REVIEW

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Targeting B-cell receptor signaling in leukemia and lymphoma: how and why?

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Practice points

- This review dissects the signaling pathways activated downstream of the B-cell receptor (BCR) in normal and malignant B cells and discusses how a greater understanding of such pathways has led to the discovery of numerous drug targets for the treatment of B-cell lymphomas.
- This review discusses the BCR signaling response, describing in detail how this pathway constantly maintains its initiation, amplification and termination through a regulatory mechanism combining protein kinases, protein scaffolds, immuno-receptor tyrosine-based activation/inhibitory motifs, protein phosphatases and ubiquitination.
- Mature B-cell lymphoproliferative disorders such as chronic lymphocytic leukemia, diffuse large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and Waldenström's macroglobulinemia show active BCR signaling that contributes to behavior of the malignant clone in these diseases.
- This review considers various targets within the BCR signaling pathway. It relates how inhibiting Lyn is controversial because of its positive and negative role in BCR signaling, reviews how Lck is potentially a good target for chronic lymphocytic leukemia treatment because of its unique role in initiating BCR signaling within the malignant cells of this disease, discusses the benefits of targeting Syk, and examines Ibrutinib, a first-in-class BTK inhibitor, and Idelalisib, a first-in-class PI3Kδ inhibitor, as recently approved drugs useful in the treatment of mantle cell lymphoma, chronic lymphocytic leukemia and Waldenström's macroglobulinemia.
- BCR signaling pathway inhibitors have proven very successful in the treatment of B-cell malignancies and offer a less toxic alternative to conventional chemotherapeutic approaches. However, some patients have displayed adverse reactions to these inhibitors and therefore research is ongoing to help develop current and new therapeutic targets.

B-lymphocytes are dependent on B-cell receptor (BCR) signaling for the constant maintenance of their physiological function, and in many B-cell malignancies this signaling pathway is prone to aberrant activation. This understanding has led to an ever-increasing interest in the signaling networks activated following ligation of the BCR in both normal and malignant cells, and has been critical in establishing an array of small molecule inhibitors targeting BCR-induced signaling. By dissecting how different malignancies signal through BCR, researchers are contributing to the design of more customized therapeutics which have greater efficacy and lower toxicity than previous therapies. This allows clinicians access to an array of approaches to best treat patients whose malignancies have BCR signaling as a driver of pathogenesis.

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Background

B-lymphocytes are dependent on B-cell receptor (BCR) signaling for the constant maintenance of their physiological function, and in many B-cell malignancies this signaling pathway is prone to aberrant activation. This understanding has led to an ever-increasing interest in the signaling networks activated following ligation of the BCR in both normal and malignant cells, and has been critical in establishing an array of small molecule inhibitors targeting BCR-induced signaling. By dissecting how different malignancies signal through BCR, researchers are contributing to the design of more customized therapeutics which have greater efficacy and lower toxicity than previous therapies. This allows clinicians access to an array of approaches to best treat patients whose malignancies have BCR signaling as a driver of pathogenesis. This review brings together old and new basic research on the pathways activated downstream of the BCR, and reports on how these pathways contribute to both B-cell homeostasis and neoplasia. We also report on how our understanding of BCR signaling has revealed drug targets both proximal and distal to the BCR that have been successfully exploited therapeutically, especially with respect to particular malignancies.

BCR signaling in normal B cells

• The importance of BCR signaling

B-cell development and maturation is dependent on a diverse range of cellular responses relayed by antigen-independent (tonic) and antigen-dependent activation of the BCR [1–4]. This begins at the very earliest stages of B-cell development where tonic signals through the pre-BCR combined with IL-7R activation function together to drive the proliferation and survival of pre-B cells in the bone marrow [5]. Expression of functional BCR helps distinguish immature B cells from pre-B cells during subsequent differentiation, and strong engagement of BCR at this stage enables negative selection (auto deletion) of self-reactive B-cell clones [6–8]. Immature (naive) B cells that survive this selection then egress from the bone marrow, and are able to receive environmental cues in the peripheral lymphoid tissues to differentiate into their various mature B-cell subsets [9]. During this period, and in the absence of antigen encounter, it is thought that tonic BCR signals maintain the survival of B cells as they recirculate in the blood and follicular regions of the lymphoid tissues [2,10] because experiments have shown that failure to express BCR on peripheral immature and mature B cells results in their death [10]. Differentiation of naive B cells does not occur without engagement of the BCR by antigen and concomitant stimulation of cytokine and co-stimulatory receptors within lymphoid tissues. Here, it is the strength of BCR signaling that determines B-cell fate, deciding whether B cells differentiate to follicular B cells, marginal zone B cells or are deleted through neglect [11,12].

