 2012).

The various structural domains not only serve to bring SHIP-1 in close proximity to its substrate at the surface membrane, but also allow SHIP-1 to perform a scaffolding role, recruiting other proteins to the surface membrane independent of its catalytic activity. Most of these interactions contribute to the negative regulation of PI3K signaling by SHIP independently of its catalytic activity. For example, binding of SHIP-1 to ITAM containing adaptor proteins via the SH2 domain, can prevent PI3K recruitment via the p85 regulatory subunit (Peng et al., 2010). Moreover, SHIP-1 has also been shown to block, independently of its catalytic activity, the recruitment of the tyrosine phosphatase SHP1 to the SLAM family receptor 2B4 in NK cells (Wahle et al., 2007). Indeed, there is a profound increase in SHP1 recruitment in SHIP null cells that tips the balance toward constitutive inhibitory signaling via 2B4 (Wahle et al., 2007). Other protein interactions allow SHIP-1 to negatively influence different signaling pathways. For example, in B lymphocytes following BCR and FcγRIIB co-ligation, SHIP-1 interactions with Shc inhibit Ras/MAPK activation either by displacing Grb-2 and Sos from their interaction with Shc and/or by recruiting Dok1 and RasGAP to FcγRIIB at the plasma membrane. In T cells, SHIP-1 interacts with the Tec kinase and inhibits its function in T cells (Tomlinson et al., 2004a) and participates in a negative signaling complex comprising Grb-2-SHIP-1 and Dok1/2 that is recruited to LAT and inhibits Akt and PLCγ activation (Dong et al., 2006).

There is evidence that the scaffolding role of SHIP-1 is not restricted to facilitating negative regulatory mechanisms. For example, adaptor functions of SHIP-1 potentiate EGF-induced PLC-gamma1 activation in COS cells over-expressing SHIP-1 (Song et al., 2005). In addition, SHIP-1 facilitates a positive regulatory role in TLR-induced cytokine production from mucosal mast cells (Ruschmann et al., 2012). Recently, SHIP-1 has been reported to interact via its proline rich region with the Cbl-interacting 85-kDa protein CIN85 (Buchse et al., 2011) and the related CD2-associated adaptor protein (CD2AP) in Bao et al. (2012). In T cells, CIN85 binds to the adaptor molecule SH3 domain-binding protein-2 (3BP2), which is involved in leukocyte signaling downstream of Src/Syk-kinase coupled immunoreceptors and formation of the immunological synapse (Le Bras et al., 2007), though its role in B cells is unclear. In plasmacytoid dendritic cells, the CDAP-SHIP-1 complex positively regulates BDCA2/FcεR1γ signaling by inhibiting Cbl-mediated ubiquitination and degradation of the activated Syk and FcεR1γ in plasmacytoid dendritic cells (Bao et al., 2012). Finally, it appears that the SH2 domain of SHIP-1 interacts (both intra- and inter-molecularly) with phosphorylated $NPXY^{1020}$ within the SHIP-1 C-terminus that leads to dimerization and oligomerization (Mukherjee et al., 2012). SHIP-1 lacking its C-terminus is activated 8–10-fold more than full length SHIP-1 (Zhang et al., 2010), suggesting that the C-terminus not only controls interactions of SHIP-1 with respective binding partners, but also catalytic activity of SHIP-1.

