Published in final edited form as:

Crit Rev Biochem Mol Biol. 2016 September ; 51(5): 359–378. doi:10.1080/10409238.2016.1215288.

The PI3K Pathway in B Cell Metabolism

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Abstract

B cell growth and proliferation is tightly regulated by signaling through the B cell receptor and by other membrane bound receptors responding to different cytokines. The PI3K signaling pathway has been shown to play a crucial role in B cell activation, differentiation and survival. Activated B cells undergo metabolic reprogramming in response to changing energetic and biosynthetic demands. B cells also need to be able to coordinate metabolic activity and proliferation with nutrient availability. The PI3K signaling network has been implicated in regulating nutrient acquisition, utilization and biosynthesis, thus integrating receptor mediated signaling with cell metabolism. In this review, we discuss the current knowledge about metabolic changes induced in activated B cells, strategies to adapt to metabolic stress and the role of PI3K signaling in these processes.

Keywords

Akt; mTOR; lymphocyte; glycolysis; mitochondria

The PI3K signaling pathway

Phosphoinositide 3-kinases (PI3Ks) are a family of kinases that generate lipid second messengers to recruit cytoplasmic proteins to cellular membrane. The PI3K enzymes can be divided into three classes according to their substrate specificity, structure and function. Class IA PI3Ks are heterodimers consisting each of a catalytic subunit (p110 α , β , δ) and a regulatory subunit (p50, p55, p85). These three different isoforms have been shown to play essential but partially redundant roles in B cell biology (Pauls et al., 2012). The production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by class I PI3Ks is induced by a variety of receptors on B cells. PI3K signaling is initiated downstream of the B cell receptor (BCR) and enhanced by the co-receptor CD19 and the cytosolic adapter proteins B cell adaptor for PI3K (BCAP) and TC21 (Okada et al., 2000, Delgado et al., 2009, Otero et al., 2001,

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Declaration of interest

The authors report no declarations of interest.

Tuveson et al., 1993). Toll like receptor (TLR) activation, chemokine signaling and B cell activating factor receptor (BAFF-R) mediated signaling also induce PI3K activation, but it is less clear how PI3K is recruited to these receptors (Jellusova et al., 2013, Dil and Marshall, 2009, Morbach et al., 2015, Reif et al., 2004). Phosphatidylinositol-3,4,5-trisphosphate binds proteins containing pleckstrin homology (PH) domains. One of the effector molecules recruited to the plasma membrane after BCR stimulation is Bruton's tyrosine kinase (Btk), which contributes to the activation of phospholipase C- γ 2 (PLC γ 2) and the production of diacylglycerol and inositol-1,4,5-trisphosphate (Rickert, 2013). These second messengers promote calcium mobilization and protein kinase C (PKC) activation, ultimately leading to the translocation of nuclear factor- κ B (NF κ B) transcription factors to the nucleus. In addition to Btk mediated signaling, the serine/threonine kinase Akt (also known as protein kinase B) is a major effector molecule downstream of PI3K activation (Werner et al., 2010, Baracho et al., 2011). Upon PI3K activation, Akt is recruited to the plasma membrane, where it is activated following phosphorylation of Thr308 and Ser473 by 3-phosphoinositide-dependent kinase 1 (PDK1) and the mammalian target of rapamycin complex 2 (mTORC2), respectively (**Fig. 1**) (Limon and Fruman, 2012). mTOR kinase is present in two complexes, mTORC1, which contains raptor and mTORC2, which contains rictor and Sin1. The two mTOR complexes differ not only by composition, but also by upstream signaling pathways regulating their activity, substrate specificity and engagement of downstream pathways. The main function of mTORC1 is to integrate cell growth and macromolecule biosynthesis with metabolic cues and growth factor mediated signaling (**Fig. 1**) (Laplane and Sabatini, 2012). mTORC1 regulates translation initiation by phosphorylating and activating p70S6K. Additionally, mTORC1 phosphorylates eIF4E-binding protein 1 (4E-BP1), which inhibits mRNA translation by binding to the eukaryotic translation initiation factor-4E (eIF4E) (Laplane and Sabatini, 2012). Phosphorylation of 4E-BP1 reduces its affinity for eIF4E, promoting assembly of the eIF4F complex and initiation of cap-dependent translation. mTORC1 regulates many other aspects of cell metabolism including nutrient sensing, nutrient uptake and autophagy (Duvel et al., 2010, Inoki et al., 2003, Yuan et al., 2013, Jewell and Guan, 2013). mTORC2 phosphorylates AGC kinases, including Akt on serine 473 (Oh and Jacinto, 2011).

Akt has many targets relevant to B cell biology including, glycogen synthase kinase 3 (GSK3), forkhead box protein 1 (Foxo1) and the tuberous sclerosis complex (TSC) (**Fig. 1**). Akt-mediated phosphorylation of Foxo1 induces its nuclear export, thereby preventing its transcriptional activity. GSK3 is a constitutively active kinase that has been implicated in regulating the stability and/or activation of many molecules involved in cell growth, division and metabolism such as mTOR (Inoki et al., 2006), cMyc (Gregory et al., 2003), p27 (Surjit and Lal, 2007), cyclin D3 (Cato et al., 2011) and others. Unlike other kinases, GSK3 is inhibited upon phosphorylation (on residues Ser9 and Ser21 of the beta and alpha isoforms, respectively (Cross et al., 1995)). This phosphorylation event can be mediated by many different kinases such as Akt (Cross et al., 1995), protein kinase (PKA) (Fang et al., 2000), p70S6 kinase (p70S6K) (Sutherland et al., 1993) and PKC (Zhao et al., 2007)(**Fig. 1**). In addition to the activation of mTOR through GSK3 inactivation, Akt has also been reported to activate mTOR signaling by phosphorylation of the TSC complex (consisting of TSC1 and TSC2), which is a major inhibitor of mTOR signaling (Inoki et al., 2002).

The PI3K signaling pathway in B cell development and differentiation

The role of the PI3K signaling pathway in B cell development, differentiation and survival has been addressed in numerous studies using mouse models to decipher the biological function of specific effector molecules. PI3K signaling is essential for the differentiation and expansion of pro-B and pre-B cells. In the absence of p110 α and p110 δ , B cell development is arrested at the pre-B cell stage, due to a failure to downregulate the recombination activating gene (RAG) proteins (Ramadani et al., 2010). The mechanism by which PI3K regulates RAG is dependent on Akt-mediated Foxo1 inactivation, preventing Foxo1-dependent expression of the *Rag1/2* genes (Amin and Schlissel, 2008, Dengler et al., 2008). Hence, *Rag* expression is repressed by pre-BCR/PI3K-mediated nuclear Foxo1 export. Foxo1 is however needed for early B cell development to drive expression of *Ii7ra*, in addition to *Rag*, in pro-B cells (Dengler et al., 2008). Phosphorylation and inactivation of Foxo1 is dependent on the presence of a functional mTORC2 signaling complex. The ablation of either Sin1 or Rictor, both of which are components of mTORC2, results in decreased Akt-(Ser473) and Foxo1-phosphorylation associated with a failure to downregulate *rag* and *il7r* gene expression (Lazorchak et al., 2010, Zhang et al., 2014). In addition to mTORC2 mediated phosphorylation of Akt on Ser473, Akt is also phosphorylated on Thr308. This phosphorylation event is mediated by PDK1. B cell progenitors lacking PDK1 show decreased Akt- (Thr308) and Foxo1- phosphorylation and their development is arrested at the transition from the pro- to pre-B cell stage (Baracho et al., 2014, Venigalla et al., 2013). Thus, dual phosphorylation of Akt by PDK1 and mTORC2 is needed for a complete inactivation of Foxo1 and normal B cell development.

P110 δ single deficient mice display a mild early B cell phenotype and a decreased population of marginal zone B cells and B1 cells in the periphery; however mature follicular B cells are present (Clayton et al., 2002, Jou et al., 2002). Mice with a lymphocyte specific deletion of p110 α or p110 β show normal development of B cells (Ramadani et al., 2010). Moreover, combined deficiency of p110 β with p110 δ did not have a greater impact on B cell differentiation (Ramadani et al., 2010). These studies suggest that p110 α and p110 δ play essential, but partially redundant functions in B cell development.

