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Targeting pathological B cell receptor signalling in lymphoid malignancies

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Abstract

Signalling through the B cell receptor (BCR) is central to the development and maintenance of B cells. In light of the numerous proliferative and survival pathways activated downstream of the BCR, it comes as no surprise that malignant B cells would co-opt this receptor to promote their own growth and survival. However, direct evidence for BCR signalling in human lymphoma has only come to light recently. Roles for antigen-dependent and antigen-independent, or tonic, BCR signalling have now been described for several different lymphoma subtypes. Furthermore, correlative data implicate antigen-dependent BCR signalling in many other forms of lymphoma. A host of therapeutic agents targeting effectors of the BCR signalling pathway are now in clinical trials and have shown initial success against multiple forms of lymphoma.

Lymphoma is a malignancy of mature lymphocytes that manifests in secondary lymphoid organs or extranodal tissues. In the United States, lymphoma is the sixth most common form of cancer, affecting 22.5 per 100,000 people (see the SEER Cancer Statistics Review 1975–2009). Lymphoma itself is an umbrella term applied to over 30 distinct clinical entities[1] (TABLE 1. Most lymphomas originate from B lymphocytes, and their diversity can often be traced to the developmental stage of a normal precursor B cell, with the bulk being derived from antigen-experienced germinal centre or post-germinal centre B cells. Although the standard of care for each type of lymphoma varies, most regimens use combinations of cytotoxic drugs along with the anti-CD20 monoclonal antibody rituximab. For example, the most common type of lymphoma, diffuse large B cell lymphoma (DLBCL), is treated with rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP). Owing to the inherent heterogeneity of lymphoma, there are very different responses to treatments. Generally, indolent lymphomas such as follicular lymphoma and chronic lymphocytic leukaemia (CLL) have less robust responses to immunochemotherapies than aggressive lymphomas such as Burkitt's lymphoma and DLBCL. However, many patients with aggressive lymphomas do not show a durable response to treatments, necessitating salvage therapies such as autologous stem cell transplant that often have poor patient outcomes. Moreover, many of the first-line immunochemotherapy regimens are too toxic to be tolerated by the elderly or by people in the developing world, where infectious diseases

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are more difficult to manage following treatment. Clearly, novel approaches to lymphoma treatment are needed.

Recent advances spurred by functional and structural genomics have enriched our understanding of the pathogenesis underlying various lymphomas. Half a century after the notion that antigenic stimulation could induce lymphomagenesis was first articulated[2], B cell receptor (BCR) signalling has now emerged as a central oncogenic pathway that promotes growth and survival in various lymphoma types. The cascade of effectors downstream of the BCR includes many kinases that are amenable to therapeutic intervention. Here we discuss how various lymphoma subtypes use qualitatively different modes of BCR signalling that engage different downstream pathways, necessitating a nuanced approach to the therapeutic inhibition of oncogenic BCR signalling. We first review the basics of BCR signalling in normal B cells and highlight how lymphomas adopt and pervert this signalling pathway. We then focus on BCR-pathway-targeting small molecules that are entering clinical trials, which have the potential to transform the standard of care for patients with lymphoid malignancies.

B cell receptor signalling in normal lymphocytes

Every normal B cell, and consequently every lymphoma, has a unique BCR consisting of pairs of immunoglobulin heavy (IgH) and light (IgL) chains. Each IgH and IgL has a unique variable (V) region that allows the BCR to bind to diverse antigens in the extracellular environment. This antibody portion of the BCR is coupled non-covalently with an invariant, disulphide-linked heterodimer of the CD79A and CD79B (Igα and Igβ) subunits, which mediates plasma membrane expression, signal transduction and receptor internalization[3]. Both CD79A and CD79B have similar signalling modules, termed immunoreceptor tyrosine-based activation motifs (ITAMs), each containing two tyrosine residues[4]. Antigen-induced aggregation of the BCR leads to phosphorylation of ITAM tyrosines by the SRC-family kinases, including LYN, FYN and BLK[5]. The tyrosine kinase SYK is then recruited to the dually phosphorylated ITAMs through its tandem SRC homology 2 (SH2) domains, resulting in SYK phosphorylation and activation by SRC-family kinases and by autophosphorylation[6]. The SRC-family kinases and SYK nucleate a signalosome composed of various other kinases and adaptor proteins. SYK recruits a complex of Cbl-interacting protein of 85 kDa (CIN85; also known as SH3KBP1) and B-cell linker protein (BLNK), which in turn coordinates the phosphorylation and activation of Bruton tyrosine kinase (BTK) and phospholipase Cγ2 (PLCγ2)[7]. PLCγ2 then catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$; also known as $PtdIns(4,5)P_2$) into diacylglycerol (DAG) and inositol trisphosphate (IP_3), resulting in increased intracellular calcium levels. The combination of DAG and increased intracellular calcium activates protein kinase Cβ (PKCβ), which in turn phosphorylates many substrates, including caspase recruitment domain-containing protein 11 (CARD11), a key signalling adaptor that coordinates a signalling complex that activates the nuclear factor-κB (NF-κB) pathway[8]. The transmembrane protein CD19 is phosphorylated by the SRC family kinase LYN during BCR signalling, recruiting phosphoinositide 3-kinase (PI3K) to the BCR. PI3K phosphorylates $PI(4,5)P_2$ to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3; also known as $PtdIns(3,4,5)P_3$), which recruits effectors including BTK and AKT to the inner

leaflet of the plasma membrane via interactions with the plextrin homology (PH) domains of these effectors[9]. The net result of proximal BCR signalling is the activation of the NF-κB, PI3K, mitogen-activated protein kinase (MAPK), nuclear factor of activated T cells (NFAT) and RAS pathways, which promote proliferation and survival of normal and malignant B cells[10] (FIG. 1).

The assembly of the IgH and IgL components of the BCR begins with the V(D)J recombination-activating protein 1 (RAG1) and RAG2-mediated recombination of V, D (diversity) and J (joining) gene segments to create a complete V region. The assembled V regions include three hypervariable subregions, termed complementarity-determining regions (CDRs), which have diverse amino acid sequences and hence can recognize diverse antigens. The V regions of the IgH and IgL chains are fused to constant (C) regions. In the case of IgH, the C region belongs to one of several 'classes' — including IgM, IgD, IgG, IgA and IgE — that can be interchanged by a DNA rearrangement process known as class switch recombination. The IgH class influences the signalling output of the BCR (see below).

Non-self foreign antigens induce a productive immune response by engaging both helper T cells and mature B cells, thereby causing the formation of a new microenvironmental niche, known as the germinal centre, which consists of germinal centre B cells, T follicular helper (TFH) cells and follicular dendritic cells (reviewed in REF. 11). B cells within the germinal centre have a distinct developmental phenotype dictated by a set of characteristic transcription factors (reviewed in REF. 12). Whereas naive mature B cells have BCRs with IgM or IgD heavy chains, the germinal centre response triggers IgH class switching to IgG, IgA or IgE. In addition, germinal centre B cells diversify their IgH and IgL V regions by somatic hypermutation (SHM), a specialized mutational mechanism that is catalysed by activation-induced cytidine deaminase (AID). Antigen trapped on follicular dendritic cells is grabbed by the germinal centre B cells and presented to the TFH cells, producing an immunological synapse that activates the B cell. Germinal centre B cells that have mutated their BCRs to bind antigen with high affinity are the evolutionary winners; they are positively selected to differentiate terminally into antibody-secreting plasma cells.

In contrast to the 'active', antigen-dependent form of BCR signalling that initiates the germinal centre response, mature B cells utilize the BCR in a qualitatively distinct fashion known as 'tonic' BCR signalling, a process that is required for B cell survival. Evidence for this comes from experiments in which IgM or CD79A was conditionally inactivated in mature mouse B cells, resulting in a profound reduction of peripheral B cells numbers over several weeks[13,14]. Provision of a truncated CD79A construct lacking an ITAM was unable to rescue the CD79A-deficient B cells, indicating a requirement for signals emanating from the BCR to sustain B cell survival[14]. Such tonic signalling is likely to be antigen-independent as it affects all B cells, regardless of their IgH and IgL V regions, although formal proof of this hypothesis is lacking. Transgenic expression of a constitutively active form of the PI3K catalytic subunit was able to rescue mature B cells following conditional BCR ablation, but constitutively active forms of inhibitor of NF-κB kinase-β (IKKβ), MAPK/extracellular signal-regulated kinase (ERK) kinase 1 (MEK1; also known as MAPKK1) or Ras-related C3 botulinum toxin substrate 1 (RAC1) were ineffective[15].

