The role of mTOR-mediated signaling in the regulation of cellular migration

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Abstract

Mechanistic target for rapamycin (mTOR) is a serine/threonine protein kinase that forms two distinct complexes mTORC1 and mTORC2, integrating mitogen and nutrient signals to regulate cell survival and proliferation; processes which are commonly deregulated in human cancers. mTORC1 and mTORC2 have divergent molecular associations and cellular functions: mTORC1 regulates in mRNA translation and protein synthesis, while mTORC2 is involved in the regulation of cellular survival and metabolism. Through AKT phosphorylation/activation, mTORC2 has also been reported to regulate cell migration. Recent attention has focused on the aberrant activation of the PI3K/mTOR pathway in B cell malignancies and there is growing evidence for its involvement in disease pathogenesis, due to its location downstream of other established novel drug targets that intercept B cell receptor (BCR) signals. Shared pharmacological features of BCR signal inhibitors include a striking “lymphocyte redistribution” effect whereby patients experience a sharp increase in lymphocyte count on initiation of therapy followed by a steady decline. Chronic lymphocytic leukemia (CLL) serves as a paradigm for migration studies as lymphocytes are among the most widely travelled cells in the body, a product of their role in immunological surveillance. The subversion of normal lymphocyte movement in CLL is being elucidated; this review aims to describe the migration impairment which occurs as part of the wider context of cancer cell migration defects, with a focus on the role of mTOR in mediating migration effects downstream of BCR ligation and other microenvironmental signals.

Highlights

- mTOR forms two distinct complexes mTORC1 and mTORC2, responsible for integrating mitogen and nutrient signals to regulate cell survival, proliferation and migration; processes which are commonly deregulated in human cancers.
- In this review we summarize the role played by mTOR kinase in regulating migration, using chronic lymphocytic leukemia as a paradigm for migration studies.
- BCR-signaling antagonists in combination with dual mTOR inhibitors may provide a novel therapeutic strategy for overcoming drug resistance in B cell malignancies.

mTOR-mediated signaling

Mechanistic target for rapamycin (mTOR) resides in two distinct signaling complexes mTORC1 and mTORC2, which integrate growth factor and nutrient signals to promote cell survival, growth and proliferation (1). mTORC1 contains RAPTOR, mLST8, PRAS40 and DEPTOR and mTORC2 contains RICTOR, mLST8, mSIN1 and DEPTOR (1-4). Originally defined in yeast (5, 6), the 250kDa protein kinase is both growth factor- and nutrient-sensitive with respect to mTORC1 however the "rapamycin-insensitive" mTORC2 responds only to growth factor signals and its existence explains the evident mTOR activity upon treatment with rapamycin (7). Positioned downstream of the phosphatidylinositol-3-kinase (PI3K)-mediated signaling, mTOR integrates signals from binding of chemokine receptors, such as CXCR4 and CXCR5, and BCR-mediated signals in addition to the more frequently reported metabolic and growth factor responses ((8) & Figure 1). The tuberous sclerosis (TSC1-TSC2) protein complex acts as an intermediary between the PI3K/AKT and mTOR signal, and its phosphorylation by AKT causes disassociation of the complex from GTPase Rheb which permits mTOR activation (9). Alternatively, in conditions of oxidative stress, hypoxia or starvation, mTOR may be inhibited by TSC1/2, activated by the conversion of Rheb-GTP to Rheb-GDP by 5'adenosine monophosphate-activated protein kinase (AMPK). While mTORC1 phosphorylates p70S6kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) to increase protein synthesis, mTORC2 directly phosphorylates Akt^{S473}, enhancing its kinase activity 5 – 10 fold to promote cell survival and initiate cell cycle (10, 11). Through AKT regulation, mTORC2 is considered to play a role in migration, however the precise molecular mechanisms have yet to be elucidated (11, 12).