In the periphery, PI3K signaling is an essential component of tonic BCR signaling and is required for B cell maintenance (Srinivasan et al., 2009). Furthermore, PI3K signaling has recently been shown to contribute to BAFFR-mediated signaling, thereby further supporting the survival of naïve mature B cells (Jellusova et al., 2013). The role of PI3K in the adaptive immune response is multifaceted and affects different aspects of the humoral immune response. Germinal center (GC) development is strongly impaired in *Cd19*^{-/-} (Rickert et al., 1995) and *p110 δ* ^{-/-} mice (Jou et al., 2002), or mice lacking PDK1 in mature B cells (Baracho et al., 2014). Furthermore, GC B cell generation is abolished in CD19-deficient mice but restored by phosphatase and tensin homolog deleted on chromosome ten (*Pten*) inactivation (Anzelon et al., 2003). PTEN is an inositol phosphatase and tumor suppressor that directly counters PI3K activity by dephosphorylating PIP3. In summary, there is compelling data to suggest that PI3K signaling supports the survival and/or generation of GC B cells. In addition, PI3K signaling in GC B cells affects cell fate decisions by inhibiting class switch recombination (CSR) and favoring plasma cell development (Omori et al.,

2006, Suzuki et al., 2003a). PI3K signaling can suppress class switch recombination through Akt-dependent Foxo1 inactivation, which is needed for activation induced cytidine deaminase (AID) expression, although Foxo1 appears to have AID-independent functions in the GC as well (Sander et al., 2015, Dominguez-Sola et al., 2015).

mTOR signaling can also be activated downstream of Akt via suppression of the TSC complex (**Fig. 1**) (Inoki et al., 2002). mTOR signaling is known to play an important role in B cell growth and proliferation, as evidenced by a nearly complete block of anti-IgM induced B cell proliferation after treatment with the mTOR inhibitor rapamycin (Donahue and Fruman, 2003, Wicker et al., 1990). Rapamycin treatment *in vivo* decreases GC formation and reduces B cell class switching (Keating et al., 2013). Furthermore, mice with a B cell specific mTOR deletion show reduced generation of GC B cells and impaired antibody production to thymus dependent antigens (Zhang et al., 2013). Thus, mTOR signaling plays an important role in GC B cell differentiation. In addition to the GC response, mTOR signaling appears to be required for B cell development, since mTOR hypomorphic mice show a partial block in B cell development (Zhang et al., 2011). Since mTOR reduction/deletion affects both mTOR signaling complexes, it is important to further analyze the individual contributions of mTORC1 and mTORC2 to B cell function. A recent study analyzing mice with a B cell specific deletion of Rictor, a crucial component of mTORC2, showed that it is required for B cell maintenance and the humoral immune response (Lee et al., 2013). Decreased Foxo1 phosphorylation after anti-IgM stimulation and impaired NFkB2/p100 cleavage after BAFF stimulation could partially explain this phenotype (Lee et al., 2013). A similar study analyzing B cell function after mTORC1 (Raptor) disruption is lacking thus far. However, the inducible deletion of Raptor has been shown to result in decreased B cell progenitor numbers (Hoshii et al., 2012), suggesting that mTORC1 may play a role in the rapid growth and proliferation associated with early B cell development. In addition, mature Raptor-deficient B cells show impaired proliferation after *in vitro* stimulation, as well as decreased frequencies of plasma cells and isotype switched B cells in culture (Limon et al., 2014). Nevertheless a thorough analysis of mice with a B cell specific raptor deletion is needed to shed light on the role of mTORC1 in late B cell differentiation and the humoral immune response.

Most of our insight into mTOR function has been gained from loss of function experiments. However, aberrant activation of the mTORC1 pathway is a hallmark of many cancer types, including Hodgkin and non-Hodgkin B cell lymphomas (Argyriou et al., 2012). Thus it is of crucial importance to understand the effect enhanced mTORC1 signaling has on B cell survival and function. Given that mTORC1 supports protein synthesis, cancer cells benefit from the anabolic processes enhanced by mTORC1 signaling. Surprisingly, however, if mTORC1 activity is increased by deleting TSC1, B cell maturation/survival is slightly impaired (Ci et al., 2015, Benhamron and Tirosh, 2011). The biochemical basis of this effect has not been fully elucidated, however it has been shown that whereas S6K1, S6 and 4EBP1 phosphorylation is increased in TSC1 deficient cells, Akt phosphorylation is decreased (Ci et al., 2015, Benhamron and Tirosh, 2011). Decreased Akt phosphorylation could thus impair B cell survival. While TSC1 deletion impairs B cell maturation, whether GC formation is affected is unresolved (Benhamron and Tirosh, 2011, Ci et al., 2015).

Nevertheless these studies suggest that enhanced mTORC1 signaling alone does not lead to B cell transformation. Since cancer cells often show simultaneous activation of mTORC1 and PI3K signaling, it would be important to analyze B cell survival and proliferation under these conditions.

Activation induced metabolic changes in B cells

In lymphocytes, glucose seems to be the prevalent source of energy, however other sources such as glutamine and fatty acids can also be catabolized as alternate carbon sources. In the cytosol, glucose is converted to pyruvate, which is subsequently metabolized in the mitochondria via the tricarboxylic acid cycle (TCA) to generate NADH, a coenzyme to be used in oxidative phosphorylation (electron transport pathway) to generate adenosine triphosphate (ATP) (**Fig. 2**). Alternatively, pyruvate can be converted into lactate, yielding much lower levels of ATP per molecule glucose than oxidative phosphorylation (**Fig. 2**). While oxygen is required for oxidative phosphorylation, it is not used during glycolysis. However, lymphocytes have been shown to catabolize glucose to lactate even if oxygen is available, hence this process is often referred to as aerobic glycolysis. Additionally, glucose is also needed for the pentose phosphate pathway to produce NADPH and 5-carbon ribose, essential molecules for maintaining redox balance and for nucleic acid and lipid synthesis.

Resting lymphocytes display low metabolic requirements. Upon co-stimulation by the T cell receptor (TCR) and CD28, quiescent T cells transition to an activated state characterized by increased glucose uptake and aerobic glycolysis (Frauwirth et al., 2002). Activated T cells disproportionally increase glycolysis in comparison to oxidative phosphorylation (OXPHOS) (Frauwirth et al., 2002), which is similar to the metabolic characteristics of cancer cells and termed the Warburg effect. Although OXPHOS is more energy efficient, enhanced glycolysis and subsequent conversion of pyruvate to lactate can potentially yield ATP faster. More importantly, glycolysis and a truncated TCA cycle can provide the cell with substrate molecules needed for biosynthesis, and help to maintain redox balance (Buck et al., 2015). Interestingly, functionally distinct T cells possess unique metabolic profiles that are essential for their function. CD4⁺ effector T cells (Th1, Th2 and Th17) express increased levels of the glucose transporter Glut1 and rely mainly on glycolysis (Michalek et al., 2011a, Gerriets et al., 2015). CD4⁺ regulatory T cells (Tregs) show higher rates of glycolysis in comparison to naïve T cells, but are less glycolytic than effector T cells (Michalek et al., 2011a). Instead, Tregs show elevated levels of lipid oxidation and mitochondrial membrane potential (Michalek et al., 2011a). Furthermore it has recently been shown that follicular helper T cells (Tfh) exhibit less proliferation, glycolysis and mitochondrial respiration in comparison to Th1 cells due to decreased mTORC1 and Akt signaling (Ray et al., 2015). Signaling via Akt and mTORC1 was demonstrated to promote Th1 cell differentiation at the expense of Tfh cells (Ray et al., 2015). CD8⁺ memory T cells rely on fatty acid oxidation (Pearce et al., 2009, van der Windt et al., 2012). Enforcing glycolysis has been shown to impair the formation of long-lived memory cells by favoring effector differentiation (Sukumar et al., 2013, Michalek et al., 2011a). Thus, metabolic reprogramming is not simply a consequence of activation, but is intricately linked to T cell fate and function. Although accumulating evidence shows that the regulation of cell metabolism is fine-tuned

to the specific needs of different T cell subsets, comparably little is known about the regulation of cell metabolism in B cells.