ROLE OF SHIP-1 IN REGULATING IMMUNE FUNCTION

SH2 domain-containing inositol-5-phosphatase-1 is recruited to the surface membrane following ligation of a variety of receptors including, chemokine, Toll-like receptors (TLR), antigen, co-stimulatory, and cytokine receptors as well as IgG engagement by FcγRIIB (Harris et al., 2008; Keck et al., 2010; **Table 2**). SHIP-1 knock-out mice have proven invaluable in identifying the crucial role of SHIP-1 in the regulation of mast cell degranulation, BCR signaling and auto-antibody production, dendritic cell function, and NK cell cytolytic function (**Table 3**). SHIP-1 also regulates TLR signaling (Sly et al., 2004; Gabhann et al., 2010; Ruschmann et al., 2012), leukocyte polarization, and migration (Nishio et al., 2007; Harris et al., 2011; Mondal et al., 2012). It has also been shown to play a central role in CD4-mediated inhibitory signaling activated by HIV-1 gp120 that leads to disarmament of the immune systems (Waterman et al., 2012). Remarkably, there is evidence that SHIP is able to influence PI3K signaling not only at receptors it is recruited to (in *cis*) but also at other receptors where it is not recruited directly (in *trans*; Brauweiler et al., 2007; Fortenbery et al., 2010). For example, CXCL12/CXCR4-induced calcium mobilization and cell migration is impaired by prior activation of FcγRIIB and this inhibition is reduced in SHIP-deficient B cells (Brauweiler and Cambier, 2003; Brauweiler et al., 2007). Consistent with a role for SHIP-1 in inhibition, signaling through CXCR4 by CXCL12 is dependent on $PI(3,4,5)P_3$ (Brauweiler et al., 2007). Similarly, SHIP-1 acting in *trans* from the 2B4 has been proposed to oppose PI3K activity at other receptors such as the MHC-I receptor in NK cells (Fortenbery et al., 2010).

It is also clear that SHIP-1 has a pivotal role in regulating the balance between pro-inflammatory and anti-inflammatory myeloid and lymphoid cells (Ghansah et al., 2004; Locke et al.,

Table 2 | Key receptors in the immune system that are known to recruit and/or be regulated by SHIP-1.

Receptor	Reference
B cell receptor	Okada et al. (1998)
CD16	Galandrini et al. (2002)
CD22	Poe et al. (2000)
CD28	Edmunds et al. (1999)
TCR/CD3 complex	Dong et al. (2006) and Osborne et al. (1996)
CXCR4	Wain et al. (2005)
FcεR1	Gimborn et al. (2005), Huber et al. (1998), and Kimura et al. (1997)
FcγRIIa	Nakamura et al. (2002)
FcγRIIb	Ono et al. (1996)
Granulocyte colony-stimulating factor receptor	Hunter and Avalos (1998)
IL-3 receptor	Liu et al. (1999)
KLRG1	Tessmer et al. (2007)
TLR2	Keck et al. (2010)
TLR3	Gabhann et al. (2010) and Ruschmann et al. (2012)
TLR4	Keck et al. (2010) and Sly et al. (2004)
TLR9	Ruschmann et al. (2012)
2B4	Wahle et al. (2007)

See main text for further details regarding whether SHIP-1 mediated negative or positive impact on signal transduction events elicited via each receptor.

Table 3 | Impact of SHIP-1 gene targeting on leukocytes.

Cell type	Phenotype of SHIP-1 KO
Basophils	SHIP-1$^{-/-}$ mice show increased Th2 skewing due to increased IL-4 secretion from basophils (Kuroda et al., 2011)
B cell	Btk membrane association increased. Hyper-responsive to cross-linking of BCR (Bolland et al., 1998; Helgason et al., 2000) Loss of anergy, production of auto-antibodies (O'Neill et al., 2011)
Dendritic cell	Enhanced survival and proliferation, but impaired maturation (Antignano et al., 2010) Reduced nitric oxide production; SHIP-1 null DC's suppress T cell proliferation (Antignano et al., 2011)
Mast cell	Enhanced maturation of BMMC, CTMC, and MMC; reduced IgE-induced BMMC survival; enhanced degranulation of BMMCs, CTMC, and MMC (Kalesnikoff et al., 2002; Ruschmann et al., 2012) Enhanced TLR expression and TLR-induced cytokine production from CTMCs via adaptor-mediated pathway (Ruschmann et al., 2012)
Myeloid cell	Increased myeloid suppressor cell numbers (Ghansah et al., 2004) Increased M2 macrophage skewing (indirect mechanism via increased IL-4 secretion from basophils; Kuroda et al., 2009) Increased ratio of PI(3,4,5)P_3 to PI(3,4)P_2 on phagosomal membrane. Decreased early NADPH oxidative activity in phagosomes (Kamen et al., 2008)
Natural killer cells	Deficient receptor repertoire. Defective IFNγ secretion. Increase in peripheral number. Defective cytolytic function (Fortenbery et al., 2010)
T cell	Increased regulatory T cell differentiation, decreased Th17 development (Locke et al., 2009) Enhanced Th1 differentiation and CD8 cytotoxic activity. Decreased Th2 differentiation (Tarasenko et al., 2007)[a]