The activation of mTORC1 may be further regulated by localization of the complex; mTOR is largely cytoplasmic but can associate with cellular organelle membranes, affecting its ability to phosphorylate S6K1. The functions of mTORC1 involve regulation of cellular proliferation, cell size, ribosomal biogenesis and angiogenesis by phosphorylation of S6K1 and 4E-BP1, which are directly responsible for mRNA translation (13). After mTORC1 activation, S6K1 triggers protein synthesis by phosphorylating PDCD4, marking it for degradation with secondary effects of binding and preventing eIF4A helicase from fulfilling its function in mRNA unwinding. S6K1 can also function to dampen signaling via PI3K by inhibition of insulin receptor substrate 1 (IRS1) and 2 (IRS2) expression, providing a negative feedback loop within the mTORC1 side of the complex, thus explaining the non-aggressive nature of the hamartomatous tuberous sclerosis syndrome, a product of mutations in *tsc1/2* (14). Upon phosphorylation by mTORC1, 4E-BP1 is released from inhibiting the elongation initiation factor 4E (eIF4E).

mTORC2 plays a major role in signaling via the PI3K/AKT pathway, through phosphorylation and activation of AKT making it of interest as a therapeutic target in blocking the AKT signal, as mTORC2 has been demonstrated to be necessary for the development of activated PI3K signaling in tumors (15). Of note, an inhibitory feedback loop exists between mTORC1 and mTORC2, enabling an upregulation in AKT signaling with the removal of the mTORC1 signal, suggesting a complex regulatory role for mTOR in AKT-dependent malignancies (16, 17).

mTOR regulation of migration

To delineate the relative contribution of mTOR complexes to the cellular migration process we still refer to evidence from studies utilizing rapamycin. The regulation of migration by inhibition of S6K1 and 4EBP1 has been shown in a variety of cell types, by the administration of rapamycin, to delineate the mechanisms by which control is exerted, including formation of the F-actin cytoskeleton, extracellular matrix remodeling (e.g. MMP9) focal adhesion formation, and Rho GTPase activation all regulated via S6K1 (18-20). Furthermore, there is evidence derived from inhibitor studies that support both mTORC1 and mTORC2 as regulators of cell adhesion (21). Extracellular matrix changes and GTPase activity are also regulated by 4EBP1 with evidence that it regulates VEGF/TGFβ levels in a rapamycin-sensitive manner in mouse models of solid organ tumors and metastasis (22). Rapamycin appears to exert its effect downstream of S6K1 and 4EBP1 via regulation of small GTPase RhoA activation (23). In contrast, mTORC2 mainly modulates the actin cytoskeleton to regulate migration (24, 25). Downstream effectors appear to be species and cell-type specific. PKA and Ras signaling pathways appear to be pivotal in amoebae (26), while, in mammals PKC, PKA signaling and Rac/RhoA are the main regulators of mTORC2-mediated migration (27). Studies using amoebae demonstrate that TORC2 exerts its influence via AKT and PKA regulation of adenylyl cyclase –based production of cyclic AMP (28). In mammalian systems, it appears that mTORC2 regulates neutrophil chemotaxis by regulation of F-actin polarization and myosin II phosphorylation, again regulated by cAMP production but via PKC in a RhoA/ROCK dependent fashion (29). There are also putative mTORC2-independent effects of RICTOR on migration that have yet to be fully explored (30).

It is hypothesized that mTORC1/2 inhibitors would also have effects on cytoskeletal formation with downstream effects of cAMP production mediated by PKC and GTPases and this is supported by studies from a variety of model organisms and cell types. Indeed, the first description of the RICTOR homologue pianissimo in *Dictyostelium discoidieum* is as a regulator of chemokine and GTPase signals. In the described piaA knock-out model, cells are unresponsive to chemokine stimulation or GTPγS activation, defects that are restored with constitutive activation of piaA (31). Cytoskeletal regulation was attributed to TORC2 in *Saccharomyces cerevisiae* and amoebae through regulation of the Rho GTPase family and downstream mediators including pkc1 (32). In mammalian systems, a link between RICTOR and PKCα was established first (25) followed by a link between mTORC2 and RhoA family in regulation of neutrophil chemotaxis via mechanisms which are independent of actin cytoskeletal reorganization (33). It appears that Rac/cdc42 GTPases are responsible for actin cytoskeleton formation and these effects are driven by RICTOR-mediated inhibition of Rho-GDP dissociation inhibitor 2 (29, 34).

CLL and migration

Cancer cell rehoming and the mechanisms by which it is governed are central to tumor spread and clonal expansion (35). The processes of cytoskeletal protein assembly, actin remodeling and integrin formation are amongst a range of individual events that precede cellular migration and are tightly regulated by chemokine and adhesion molecule interactions. In normal lymphocyte migration, selectin and integrin binding of lymphocytes at the vascular endothelium initiates lymphocyte rolling, arrest and firm adhesion. Subsequent transendothelial migration is triggered by integrin activation and chemokine

receptor/ ligand binding as the lymphocyte is exposed to local chemokine release from the vascular lumen (36). B cell positioning within the lymph node (LN) germinal center is determined by chemokine surface receptor expression of CXCR4 and CXCR5 and secretion of ligands CXCL12, CXCL13 and CCL19/CCL21 by the LN stromal cells. B cells migrate along chemokine gradients to the appropriate microanatomical site where they mature through phases of somatic hypermutation and clonal selection on the basis of antigen affinity (35).