During an immune response, B cells are activated through the B cell receptor, but also receive other signals through CD40, BAFF-R, interleukin-4 receptor (IL4-R) and others. Over the years, considerable effort has been invested into understanding how these signals affect B cell differentiation, proliferation and survival and which intracellular signaling pathways contribute to these processes. As a first step in understanding the regulation of B cell metabolism, it is important to elucidate how different receptor induced signals regulate macromolecule biosynthesis, energy production and nutrient acquisition. Several stimuli such as IL4, BAFF (Woodland et al., 2008, Patke et al., 2006), anti-CD40 (Woodland et al., 2008, Blair et al., 2012), lipopolysaccharide (LPS) (Caro-Maldonado et al., 2014) or anti-BCR stimulation (Doughty et al., 2006, Caro-Maldonado et al., 2014) lead to increased glucose uptake and metabolism. IL4 and BAFF are two stimulants that do not induce proliferation, yet affect cell size and survival (Otipoby et al., 2008, Patke et al., 2006, Hudak et al., 1987, Woodland et al., 2008). BAFF stimulation *in vitro* has been shown to induce an increase in cell size, mitochondrial potential and enhanced transcription of genes involved in carbohydrate metabolism (Patke et al., 2006). Similarly, interleukin 4 (IL4) stimulated cells upregulate glucose transporter 1 (Glut1) expression and show an increased glycolytic flux (Dufort et al., 2007). Interestingly, the acquired glucose does not seem to substantially fuel the pentose phosphate pathway (Dufort et al., 2007), nor is it converted to lactate (Cho et al., 2011). Instead, glucose is oxidized to CO₂ in IL4-stimulated B cells (Cho et al., 2011). Furthermore, glucose metabolism is central to IL4-mediated survival and glycolysis inhibition with 2-deoxy-D-glucose (2-DG) leads to decreased IL4-mediated survival *in vitro* (Dufort et al., 2007). Both LPS and BCR stimulation have been shown to lead to increased glycolysis and OXPHOS. Interestingly, although both extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were increased in LPS or BCR stimulated B cells, the ratio remained comparable to unstimulated cells, suggesting that B cells upregulate their metabolic activity in a balanced fashion (Caro-Maldonado et al., 2014). This is in stark contrast with T cells as anti-CD3+anti-CD28 stimulated T cells show a shift towards lactate production. LPS mediated B cell proliferation and antibody secretion has been shown to be inhibited by 2-DG and dichloroacetate (DCA). 2-DG and DCA affect glycolysis at two different steps. 2-DG inhibits hexokinase, an enzyme responsible for the first step of glycolysis, and DCA suppresses pyruvate dehydrogenase phosphorylation by pyruvate dehydrogenase kinase (PDHK), thus promoting pyruvate entry into the TCA cycle rather than conversion to lactate (Papandreou et al., 2011) (**Fig. 2**). Thus, glycolysis is important for LPS-mediated B cell proliferation and antibody secretion. Curiously, although oxygen consumption was also increased in LPS-stimulated B cells, inhibition of the mitochondrial respiratory chain complex I with rotenone did not lead to decreased IgM secretion (Caro-Maldonado et al., 2014). Similarly to LPS treated cells, BCR stimulated cells increase Glut1 expression, glucose uptake and glucose catabolism. Interestingly, prior to S-phase entry anti-IgM stimulated cells shift from a primarily glycolytic metabolism to include the pentose phosphate pathway (Doughty et al., 2006), which favors nucleotide synthesis.

To support proliferation, activated cells not only need ATP, but also require precursor molecules for biosynthesis. As mentioned, stimulation with anti-IgM, LPS, IL4, anti-CD40 and BAFF leads to increased glucose uptake and utilization. A recent study has shown that in LPS-stimulated B cells, fatty acid synthesis increases (Dufort et al., 2014). The majority of acetyl CoA used for fatty acid synthesis is derived from glucose, a smaller portion originates from glutamine and only a small fraction is derived from other sources. The expression and activity of ATP citrate lyase (ACLY) is increased in LPS stimulated cells. ACLY plays an important role in *de novo* lipogenesis by cleaving citrate to generate Acetyl-CoA and oxaloacetate (**Fig. 2**). ACLY inhibition in LPS stimulated B cells leads to decreased proliferation and survival. In addition, several markers of plasma cell differentiation are decreased after ACLY inhibition, including expansion of the endoplasmic reticulum (ER) and CD138 and B lymphocyte induced maturation protein 1 (Blimp1) expression (Dufort et al., 2014). Although it is not clear whether these plasma cell-related defects are secondary to decreased survival and proliferation, it is possible that *de novo* lipogenesis is required for the expansion of the ER needed to support antibody secretion.

Taken together glucose metabolism seems to be enhanced by many different stimuli *in vitro*. However little is known about the metabolic requirements of activated B cells and specific B cell subpopulations *in vivo*. During B cell development and differentiation, B cells exhibit distinct proliferation and biosynthetic needs. It is currently unknown which metabolic pathways are essential to drive the differentiation of the two main proliferative subsets of B cells: the pre-B cells in early development and GC B cells in antigen-driven responses. Although many stimuli lead to an increase in glycolytic flux, it is not known whether these cells require glucose for survival and proliferation, or whether they are able to switch to other metabolic pathways. A study analyzing glucose dependence during B cell development by Kojima et al. suggests that B220⁺CD43⁺ B cell precursors (pro B cells, large pre B cells) and immature B cells (B220^{hi}CD43⁻) express more Glut1, consume increased levels of glucose and are more dependent on glucose for their survival *in vitro* than small pre-B cells (B220⁺CD43⁻) (Kojima et al., 2010). Interestingly, glucose addiction during B cell development seems to be mediated by the transcription factor hypoxia inducible factor-1 α (Hif1 α). Hif1 α deficiency in B cells leads to decreased Glut1 expression, but increased survival after 2-DG inhibition or in glucose free media (Kojima et al., 2010). Since genes associated with the respiratory chain (*Atp5k*, *Cyba*, *Cyc*, *Cox7c*) are upregulated in Hif1 α deficient B cell precursors, it is possible that Hif1 α deficient cells are more flexible in their choice of energy sources (Kojima et al., 2010). Although valuable, these insights into glucose metabolism during B cell development were mainly gained from cells cultured *in vitro* and need to be validated in *in vivo* models. New insight could be gained by analyzing the metabolic profile of B cells from mice in which proteins involved in nutrient uptake and utilization (e.g. HKII, SLP-2, RISP) or known regulators of cell metabolism (e.g. c-Myc, Foxo1, GSK3, mTOR, Hif1 α) have been deleted or overexpressed at different stages of B cell development. These approaches could be combined with *in vivo* inhibition of specific metabolic pathways by injecting mice with various compounds such as 2-DG (glycolysis inhibitor) or metformin (inhibits mitochondrial complex 1). By combining these approaches, the metabolic requirements during B cell differentiation in distinct microenvironments could be revealed.

To understand how cell metabolism is regulated in GC B cells, it is important to take into consideration that GC B cells are not a homogeneous cell population. B cells in the GC cycle between the dark zone and the light zone. While the dark zone is characterized by large proliferating cells (centroblasts), the light zone is populated by small centrocytes that exhibit a transcription profile of cells responding to T cell help (Victora et al., 2010). Bcl6, which has been shown in T cells to repress genes encoding molecules involved in the glycolytic pathway (Oestreich et al., 2014), is expressed by GC B cells and is essential for the GC response (Fukuda et al., 1997). On the other hand, cMyc a transcription factor known to induce a glycolytic transcriptional program is also essential for the GC response, but is only transiently expressed in a small fraction of GC B cells in the light zone (Dominguez-Sola et al., 2012, Calado et al., 2012). These findings suggest that metabolism exerts a strong influence on B cell fate in the GC. Furthermore, pro B cells, marginal zone B cells, B1 cells and plasma cells are significantly larger than naïve follicular B cells, yet it is not clear how they differ in their metabolic requirements from naïve B cells. In addition, B1a cells and plasma cells secrete antibodies that require enhanced protein synthesis. However, little is known about the metabolic pathways supporting protein synthesis in these cells.