Although these experiments implicated the PI3K pathways as a key component of tonic BCR signalling, the biochemical basis of PI3K activation is less clear. SYK may have a role in PI3K activation, as it does during strong antigenic stimulation[16], but other evidence suggests that the small GTPase TC21 (also known as R-RAS2) may directly bridge CD79A to PI3K activation in tonic BCR signalling[17]. PI3K also associates directly with non-phosphorylated CD79A, suggesting yet another avenue for tonic BCR signalling through PI3K[18]. The biophysical basis of tonic BCR signalling is still debated. Early studies suggested that before antigen exposure, the BCR of mature B cells is pre-associated with SRC-family kinases as well as protein tyrosine phosphatases such as SHP1 (also known as PTPN6), which constantly extinguish most but not all signalling from the BCR[19]. Recent evidence suggests that the BCRs on a resting B cell do not exist as monomers but rather as auto-inhibited oligomers[20], which might produce a tonic BCR signal.

Several mechanisms exist to prevent B cells from reacting with self antigens, which is a substantial threat because most developing B cells express a BCR that is autoreactive[21]. Exposure of immature B cells in the bone marrow to self antigens triggers an attempt to alter the specificity of the BCR, known as ‘receptor editing’, or results in cell death (reviewed in REF. 22). Autoreactive B cells that escape these ‘central tolerance’ mechanisms can be rendered functionally inactive by encounter with self antigens in the periphery, a process known as ‘anergy’ (reviewed in REF. 23). The hallmarks of anergic B cells are low expression of IgM on the cell surface and reduced responsiveness to further stimulation through the BCR. In humans, perhaps 30% of mature peripheral B cells are anergic[24], and failures in maintaining the anergic phenotype are thought to contribute to the development of human autoimmune diseases, including systemic lupus erythematosis and type 1 diabetes. Continuous exposure to auto-antigens causes abnormally high activation of the SRC-family kinase LYN[25]. Sustained LYN activity attenuates BCR signalling by activating the lipid phosphatase SH2 domain-containing inositol 5′-phosphatase 1 (SHIP1; also known as INPP5D and PIP_3 5-phosphatase 1), which can reduce PI3K activity[26], causing the protein phosphatase SHP1 to be recruited to the inhibitory BCR co-receptors CD22 and CD72, culminating in CD79A and CD79B ITAM dephosphorylation.

BCR signalling in lymphoid malignancies

Most B cell lymphomas maintain BCR expression on the cell surface (reviewed in REF. 27). This is surprising as the enzymes that generate and refine the BCR — AID, RAG1 and RAG2 — also cause chromosomal translocations that disrupt the immunoglobulin loci, placing oncogenes under the control of the powerful immunoglobulin enhancers (reviewed in REF. 28). Despite these genetic assaults on the immunoglobulin loci, lymphomas retain one intact set of IgH and IgL alleles, allowing the lymphoma to form a BCR. These observations suggest that malignant B cells benefit from the proliferation and survival pathways that are triggered by the BCR.

Many B cell lymphomas utilize IgM constant regions to form their BCR despite the fact that most of these lymphomas are derived from germinal centre cells that typically switch their BCRs from IgM to IgG. This observation may be explained by the fact that IgM and IgG BCRs produce qualitatively distinct signalling outputs: IgM-BCR signalling promotes the

survival and proliferation of B cells by activating many pathways, including NF-κB, whereas IgG-BCR signalling favours plasmacytic differentiation through the activation of ERK and MAPK pathways[29-31].

The selective pressure to retain IgM-BCR expression is exemplified by follicular lymphoma (reviewed in REF. 32). Follicular lymphoma is characterized by a t(14;18) translocation that places BCL-2 expression under control of the IgH locus enhancers, destroying expression of immunoglobulin from the translocated allele in the process. In most B cells, one IgH allele is termed ‘productive’ because it encodes an in-frame V-D-J region, whereas the other is termed ‘non-productive’ because its V-D-J region is out of frame and cannot make a full-length IgH chain. In follicular lymphoma, the productive allele is never translocated to BCL-2 and remains as IgM, but the non-productive allele is translocated and undergoes class switch recombination to IgG[33], demonstrating the selective pressure to maintain surface IgM expression in follicular lymphoma.

A second example is provided by DLBCL, an aggressive lymphoma diagnostic category that is comprised of two major molecular subtypes: germinal centre B cell-like (GCB) DLBCL and activated B cell-like (ABC) DLBCL[34]. GCB DLBCLs typically express an IgG-BCR[35] and do not require BCR signalling for survival[36]. By contrast, ABC DLBCLs rely on BCR signalling (see below) and retain expression of an IgM-BCR[36] as a consequence of genetic deletions within the IgH ‘switch’ regions (Sμ and Sγ), which are necessary for class switch recombination[35]. These deletions in the switch regions occur selectively on the productive IgH allele, blocking class switch recombination, whereas the non-productive allele lacks these mutations and can undergo class switch recombination[37].

Chronic active BCR signalling

Genomic investigations of ABC DLBCL have provided clear genetic evidence that BCR signalling is central to the pathogenesis of human lymphoma[36,38,39]. Early studies noted that ABC DLBCL tumours rely on the anti-apoptotic NF-κB pathway for survival, unlike GCB DLBCLs, but the mechanisms responsible for this constitutive NF-κB pathway activation were unclear[40]. Evidence that BCR signalling might play a part in this constitutive NF-κB activity came from an RNA interference screen, which revealed that the CBM (CARD11–BCL-10–mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)) complex is required for NF-κB activity and survival in ABC DLBCL[38]. In normal B cells, the CBM complex is an essential signalling intermediate between the BCR and IKK, the central regulatory kinase of the NF-κB pathway (reviewed in REF. 41). In ~10% of ABC DLBCL cases, activation of the CBM complex could be traced to mutant isoforms of CARD11 (REF. 39). These tumours acquire somatic mutations affecting the coiled-coil domain of CARD11, causing it to form spontaneous aggregates that recruit all downstream signalling components of the NF-κB pathway.

However, in most ABC tumours CARD11 is not mutated, but the tumour cells still require CARD11 for survival[38]. In these cases, RNA interference screening revealed that BTK is essential for NF-κB activation and cell survival[36]. In normal B cells, BTK is activated by BCR stimulation and is required for downstream NF-κB pathway engagement[42,43], raising

the possibility that its activity in ABC DLBCL was caused by BCR signalling. Indeed, these ABC DLBCLs were killed by knockdown of any BCR component (IgH, Igκ, CD79A or CD79B) or proximal BCR signalling effectors (SYK, BLNK, PLCγ2, PI3Kδ or PKCβ)[36]. Genetic or pharmacologic disruption of BCR signalling in ABC DLBCL cell lines abrogated multiple downstream effectors of BCR signalling, including NF-κB, AKT, ERK and NFAT signalling, underscoring the potent consequences of BCR signalling on cellular growth and survival pathways[36,44]. Total internal reflection fluorescence (TIRF) microscopy showed that the BCRs in ABC DLBCL are organized in clusters on the cell surface, which is reminiscent of BCR clusters that form on normal B cells when they are exposed to antigen[36,45]. Phosphotyrosine was localized underneath these BCR clusters, suggesting that they are the sites of constitutive BCR signalling.

By several criteria, the BCR signalling in ABC DLBCL resembles active rather than tonic BCR signalling. First, the NF-κB dependence of ABC DLBCLs is not a feature of tonic BCR signalling, which instead relies on PI3K signalling[15]. Second, the CBM complex is required in ABC DLBCL but is not essential for tonic BCR signalling, as knockout of CBM components does not affect the survival of mouse follicular B cells (reviewed in REF. 46). Third, the BCR clusters in ABC DLBCL are present in antigen-stimulated B cells but not in naive B cells, even though they rely on tonic BCR signalling for survival. For these reasons, the term ‘chronic active BCR signalling’ was used to describe the constitutive BCR activity in ABC DLBCL[36] (FIG. 2).

Mutations affecting the ITAM motifs of CD79A and CD79B in ABC DLBCL establish a genetic basis for chronic active BCR signalling. Over 20% of ABC DLBCL tumours have ITAM mutations, most of which change the first amino-terminal ITAM tyrosine of CD79B to another residue[36]. Mutations affecting other ITAM residues of CD79B are less common, and mutations in the CD79A ITAM are rarer still. By contrast, CD79 mutations are rare or absent in other lymphoma subtypes, highlighting the importance of BCR signalling in ABC DLBCL.