In CLL, the pathophysiological process hijacks the innate properties of normal B cells not only to retain the function of recognized surface markers which interact with chemokines to regulate migration such as CXCR4 – CXCL12 and CXCR5 – CXCL13, but also to utilize the enhanced expression of these markers, facilitating increased migration to the secondary lymphoid organs. Understanding the regulation of these events offers potential candidates for *in vitro* study of oncogenic signaling: molecules of interest in CLL can be broadly categorized as chemokine receptors, adhesion molecules and other transmembrane signaling molecules. CXCR4 surface molecule function is preserved in CLL cells with high expression of CXCR4 on CLL cells in peripheral blood and downregulated expression after migration underneath a stromal cell layer (37). Also, CXCR5 surface expression is found to be present and functional on CLL cells (38). The molecule CD49d, which is the α4 component of the $\alpha 4\beta 1$ integrin complex, has been implicated in CLL cell migration for some time (39) and its expression has been found to co-segregate with prognostic entities that have a tendency for increased migration (40, 41). Interestingly, those cells carrying trisomy 12 in association with the poor prognostic marker *NOTCH1* mutation display further upregulation of integrin signaling by way of increased β2 signaling (40). The surface marker CD38 is established as a marker of negative prognostic significance and this could be in part due to increased BCR signaling and tendency for cellular migration to the LN (42). CD44 interaction with its extracellular matrix ligand hyaluronan (HA) plays a role in cell positioning within the secondary lymphoid organs to receive pro-survival signals, which have been implicated in cell activation, migration and tissue retention of CLL cells. Properties of the CLL microenvironment including the presence of CCL21 secretion and the strength of CD40-CD40L interactions with T cells determine the balance of the equilibrium of CD44-HA binding; CCL21 induces motility whereas CD40L stimulation shifts the balance towards tissue retention of CLL cells (43). The surface receptor CD62L is implicated in homing of CLL cells and is upregulated in cells localized to LN and BM tissue (44). A similar pattern of increased expression of MMP9 on cells localized to the LN and BM formed the basis of studies of MMP9 in CLL migration and invasion. *In vitro* modeling of migration demonstrated an upregulation in MMP9 with increased migration under regulation by $\alpha 4\beta 1$ integrin and PI3K/AKT signaling (45).

GTPases are also dysregulated in CLL cell migration, elucidated by two studies with a specific focus on the role of Rap1 GTPase. Chemokine-induced transendothelial migration has been shown to be defective in CLL and pathological B cells lose their chemokine-responsiveness in the disease. GTP-loading of Rap1 fails due to impaired endosomal recycling to the plasma membrane and this has been attributed to defective activation of phospholipase D1 and Arf1, its regulator (46). Additionally, chemokine-induced activation of integrin clustering fails due to an inability of polar clustering of $\alpha L\beta 2$ integrin which is Rap1- induced. Binding of $\alpha 4\beta 1$, VEGF and chemokine are all needed for $\alpha L\beta 2$

activation, as a result of failure to GTP-load Rap1 in CLL cells (47).

More recent studies of cellular migration have described surface changes in chemokine receptor profile and have derived an emigrant lymphocyte phenotype ($CXCR4^{dim}/CD5^{hi}$) (48). Proliferation studies have established a 2-compartment model of CLL with those cells in the peripheral blood demonstrating a low proliferation rate and those with a higher birth rate being derived from the secondary lymphoid tissue. Cells traffic between compartments and display distinct surface immunophenotype along with gene expression profile (49). Studies that demonstrate the immunophenotypic differences employed deuterium labeling of resting and proliferative fractions of CLL cells (50) whereas later work examined paired fine needle aspirates and peripheral blood CLL samples and used functional assays to demonstrate phenotypic differences. An *in vitro* circulation system of migration was utilized to lend further support to the proposed model of migration in which the CLL microenvironment exerts a phenotype upon cells according to their positioning (48).