Early studies suggested that glycolysis is essential for an effective humoral immune response. Mice treated with DCA showed decreased antibody production after NP-KLH immunization (Caro-Maldonado et al., 2014). However, other cell types will be affected by this treatment, making it unclear whether glycolysis is essential for B cells or supporting cells such as Tfh cells. The specific role of glucose metabolism in B cells has been addressed by Caro-Maldonado et al. using mice with a B cell-specific deletion of *Glut1* (CD19cre × *Glut1*flox) (Caro-Maldonado et al., 2014). In these mice, mature B cell numbers are reduced and the mice respond poorly to antigenic challenge. However, it was not clarified which B cell differentiation stages or processes were affected by *Glut1* deficiency. A comprehensive analysis of plasma cell metabolomics by Garcia-Manteiga et al. has shown that while proliferating B cells show a high rate of glycolysis, plasma cell differentiation is accompanied by decreased use of glucose, sustained glutamine consumption and the release of glutamate and alanine (Garcia-Manteiga et al., 2011). Thus, plasma cells show a distinct metabolic profile and it would be interesting to analyze how these metabolic pathways affect long-term plasma cell survival and function in the bone marrow. And lastly it is not known whether memory B cells rely upon specific metabolic pathways to support their survival as quiescent cells and then adapt rapidly to respond to antigenic re-challenge.

The role of PI3K signaling in activation-induced metabolic changes

In several cell types, the PI3K/mTOR signaling pathways have been found to be central to the regulation of cell growth and metabolism. Akt regulates various processes in glucose metabolism including localization of the glucose transporter *Glut1* to the cell membrane (Rathmell et al., 2003), pentose phosphate shuttle activity and activation of several glycolytic enzymes such as hexokinase and phosphofructo kinase (Rathmell et al., 2003, Gottlob et al., 2001) (**Fig. 2**). Cells transfected with constitutively active myristoylated Akt show increased ATP levels through enhanced glucose metabolism and oxidative phosphorylation. Furthermore, in these experiments glucose was required for the inhibition of cytochrome c release and apoptosis by Akt upon growth factor withdrawal, possibly

through stimulating hexokinase activity (Gottlob et al., 2001, Rathmell et al., 2003). Interestingly, prosurvival effects of Bcl2 and BclXL in this setting were glucose independent (Gottlob et al., 2001, Rathmell et al., 2003). Akt has been shown to phosphorylate HK-II at Thr-473 leading to increased mitochondrial association (Roberts et al., 2013) (**Fig. 1**). Mitochondria-bound HKII phosphorylates glucose to glucose-6-phosphate, using ATP derived from the mitochondria, thereby coupling glucose metabolism and oxidative phosphorylation. In addition, HKII association with the voltage-dependent anion channel (VDAC) protein on the outer mitochondrial membrane facilitates evasion of apoptosis (Krasnov et al., 2013).

In addition to directly affecting enzymes involved in glycolysis, Akt can regulate the metabolic activity of cells by modulating mTORC1 and GSK3 activity (**Fig. 1**). mTORC1 is a known regulator of cell metabolism and mTORC1 activation has been shown to stimulate different metabolic processes such as glycolysis, the oxidative arm of the pentose phosphate pathway and lipid biosynthesis (Duvel et al., 2010). GSK3 is a ubiquitously expressed kinase with more than 50 known substrates, suggesting that GSK3 is involved in many diverse processes in the cells. Unlike many other kinases, GSK3 is constitutively active and inhibited upon extracellular stimulation. Among other targets, GSK3 has been implicated in regulating the stability/activation of cMyc (Gregory et al., 2003), β -catenin (Tejeda-Munoz and Robles-Flores, 2015) and mTOR signaling (Inoki et al., 2006). In several cell types, GSK3 inhibition/deletion has been shown to augment anabolic processes such as proteinsynthesis, glycogenesis and lipogenesis (Kim et al., 2010, Kim et al., 2013, MacAulay et al., 2005). Thus PI3K signaling can indirectly affect many aspects of cell metabolism by regulating GSK3 and mTORC1. However, it should be noted that both GSK3 and mTORC1 are regulated by other mechanisms independent of Akt activity.

In B cells, BAFF treatment leads to an increase in cell size, cellular protein content, mitochondrial membrane potential and induces the transcription of genes related to glucose metabolism (Patke et al., 2006). BAFF receptor mediated signaling is characterized by the activation of the alternative NF κ B pathway. However BAFF treatment also induces Akt phosphorylation (Otipoby et al., 2008, Woodland et al., 2008) and recent studies suggest that PI3K mediated signaling plays a role in BAFF mediated survival (Jellusova et al., 2013). BAFF treatment has also been reported to induce 4E-BP1 and S6 phosphorylation, which are typical mTORC1 targets (Patke et al., 2006). Furthermore, rapamycin, an mTORC1 inhibitor, decreases BAFF-mediated cell growth, but does not affect survival (Woodland et al., 2008). Since TSC2 has been shown to be phosphorylated after BAFF stimulation (Patke et al., 2006), and TSC2 is an Akt target, it is possible that PI3K signaling is involved in the regulation of cell size through mTORC1. However, direct evidence is lacking that PI3K signaling is responsible for BAFF induced protein synthesis and cell growth.

As discussed, the stimulation of many membrane receptors in B cells induces an increase in glycolytic flux. Interestingly, while IL-4-mediated glycolysis is PI3K independent (Dufort et al., 2007), glucose catabolism following anti-Ig stimulation is reduced in p85 α deficient B cells (Dufort et al., 2007). In addition, it has been shown that Glut1 expression and glucose uptake are reduced after anti-IgM stimulation in the presence of the PI3K inhibitor Ly294002. By comparison, mTORC1 inhibition by rapamycin showed little effect, while

enhanced Akt activation leads to increased glucose uptake and glycolytic flux. In conclusion, these data suggest that glucose utilization in BCR-stimulated cells is regulated by PI3K signaling (Doughty et al., 2006). PI3K signaling appears to be important for BCR induced biosynthesis as well. In the presence of LY294002, S6K phosphorylation and cell mass accumulation is inhibited in anti-IgM stimulated B cells (Donahue and Fruman, 2003). In anti-IgM/LPS stimulated B cells, PI3K inhibition leads to impaired c-Myc accumulation and impaired cell growth. PI3K seems to regulate c-Myc levels by affecting I κ B α stability, and thus NF κ B/Rel mediated c-Myc translation, and also by regulating c-Myc post-translationally (Grumont et al., 2002). In addition, PI3K inhibition in anti-IgM+IL4 stimulated cells leads to decreased ACLY phosphorylation, suggesting that PI3K is also involved in lipogenesis regulation. Thus, PI3K signaling appears to regulate many aspects of cell metabolism and biosynthesis in activated B cells (Dufort et al., 2014). However it should be noted that the PI3K inhibitor Ly294002 has also been shown to directly inhibit other kinases including mTOR (Brunn et al., 1996) thus conclusions drawn from these experiments might not be accurate and should be verified by other experimental approaches.

Mitochondrial metabolism and reactive oxygen species in B cell signaling and differentiation

Mitochondria are organelles that play a major role in cell metabolism by generating ATP through oxidative phosphorylation, and also participate in biosynthesis of macromolecules such as lipids, heme and iron-sulfur clusters (Lill et al., 2012, Ahn and Metallo, 2015, Wallace et al., 2010, Mena et al., 2015). In addition, mitochondria integrate death signals such as DNA damage or low nutrient levels with apoptosis by releasing cytochrome c into the cytoplasm (Green et al., 2014, Norbury and Zhivotovsky, 2004). Interestingly, mitochondria appear to also link metabolic processes with B cell activation, and regulate cell differentiation by the production of reactive oxygen species (ROS). Reactive oxygen species are a natural byproduct of mitochondrial respiration. However mitochondrial dysfunction or an imbalance between ROS production and the availability of antioxidants can lead to excessive ROS accumulation. ROS are highly reactive molecules and have the potential to cause oxidative damage to DNA, proteins and lipids. Oxidative stress can lead to a further decline in mitochondrial function and ultimately result in cell death. However, mitochondrial ROS appears to also support BCR mediated proliferation and regulate B cell differentiation under physiological conditions (Wheeler and Defranco, 2012, Jang et al., 2015). Engagement of the BCR induces ROS production, with the early phase being dependent on the Nox2 isoform of NADPH oxidase and the late phase likely being mediated by mitochondrial respiration (Wheeler and Defranco, 2012). Increased ROS production has also been observed in the GC (Vene et al., 2010). ROS inhibition with the ROS scavenger N-acetyl-L-cysteine (NAC) decreases BCR mediated proliferation (Wheeler and Defranco, 2012). Ncf1-deficient B cells, which have a defective Nox2 complex, fail to generate ROS in the early phases of BCR stimulation. However, BCR mediated signaling, proliferation and *in vivo* responses are normal. Late ROS production (>6h) after BCR engagement is independent of the Nox2 complex and appears to be mediated by mitochondria (Wheeler and Defranco, 2012). Mitochondrial function and ROS production have been recently shown to play a role in fate decision of activated B cells. Jang et al. showed that after *in vitro*

stimulation (LPS+IL4 or CD40+IL4) or *in vivo* immunization, two populations of activated B cell can be distinguished, with one characterized by increased mitochondrial mass, oxygen consumption, H⁺ production, ATP levels and membrane potential. Interestingly, while this population is enriched for isotype-switched B cells, it contains fewer plasma cells. Consistently, this population exhibited increased levels of Pax5, AID and cMyc, but less Blimp1, Irf4, and Xbp1 (Jang et al., 2015).