CD79A and CD79B mutations do not initiate BCR clustering or chronic active signalling in ABC DLBCL, but rather augment chronic active BCR signalling in two ways[36]. First, the mutations prevent BCR endocytosis, leading to increased expression of the BCR on the cell surface. Second, the CD79B mutants blunt the activity of LYN, a SRC-family tyrosine kinase that delivers negative feedback signals that attenuate BCR activity (see above). Indeed, LYN knockout mice develop a severe autoimmune disease stemming from aberrant BCR-dependent activation of B cells[47].

The role of antigen in lymphomas

Many lymphomas may sustain BCR signalling by responding to foreign or self antigens in the tumour microenvironment. A foreign antigen has clearly been implicated in splenic marginal zone lymphoma (SMZL) arising in patients with hepatitis C virus (HCV) infection[48]. Treatment of these patients with type I interferon clears HCV and eradicates the lymphoma in over 75% of cases[49]. In one case, the BCR from HCV-associated lymphoma

could directly bind the HCV-E2 envelope glycoprotein[50], demonstrating a potential role for antigen-dependent BCR signalling in this case.

However, in most lymphomas, evidence favours BCR reactivity to a self antigen. In ABC DLBCL, the presence of BCR clusters on the cell surface raises the possibility that the BCR is engaged by a self antigen[51]. If this cancer indeed derives from autoreactive B cells, the presence of CD79A and/or CD79B mutations may aid and abet malignant transformation. As described above, normal B cells with a low avidity for a self antigen persist as anergic B cells, and high LYN kinase activity characterizes this state[23]. Conceivably, the CD79A and CD79B mutations in ABC DLBCL may quell LYN activity in such anergic cells, redirecting BCR signalling towards BTK activation and NF-κB engagement, thereby breaking the anergic state. In epidemiological studies, several systemic autoimmune diseases are risk factors for the development of DLBCL, supporting the hypothesis that ABC DLBCL is a malignancy that may utilize some of the same pathological mechanisms as the autoreactive B cells in these diseases[52].

Strong circumstantial evidence for antigenic signalling in CLL comes from immunoglobulin V region analysis. Two main forms of CLL have been discerned: an indolent subtype with highly mutated V regions in the CLL BCR (M-CLL) and an aggressive subtype with few or no BCR mutations (U-CLL)[53,54]. ZAP70, a paralogue of SYK that can augment BCR signalling, is expressed in U-CLL but not M-CLL[55,56]. Of the ~65 functional human IgH V gene segments, only a handful are found in CLL (for example, variable heavy (VH)1-69, VH3-21, VH3-07 and VH4-34)[57,58]. In one-third of CLL cases, including both U-CLL and M-CLL, the malignant cells express ‘stereotyped’ BCRs in which the V regions from unrelated patients are nearly identical[58]. Stereotypical BCRs converge on these identical sequences despite the V region diversification that occurs during V(D)J recombination and during SHM.

The limited repertoire of V regions in CLL and the selection of stereotyped BCRs imply a role for antigen, and many candidates have been proposed. Both U-CLL and M-CLL seem to be derived from normal B cells with BCRs that react weakly with diverse self antigens[59] and are often reactive with antigens that are exposed on apoptotic cells[60,61]. It is likely that CLL cells encounter these self antigens at high concentration when they reside in the lymph node microenvironment, as it is at this site, rather than in the peripheral blood, that BCR signalling to NF-κB is detectable in the malignant clone[62]. A recent study proposed a novel form of autoreactivity in CLL, in which the BCR reacts with an invariant epitope on its own V region located in the framework 2 region[63]. This reactivity caused all CLL-derived BCRs tested to spontaneously signal when introduced into BCR-deficient mouse B cells, a property not shared by BCRs derived from other lymphoid malignancies or from normal B cells. It is not yet clear whether the same CLL-derived BCRs that recognize their own framework 2 region can also recognize other auto-antigens, but these two reactivities are not mutually exclusive. It may be the case that the malignant B cell clone relies on BCR recognition of its own framework 2 regions to sustain itself initially, but that BCR recognition of more classical self antigens, which may help to break tolerance by integrating additional signalling in the malignant B cell, is required to develop CLL.

An unusual form of BCR signalling may occur in follicular lymphoma owing to the frequent introduction of *N*-linked glycosylation acceptor sites in the V region by SHM[64] Consequently, these BCRs are modified by high-mannose oligosaccharides[65], leading to the proposal that mannosylated BCRs can interact with mannose-binding lectins present on stromal cells in the tumour microenvironment, thereby crosslinking the BCR and initiating BCR signalling[66]. Consistent with this, analysis of somatic mutations in the IgH V regions of follicular lymphoma reveals a selection against deleterious mutations in the conserved framework region, suggesting a pressure to maintain a structurally intact BCR on the cell surface[67]. More recently, one-quarter of follicular lymphoma BCR clones were shown to have some autoreactivity despite ongoing SHM[68]. However, BCR signalling in follicular lymphoma appears to be more complicated, as malignant subclones exist that cannot not be activated through the BCR upon receptor crosslinking[69]. Phosphatase inhibition with hydrogen peroxide could reverse this deficit, suggesting an active suppression mechanism in the tumour subclone. Future work will be needed to determine whether this phenotype is a form of B cell anergy or whether this subclone represents a different stage of B cell differentiation, as it also has low CD20 expression[69]. Importantly, follicular lymphoma tumours with a high proportion of these BCR-inhibited cells were associated with a shorter overall survival[69]. It will be interesting to investigate whether this subpopulation of BCR-inhibited cells emerges as a mechanism of resistance in patients treated with BCR pathway inhibitors.

Tonic BCR signalling in lymphoma

As tonic BCR signalling to the PI3K pathway is required to maintain the viability of all peripheral B cells in the mouse[13-15], it would seem plausible that lymphoid malignancies would take advantage of this mechanism. The clearest example is Burkitt's lymphoma, an aggressive malignancy derived from germinal centre B cells[70]. MYC translocations to the heavy chain locus are a hallmark of Burkitt's lymphoma. However, conditional activation of MYC in mouse germinal centre B cells is insufficient to cause Burkitt's lymphoma, suggesting that cooperating oncogenic mechanisms are required[71]. Despite their derivation from the germinal centre, where most B cells undergo class switch recombination, Burkitt's lymphoma tumour cells almost invariably express IgM-BCRs[72], suggesting selection against class switch recombination in Burkitt's lymphoma and a possible role for signalling from the IgM-BCR. Consistent with this hypothesis, most Burkitt's lymphoma cell lines die upon knockdown of the BCR component CD79A and the associated kinase SYK[70]. However, the survival of BCR-dependent Burkitt's lymphoma lines does not depend on CARD11 or BTK, demonstrating that the BCR signal in Burkitt's lymphoma is qualitatively distinct from chronic active BCR signalling in ABC DLBCL (FIG. 2). BCR signalling in Burkitt's lymphoma activates the PI3K pathway, as detected by phosphorylation of AKT and p70 S6 kinase[70]. Moreover, pharmacologic inhibition of PI3K or treatment with the mammalian target of rapamycin complex 1 (mTORC1) inhibitor rapamycin kills Burkitt's lymphoma cell lines[70]. Thus, tonic BCR signalling to PI3K may provide the necessary survival signals to all Burkitt's lymphoma cells to allow them to tolerate ectopic MYC expression, which is lethal to many primary cells in the absence of growth factors[73]. Consistent with this

hypothesis, conditional activation of PI3K along with MYC in mouse germinal centre B cells causes lymphomas that phenocopy human Burkitt's lymphoma[71].

A genetic basis for tonic BCR signalling in Burkitt's lymphoma was revealed by genomic resequencing of human Burkitt's lymphoma tumours, which uncovered many recurrent somatic mutations that distinguish Burkitt's lymphoma from other lymphoma subtypes[70]. In particular, ~70% of Burkitt's lymphoma cases harbour mutations affecting the transcription factor TCF3 (also known as E2A) or its negative regulator inhibitor of DNA binding 3 (ID3). Most of these mutations foster TCF3 transcriptional activity by preventing ID3 from inhibiting TCF3. TCF3 was originally identified and cloned as a factor required for expression of all immunoglobulin genes and TCF3 has been shown to increase expression of the BCR in Burkitt's lymphoma[70]. Moreover, TCF3 represses expression of the phosphatase SHP1, a potent negative regulator of BCR signalling. Hence, TCF3 augments BCR signalling in two distinct ways and, accordingly, knockdown of TCF3 decreased PI3K activity in Burkitt's lymphoma cell lines and was lethal.