• Proximal BCR signaling

The BCR is comprised of a membrane anchored ligand binding unit formed by immunoglobulin (Ig), and a signaling unit consisting of a heterodimer of Igα (CD79a) and Igβ (CD79b) [13–15]. Antigen binds to hypervariable regions present on the Ig component of the BCR causing receptor clustering and juxtapositioning into areas rich in signaling components [16,17]. **Figure 1** illustrates the signaling networks activated following BCR stimulation. Upon antigen binding, two conserved tyrosine residues within the immuno-receptor tyrosine-based activation motifs (ITAMS) of CD79 become phosphorylated by Src-family kinases (SFK) such as Lyn **(Figure 1A)** [18,19]. Phosphorylated ITAMS act as docking sites for SH2 domains present in Syk, leading to its phosphorylation and subsequent activation **(Figure 1B)** [20,21]. SFK can also phosphorylate tyrosine residues outside the ITAM motifs of CD79 as well as tyrosine residues within the co-receptor protein CD19 **(Figure 1A)**. Adaptor proteins such as BLNK, LAB and Nck are recruited to these extra-ITAM phosphotyrosine motifs in CD79 **(Figure 1B)** [22–24]. Active Nck recruits B-cell adapter for PI3K (BCAP) which becomes phosphorylated by Syk and attracts PI3Kδ via its p85α regulatory domain **(Figure 1C)** [25,26]. Additionally, the SFK-phosphorylated tyrosines within CD19 are additional binding sites for p85α and activation of PI3Kδ **(Figure 1C)** [27–29]. However, it is suggested that PI3K can be activated independently of BCR engagement because the introduction of exogenous PI3K has been shown to rescue BCR-negative cells from apoptosis [30,31]. A proposed mechanism for this rescue is the recruitment of PI3K to unphosphorylated motifs present on CD79a by the GTPase TC21 [32]. Whatever the mechanism of activation, PI3Kδ converts PIP_2 to PIP_3 on the plasma membrane, and, in doing so, generates a binding site for the pleckstrin

homology (PH) domain of a collection of proteins normally residing in the cytosol of resting B cells. Btk is one such protein, that additionally binds BLNK and becomes phosphorylated by Syk [33], exposing an autophosphorylation site within Btk that culminates in its full activation **(Figure 1C & D)** [34]. PLCγ2 is also recruited to the plasma membrane via its PH domain and BLNK **(Figure 1C & D)**, and is sequentially activated through phosphorylation by Btk and Syk [35]. Similarly, the Rac GTP-exchange factor Vav is also attracted to the BCR signalosome where it can be phosphorylated and activated by Syk **(Figure 1C & E)** [36–38]. Finally, PIP_3 also attracts the kinases PDK1 and Akt to the plasma membrane **(Figure 1C)**. Once recruited, PDK1 phosphorylates Akt on T308 to increase the kinase activity of the latter. Stabilization of Akt kinase activity then occurs when it is phosphorylated on S473 by mTORC2 through a mechanism involving Akt-mediated phosphorylation of SIN1 [39].