BCR signal transduction and migration

Further evidence for migratory effects as part of the neoplastic process can be derived from recent advances in CLL therapy and the function of inhibitors which act downstream of the BCR signaling cascade. It has long been understood that the ability of B cells to respond to antigenic binding of BCR molecules is retained in CLL but the reliance on differential methods of activation of signaling downstream of the BCR varies by B cell malignancy. Both antigen-dependent and autonomous BCR signaling are at play in CLL and recruit similar downstream effectors in signal mediation. Antigen binding of the BCR molecule, through the surface immunoglobulin (sIg) leads to Igα/β (CD79a/b) phosphorylation of tyrosine residues on immune-receptor tyrosine-based activation motifs (ITAMs) by Src-family kinases, such as LYN, FYN or BLK. Binding of tandem Src homology 2 (SH2) domains within the spleen tyrosine kinase (SYK) molecule, a non-receptor tyrosine kinase, allows further propagation of the signal by recruitment of BLNK to the phosphorylated tyrosine residues. BLNK, the B cell linker protein also known as SLP-65, acts as a substrate for SYK to recruit proteins such as the enzyme phospholipase C gamma 2 (PLCγ2) and TEC family kinase Bruton's tyrosine kinase (BTK) to the signaling complex (Figure 1). Active PI3K generates phosphatidylinositol (3,4,5) triphosphate (PIP_3) to enable membrane recruitment of oncogenic serine/threonine kinase AKT, BTK and PLCγ2 and permit signal transduction to the nucleus. The PI3K family of enzymes comprises three classes, regulating cell growth, differentiation and survival. Class IA PI3K are dysregulated in human disease, including the isoform PI3Kδ whose expression is restricted to hematopoietic cells and implicated in the pathogenesis of B cell malignancies (51).

The impact of BCR signaling inhibitors on CLL migration

Ibrutinib, fostamatinib and idelalisib, which target BTK, SYK and PI3Kδ respectively, all affect CLL cell migration mediated by tissue-specific expression of chemokines and ligands; modulating processes of cellular adhesion, transendothelial migration and tissue retention via effects on integrins and adhesion molecules (52-54). These inhibitors, as well as targeting BCR-mediated signals, also have effects on microenvironmental signaling outwith the BCR signaling pathway. Paracrine secretion of chemokines

CCL3 and CCL4 by CLL cells is blocked, reducing support from monocyte-derived nurse-like cells and accessory T cells (52, 53). Pre-clinical studies of BCR signaling inhibitors detail a striking lymphocytosis effect with individual therapies in terms of plasma chemokine levels and surface receptor expression. Using primary CLL samples De Rooij *et al*. demonstrated a reduction in signaling via CXCL12, CXCL13 and CCL19 upon ibrutinib treatment, with a reduction in adhesion and migration of CLL cells (54). In parallel studies of ibrutinib, Ponader *et al*. identified a reduction in migration toward CXCL12 and CXCL13, which inhibited CCL3 and CCL4 secretion. Targeting of SYK with fostamatinib also displayed an inhibitory effect on CCL3 and CCL4 secretion in primary disease samples, with a reduction in homing towards CXCL12 and CXCL13 and a reduction in CLL cell adhesion to VCAM-1 (53, 55). Our group investigated the effects of LYN kinase inhibition with dasatinib revealing inhibitory effects on the CXCR4-CXCL12 signaling axis in CLL samples *in vitro* (56). PI3Kδ inhibition with idelalisib revealed a downregulation of chemokine levels *in vitro*, a finding that was corroborated by data from patients treated with the drug during clinical trial. Chemokine levels in patient plasma samples exhibited a reduction in secretion of CCL3, CCL4 and CXCL13 and a concomitant surge in the lymphocyte count in the peripheral blood (52).

The TCL-1-transgenic mouse model develops a leukemia analogous to poor prognostic CLL, due to the overexpression of the oncogene *T cell leukemia/lymphoma 1* (*TCL-1*) in B lineage cells (57), and has been used to delineate the molecular mechanisms that govern CLL cell migration. Pre-clinical data using ibrutinib and fostamatinib in these mice showed *in vivo* effects of an initial increase in lymphocytosis with therapy and a reduction in lymphadenopathy (58, 59). In studies with TCL-1 mice, dasatinib was found to have inhibitory effects on LYN substrate hematopoietic lineage cell-specific protein 1 (HS1) which has targeted effects on regulation of migration via cytoskeletal formation (60). Further work to elucidate the mechanisms of migration control reveal that the TCL-1 GTPase *Rho* knockout model displays a delayed disease onset, with a reduction in homing of cells to the BM, a reduction in interactions with CXCL12 and CXCL13 and a reduction in the polarization of phosphorylated focal adhesion kinase (FAK) with defective integrin function (61).