N.B data is derived from germline SHIP-1 knock-out cells, except where denoted.
[a]The reported phenotype is derived from a T cell-specific SHIP-1 knock-out.
BMMC, bone marrow-derived mast cells; CTMC, connective tissue mast cells; MMC, mucosal mast cells.

2009; Kuroda et al., 2011). For example, SHIP-1 deficient mice exhibit more myeloid-derived suppresser cells (MDSCs) than their wild type counterparts (Ghansah et al., 2004). Selective ablation of SHIP expression in either myeloid or T lymphoid lineage cells, has revealed that myeloid-specific ablation of SHIP leads to the expansion of both MDSC and Treg-cell numbers, indicating SHIP-dependent control of Treg-cell numbers by a myeloid cell type. Conversely, T-lineage specific ablation of SHIP leads to expansion of Treg-cell numbers, but not expansion of the MDSC compartment, indicating SHIP also has a lineage intrinsic role in limiting Treg-cell numbers (Collazo et al., 2012). G-CSF is required for expansion of the MDSC splenic compartment in mice rendered SHIP-deficient as adults. Thus, SHIP controls MDSC numbers, in part, by limiting production of the myelopoietic growth factor G-CSF (Collazo et al., 2012).

SH2 domain-containing inositol-5-phosphatase-1 also plays a role in regulating the balance of M1 macrophages (implicated in the first inflammatory response) and M2 macrophages (implicated in inflammatory response termination, tissue repair, regeneration, and remodeling). SHIP-1 deficiency leads to increased macrophage skewing toward M2 macrophages. This indicates that PI(3,4,5)P_3 drives macrophage progenitors toward an M2 phenotype and that SHIP-1 blocks this skewing (Rauh et al., 2005; Kuroda et al., 2009). Moreover, SHIP-1 is essential for normal Th17 cell development and plays a key role in the reciprocal regulation of Tregs and Th17 cells (Collazo et al., 2009; Locke et al., 2009). Germline SHIP deficiency promotes a preferential expansion and/or accumulation of conventional Tregs that have increased expression of FoxP3 indicating that SHIP limits Treg-cell function *in vivo* and limits FoxP3 acquisition by naïve CD4$^+$ T cells (Collazo et al., 2009). Mice carrying a T cell-specific deletion of SHIP-1 uncovered a regulatory role for SHIP-1 in controlling Th1/Th2 bias and cytotoxic responses as a result of its inhibitory effect on T-bet expression. Hence, SHIP-1 null T cells do not skew efficiently to a Th2 phenotype and display Th1-dominant immune responses *in vitro* and *in vivo* (Tarasenko et al., 2007). This is in contrast to evidence from germ line SHIP-1 null mice, which indicates that SHIP-1 can also repress Th2 skewing by inhibiting IL-4 production from basophils (Kuroda et al., 2011).

T cell-specific deletion of SHIP-1 using CD4CreSHIP$^{flox/flox}$ mice, had no affect on T-cell development, activation state, or Treg-cell numbers (Tarasenko et al., 2007). However, a recent study using in LckCreSHIP$^{flox/flox}$ mice reported significant reduction in the frequency of splenic CD3$^+$ T cells and CD4$^+$ and CD8$^+$ T cells in the peripheral blood relative to SHIP$^{flox/flox}$ controls (Collazo et al., 2012). The discrepancy may be because deletion of SHIP in CD4CreSHIP$^{flox/flox}$ mice may occur at a different time point during T-cell development compared to SHIP deletion in LckCreSHIP$^{flox/flox}$ mice.