• Distal BCR signaling

The proximal BCR signaling response quickly transcends to activate numerous signaling pathways summarized in **Figure 1D**. Active PLCγ2 is able to hydrolyze PIP_2 to generate two second messengers, IP_3 and DAG. IP_3 induces the release of Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER) and, together with DAG, this leads to the activation of PKCβ [40]. PKCβ is then able to mediate the stimulation of multiple downstream signaling pathways. For example, PKCβ together with DAG and Sos can generate active Ras (GTP-Ras), leading to activation of the cRaf-MEK-ERK pathway [41–43]. PKCβ can also phosphorylate CARMA1 (CARD11) [44] to initiate the recruitment of MALT1 and Bcl-10, which forms the CBM complex [45,46]. This complex activates TAK1, a kinase that can catalyze the activation of both NF-κB and JNK [47]. TAK1 activates NF-κB by initially phosphorylating IKKβ [47], which in turn phosphorylates IκBα, flagging it for ubiquitination and degradation by the proteasome [48]. This alleviates its inhibition of NF-κB and allows it to translocate to the nucleus. Calmodulin (CaM) detects increased Ca^{2+} mobilization from intracellular stores and activates the S/T phosphatase calcineurin (CN). CN dephosphorylates regulatory regions within NFAT causing a conformational change that exposes its nuclear localization sequence and results in its activation [49].

The BCR signal can also be relayed to downstream signaling pathways independently of calcium release from the ER. As mentioned previously, generation of PIP_3 by activation of PI3Kδ causes the recruitment of Vav and generation of active GTP-loaded Rac1 and Rac2 **(Figure 1E)**. These small G-proteins can facilitate F-actin reorganization as well as activation of p38MAPK and JNK [24,50–51]. Active Akt plays an important role in stimulating the mTORC1 pathway and in deactivating GSK3 and FoxO to facilitate increased cell growth and survival **(Figure 1E)** [52–54]. Collectively, these pathways are responsible for the control of a whole host of cellular functions such as; survival, proliferation, oxidative stress response, differentiation and DNA damage repair.

• Negative regulation of BCR signaling

BCR signaling is highly regulated by a complex network of phosphatases, regulatory receptors and negative feedback signals in order to prevent its inappropriate activation. A primary example of this is how the SFK Lyn mediates the BCR signaling response. The protein tyrosine phosphatases (PTP) CD45 and CD148 dephosphorylate Y508 on the C-terminus of Lyn causing a conformational change that allows for its autophosphorylation and activation [55,56]. Then, depending on the context of the BCR signal, active Lyn can moderate the BCR signal by phosphorylating immuno-receptor tyrosine-based inhibitory motifs (ITIM) present on the regulatory receptors CD5, CD22, CD32 and CD72 [57–62]. Phosphorylated ITIMs recruit phosphatases such as SHP-1, SHIP-1, SHIP-2, PTP-PEST, PTPN2, PTPROt, PTEN and PTPN22 which act on proximal targets to downregulate the BCR signaling response. Lyn controls its own inactivation by recruiting the phosphatase PTPN22, which forms a complex with the C-terminal Src kinase (Csk) [63]. Active Lyn binds and phosphorylates the Csk binding protein (Cbp) on Y314 and this serves to recruit Csk. PTPN22 then dephosphorylates the activating tyrosine residue on Lyn and allows Csk access to phosphorylate Lyn on Y508, resulting in the restoration of its closed inactive conformation [64,65]. Additionally, PTPN22 can regulate the stability of SFK and SYK by activating the E3-ubiquitin ligase c-Cbl, which ubiquitinylates these proteins and marks them for degradation [66–68]. Interestingly, SFK and Syk can recruit SOCS-1 which seems to augment their polyubiquitination and degradation