Targeted inhibition of mTOR complexes

mTOR kinase was discovered by virtue of research gaining an understanding of the pharmacological activity of rapamycin (62). Originally discovered in the soil of Easter Island and derived from *Streptomyces Hygropicus,* this macrolide was found to possess both immunosuppressive and anti-neoplastic properties (63, 64). The two mTOR complexes are defined by their differential responses to rapamycin; mTORC1 binds rapamycin allosterically by its association with FKBP12 whereas mTORC2 was first thought to be rapamycin-insensitive thus making mTORC1 the obvious choice as a drug development target. Thereafter, CCI-779 (temsirolimus) and RAD001 (everolimus) which have greater clinical tolerability than rapamycin, were developed for clinical use and have been trialed as therapies in both solid organ and hematological cancers with some efficacy (65-68). In CLL, everolimus elicited similar lymphocyte redistribution effects to that of PI3K and BTK inhibitors however overall clinical responses were not as promising (68), potentially due the existence of the negative feedback loop allowing preservation of AKT signaling in the absence of mTORC1 signal (16, 17).

Dual mTOR inhibitors can be divided into two broad categories (Table 1). Firstly, there are small molecules that selectively inhibit both mTORC1 and mTORC2. First-in-class drug AZD8055 and its formulation for clinical use AZD2014 (Vistusertib) is an ATP-competitive inhibitor of mTOR, which selectively blocks phosphorylation of mTOR substrates (69). Dual mTOR inhibitors are ATP-competitive inhibitors and their superior clinical results and depth of mTORC1/2 inhibition was originally attributed to their ability to inhibit mTORC2 however some of their properties may a result of their ability to block rapamycin-resistant components of mTORC1. Upon inhibition of PRAS40 phosphorylation by dual mTOR inhibitors, this protein has enhanced binding of the RAPTOR/mTOR complex with resultant inhibition of the mTORC1/4EBP1 signal (70). Furthermore, there are differential effects upon cyclin D1 and D3 by rapamycin and dual mTOR inhibitors, with a reduction in protein expression of these and an increase in p27 with dual mTOR inhibition (71). Pitfalls with these agents include the existence of alternative activating signals or negative feedback loops. Other types of dual mTOR inhibitor aim to overcome the potential deficiencies of specific inhibitors and inhibit PI3K signals as well as mTOR (Table 1); these have the advantage of inhibiting the three proto-oncogenic kinases PI3K, AKT and mTOR, closing some of the feedback loops. The pan PI3K/mTOR inhibitor SAR245409 (voxtalisib) was demonstrated to inhibit BCR-mediated adhesion to a similar extent as idelalisib, while the PI3Kα inhibitor was less effective, supporting a key role for PI3Kδ in regulating CLL adhesion. SAR245409 was also shown to partially reduce chemokine-mediated migration in CXCL12-transwell migration studies, with a modest but significant reduction in migration (72). The pan PI3K/mTOR inhibitor PF-04691502 exhibited significant inhibition of BCR- and CXCR4-mediated signaling, and potent reduction in migration towards CXCL12 in transwell assays (73). Furthermore, PF-04691502 induced a lymphocytosis in TCL-1 mice, and blocked leukemia development in these mice, similar to that observed in TCL-1 mice treated with ibrutinib and fostamatinib (58, 59, 73). Therefore, pan PI3K/mTOR inhibitors have fared well in pre-clinical tests in CLL and clinical data is awaited. To augment the approach to mTOR inhibition further CC-115, a dual mTOR/DNA-PK inhibitor has shown promise *in vitro* inducing more robust caspase-dependent cell killing, and inhibition of proliferation and BCR-mediated signaling in comparison to specific mTOR inhibitor CC-214 and BTK inhibitor CC-292. CC-115 has progressed further to preliminary clinical testing, with promising early clinical results, with the majority of patients exhibiting decreased lymphadenopathy (74).