THE SHIP NAVY: A FORCE FOR DIVERSITY AND COMPLEXITY OF FUNCTION

Multiple forms of the INPP5D gene product can occur via post-translational modification, degradation, or alternative mRNA splicing. This produces SHIP-1 proteins of 145 kDa (SHIPα), 135 kDa (SHIPβ), and 110 kDa (SHIPδ) in size. In addition, other 130, 125, and 110 kDa forms of SHIP-1 have been reported (Hamilton et al., 2011; Kerr, 2011). Truncated SHIP-1 proteins exhibit differential protein binding properties owing to the lack of/altered expression of certain protein binding domains (**Figure 3**). For example, s-SHIP and its human homolog SIP-110, are truncated at the N-terminus and lack the SH2 domain, but retain the catalytic, C2 and proline rich domains. This limits the repertoire of binding proteins available for interaction and hence, these forms cannot interact with Shc, yet can still interact with Grb-2. Moreover, s-SHIP is mostly localized at the plasma membrane rather than the cytoplasm (Hamilton et al., 2011; Kerr, 2011). Although originally thought to be restricted to embryonic stem cells, s-SHIP expression has been reported in adult hematopoietic cells and synergizes

with SHIP-1 to regulate the activation of macrophages (Nguyen et al., 2011).

SHIP-2 is a 142-kDa protein encoded by a separate gene, yet still retains approximately 65% homology with SHIP-1 within the catalytic domain. Divergence between SHIP-1 and SHIP-2 occurs largely within the proline rich domains as well as within the SH2 domain. In addition, SHIP-2 contains a unique sterile alpha motif (SAM) domain that can be involved in SAM–SAM domain interactions with other proteins, for example ARAP3 (Raaijmakers et al., 2007). SHIP-2 also shows the presence of an ubiquitin interacting motif at the C-terminal end and (unlike SHIP-1), it can hydrolyze $PI(4,5)P_2$ *in vitro*. SHIP-2 expression is not restricted to hematopoietic cell lineages and can be detected in heart, skeletal muscle, and brain tissues. SHIP-2 appears to have a major role in the negative regulation of insulin signaling in non-immune cells (Ooms et al., 2009). SHIP-1 and SHIP-2 are co-expressed in T cells and both are potent negative regulators of $PI(3,4,5)P_3$-mediated signals (Bruyns et al., 1999; Brauweiler et al., 2001). Tyrosine phosphorylation of SHIP-2 in T lymphocytes has not been reported, but it may still be enzymatically active and hence, the SHIP-1 knock-outs may have an incomplete phenotype. Interestingly, it is becoming clear that although SHIP-1 and SHIP-2 can interact with common binding partners, they additionally have their own unique profile of interacting partner proteins (**Table 4**), that possibly reflects the differences in their non-catalytic domains (Erneux et al., 2011; Mehta et al., 2011).

THE ROLE OF SHIP-1 IN HEMATOLOGICAL MALIGNANCIES

Over-activation of PI3K-dependent signaling cascades is a common occurrence in many human cancers (Engelman, 2009). The lipid phosphatase PTEN which also negatively regulates $PI(3,4,5)P_3$ accumulation by de-phosphorylating the D3 position of the inositol ring, is a well characterized tumor suppressor gene (Hollander et al., 2011). Likewise, evidence for mutations of SHIP-1 have also been shown in acute lymphoblastic leukemia (Luo et al., 2003) and in acute myeloid leukemia (Luo et al., 2004).