Modulation of mitochondrial activity and B cell metabolism *in vitro* was shown to affect the frequency of class switched cells vs plasma cells. Rotenone, metformin and oligomycin suppressed plasma cell differentiation, while proliferation-coupled isotype switching was unaffected. Similarly, 2-DG decreased plasma cells, but the frequency of isotype-switched cells was not affected. Inhibition of fatty acid oxidation with etomoxir decreased both processes. Methyl malate, which provides NADPH and methyl pyruvate, which provides substrates for the TCA cycle, decreased plasma cell frequency, but isotype switching was normal. The interpretation of these results is made difficult by the fact that these reagents may affect cell proliferation and survival, which impacts plasma cell differentiation and isotype switching. Nonetheless, it is interesting that although both plasma cell generation and class switching depend upon proliferation, these two processes are affected differently. Furthermore, ROS were induced by reagents supporting class switch recombination, whereas their levels were reduced by agents promoting plasma cell development. At least some of the ROS were shown to be produced by mitochondria, as determined by mitoSOX staining (Jang et al., 2015). The anti oxidant ascorbic acid increased plasma cell formation and decreased isotype switching. Similarly, MitoTEMPO, a mitochondria-targeted antioxidant promoted plasma cell generation (Jang et al., 2015). Thus, mitochondrial ROS (mROS) appears to affect the fate of activated B cells. In B cells committed to CSR, mROS has been shown to attenuate heme synthesis (Jang et al., 2015) (Fig.3). Heme is known to bind to Bach2 and to inhibit its function. Bach2 is a transcription factor, which represses Blimp-1 expression, thus delaying plasma cell differentiation and favoring class switch recombination during an immune response. Both DNA binding activity and protein stability of Bach2 are inhibited by heme, resulting in increased *Prdm1* expression. Heme addition during B cell stimulation *in vitro* results in decreased class switch recombination and increased production of antibody secreting cells (Watanabe-Matsui et al., 2011). Thus, mROS may affect cell fate decision by modulating heme synthesis and thereby Bach2 function (Jang et al., 2015) (Fig.3.).

The role of PI3K in mitochondrial metabolism

In several cell types PI3K signaling has been shown to regulate various aspects of mitochondrial function (Fig.3). PTEN deletion in hepatocytes has been shown to cause increased ROS production and oxidative stress (Li et al., 2013). Mitochondrial mass, glycolysis and oxidative phosphorylation, as measured by extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), respectively, were increased in PTEN-deficient hepatocytes due to elevated PI3K activity (Li et al., 2013). Furthermore, expression of the estrogen related receptor α (ERR α) and ERR α target genes such as *Cyts* and *Acadm* are upregulated in PTEN deficient hepatocytes. ERR α is a central transcription factor that is responsible for adaptive mitochondrial biogenesis in response to different stimuli. ERR α is

crucial for mitochondrial function and may be responsible for the observed increase in respiration. $ERR\alpha$ has also been shown to play a role in regulating ROS. PI3K inhibition was shown to suppress $ERR\alpha$ expression in PTEN deficient hepatocytes. Similarly, constitutively active Akt was demonstrated to induce $ERR\alpha$ expression and to lead to increased oxygen consumption. Furthermore, oxygen consumption was reduced to normal levels by $ERR\alpha$ knockdown. Thus, PI3K/Akt modulates mitochondrial function through the regulation of $ERR\alpha$ expression (Li et al., 2013). In T cells, $ERR\alpha$ expression is induced upon stimulation and facilitates expression of proteins involved in glucose and mitochondrial metabolism. $ERR\alpha$ inhibition in activated T cells leads to decreased levels of glucose uptake and respiration. Thus, similarly to hepatocytes, $ERR\alpha$ regulates metabolism in T lymphocytes as well (Michalek et al., 2011b). Similar to hepatocytes, oxygen consumption is accelerated in mouse embryonic fibroblasts (MEFs) expressing activated Akt or PTEN and decreased in Akt1/2 double-deficient MEFs (Goo et al., 2012, Nogueira et al., 2008). ROS levels are decreased in Akt1/2 deficient MEFs due to increased transcription activity of FoxO factors. Akt1/2 deficiency leads to increased resistance to replicative senescence and oxidative stress induced cell death. The expression of ROS scavengers such as sestrin 3 is increased in Akt1/2 deficient MEFs. Thus, Akt accelerates cell senescence by inhibiting FoxO activity, resulting in increased ROS production (Nogueira et al., 2008).

FoxO deficiency has been shown to result in increased ROS production and a significant reduction in hematopoietic stem cells. This phenotype could be rescued by the administration of the ROS scavenger N-acetyl cysteine (Tothova et al., 2007). Furthermore, increased mTOR activity in TSC1-deficient hematopoietic stem cells is also associated with increased mitochondrial biogenesis and higher ROS levels (Chen et al., 2008). Taken together, PI3K signaling has the potential to affect mitochondrial function and ROS production via diverse pathways (Fig.3). Thus, while PI3K signaling generally supports proliferation and cell survival, activation of this pathway may antagonize these processes under specific conditions through increased respiration and ROS production.

In B cells, ROS are not only produced in a pathologic state, but are also an integral part of receptor mediated signaling. BCR stimulation has been shown to lead to ROS production and contribute to signal transduction (Reth, 2002). The mechanisms by which ROS affects cell signaling are not fully understood. However it has been suggested that hydrogen peroxide can modulate the activity of phosphatases through oxidation of cysteine residues (Reth, 2002, Tonks, 2005), thus affecting B cell signaling in a global manner. A recent study suggests a more specific function for ROS signaling and places hydrogen peroxide upstream of Syk mediated signaling (Patterson et al., 2015). It has been shown that H_2O_2 -induced phosphorylation of Btk, PLC γ 2 and JNK are Syk dependent. Interestingly, while phosphorylated Syk is mainly localized in proximity to the plasma membrane after anti-IgM treatment, a prominent pool of Syk can be found in the mitochondrial membrane after H_2O_2 treatment. Syk-deficient B cells are more sensitive to H_2O_2 -induced apoptosis (Patterson et al., 2015). Akt is also activated in H_2O_2 treated B cells (Qin and Chock, 2003) and it has been shown that this activation is Syk dependent (Ding et al., 2000). Taken together ROS can affect B cell signaling and function in multiple ways. While evaluating these findings, one needs to take into consideration that B cells can be exposed to ROS from different sources, which can be extracellular, such as ROS produced by adjacent neutrophils, or

intracellular, originating from damage to the mitochondria and receptor mediated signaling (Bertolotti et al., 2012). Thus, the amount and source of ROS can possibly have different effects on B cells and can vary from supporting proliferation and signaling to cell damage and cell death. Similarly, while H₂O₂ treatment has been shown to induce PI3K signaling, the functional outcome of this induction is not very well defined. In general, increased PI3K signaling results in enhanced plasma cell generation, yet ROS levels seem to inversely correlate with plasma cell generation. This dichotomy highlights the need for a better understanding of the role ROS plays in B cell signaling and fate decision.

Metabolic stress and autophagy in B cells

The coordination of nutrient and energy availability with anabolic processes such as protein synthesis, cell growth and division is essential for proper cell function and survival. Thus, mechanisms need to be in place to respond to metabolic and energetic cues to regulate cell function and avoid cell death. It is expected that these mechanisms are of primary importance in highly proliferating cell populations such as pre-B cells and GC B cells as the transcriptional program of these cells directs increased biosynthesis and therefore quickly depletes intracellular energy supplies. Additionally, cell populations secreting high levels of protein, such as antibody-producing plasma cells and B1a cells, also need to counterbalance the stress imposed by increased protein synthesis.