A role for tonic BCR signalling has been postulated in GCB DLBCL based on the sensitivity of certain cell lines of this lymphoma subtype to R406, a small molecule inhibitor of SYK[74]. However, R406 targets several other kinases besides SYK[75,76] and thus is not a perfect tool for probing tonic BCR signalling. Genetic knockdown of BCR components (IgM, Igκ, CD79A or CD79B) did not kill various GCB DLBCL cell lines[36], suggesting that if SYK is indeed essential in GCB DLBCL, other receptors may have a role in its activation (reviewed in REF. 77).

BCR pathway inhibitors

As described above, many lymphoma subtypes subvert BCR signalling to their malignant purpose, suggesting that pharmacological inhibition of this pathway holds promise in these cancers. However, correct deployment of these inhibitors will require a careful understanding of the type of BCR signalling that is used in each lymphoma subtype (TABLE 2). For example, chronic active BCR signalling in ABC DLBCL engages the SRC-family kinases SYK and BTK to activate downstream NF-κB and PI3K pro-survival pathways. Although less certain, the same pathways are likely to be triggered in other lymphomas in which a foreign or self antigen stimulates the BCR. By contrast, lymphomas that rely upon tonic BCR signalling, such as Burkitt's lymphoma, depend upon SRC-family kinases and SYK to activate the PI3K pathway, but BTK and the NF-κB pathway are dispensable.

As with any targeted agent in cancer, a crucial goal is to identify molecular predictors of therapeutic response so that the right drug can be chosen for each patient. In this regard, an important conceptual distinction exists between the development of kinase inhibitors in epithelial cancers and BCR signalling inhibitors in lymphoma. Whereas the addiction of epithelial tumours to a signalling pathway stems from oncogenic gain-of-function mutations in the pathway, such as in the epidermal growth factor receptor or B-RAF, the addiction of lymphomas to BCR signalling may not be directly related to oncogenic mutations but rather a consequence of BCR engagement by an antigen. Indeed, although CLL appears to be responsive to BCR pathway inhibitors (see below), genomic resequencing has failed to

identify genetic aberrations that activate the BCR pathway[78-80]. Likewise, although CD79A and CD79B mutations are likely to identify ABC DLBCL tumours with chronic active BCR signalling, some ABC DLBCL cell lines with chronic active BCR signalling have wild-type CD79A and CD79B[36].

SYK inhibitors

SYK is a non-receptor tyrosine kinase that is recruited and activated by phosphorylated ITAMs following BCR engagement (reviewed in REF. 81). As such, SYK is crucial to antigen-dependent BCR signalling, which includes chronic active BCR signalling in ABC DLBCL. Genetically, SYK is required for tonic BCR signalling in Burkitt's lymphoma, but the role of SYK kinase activity in this setting has not been addressed. SYK has additional diverse roles in several haematopoietic lineages that can be traced to its involvement in integrin and selectin signalling and in certain innate immune receptors that sense fungal pathogens[81]. Thus, SYK inhibitors may have on-target side effects outside the B cell lineage that may or may not limit their clinical utility.

Rigel Pharmaceuticals and AstraZeneca developed fostamatinib (R788), the first SYK inhibitor to enter clinical trials for lymphoma and certain autoimmune and inflammatory disorders. Fostamatinib is an orally available pro-drug version of the active R406 compound, which is an ATP-competitive inhibitor. *In vitro* kinase assays demonstrated that R406 is only somewhat selective for SYK, and can inhibit Fms-like tyrosine kinase 3 (FLT3), KIT, lymphocyte cell-specific protein-tyrosine kinase (LCK), Janus kinase 1 (JAK1), JAK3, RET and the adenosine A3 receptor with similar potencies[75,76]. Constitutive phosphorylation of SYK on Y352 was observed in CLL cells, which is consistent with BCR signalling. R406 treatment induced apoptosis of CLL cells[82] and also antagonized the beneficial influence of stromal cells on CLL viability[83]. Likewise, R406 is toxic to ABC DLBCL cell lines with chronic active BCR signalling[36]. *In vitro* studies determined that R406 could induce apoptosis in certain GCB DLBCL cell lines[74], although as noted above, GCB DLBCL lines do not appear to rely upon BCR signalling.

In preclinical studies of mouse lymphoma models with antigen-dependent BCR signalling, fostamatinib prolonged survival[84]. Likewise, fostamatinib inhibited lymphoma growth in the Eμ-TCL1 model, which recapitulates certain aspects of CLL, including the use of stereotyped BCRs and BCRs that recognize antigens from dying cells[85].

Fostamatinib has completed Phase I and Phase II clinical trials examining its toxicity and efficacy in the context of autoimmune diseases and haematological malignancies. A Phase I/II clinical trial of fostamatinib in relapsed or refractory non-Hodgkin's lymphoma of various subtypes indicated significant clinical activity[86]. Common toxicities included diarrhoea, fatigue, leucopoenia, neutropoenia and anaemia. The study reported objective response rates of 55% in CLL, 24% in DLBCL, 11% in mantle cell lymphoma (MCL) and 10% in follicular lymphoma. It was noted in this study that all patients with CLL experienced an increase in circulating lymphocytes following initial treatment, a phenomenon that has also been noted in patients with CLL who were treated with other BCR pathway inhibitors[87]. The mutational status of the patients with CLL was not discussed

in this study, but it would be of interest to know whether fostamatinib was selectively toxic to either U-CLL or M-CLL. The DLBCL tumours were not classified into ABC and GCB subtypes, so it is not clear whether fostamatinib was inhibiting chronic active BCR signalling in ABC DLBCL or exerting some other antitumour effect.

A second SYK inhibitor from Portola Pharmaceuticals, termed PRT062607, is being developed collaboratively with Biogen IDEC and is currently in Phase I trials. Initial trials will target autoimmune and inflammatory diseases based on preclinical activity in a mouse collagen antibody-induced arthritis model[88]. PRT062607 also has *in vitro* anti-proliferative activity against DLBCL cell lines and can inhibit BCR signalling[89].

BTK inhibitors

BTK is a member of the TEC family of non-receptor kinases and contains an N-terminal PH domain followed by a proline-rich domain, an SH3 domain, an SH2 domain and a carboxy-terminal kinase domain. Inactivating mutations in *BTK* are the cause of X-linked agammaglobulinaemia (XLA)[90], in which B cell development and antibody production is severely attenuated, rendering these patients susceptible to infection. Similar mutations in the mouse *Btk* gene cause X-linked immunodeficiency (XID)[91], which is characterized by a partial block in B cell development, and the same phenotype occurs with a full *Btk* knockout[92,93]. Outside the B cell lineage, BTK deficiency has minimal consequences, suggesting that BTK inhibitors should have an excellent safety profile.

BTK is essential for antigen-stimulated BCR signalling. BTK is recruited to the BCR signalosome through two mechanisms. First, the BTK PH domain recognizes the lipid PIP_3, which is produced by PI3K following BCR crosslinking, causing BTK to interact with the inner leaflet of the plasma membrane. Second, SYK phosphorylation of the adaptor BLNK causes BTK recruitment via the SH2 domain of BTK (reviewed in REF. 94). Once in the BCR signalosome, SRC-family kinases phosphorylate BTK on Y551 in its activation loop[95,96], which in turn stimulates autophosphorylation of Y223 for full activation of BTK[97]. Active BTK phosphorylates and activates PLCγ2 (REFS 98,99), activating many downstream pathways, including NF-κB (reviewed in REF. 97). Thus, therapeutic targeting of BTK is rational in ABC DLBCL and other lymphoid malignancies that use an antigen-dependent chronic active form of BCR signalling. BTK inhibitors are likely to have little effect on tonic signalling, which relies on PI3K signalling, not NF-κB signalling. Indeed, the tonic BCR signalling in Burkitt's lymphoma cell lines is not inhibited by genetic or pharmacologic inactivation of BTK (R.M.Y. and L.M.S., unpublished results).

Several kinase inhibitors capable of blocking BTK have recently entered clinical trials in lymphoid malignancies. Dasatinib (BMS-354825; Spycell) is a multi-kinase inhibitor used to target the BCR-ABL fusion protein produced by the t(9;22) translocation in chronic myelogenous leukaemia. Dasatinib also efficiently targets SRC-family kinases and BTK[100]. Treatment of ABC DLBCL lines with dasatinib blocked chronic active BCR signalling and induced cell death[36]. Although promising, trials of dasatinib in DLBCL have not been reported. A Phase II trial of dasatinib in CLL yielded a 20% objective response rate[101].