The loss of SHIP-1 has also been shown to promote the development of erythroleukemia, with SHIP-1 identified as a target gene of the oncogene fli-1 (Lakhanpal et al., 2010). There are at least two mechanisms by which SHIP-1 expression may be down-regulated. The first involves targeting of SHIP-1 by miR-155 in B cells, where high levels of miR-155 and reduced SHIP-1 expression have been linked to the development of acute lymphoblastic leukemia in mice (Costinean et al., 2009). miR-155 levels were also found to be significantly increased in human patients with diffuse large B cell lymphoma (Eis et al., 2005). The second involves BCR-ABL (the oncogene responsible for chronic myeloid leukemia), which either directly or via a Src kinase family member, tyrosine phosphorylates SHIP-1. This leads to polyubiquitination of SHIP-1 and subsequent STAT6 dependent-proteasomal degradation (Ruschmann et al., 2010). Interestingly, there is an inverse relationship between expression of SHIP-1 and BCR-ABL (Martino et al., 2001; Jiang et al., 2003). Thus, reduced SHIP-1

Table 4 | SHIP-1 and SHIP-2 interacting proteins.

SHIP-1 interacting proteins	SHIP-2 interacting proteins	SHIP-1 and SHIP-2 interacting proteins
CD2AP (Bao et al., 2012)	Actin, non-muscle[a] (Mehta et al., 2011)	Btk[d] (Tomlinson et al., 2004b; Xie et al., 2008a)
Ezrin, Radaxin, and Meosin[a] (Mehta et al., 2011)	APS[a] (Onnockx et al., 2008)	CIN-85[a,c] (Havrylov et al., 2009; Buchse et al., 2011)
FUBP2[a] (Mehta et al., 2011)	ARAP3[b] (Raaijmakers et al., 2007)	DOk1a (Tamir et al., 2000; Havrylov et al., 2009; Cunningham et al., 2010)
Grb-2[a] (Mehta et al., 2011)	c-Cbl[b] (Vandenbroere et al., 2003)	Filamin[a,c] (Dyson et al., 2001; Lesourne et al., 2005)
KLRG1[a] (Tessmer et al., 2007)	Glucose-regulated protein precursor[a](Mehta et al., 2011)	Shc[a] (Mehta et al., 2011)
LyGDI[a] (Mehta et al., 2011)	Heat shock protein 90-beta[a] (Mehta et al., 2011)	Tec[d] (Tomlinson et al., 2004a)
PKC-δ[a] (Chari et al., 2009)	Hematopoietic cell specific Lyn substrate[a](Mehta et al., 2011)	
PLC-γ1[a] (Song et al., 2005)	HSP90β[a] (Mehta et al., 2011)	
	Intersectin 1[b] (Xie et al., 2008b)	
	Protein disulfide-isomerase A3 precursor[a] Mehta et al., 2011)	
	PR130[c] (Zwaenepoel et al., 2010)	
	PTP1B[c] (Mertins et al., 2008)	
	p130Cas[a] (Prasad et al., 2001)	
	JIP1[b] (Xie et al., 2008a)	
	Tubulin beta-2A chain[a] (Mehta et al., 2011)	
	Vinexin[c] (Paternotte et al., 2005)	

Methods by which interactions have been identified are indicated: [a] immunoprecipitation; [b] yeast two hybrid; [c] mass spectrometry; [d] GST-SH3 pull-down. Where known, the domains within SHIP-1 that interact with these proteins are indicated in ***Figure 3****. Protein interactions with SHIP-1 that are known to result in positive signaling outcomes are indicated in red. Refer to main text for further detail.*

ARAP3, Arf-GAP, Rho-GAP, ankyrin repeat and PH domain-3; CIN85, Cbl-interacting 85 kDa protein; FUBP2, far upstream element binding protein-2; JIP1, c-Jun NH_2 terminal kinase (JNK)-interacting protein-1; KLRG1, killer cell lectin-like receptor G1; LyGDI, a Rho guanine nucleotide dissociation inhibitor originally identified in lymphocytes; PTPB1, protein-tyrosine phosphatase 1B.

activity might be a prerequisite for the proliferative advantage of some chronic myeloid leukemia clones. A similar inverse relationship exists between a constitutively active oncogenic c-kit receptor and SHIP-1, whereby inhibition of c-kit's intrinsic tyrosine kinase activity with Imatinib reversibly raises SHIP-1 levels (Vanderwinden et al., 2006).