AMP activated protein kinase (AMPK) and mTOR are central players in monitoring the metabolic and energetic state of the cell. mTORC1 is activated downstream of several kinases such as Akt and GSK3 and also responds to amino acid availability. AMPK is activated in response to diminishing energy levels and inhibits anabolic processes such as lipid biosynthesis, mTOR-induced protein synthesis and cell proliferation (**Fig.1**), thus supporting survival in response to metabolic stress. AMPK regulates mTOR signaling by phosphorylating TSC2, but also through direct phosphorylation of raptor (Gwinn et al., 2008). However, little is known about the specific role of AMPK in B cells. Mice deficient for the catalytic subunit AMPK α 1 showed normal B cell numbers and produce comparable levels of antigen-specific IgG antibodies after immunization with a thymus-dependent antigen (Mayer et al., 2008). However, antibody affinity maturation and memory responses were not assessed in this study. B cells from AMPK α 1-deficient mice showed normal proliferation to BCR stimulation, but displayed increased apoptosis after oligomycin-induced disruption of mitochondrial function (Mayer et al., 2008). Thus, it would be important to also assess viability of B cell subsets such as germinal center B cells, plasma cells, memory B cells and B1 cells known to be exposed to increased metabolic stress. Interestingly, B lymphoma cells in the E μ Myc model were shown to require AMPK to maintain cell viability in the presence of 2-DG inhibition. Furthermore, the absence of AMPK leads to increased lactate production, enhanced mTOR signaling and faster tumor development *in vivo*. Thus, loss of AMPK favors cell growth pathways, thereby supporting tumor growth but decreasing viability under metabolic stress (Faubert et al., 2013).

Folliculin interacting protein 1 (Fnip1), which is a factor known to interact with folliculin and AMPK, has been shown to impose a metabolic checkpoint on pre-B cells (Park et al., 2012). While pre-BCR signaling seems to be normal in *Fnip1*^{-/-} cells, they exhibit increased

mTOR activation, mitochondrial biogenesis and cell growth. Furthermore, while activation of AMPK leads to mTORC1 inhibition in wildtype B cells, AMPK activation failed to inhibit S6 phosphorylation in *Fnip1*^{-/-} B cells. Fnip1 deficient B cell progenitors showed increased sensitivity to developmental arrest induced by glucose/glutamine or essential amino acid deprivation. Strikingly, unlike AMPK, Fnip1 deficiency inhibited the development of lymphomas in the EμMyc model (Park et al., 2012). It is surprising that two molecules which are both involved in the regulation of mTOR signaling and metabolic stress responses have such disparate roles in lymphoma cells. This enigma highlights the need to better understand how B cells adapt to low nutrients/energy and elucidate the mechanisms by which lymphoma cells avoid metabolic collapse.

A distinct cellular response to low nutrient availability is activation of autophagy. Autophagy is a catabolic process in which cytoplasmic contents are engulfed in double-membrane vesicles and delivered to the lysosome for degradation. Thus, autophagy serves as a recycling pathway, providing substrates for biosynthesis under conditions of nutrient deprivation. Autophagy also serves the function of clearing damaged proteins and organelles, thereby contributing to the maintenance of cellular homeostasis. In the B cell lineage, autophagy has been shown to play an important role in the maintenance of plasma cells (Pengo et al., 2013, Conway et al., 2013) and memory B cells (Chen et al., 2015, Chen et al., 2014). Deletion of Atg5, which is essential for the formation of the autophagosome, results in a dramatic reduction in B1 cell numbers (Miller et al., 2008, Arnold et al., 2015). However the role of autophagy in conventional B cell development and maintenance remains controversial. Chimeric mice generated from Atg5-deficient fetal liver cells displayed a partial block at the pro- to pre- B cells stage, but mature B cells were not affected if Atg5 is deleted later in B cell development using CD19-cre (Miller et al., 2008, Pengo et al., 2013). In contrast, B cell development was normal in mice in which Atg5 is deleted in B cells using the Mb1-cre system. Although no impact on early B cell development was observed in these mice, mature B cell numbers were slightly, but significantly reduced. A similar decrease in mature B cell numbers was observed if Atg5 is deleted at the transitional B cell stage using CD21-cre, suggesting that Atg5 is needed for B cell maturation and/or survival in the periphery (Arnold et al., 2015). Discrepancies observed in these two mouse models could possibly be attributed to the different developmental stages of Atg5 deletion. B cells in Atg5 fetal liver chimeric mice originate from Atg5 deficient hematopoietic stem cells, thus potential defects such as a failure to clear damaged organelles or excessive ROS production can accumulate over a longer period of time and thus have a stronger effect on B cell development. To better understand the role of autophagy in B cell development and maintenance, the mechanism of cell death needs to be thoroughly investigated in these mouse models. Moreover, technical differences between these systems need to be taken into consideration. Deletion efficiency needs to be monitored carefully when using Cre-recombinase based deletion, since B cells escaping deletion might obscure the impact of Atg5 deletion on B cell development and survival. Moreover, B cells derived from fetal liver precursors may differ from bone marrow-derived B cells. Because of the high rate of immunoglobulin synthesis, plasma cells are exposed to severe ER stress, oxidative stress and proteasome stress. Plasma cells display increased autophagic activity, which seems to be important for their survival (Pengo et al., 2013). Plasma cells from CD19cre × Atg5floxed

mice display a larger endoplasmic reticulum and enhanced ER stress. Interestingly, Atg5-deficient cells have increased Blimp-1 levels and immunoglobulins production. Blimp-1 is induced by ER stress, suggesting that in absence of autophagy increased ER stress signaling may be responsible for higher immunoglobulin secretion. By contrast, antigen-specific antibody production is reduced *in vivo* despite normal GC B cell differentiation (Pengo et al., 2013). Memory B cells have also been found to require autophagy for their long term survival. Memory B cells are a heterogeneous population of antigen experienced cells that persist without the need for continued antigen exposure. Memory B cells have been found to display increased autophagic activity, and mice with a B cell specific deletion of Atg7 showed a rapid loss of memory B cells and impaired secondary antibody responses. Clearing damaged mitochondria may provide an explanation for why memory B cells need autophagy, since Atg7-deficient memory B cells show decreased mitochondrial membrane potential, increased ROS production and lipid peroxidation. The anti-oxidant α -TOC has been shown to inhibit cell death in Atg7 deficient memory B cells, suggesting that oxidative stress impairs cell survival of memory B cells (Chen et al., 2014)(Chen et al., 2015). Although accumulating evidence suggests that autophagy plays an important role in the survival of plasma cells and memory B cells, the extent to which these defects affect antibody levels in immunized mice vary between studies (Pengo et al., 2013, Arnold et al., 2015, Chen et al., 2014). As discussed above, the chosen mouse models and immunization protocols could account for some of the differences between the studies. It should also be noted that Atg5 and Atg7 might have some unique functions that differentially impact aspects of B cell biology. For example, Atg5 has been shown to contribute to the regulation of TLR signaling in marginal zone and B1 B cells, and Atg5 loss in these cells leads to increased LPS-mediated NF- κ B signaling and proliferation (Acharya et al., 2016). In addition, an autophagy pathway that is independent of Atg5 or Atg7 has also been described (Nishida et al., 2009). Thus many questions concerning the role autophagy plays in B cells remain to be elucidated.