A more selective and potent BTK inhibitor, ibrutinib (PCI-32765), that covalently modifies the enzyme has recently been developed by Pharmacyclics[102]. Ibrutinib is an irreversible BTK inhibitor because it forms a covalent bond with cysteine 481 near the active site of BTK. Only nine other kinases in the human genome have a similarly placed cysteine residue, lending ibrutinib a high level of specificity[102]. Celgene is developing another BTK inhibitor, AVL-292, that covalently attaches to the same cysteine in BTK as ibrutinib. *In vitro* studies of ibrutinib determined an IC_{50} of 0.5 nM for BTK[102,103]. In cell-based assays, ibrutinib was toxic for ABC DLBCL cell lines at low nanomolar concentrations[36]. Moreover, ibrutinib showed preclinical activity in canine lymphomas[103]. Preclinical studies in mouse models of lymphoma determined that ibrutinib treatment only affected mouse tumours that use chronic active BCR signalling and not tumours dependent on tonic BCR signals[104].

In CLL, ibrutinib decreased DNA synthesis and viability of *ex vivo* cultured primary cells, whether or not supporting stromal cells or cytokines were present[105,106]. In a mouse model using Eμ-TCL1 transgenic CLL-like cells, ibrutinib prevented disease progression[106]. In early phase studies of patients with CLL, ibrutinib decreased plasma expression of the chemokines CLL3 and CLL4 (REF. 106), which is thought to be the consequence of BCR signalling in CLL cells within the lymph node microenvironment[62]. Ibrutinib inhibition of BTK may also block the ability of chemokines within the lymph node microenvironment to induce homing through C-X-C chemokine receptor type 4 (CXCR4) and CXCR5 and adhesion of CLL via integrins[87], potentially explaining why ibrutinib treatment of patients with CLL induces a transient increase of CLL cells in the circulation[107].

Both ibrutinib and AVL-292 are in clinical trials in lymphoid malignancies, but published data only exist for ibrutinib. An initial Phase I study used escalating doses of ibrutinib in a cohort of 47 patients with various forms of relapsed/refractory non-Hodgkin's lymphoma[108]. No cumulative haematological toxicities were noted, and ibrutinib was generally well tolerated with some side effects that included neutropoenia and hypersensitivity reactions. An objective response (complete response (CR) or partial response (PR)) was observed in 20 of the 47 patients, including patients with CLL (9/15), MCL (3/4), follicular lymphoma (4/15), DLBCL (3/8) and marginal zone lymphoma (1/3).

Given the strong preclinical evidence that ibrutinib inhibits chronic active BCR signalling in ABC DLBCL, the Phase I trial was extended at the 560 mg per day dose to include ten patients with relapsed/refractory ABC DLBCL[109]. In this cohort, two patients achieved CR and one achieved PR, and the character of the responses was exceptional. One patient has remained in CR for over 2 years, taking ibrutinib daily as monotherapy without discernable side effects. Another patient with primary refractory disease that had not responded to any chemotherapy regimen had virtually complete resolution of massive abdominal disease.

Based on these results, a Phase II trial is being conducted at 560 mg per day to evaluate the efficacy of ibrutinib in patients with relapsed refractory DLBCL ($n = 70$), with the ABC and GCB subtypes identified by gene expression profiling[110]. Among patients with ABC DLBCL, an objective response rate of 40% was observed, including both complete and partial responses, suggesting that a substantial fraction of ABC DLBCL tumours depend

upon BCR signalling. By contrast, only one partial response was observed among patients with GCB DLBCL (5%), which is consistent with the view that this subtype of DLBCL has minimal dependence on BCR signalling[36]. Sequence analysis was performed for genes in the BCR pathway that are frequently mutated in ABC DLBCL. Responses were more frequent among ABC DLBCLs with a CD79B ITAM mutation (60%), as expected. Importantly, responses were also frequent in patients whose tumours were wild type for CD79B (37%), demonstrating that chronic active BCR signalling in ABC DLBCL may not require oncogenic mutations. CARD11 mutant ABC DLBCL tumours did not respond to ibrutinib, consistent with CARD11 sitting downstream of BTK in the BCR signalling cascade. Together, these results argue for the use of the ABC versus GCB DLBCL distinction as a biomarker of ibrutinib response, with additional predictive value provided by mutational analysis.

As discussed above, abundant circumstantial evidence exists for BCR signalling in the pathogenesis of CLL, and clinical trials with ibrutinib provide strong support for this hypothesis. Phase II trials have administered ibrutinib with a median follow-up of 17.3 months with minimal and acceptable toxicity[107,111]. An objective response rate of 67% was seen in relapsed/refractory disease[107,111]. In elderly, treatment-naive patients for whom standard treatment is too toxic, a response rate of 71% was observed, with 61% PR and 10% CR[111]. Ibrutinib was effective in both the aggressive U-CLL subtype and the indolent M-CLL subtype. Importantly, ibrutinib responses occur in patients with known molecular risk factors, including inactivation of p53 (that is, del(17p)) and ataxia telangiectasia mutated (ATM; that is, del(11q))[107].

Although less evidence exists for BCR signalling in MCL, recent support for this possibility came from V region sequencing, which revealed a restricted repertoire with stereotyped BCRs in some cases[112]. A Phase II trial of ibrutinib in relapsed/refractory patients with MCL at 560 mg per day yielded an objective response rate of 65.1% ($n = 63$) in a bortezomib-naive cohort and 67.4% ($n = 46$) in a cohort of patients previously treated with bortezomib[113]. This striking response rate suggests that further investigation is warranted into the nature of BCR signalling in MCL.

PI3K, AKT and mTOR inhibitors

The PI3K pathway is emerging as a promising therapeutic target in B cell lymphomas that utilize pathogenic chronic active or tonic BCR signalling. PI3K activity provides a direct conduit from diverse stimuli, including cell surface receptors and metabolic changes within the cell, to downstream pathways affecting cellular growth, size, survival and angiogenesis, among others (reviewed in REFS 9,114). It comes as no surprise then that PI3K and its downstream effectors and regulators, such as phosphatase and tensin homologue (PTEN), are frequently deregulated in human cancer[114], and hence the development of PI3K pathway inhibitors has been an active area of research for many years.

PI3K initiates downstream signalling by recruiting signalling effectors to the plasma membrane (reviewed in REF. 115). PI3K is a lipid kinase that catalyses the phosphorylation of the 3-hydroxy position of phosphatidylinositols and phosphoinositides in the inner leaflet

of the plasma membrane, most significantly phosphorylating $PI(4,5)P_2$ to form PIP_3. Conversely, PI3K activity is antagonized by PTEN, which is a PIP_3 3-phosphatase, and by SHIP1, which is a PIP_3 5-phosphatase. 3-phosphorylated phosphoinositides recruit cytosolic proteins containing PH, FYVE (FAB1, YOTB, VAC1 and EEA1) or PX (phox homology) domains to the plasma membrane, where they affect signalling. The PH domain of the kinase AKT mediates AKT recruitment to the plasma membrane in response to BCR signalling, resulting in AKT phosphorylation and activation. AKT in turn interacts with a multitude of signalling effectors that affect growth, proliferation and survival[114]. Prominent among these is mTORC1, a kinase that increases protein and lipid synthesis and energy production (reviewed in REF. 116). Besides the AKT–mTOR pathway, the generation of PIP_3 by PI3K causes BTK to translocate to the plasma membrane, facilitating its activation during BCR signalling.

In vitro studies of lymphoma cell lines and primary cells have revealed PI3K pathway dependence in several subtypes. ABC DLBCL cell lines with chronic active BCR signalling engage PI3K, which augments anti-apoptotic NF-κB signalling and may also provide additional survival signals[36,117]. The tonic BCR signalling that characterizes some Burkitt's lymphoma cell lines activates the PI3K pathway and downstream AKT and mTORC1 signalling[70,71]. In MCL, primary biopsy samples from highly proliferative (blastoid) cases showed AKT phosphorylation in all cases, and PI3K inhibition was toxic to MCL cell lines[118]. Although the molecular mechanisms that activate PI3K in MCL have not been fully elucidated, BCR signalling is plausibly involved given the efficacy of the BTK inhibitor ibrutinib in many cases (see above).