The role of SHIP-1 as a tumor suppressor is also evident in the ability of SHIP-1 to restrict myeloid suppressor cells and regulatory T cells (Ghansah et al., 2004; Locke et al., 2009). Therefore the loss of SHIP-1 expression/function may lead to increased suppression of T-cell mediated anti-tumor immunity. Indeed, in murine pancreatic cancer SHIP-1 expression was shown to be reduced in splenocytes which also correlated with an increase in myeloid suppressor cell numbers (Pilon-Thomas et al., 2011). Decreased SHIP-1 expression has also been shown in myelodysplastic syndrome progenitor cells, whereas over-expression of SHIP-1 inhibited myeloid leukemic growth (Lee et al., 2012).

The role of SHIP-1 in leukemia however, appears more complex than initially thought. For example, while PTEN can suppress growth and apoptosis, SHIP-1 was shown not to act as a tumor suppressor in myeloma cells (Choi et al., 2002). The use of a small molecule SHIP-1 inhibitor demonstrated that catalytically active SHIP-1 is required for the survival of multiple myeloma cells (Brooks et al., 2010) and that therefore, in certain cases, SHIP-1 actually supports cancer cell survival. This would be consistent with increased levels of the SHIP-1 enzymatic product $PI(3,4)P_2$ promoting Akt activation and survival/proliferation (Manning and Cantley, 2007). Indeed, another group has shown that SHIP-1 inhibits CD95/Fas-mediated apoptosis of T cells, albeit independently of its catalytic activity (Charlier et al., 2010).

SHIP-1 AND THE STEM CELL NICHE

The crucial role of PTEN/Akt in the maintenance of stem cell homeostasis is now evident (Hill and Wu, 2009; Song et al., 2012). It is now becoming clear that SHIP-1 also has an important role in maintaining the stem cell niche. Hemopoietic stem cell (HSC) proliferation is increased in SHIP-1 null mice. Despite expansion of the compartment, SHIP-1 deficient HSCs exhibit reduced capacity for long-term repopulation and home inefficiently to bone marrow (Desponts et al., 2006; Hazen et al., 2009). The role of SHIP-1 in the biology of both HSC and the hematopoietic stem cell niche, suggests that it may be a useful target for treatment of bone marrow failure syndromes caused by viruses, radiation, chemotherapy, or malignancy. As already mentioned, MDSCs are a type of immunoregulatory cell that can repress allogeneic T cell responses. A common complication arising after bone marrow transplantation is Graft-versus-host disease (GVHD) which involves priming of allogeneic T cells. Remarkably, SHIP-1 deficient mice express more myeloid suppressor cells than their wild type counterparts and accept allogeneic bone marrow grafts with a reduced incidence of GVHD (Ghansah et al., 2004; Kerr, 2008). In addition SHIP-1 null mice are better able to accept bone marrow transplants compared to controls (Wang et al., 2002) and SHIP-1 deficient mice have shown reduced cardiac graft rejection compared to controls (Collazo et al., 2009).