The role of PI3K signaling in metabolic stress responses and autophagy

Activation of the PI3K and the mTOR signaling pathways leads to the induction of many anabolic processes (**Fig. 1**). As discussed, mTOR activity is inhibited in metabolically stressful conditions by AMPK to conserve energy. AMPK phosphorylates TSC2 and enhances its activity. Activated TSC2 then inhibits mTOR-mediated cell growth and protects cells from cell death (Inoki et al., 2003). In contrast, Akt mediated phosphorylation of TSC2 decreases its ability to inhibit mTORC1 signaling (Inoki et al., 2002). The role of Akt signaling under nutrient stress remains controversial. Several studies suggest that the pro-survival role of Akt is dependent on glucose, and constitutively active Akt signaling accelerates death of glucose starved cells (Bruno et al., 2007, Coloff et al., 2011, Buzzai et al., 2005, Elstrom et al., 2004). However, it has also been shown that glucose deprivation leads to Akt phosphorylation in some cell types and selective phosphorylation of some Akt targets such as GSK3 (Chopra et al., 2012, Gao et al., 2014). Surprisingly, Akt activation seems to support cell survival under glucose deprivation despite the general role of Akt in promoting anabolic processes (Gao et al., 2014, Suzuki et al., 2003b). Thus, PI3K signaling may play a dual role in nutrient deprived cells and altered Akt activity, rather than Akt

inhibition might be needed for cell survival. Akt is also known to play a role in autophagy. Induction of autophagy is regulated mainly by nutrient availability. If nutrients are abundant, mTORC1 phosphorylates ULK1 and ATG13 to block autophagy. When nutrients are scarce, mTORC1 dissociates from the unc-51-like kinase 1 (ULK1) complex and autophagy is initiated (Dunlop and Tee, 2014). mTOR is also activated by Akt in response to mitogens. Thus, Akt can inhibit autophagy through mTORC1 activation. Furthermore, Akt is able to inhibit autophagy in an mTOR independent manner as well. Akt has been shown to phosphorylate Beclin1, leading to increased association of Beclin1 with 14-3-3 proteins, resulting in suppressed autophagy (Wang et al., 2012). Although Akt has been suggested to regulate different processes in metabolically stressed cells, it remains to be determined whether this also applies to B cells.

Metabolic signatures of B cell derived malignancies

Although the regulation of metabolism in normal B cells is not very well understood, considerably more effort has been put into studying cancer cell metabolism. Lymphoid neoplasms are a heterogeneous group of malignancies and can be broadly divided into two major groups: neoplasms derived from progenitor cells - acute lymphoblastic leukemia/lymphoma (ALL) and neoplasms originating from mature lymphocytes or plasma cells, including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL) and others. A separate type is the Hodgkin lymphoma, in which tumor cells are derived from GC B cells, but lack most phenotypic markers of B cells. Cancer cells often exhibit altered metabolism that ensures a continued supply of ATP and precursor molecules to promote ongoing proliferation and cell growth. One of the main metabolic alterations identified in cancer cells is increased glycolytic rate, even in the presence of sufficient oxygen (Warburg effect). Similar to other cancer types, B cell-derived cancer cells often show increased glucose uptake and enhanced rate of anaerobic glycolysis. Indeed, the increase in glucose uptake forms the basis for fluoro-deoxyglucose positron emission tomography (FDG-PET), which is diagnostic of aggressive lymphomas (El-Najjar et al., 2012). Correspondingly, lymphoma cells often show increased levels of glucose transporters and glycolytic enzymes. Burkitt lymphoma, follicular lymphoma, DLBCL and primary effusion lymphoma (PEL) lines show increased glycolytic flux (Bhatt et al., 2012). In many patients, Hodgkin and Reen Stenberg cells express high levels of the glucose transporter Glut1 and glycolytic enzymes such as lactate dehydrogenase (LDHA) (Hartmann et al., 2012). In comparison to B cells from healthy donors, primary B cell acute lymphoblastic leukemia (B-ALL) cells show increased rates of aerobic glycolysis and decreased survival after 2-DG inhibition (Liu et al., 2014). Multiple myeloma patient samples exhibit Glut8 and Glut11 overexpression in comparison to normal plasma cells. Multiple myeloma cell lines overexpress Glut4, Glut8 and Glut11 and show increased glucose uptake and decreased survival in glucose free medium, while the survival of normal B cells is unaffected. Glut4 has been shown to be essential for survival of multiple myeloma lines, and Glut4 knockdown leads to decline in Mc11 mRNA (McBrayer et al., 2012). Thus, glucose uptake and utilization is increased in many malignancies of B cell origin and often supports cancer cell survival. Although glycolysis is increased in many cancer cells, the increase is not always coupled to a decrease in mitochondrial respiration.

For example, leukemia cells also have the ability to carry out oxidative phosphorylation, although often using electrons from non-glucose carbon sources (Velez et al., 2013).

In addition to glycolysis, other metabolic pathways might be essential for the proliferation and survival of cancer cells. It has been demonstrated that the inhibition of fatty acid oxidation with etomoxir or fatty acid synthesis with orlistat decreases proliferation of multiple myeloma cells (Tirado-Velez et al., 2012). Thus, analyzing the metabolic requirements of cancer cells can help to develop new treatment strategies. It is also important to acknowledge that metabolic features vary not only between different types of cancers, but can diverge significantly within a specific cancer type. For example, a subset of diffuse large B cell lymphoma has been found to be characterized by elevated oxidative phosphorylation of fatty acids and increased lipogenesis (Caro et al., 2012). Diffuse large B cell lymphomas are a genetically heterogeneous group of tumors, often classified into germinal center B cell –like (GCB) or activated B cell –like (ABC) subsets based on their cell of origin. The GCB subset has a transcriptional profile similar to normal GC B cells, the ABC subset is similar to BCR-activated B cells. The GCB subtype is characterized by the loss of PTEN expression, while PTEN is expressed in most of the ABC subtypes (Pfeifer et al., 2013). A different classification framework identifies BCR-DLBCL, OxPhos DLBCL and host response (HR) subsets. BCR subsets display up-regulation of genes encoding B-cell receptor signaling components, the HR tumors are characterized by host inflammatory infiltrate and the OxPhos subset is enriched in genes involved in mitochondrial oxidative phosphorylation. Cells of the OxPhos type show increased fatty acid oxidation in comparison to BCR type lymphoma (both ABC and GCB). Interestingly, Syk knockdown in BCR type lymphomas leads to increased palmitate oxidation, suggesting that silencing of BCR signaling is responsible for the different metabolic profiles in BCR- and OxPhos-Lymphoma subtypes (Caro et al., 2012).

PI3K mediated regulation of cancer cell metabolism

Aberrant PI3K signaling is implicated in numerous aspects of cancer biology and the PI3K signaling network is activated in many different types of cancer, including lymphoid neoplasms. As the PI3K/Akt/mTOR signaling cascade plays a crucial role in many aspects of cancer biology, inhibition of these signaling molecules has become an attractive target for cancer therapy. Several compounds targeting PI3K, Akt or mTOR, or drugs with dual activities have been developed and are currently being evaluated in clinical studies (Majchrzak et al., 2014, Fang et al., 2013). Protein microarray analysis of follicular lymphoma patient samples has revealed a significant increase in Akt (Ser473) phosphorylation (Gulmann et al., 2005, Zha et al., 2004) and the dual PI3K/mTOR inhibitor NVP-BEZ235 inhibits FL cell proliferation and has shown anti-tumor activity in mouse xenograft models (Bhende et al., 2010). Dysregulation of the PI3K/Akt/mTOR signaling pathway was also observed in DLBCL, and patients with high levels of Akt phosphorylation showed decreased survival time (Hong et al., 2014, Hasselblom et al., 2010). Inhibition of the PI3K/Akt/mTOR pathway has been shown to impair proliferation of DLBCL cells (Fang et al., 2013) and several small molecule inhibitors of the PI3K signaling pathways are currently undergoing evaluation in different phases of clinical trials for lymphoma (Majchrzak et al., 2014). Increased PI3K activity was also observed in chronic lymphocytic

leukemia, and selective inhibition of the p110 δ isoform of PI3K with CAL-101 (Idelalisib, GS-1101) promotes apoptosis of primary CLL cells *in vitro* (Herman et al., 2010, Gopal et al., 2014) and has also shown promising results in clinical trials (Fruman and Rommel, 2011, Gopal et al., 2014). However, the efficacy of CAL-101 possibly arises in part from modulating the tumor microenvironment (Fruman and Rommel, 2011, Herman et al., 2010). Enhanced PI3K signaling has also been observed in cells from other B cell malignancies such as Burkitt lymphoma and mantle cell lymphoma (Fang et al., 2013). Collectively, these findings suggest that inhibition of PI3K signaling could provide a general strategy to impair cancer cell survival/proliferation. The challenge, however, remains to develop selective inhibitors with low overall toxicity and acceptable side effects in patients. In addition, to avoid the development of resistance due to the activation of alternative survival pathways or acquired mutations, inhibitors of PI3K signaling would likely be most effective when used in combination with other treatments (Fruman and Rommel, 2014).