Three classes of PI3K exist, but only class I PI3Ks can phosphorylate $PI(4,5)P_2$ to generate PIP_3, which is perhaps the most important phosphoinositide species produced downstream of BCR signalling[115]. The several class I PI3Ks are composed of a regulatory (p85) subunit and one of four catalytic (p110) subunits to yield PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ. These different PI3K isoforms have both unique and redundant functions in B cells. PI3Kδ has a non-redundant role downstream of active BCR signalling[119], whereas PI3Kα and PI3Kδ are both required for B cell development[120], and a constitutively active form of PI3Kα can substitute for tonic BCR signalling in mice[15]. As discussed above, the mechanisms that engage PI3K during tonic BCR signalling have not been elucidated, and may either involve SYK[16] or TC21 (REF. 17). Following antigen stimulation, BCR-dependent phosphorylation of CD19 and B cell adaptor for PI3K (BCAP; also known as PIK3AP1) recruits PI3Kδ via its SH2 domains, resulting in PI3Kδ activation. In addition to the BCR pathway, PI3Ks — including PI3Kδ — are effectors downstream of other cell surface receptors, including CD40, tumour necrosis factor receptors (TNFRs) and B cell activating factor (BAFF) receptors.

A variety of PI3K pathway inhibitors are available in the clinic, or are now in clinical trials. GS-1101 (CAL-101) from Calistoga Pharmaceuticals/Gilead Sciences is a potent and specific PI3Kδ inhibitor, achieving an IC_{50} of 2.5 nM *in vitro* versus 500–900 nM for PI3Kβ and PI3Kα, respectively[121,122]. GS-1101 induced apoptosis in *ex vivo* CLL cultures from both U-CLL and M-CLL tumours at concentrations approaching 10 μM, but this concentration of drug did not affect normal immune cell survival *ex vivo*[123]. Moreover,

GS-1101 was preferentially toxic to CLL samples with high levels of PI3Kδ. Another study used *ex vivo* co-cultures of CLL cells and stromal cells to mimic the tumour microenvironment more closely[124]. In this setting, GS-1101 inhibited CLL cells blocking both BCR-dependent signalling and chemokine receptor signalling induced by C-X-C chemokine ligand 12 (CXCL12; also known as SDF1) and CXCL13 in the malignant cells. Interestingly, inhibition of stromal-dependent chemokine effects was achieved at a lower drug concentration than inhibition of BCR signalling[124]. GS-1101 also abolishes AKT phosphorylation (S573) in cell line models of DLBCL and in primary MCL cells *ex vivo*[122].

An early Phase I dose escalation study in relapsed/refractory CLL reported that GS-1101 was well tolerated, decreased levels of phospho-AKT in patient tumour cells and reduced lymphadenopathy in all patients (32/32)[125]. Overall, the objective response rate in patients with CLL treated with GS-1101 was 26% at 11 months[125]. The increased levels of CXCL13 in sera from patients with CLL were reduced after one dose of GS-1101, correlating with an increase in peripheral lymphocytes and CLL cells. Thus, GS-1101 may abrogate chemokine signalling that keeps normal and malignant lymphocytes within the lymph node microenvironment. As the lymph node environment is crucial for BCR signalling and survival in CLL, the egress of CLL cells from their protective niche may contribute to the efficacy of GS-1101 (REF. 62).

Another Phase I clinical trial examined the effect of escalating doses of GS-1101 in indolent and aggressive lymphomas[126]. Only partial responses were seen, with rates of 62% (15/24) for indolent non-Hodgkin's lymphoma, 62% (10/16) for MCL and 0% (0/9) for DLBCL. However, the DLBCL cases were not subclassified by their molecular subtype, so it is unclear whether PI3K-dependent ABC DLBCLs were included in this study.

In addition to isoform-specific PI3K inhibitors, several pan-PI3K inhibitors are in clinical trials for solid cancers. One of these, NVP-BKM120 from Novartis, was toxic to most Burkitt's lymphoma cell lines that rely on PI3K activity for their survival[70].

Downstream of PI3K, AKT is an attractive target, and many inhibitors are in development[127]. mTORC1 has been targeted by rapamycin and its analogues, yielding response rates of 20–38% in MCL[128,129] and 30% in relapsed/refractory DLBCL[130]. Given that all Burkitt's lymphoma cell lines were killed by rapamycin treatment *in vitro*, rapamycin analogues should be evaluated in this lymphoma as well. Of course, it should be noted that mTORC1 receives inputs from many signalling and metabolic pathways, so the activation of this kinase may not necessarily be a result of BCR signalling.

Other BCR pathway inhibitors

The initial biochemical step in BCR signalling is phosphorylation of ITAM tyrosines on CD79A and CD79B by SRC-family kinases. Moreover, SRC kinases are crucial to B cell development and have a presumed role in tonic BCR signalling[5]. As such, inhibiting these kinases would be a rational method for inhibiting BCR-dependent signalling in lymphoma. Owing to the functional redundancy of the SRC-family kinases, it is difficult to assess the contribution of individual family members to BCR signalling in lymphoma by genetic

methods. Pharmacological approaches to inhibiting SRC kinases are complicated by off-target effects of many inhibitors. As described above, dasatinib is a multikinase inhibitor that can inhibit SRC kinase activity in the 50–200 nM range in ABC DLBCL lines, but as it is also able to inhibit BTK, it is unclear whether its toxicity for these cells is due to SRC kinase inhibition[36]. A new generation of more specific SRC kinase inhibitors, including SU6656 (Sugen, Inc.), saracatinib (Biovision), KX-01 (Kinex Pharmaceuticals), CGP76030 (Novartis) and bosutinib (Pfizer), is currently entering clinical trials in solid tumours. These new inhibitors may prove effective in lymphomas that rely on either chronic active or tonic BCR signalling, and they may be useful compounds for dissecting the biochemical details of BCR signalling in these malignancies.

PKCβ functions downstream of BTK and upstream of IKK and is therefore another potential therapeutic target in lymphomas with BCR signalling. PKCβ is a classical isoform of PKC that is activated by the products of PLCγ2 activity, DAG and increased intracellular calcium[10]. Activated PKCβ phosphorylates CARD11 to promote NF-κB activation[8]. Knockdown of PKCβ is toxic to ABC DLBCL cell lines that rely on chronic active signalling[36], as is treatment with either pan-PKC inhibitors or PKCβ-specific inhibitors[44]. PKCβ may also have a role in CLL, as Eμ-TCL1 mice failed to develop a CLL-like disease when crossed with PKCβ-deficient mice[131]. The PKC inhibitor enzastaurin is in clinical development for lymphoma[132,133], but enzastaurin did not selectively kill DLBCL cells that are dependent on BCR signalling[44]. However, a PKCβ-specific inhibitor from Novartis, sotrastaurin (AEB071), was selectively toxic to ABC DLBCL cell lines that rely on chronic active BCR signalling[44], prompting Phase I trial evaluation in ABC DLBCL.

The CBM complex is required to recruit and activate IKKβ during antigen receptor signalling in normal and malignant lymphocytes. Recently, a catalytic function for MALT1 has emerged in which its caspase-like domain functions as a protease to cleave various protein substrates after arginine residues[134-136]. One substrate of MALT1 is A20, a negative regulator of NF-κB signalling, and another is RELB, an NF-κB subunit that is specific for the non-canonical NF-κB signalling cascade downstream of NF-κB-inducing kinase (NIK; also known as MAP3K14) and IKKα. As such, MALT1 catalytic activity preferentially activates the classical NF-κB pathway that is the consequence of IKKβ activity. As this limb of the NF-κB pathway is required for ABC DLBCL survival[40,137], MALT1 inhibition is selectively toxic for these lymphoma cells[138]. These considerations support the identification and development of MALT1 inhibitors. As there are no other proteins encoded in the human genome with domains that resemble the MALT1 paracaspase domain, MALT1 inhibitors could have a good safety profile.

Finally, direct inhibition of IKKβ, which is activated by BCR signalling and is required for survival in many lymphoma subtypes, could be considered. Given the essential role of IKKβ and NF-κB in normal immune cell function and in a host of other stress responses, the development of drugs targeting this kinase has proceeded slowly (reviewed in REF. 139). Although long-term administration of IKKβ inhibitors in chronic autoimmune and inflammatory diseases might be problematic, their short-term use in cancer might be manageable. Given the ability of NF-κB to block the cytotoxic activity of conventional

chemotherapeutic agents[140], IKKβ inhibitors might be useful in combination with these drugs.