SHIP IS TARGETED BY PATHOGENS TO AVOID IMMUNE RECOGNITION

The key regulatory role of SHIP-1 has been exploited by several opportunistic pathogens that target these phosphatases in order to evade immune detection. Thus, lymphocytes are particularly sensitive to the cytolethal distending toxin subunit B (CdtB), an immunotoxin produced by *Actinobacillus actinomycetemcomitans*, that can hydrolyze $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Exposure to CdtB leads to cell cycle arrest and death by apoptosis. The lipid phosphatase activity of CdtB may therefore, result in reduced immune function, facilitating chronic infection with *Actinobacillus* and other enteropathogens that express Cdt proteins (Shenker et al., 2007). The measles virus evades destruction by the immune system, at least in part, by targeting negative regulation of PI3K/Akt signaling. It induces expression of the SHIP-1 homolog SIP-110 which depletes cellular $PI(3,4,5)P_3$ pools, suggesting that the threshold for activation signals leading to induction of T cell proliferation is raised (Avota et al., 2006). The targeting of this protein by pathogens to avoid immune recognition, emphasizes the notion that SHIP-1 might offer opportunities for the design of new drugs targeting PI3K-dependent signaling.

PHARMACOLOGICAL MANIPULATION OF SHIP

ALLOSTERIC SHIP-1 ACTIVATORS

In 2005, pelorol (a product of the marine invertebrate *Dactylospongia elegans*) was described as an activator of SHIP-1 (Yang et al., 2005). More potent synthetic chemical entities have since been designed by Aquinox Pharmaceuticals (**Figure 1**). Along with $PI(3,4)P_2$, these compounds were shown to allosterically enhance catalytic activity by binding to the C2 domain of SHIP-1 (**Figure 3**). The C2 domains of SHIP-1 and SHIP-2 share 38% homology (compared to 51% homology between total SHIP-1 and SHIP-2 in humans), and it is believed that this reduced homology in the C2 domain allows these pelorol based compounds to achieve SHIP-1 selectivity. This is particularly important given the crucial role of SHIP-2 in the regulation of insulin signaling (Ooms et al., 2009). Two of these compounds, AQX-016A and AQX-MN100, exhibited potent inhibition of immune cell activation *in vitro* and were anti-inflammatory *in vivo* using mouse models of endotoxemia and acute cutaneous anaphylaxis (Ong et al., 2007). Intriguingly, these SHIP-1 activating compounds increased apoptosis of multiple myeloma cells *in vitro* and when used in combination with bortezomib (an established multiple myeloma treatment) proved more effective at inhibiting cancer cell proliferation than bortezomib alone (Kennah et al., 2009). Other compounds based on the structure of pelorol have been developed by Aquinox Pharmaceuticals as SHIP-1 activating compounds with a view for application in inflammatory disorders. AQX-1125 is the most advanced and has passed Phase 1 clinical trials in 2011, with Phase IIa clinical studies initiated in late 2011 for the treatment of mild and moderate asthma (**Table 1**). With regard to the latter, the recent finding that TLR stimulation augments IgE plus Ag-induced TNFa and IL-6 production from MMCs (Ruschmann et al., 2012) might explain the exacerbation of IgE-mediated allergic episodes by infectious agents (Qiao et al., 2006). Since IgE synergizes with TLR ligands to trigger cytokine production from SHIP-1 null mucosal mast cells,

activating SHIP-1 specifically in these cells might be useful for treating chronic inflammatory diseases like asthma.

SHIP-1 INHIBITORS

A novel small molecule selective inhibitor of SHIP-1, termed 3 α-aminocholestane (3AC, **Figure 1**) has also recently been identified using high-throughput screening, though the site of drug-protein interaction is unclear (Brooks et al., 2010). Consistent with observations from SHIP-1 deficient mice, treatment of mice with 3AC led to increased numbers of myeloid suppressor cells and reduced ability of peripheral lymphoid tissues to prime myeloid-associated responses and protected against GVHD (Brooks et al., 2010). The inhibition of SHIP-1 using pharmacological compounds may therefore offer the potential to aid transplant acceptance in patients undergoing transplant surgery. 3AC also increased levels of granulocytes, red blood cells, neutrophils, and platelets in mice and could therefore, have potential to improve blood cell number in patients with myelodysplastic syndrome or myelosuppressive infection.