In addition to the regulation of proliferation and survival, PI3K signaling has also been implicated in the regulation of various aspects of cell metabolism. PI3K or Akt inhibition has been shown to lead to Glut1 retention in the DLBCL cell line SUDHL4 and other types of lymphoma lines (Sommermann et al., 2011). Primary effusion lymphoma (PEL) cells, a subset of B cell Non-Hodgkin lymphoma (B-NHL), display increased glycolytic flux and cell viability decreased when glucose concentration in the growth medium is lowered or if glycolysis is inhibited with 2-DG. In addition, fatty acid synthesis is increased in PEL cells and tightly linked to glycolysis. If fatty acid synthesis is inhibited in these cells, glycolysis is reduced as well. Conversely, glycolysis inhibition leads to decreased fatty acid synthesis, suggesting that glucose metabolism and fatty acid synthesis are linked in PEL and glucose is possibly used to generate intermediates for fatty acid synthesis. A non-cytotoxic dose of the PI3K inhibitor LY294002 has been shown to reduce fatty acid synthesis and glycolysis in PEL cells (Bhatt et al., 2012). Thus, PI3K signaling appears to affect different aspects of cell metabolism in cancers of B cell origin. Since PI3K signaling is enhanced in most types of B cell malignancies, a better understanding of metabolic changes caused by enhanced PI3K activity could help to identify new targets that could be exploited for therapy. An important aspect of cancer cell biology that remains understudied is the flexibility of cancer cells to adapt to different types of stress over a longer period of time. Although it is tempting to target glucose metabolism to diminish cancer cell survival and proliferation, it remains to be determined how a specific signaling profile in different malignancies affects metabolic plasticity if cells are exposed to glycolysis inhibitors *in vivo* over a longer period of time. Similarly, indirect inhibition of glucose metabolism by targeting PI3K activity might lead to metabolic adaptations. Thus metabolic plasticity of cancer cells deserves further consideration.

Concluding remarks and future prospects

Studies on lymphocyte metabolism in the recent years have brought a new appreciation for the intricate interplay between metabolic and signaling pathways. It has become increasingly apparent that cell metabolism is not simply a byproduct of cell activation, but that nutrient acquisition and utilization are tightly regulated processes dependent on the developmental stage, cell function and many other aspects. Nutrient availability and other environmental

cues have been shown to not only influence cell growth and proliferation, but in many cases dictate cell fate and function. The PI3K signaling network integrates membrane receptor mediated signaling with cell metabolism and impacts various aspects of B cell biology. However many questions remain unanswered. Little is known about the metabolic profile of different B cell subpopulations. While glucose uptake and glycolysis seem to be increased by many mitogenic stimuli it remains to be determined whether glucose metabolism is essential for B cell survival and the function of different B cell subsets. Furthermore, glucose can serve many different purposes in the cell. For example, it can be used to produce energy, to generate reducing agents through the pentose phosphate pathway or for biosynthesis. However it is unclear which of these processes crucially depend on glucose in B cells, and whether B cells possess any flexibility in their choice of source metabolites. Although Burkitt lymphoma cells have been shown to be able to support a glucose independent TCA cycle with glutamine (Le et al., 2012), it is not known whether the different naïve B cell subsets are able to do so as well. The OxPhos subtype of diffuse large B cell lymphoma has been shown to oxidize fatty acids. Similarly, silencing of BCR signaling in BCR type DLBCL has been shown to lead to increased fatty acid oxidation (Caro et al., 2012). Since DLBCL cells originate from activated or GC B cells it would be important to analyze whether a similar situation is present in non transformed GC B cells and whether metabolic reprogramming affects B cell selection and survival in the GC. Furthermore, new *in vivo* models are needed to analyze the regulation of B cell metabolism during an immune response. Although *in vitro* models have brought some valuable insight a cell culture system cannot replicate the dynamic environment of the secondary lymphoid tissues, the blood and the bone marrow during an immune response. Oxygen tension, nutrient availability and the release of small molecules such as ROS or ATP from bystander cells have the potential to affect B cell function. As exemplified by the modulation of ROS levels, which are linked to mitochondrial function, B cell fate can be skewed by affecting metabolism. In this context, it is important to assess the role environmental cues play in B cell function and which signaling pathways regulate these processes. The PI3K signaling pathway has been shown to play a major role in B cell survival, differentiation and function, yet comparably little attention has been paid to deciphering its role in B cell metabolism. Cancer cells are known to reprogram their metabolism in order to support cell growth and survival in potentially oxygen and nutrient low environments. Furthermore chronically activated B cells in mice have been recently shown to possess increased respiratory capacity (Caro-Maldonado et al., 2014). Thus, a thorough understanding of how B cell metabolism is regulated could provide new therapeutic tools and treatment strategies for different diseases in the future.

Acknowledgement

This study was funded by the National Institutes of Health (AI41649) to RCR and the Excellence Initiative by the German Federal and State Governance (EXC 294) to JJ.

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Figure 1. Regulation of cell metabolism by the PI3K/Akt signaling pathway

Akt is one of the main effector molecules downstream of PI3K and regulates various metabolic processes by phosphorylating enzymes such as ACLY, which is needed for lipid synthesis, or HK2 and PFK2, which are involved in glycolysis. Akt can also indirectly affect cell metabolism by regulating the activity of mTORC1 or GSK3.

Figure 2. Major metabolic pathways that support ATP production and biosynthesis in lymphocytes

Glucose is metabolized to pyruvate through glycolysis and can be further converted to lactate, which is then secreted. This process does not require oxygen and the net gain in energy is 2 ATP molecules per molecule glucose. Alternatively, pyruvate can be oxidized to Acetyl-CoA and enter the TCA cycle. The NADH and FADH₂ molecules produced in the TCA cycle are then used as electron donors in the electron transport chain (oxidative phosphorylation) to generate ATP. If glucose is metabolized completely through oxidative phosphorylation, the net energy gain is approximately 36 ATP molecules per molecule

glucose. Carbon atoms from molecules other than glucose can also enter the TCA cycle. Fatty acids need to be activated in the cytosol and transferred across the mitochondrial membrane before being oxidized in the mitochondria. Catabolic pathways are highlighted in green. Anabolic pathways are highlighted in red. In blue are shown some of the enzymes catalyzing important steps in cell metabolism. Abbreviations: ACC= Acetyl-CoA-carboxylase, ACLY= ATP citrate lyase, F6P= Fructose 6-phosphate, F1,6BP= Fructose 1,6-bisphosphate, FAS= fatty acid synthase, G6P= Glucose 6-phosphate, HK=hexokinase, LDHA= Lactate dehydrogenase A, PDH= Pyruvate dehydrogenase, PDHK= Pyruvate dehydrogenase kinase, PFK= Phosphofructokinase, TCA cycle = Tricarboxylic acid cycle

Figure 3. The production and function of reactive oxygen species in B cells

ROS can be produced in B cells as a byproduct of mitochondrial function and by NADPH oxidases. It has been suggested that the Nox2-containing NADPH oxidase complex is responsible for the majority of ROS production in B cells. However the calcium- regulated NADPH oxidase Duox1 may play a role in specific B cell subsets (Singh et al., 2005). Furthermore, the phenotypes of mice deficient in Ncf1 (an essential part of the Nox2 complex) or gp91^{phox} (catalytic subunit of Nox2) differ from mice lacking the voltage-gated proton channel HVN1, which plays a role in maintaining NADPH oxidase activity (Wheeler and Defranco, 2012, Capasso et al., 2010, Richards and Clark, 2009). Thus, the exact mechanism of ROS production in B cells awaits further clarification. ROS produced downstream of the BCR serves as a signaling molecule and can affect B cell activation and function. Several lines of evidence suggest that Syk and the PI3K signaling pathways are activated in response to ROS, however the exact mechanism is not completely understood

yet. Mitochondrial ROS can also affect cell fate by inhibiting Heme synthesis. Heme is known to inhibit Bach2 function and thereby relieving Bach2 mediating Blimp repression. Thus, increased ROS production can inhibit plasma cell development and favor class switch recombination by modulating Bach2 function. While ROS seems to contribute to B cell signaling and is essential for BCR mediated proliferation, excessive ROS production can lead to cell damage and ultimately to cell death. Abbreviations: ALA= Aminolevulinic acid, CoPIII= coproporphyrinogen III, mtDNA= mitochondrial DNA, PIX= protoporphyrin IX, SCoA= Succinyl coenzyme A.

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