Combination therapies

Other oncogenic pathways in lymphoma might in some cases potentiate BCR signalling but could alternatively provide redundant proliferation and/or survival signals that might provide mechanisms of acquired resistance to BCR-directed therapies. A recent instructive example of these principles is oncogenic myeloid differentiation primary response protein 88 (MYD88) signalling in ABC DLBCL[141]. MYD88, a key adaptor protein in Toll receptor and interleukin-1 receptor signalling, is altered by gain-of-function somatic mutations in 39% of ABC DLBCL tumours. One highly recurrent mutation, L265P, accounts for 29% of cases. Notably, this same mutant has been identified at varying frequencies in CLL[78-80], primary cutaneous DLBCL[142], primary central nervous system lymphoma[143], marginal zone lymphomas[141,144,145] and Waldenstrom's macroglobulinaemia[146]. In a similar way to BCR signalling, MYD88 signalling in ABC DLBCL activates NF-κB, but MYD88 uses a different pathway to IKKβ involving the kinases interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4. From this perspective, oncogenic MYD88 mutations might make ABC DLBCL cells resistant to BCR pathway inhibitors by activating NF-κB in a parallel, redundant fashion. However, it is known that BCR and MYD88 signalling pathways can cooperate in normal and autoimmune B cells[147,148]. Of particular interest therefore was the significant co-occurrence in ABC DLBCL tumours of MYD88 L265P and CD79B mutations[141]. This genetic evidence suggests that the MYD88 pathway might in some way aid and abet BCR signalling. Consistent with this hypothesis, ABC DLBCL cell lines with MYD88 L265P are not resistant to inhibition of the BCR pathway[141].

Ongoing experience with targeted agents in cancer teaches that single drugs are rarely effective in achieving long-term remission owing to existing or acquired compensatory pathways. From this perspective, cures in cancer will only come from the rational combination of targeted agents, informed by a deep knowledge of signalling pathways and their interconnectedness. For example, simultaneous inhibition of the BCR and MYD88 pathways by RNA interference was more toxic than inhibition of either pathway alone, probably due to the fact that both pathways activate pro-survival NF-κB signalling. Hence, a rational therapeutic cocktail might combine a BCR pathway inhibitor with an IRAK4 inhibitor, given the essential role of IRAK4 activity in MYD88 signalling[141].

An emerging and powerful concept in cancer therapy is synthetic lethality, which describes the situation in which oncogenic mutations render cancer cells exquisitely sensitive to drugs that have little if any effect on normal cells that lack these mutations. In some instances, synthetic lethality occurs when the oncogene creates a gain-of-function signalling capability that can be exploited therapeutically. In others, a cancer mutation may remove one of two redundant pathways, making the cancer cell particularly vulnerable to drugs that attack the remaining pathway.

A surprising example of synthetic lethality was recently uncovered in ABC DLBCL with the MYD88 L265P mutation, leading to the demonstration that these cancer cells can be

efficiently killed by combining BCR pathway inhibitors with lenalidomide, a cancer drug of relatively obscure function[149]. Lenalidomide has been approved for use in multiple myeloma and myelodysplatic syndrome and has recently been reported to produce complete and partial remissions in relapsed/refractory ABC DLBCL[150]. Unexpectedly, treatment of ABC DLBCL cell lines with lenalidomide induced secretion of interferon-β and the activation of the type I interferon response. The cytotoxic effect of lenalidomide on ABC DLBCL cells was traced to this interferon-β production, and MYD88 L265P was required for this dysregulated interferon response. In other words, lenalidomide was synthetically lethal for ABC DLBCL cells owing to their oncogenic MYD88 L265P mutations. The transcription factor interferon regulatory factor 4 (IRF4) is key to this dysregulated interferon response, as it directly represses the transcription of *IRF7*, which encodes a key positive regulator of interferon-β expression. By an as yet unclear mechanism, lenalidomide causes IRF4 mRNA and protein levels to drop, thereby relieving the IRF4 blockade of interferon-β expression. IRF4 is regulated in a separate fashion by BCR signalling, as it is a direct target of the NF-κB transcription factors[137,151]. Consequently, simultaneous treatment of ABC DLBCL cell lines with ibrutinib, to inhibit BCR signalling, and lenalidomide caused the virtual disappearance of IRF4, a dramatic induction of interferon-β secretion and synergistic killing *in vitro* and *in vivo* in xenograft models[149]. These results support clinical trials testing ibrutinib plus lenalidomide in ABC DLBCL, anticipating that tumours with MYD88 L265P and chronic active BCR signalling will be the most sensitive to this combination.

Conclusions

The definition of pathological BCR signalling in lymphoma has led to the rapid development of BCR pathway inhibitors for this indication. Thus far, these therapeutic agents have had remarkable success in treating patients with cancers that were in many cases resistant to conventional chemotherapeutic agents. Moreover, the side effect profile of BCR-targeted therapies appears to be easily manageable, although it remains to be seen what long-term toxicities might emerge if patients with lymphoma need to be maintained on BCR pathway inhibitors continuously.

Two paradigms of BCR signalling in lymphoma have emerged — chronic active BCR signalling and tonic BCR signalling — and each signalling pathway offers different opportunities for therapeutic intervention. An important goal for the future will be to learn how to identify lymphomas that depend on BCR signalling and to classify them according to the type of BCR signalling that they use. It is unlikely that the analysis of oncogenic mutations will be sufficient. For example, ABC DLBCL tumours that have CD79B mutations are responsive to the BTK inhibitor ibrutinib, but so are some ABC DLBCL tumours with no mutations in the BCR[110]. Likewise, ibrutinib has high activity in CLL, but no mutations that clearly activate BCR signalling have been identified in genomic resequencing studies. Surrogate markers of BCR signalling will be quite important, therefore, which might take the form of a gene expression signature of BCR signalling[62], an analysis of phosphoproteins that are produced by BCR signalling[69], or perhaps analysis of secreted cytokines[107]. Genetic analysis will still be important, however, as some oncogenic mutations make lymphomas resistant to BCR pathway inhibitors. For example, ~10% of patients with ABC DLBCL have oncogenic mutations in CARD11 that obviate the need for

chronic BCR signalling, rendering the lymphoma cells resistant to ibrutinib[39]. Phase II trials with deep and sophisticated correlative science will be needed to optimize the promise of BCR pathway inhibitors in lymphoma.

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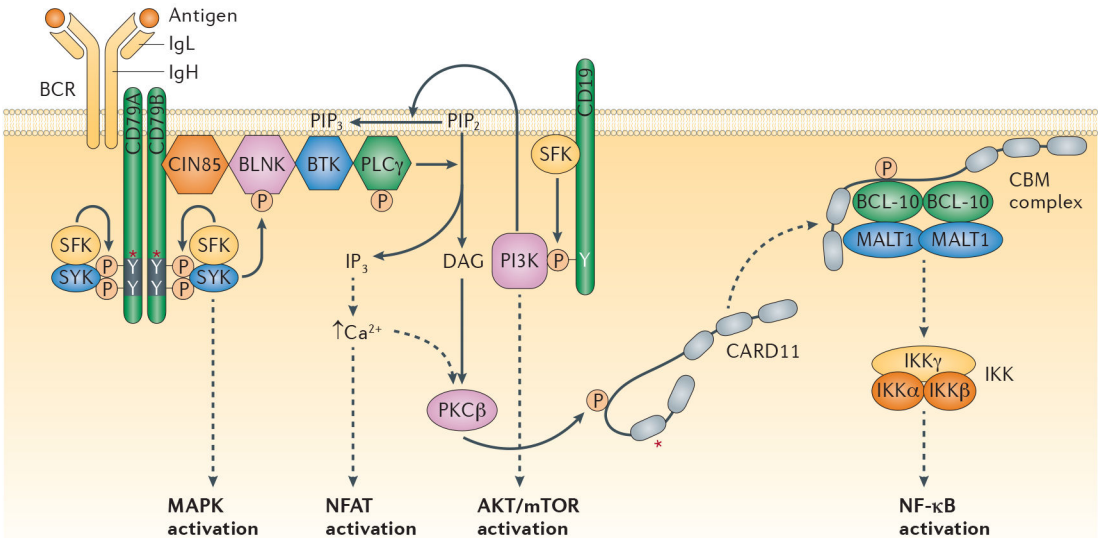

Figure 1|. B cell receptor signalling basics.

Shown are the B cell receptor (BCR), the co-receptor CD19 and various signalling intermediates that are engaged following binding of the BCR to antigen. Several downstream pathways are ultimately triggered, as indicated. The asterisk indicates protein regions affected by recurrent somatic alterations in human lymphomas. See main text for details. BLNK, B-cell linker protein; BTK, Bruton tyrosine kinase; CARD11, caspase recruitment domain-containing protein 11; CBM, CARD11–BCL-10–MALT1; CIN85, Cbl-interacting protein of 85 kDa; DAG, diacylglycerol; IKK, inhibitor of NF-κB kinase; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; IP_3, inositol trisphosphate; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T cells; PI3K, phosphoinositide 3-kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PKCβ, protein kinase Cβ; PLCγ, phospholipase Cγ; SFK, SRC family kinase.