Remarkably, SHIP-1 inhibition using 3AC induced the apoptosis of human acute myeloid leukemia cell lines which is consistent with SHIP-1 being anti-apoptotic under some circumstances (Brooks et al., 2010). Further studies showed that 3AC inhibited multiple myeloma cell growth in a tumor xenograft model in mice (Fuhler et al., 2011). Since both substrate [PI(3,4,5)P_3] and product [PI(3,4)P_2] of SHIP-1 have been shown to influence Akt activation and cell survival, this may explain in part, why both activators and inhibitors of SHIP-1 have shown efficacy against leukemic cells (Kerr, 2011).

SHIP-2 INHIBITORS

SHIP-2 is thought to be involved in type-2 diabetes and obesity (Ooms et al., 2009) as well as cancer and atherosclerosis (Suwa et al., 2010). The development of compounds which selectively target SHIP-2 has therefore been of great interest. Small molecule compounds which specifically inhibit the catalytic activity of SHIP-2 have recently been described (Suwa et al., 2009). In addition a novel biphenyl 2,3′4,5′,6-pentakisphosphate [BiPh(2,3′,4,5′,6)P_5] compound has demonstrated potent inhibition of SHIP-2 catalytic activity (Vandeput et al., 2007). BiPh(2,3′,4,5′,6)P_5 in its current form is however not cell permeable and therefore does not possess drug-like properties. The crystal structure of the phosphatase domain of SHIP-2 bound to BiPh(2,3′,4,5′,6)P_5 has identified a flexible loop which folds over and encloses the ligand (Mills et al., 2012) and may have implications for development of small molecules that target SHIP-1. The targeting of this region may allow more SHIP-2 specific drugs to be developed. Cell permeable pan-SHIP-1/2 inhibitors have also recently been identified and have been reported to kill multiple myeloma cells (Fuhler et al., 2011). The development of SHIP-2 specific compounds suggests that SHIP-2 may be a potential target with which to treat a range of diseases, in addition to allowing the poorly understood role of SHIP-2 in the immune system, to be probed in greater depth.

SUMMARY

The difficulties of developing PI3Kγ inhibitors with sufficient selectivity over other PI3K isoforms has in part, led to the search for alternative drug targets to selectively modify PI3K signaling in the immune system. This search revealed the potential for exploiting the lipid phosphatase SHIP-1, an endogenous and leukocyte-restricted regulator of PI3K signaling. Small molecule regulators of this protein have shown early promise in inflammatory, transplantation, and cancer settings, and are currently in phase IIa clinical trials to evaluate the safety, tolerability, and pharmacokinetics (**Table 1**). The selectivity profile of compounds targeting SHIP-1 is at present quite limited and while they appear to exhibit specificity for SHIP-1 versus SHIP-2 and PTEN, it remains to be seen whether there are other off-target effects. Despite this early promise, the targeting of SHIP-1 (particularly with inhibitors), is not without its potential problems. For example, SHIP-1 deficiency leads to a number of pathologies including fibrotic lung disease (Rauh et al., 2005), osteoporosis (Moon et al., 2011), and the development of spontaneous intestinal inflammation and fibrosis (Kerr et al., 2011; McLarren et al., 2011). An important factor to consider is that targeting catalytic activity may not be sufficient to inhibit all SHIP-1 mediated effects, given that SHIP-1 also fulfills key non-catalytic scaffolding functions (Song et al., 2005; Peng et al., 2010; Bao et al., 2012; Ruschmann et al., 2012). Hence, small molecule-based strategies to target catalytic activity are unlikely to affect these non-catalytic functions. This may be beneficial on the one hand, if pathological consequences are dependent on catalytic functions as such approaches will likely retain the non-enzymatic functions and hopefully limit unwanted side-effects. On the other hand, such strategies may be ineffective if pathological consequences are driven by non-enzymatic functions of SHIP-1. It is interesting to note however, that prolonged inhibition of SHIP-1 with 3AC leads to proteasome-dependent degradation of SHIP-1 (Fuhler et al., 2011).

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