Conclusion

Cellular migration is appropriated by neoplastic processes and in CLL there are demonstrable changes in the cellular phenotype which determine cell localization. As a consequence of these changes, cells may be encouraged to proliferate with subsequent clonal expansion and evolution. The molecular mechanisms that mediate the effects on migration are not fully understood however insights may be gained from knowledge of recent advances in CLL therapeutics. The pathway at the center of this review, mTOR signaling, is described and evidence is provided in support of mTOR inhibition as a means of controlling CLL cell migration. Theories as to the suboptimal clinical performance of mTORC1 inhibition include the incomplete block of the pathway or the existence of negative feedback loops. There is a need for *in vitro* study of dual mTORC1/2 inhibition in CLL incorporating all emerging data on

crosstalk between mTOR and other signaling pathways to optimize experimental design. Synergistic studies utilizing PI3K and BTK inhibitors alongside dual mTOR inhibitors may provide a basis for clinical trial designs in the use of combination therapy to overcome drug resistance.

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Compound	Target	Phase	Disease	Reference
AZD2014 (Vistusertib)	mTORC1/2	I-II	AST, GBM, HCC, Lymphomas	(69)
MLN0128 (TAK228)	mTORC1/2	I-II	Prostate, thyroid, breast, liver	Hsieh *et al.*, Nature (2012)
OSI-027	mTORC1/2	I	AST, Lymphomas	Mateo *et al*., Br J Cancer, 2016
CC-223	mTORC1/2	I-II	NSCLC, NHL, MM	Bendell *et al.*, Cancer (2015)
PF-04691502	PI3K-mTORC1/2	I-II	Breast, Endometrial, AST	(73)
VS-5584	PI3K-mTORC1/2	I	Metastatic cancer, lymphoma	Hart *et al.*, Mol Cancer Ther (2013)
SAR245409 (Voxtalisib)	PI3K-mTORC1/2	I-II	AST, GBM, Ovarian, Breast	Awan et al. Br J Haem (2016)
CC-115	mTORC1/2 & DNA-PK	I-II	AST, GBM, AML	(74)

Table 1: Table of dual mTOR inhibitors in current clinical trials

Key: AST = Advanced Solid Tumors, GBM = Glioblastoma Multiforme, HCC= Hepatocellular Carcinoma, AML = Acute Myeloid Leukemia, NSCLC= Non-Small Cell Lung Cancer, NHL = Non-Hodgkin Lymphoma

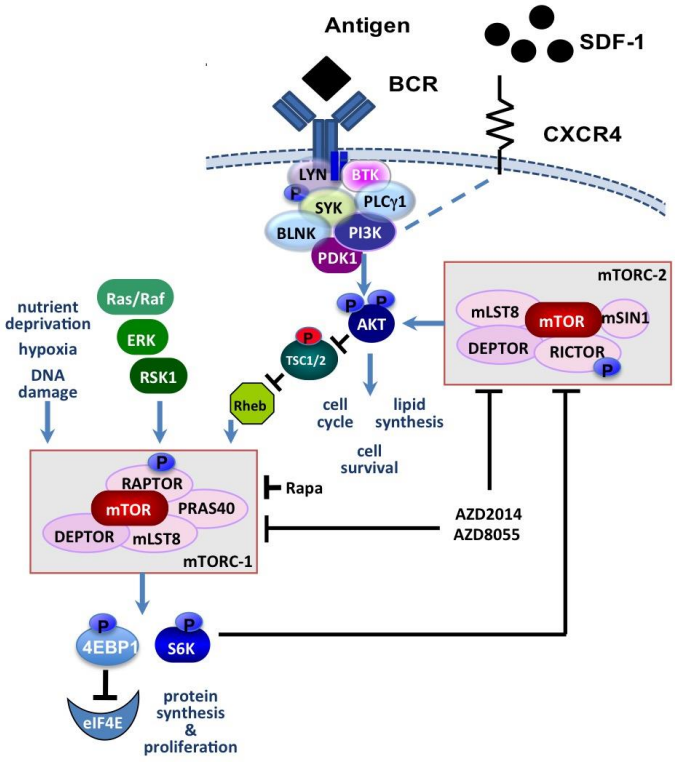

Figure 1: Diagram of B cell receptor signal transduction cascade and mTOR mediated components within normal B cells. The mTOR kinase complex is positioned downstream of the BCR and chemokine (CXCR4-CXCL12) receptors. The mTORC1 and dual mTORC1/2 inhibitors, rapamycin (Rapa) and AZD8055/AZD2014 respectively, are shown with their site of action within the mTOR cascade.