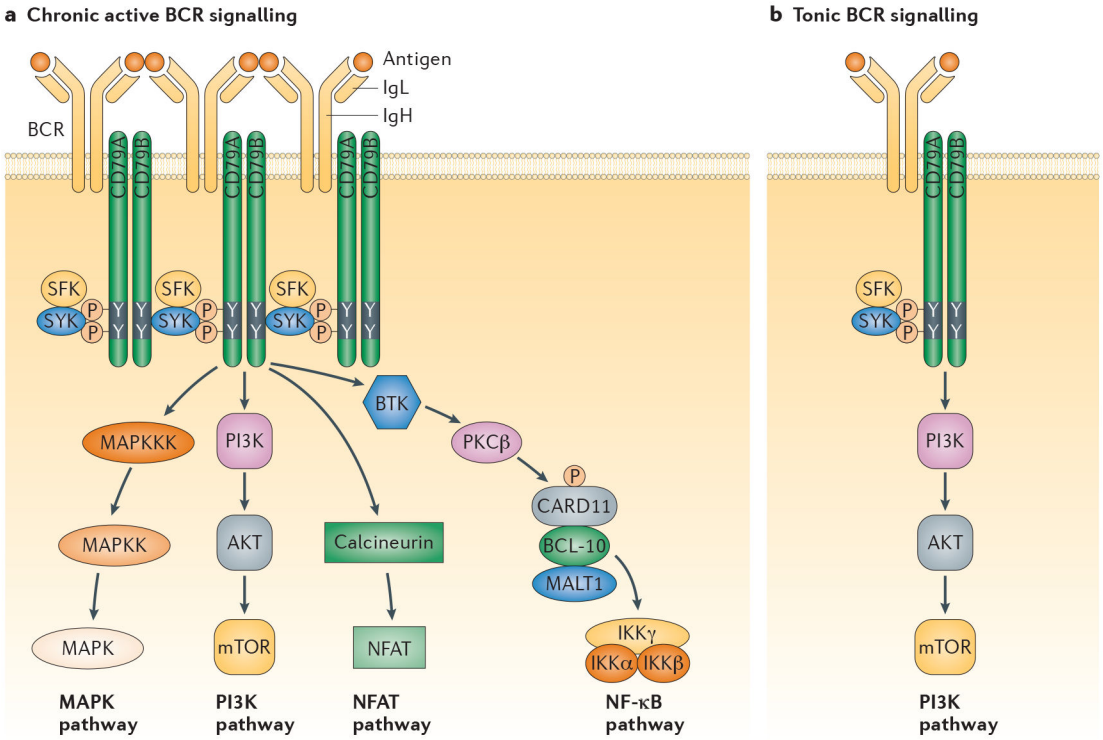

Figure 2 |. Two forms of pathological B cell receptor signalling in lymphoid malignancies. Chronic active B cell receptor (BCR) signalling, which typifies activated B cell-like diffuse large B cell lymphoma (ABC DLBCL), engages multiple downstream pathways, including nuclear factor-κB (NF-κB). In normal B cells, antigen triggers this form of signalling, and antigen may also have a role in lymphoid malignancies. Tonic BCR signalling, which typifies Burkitt's lymphoma, engages the phosphoinositide 3-kinase (PI3K) pathway only. Antigen most likely does not contribute to BCR signalling in this context. See main text for details. BTK, Bruton tyrosine kinase; CARD11, caspase recruitment domain-containing protein 11; IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain; IKK, inhibitor of NF-κB kinase; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPK kinase kinase; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; PKCβ, protein kinase Cβ.

Table 1 |

Evidence supporting BCR signalling in lymphoma

Malignancy	Frequency*	Clinical features	Presumed cell of origin	Pre-dominant IgH isotype	SHM	Putative BCR signalling	Putative antigens	Evidence supporting BCR activity
Activated B cell-like diffuse large B cell lymphoma (ABC DLBCL)	15%	Median age 66; nodal and extranodal; survival ~40% at 5 years	Post-germinal centre; plasmablast	IgM	Yes	Chronic Active‡	Auto-antigens	CD79B and CD79A ITAM mutations; RNAi: CD79A, IgM, IgL, SYK, BTK and CARD11; BCR clusters; ibrutinib response
Germinal centre B cell-like diffuse large B cell lymphoma (GCB DLBCL)	17%	Median age 61; nodal and extranodal; survival ~60% at 5 years	Germinal centre B cell	IgG	Yes	None or tonic	None	
Burkitt's lymphoma	<1%	Median age 11 (children), 24 (adult); extranodal and nodal; survival >80% at 5 years	Germinal centre B cell	IgM	Yes	Tonic‡	None	RNAi: CD79A and SYK
Follicular lymphoma	25%	Median age 57; nodal and bone marrow; survival 73% at 10 years	Germinal centre B cell	IgM	Yes	Chronic active	IgH–mannose–lectin interactions§	Nonrandom IgH V region mutation; phospho-flow analysis
Chronic lymphocytic leukaemia (CLL)	8%	Median age 65; blood, bone marrow, spleen and nodal; survival 50% at 7 years	Pre-germinal centre mature B cell or memory B cell	IgM	M-CLL: yes; U-CLL: no	Chronic active	Auto-antigens and IgH V region framework 2	IgH V segment usage and stereotyped receptors; ibrutinib response
Mantle cell lymphoma (MCL)	7%	Median age 55; nodal, intestinal, blood and bone marrow; survival 50% at 3–5 years	Pre-germinal centre mature B cell	IgM	No	Chronic active	Unknown	IgH V segment usage and stereotyped receptors; ibrutinib response
Mucosa-associated lymphoid tissue lymphoma/marginal zone lymphoma (MALT/MZL)	9%	Median age 60; extranodal and bone marrow; survival >80% at 5 years	Marginal zone B cell	IgM	Yes	Chronic active	Bacterial and viral infection	IgH V segment usage and stereotyped receptors
Primary mediastinal B cell lymphoma (PMBL)	6%	Median age 33; mediastinal thoracic; survival >60% at 5 years	Thymic B cell	IgG¶	Yes	None	None	-
Hodgkin's lymphoma	11%	Bimodal median age: 25 and 60 years; mediastinal and nodal; survival 85% at 15 years	Thymic B cell	IgG¶	Yes	None	None	-

Adapted from REF. 12. BCR, B cell receptor; BTK, Bruton tyrosine kinase; CARD11, caspase recruitment domain-containing protein 11; IgH, immunoglobulin heavy chain; ITAM, immunoreceptor tyrosine-based activation motif; M-CLL, mutated CLL; RNAi, RNA interference; SHM, somatic hypermutation; U-CLL, unmutated CLL; V, variable.

*Approximate percentage of the particular lymphoma subtype among all patients with B cell lymphoma.

‡Indicates known mechanism.

§Not an antigen, but can cause BCR crosslinking.

¶These malignancies express little or no surface BCR expression.

Table 2 |

BCR pathway inhibitors in the clinic

Target	Inhibitor	Manufacturers	Clinical trials
Chronic active BCR signalling			
BTK	Ibrutinib (PCI-32765)	Pharmacyclics/Janssen Research & Development	Phase III
	Dasatinib	Bristol-Myers Squibb	Approved
	AVL-292	Celgene	Phase I
PKCβ	Sotrastaurin (AEB071)	Novartis	Phase I
PI3Kδ	GS-1101 (CAL-101)	Calistoga Pharmaceuticals/Gilead Sciences	Phase II
Chronic active and tonic BCR signalling			
SYK	Fostamatinib (R788)	Rigel Pharmaceuticals/AstraZeneca	Phase II
	PRT062607	Portola Pharmaceuticals/Biogen Idec	Phase I
Pan-PI3K	BKM120	Novartis	Phase I*
	GDC-0941	Genentech	Phase Ib*
	XL147	Exelixis	Phase I*
	ZSTK474	Zenyaku Kogyo Co.	Phase I*
SRC family	Saracatinib	AstraZeneca	Phase II
	KX01	Kinex Pharmaceuticals	Phase II
	Dasatinib	Bristol-Myers Squibb	Approved
TORC1	Rapamycin (sirolimus)	Wyeth/Pfizer	Approved
	Everolimus	Novartis	Phase III
	Temsirolimus	Wyeth/Pfizer	Approved

BCR, B cell receptor; BTK, Bruton tyrosine kinase; PI3K, phosphoinositide 3-kinase; PKCβ, protein kinase Cβ; TORC1, target of rapamycin complex 1.

*Clinical trials in solid tumours only.