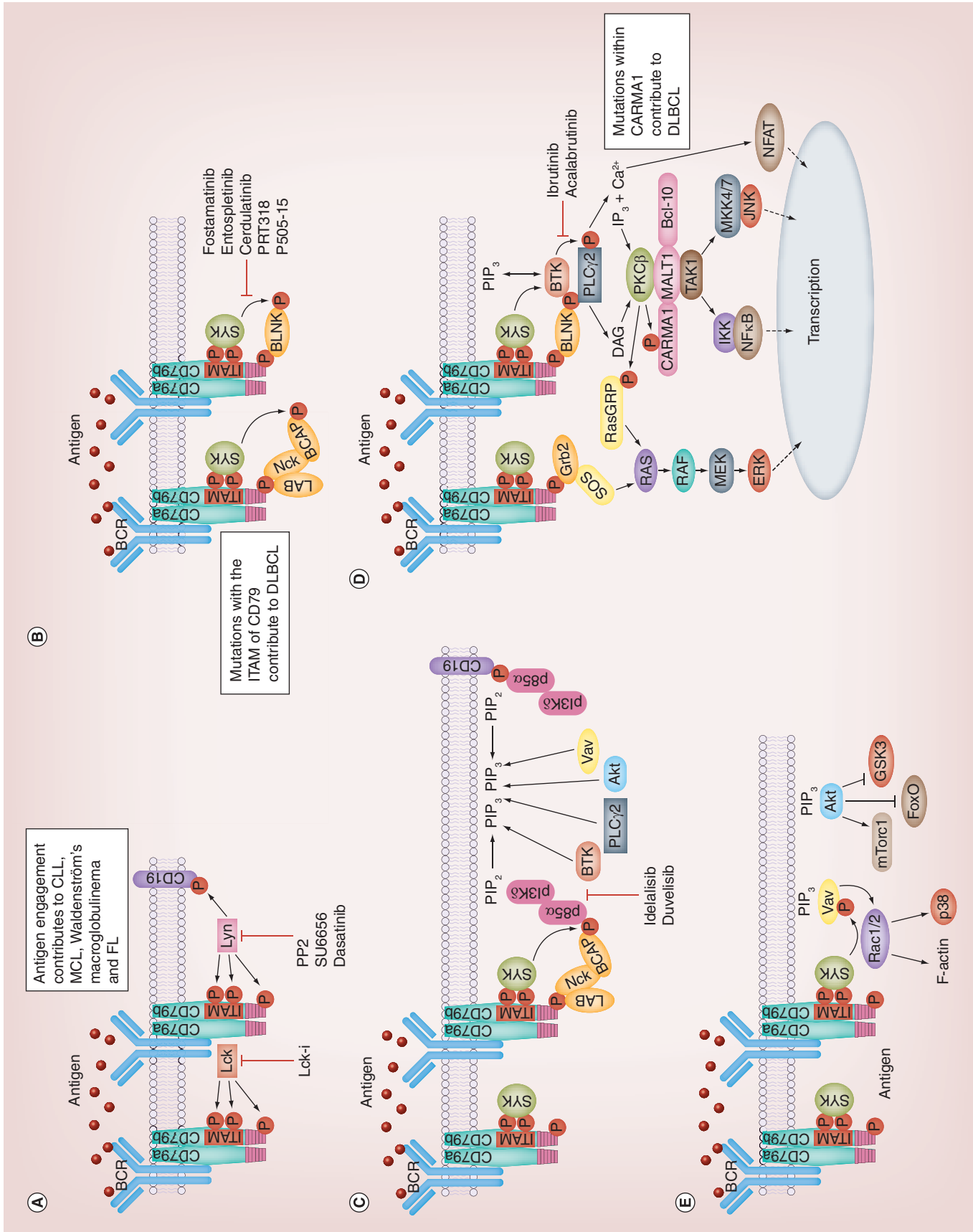
A
Antigen engagement contributes to CLL, MCL, Waldenström's macroglobulinema and FL
Antigen
BCR
CD79a
CD79b
ITAM
P
Lyn
Lck
CD19
PP2
SU6656
Dasatinib
Lck-i
B
Mutations with the ITAM of CD79 contribute to DLBCL
Antigen
SYK
BLNK
LAB
Nck
BCAP
Fostamatinib
Entospletinib
Cerdulatinib
PRT318
P505-15
C
Antigen
PIP2
PIP3
p85α
p13Kδ
BTK
PLCγ2
Vav
Akt
LAB
Nck
BCAP
Idelalisib
Duvelisib
D
Antigen
PIP3
BTK
PLCγ2
BLNK
Ibrutinib
Acalabrutinib
DAG
IP3 + Ca2+
PKCβ
CARMA1
MALT1
Bcl-10
TAK1
MKK4/7
JNK
IKK
NFκB
NFAT
Transcription
RasGRP
Grb2
SOS
RAS
RAF
MEK
ERK
Mutations within CARMA1 contribute to DLBCL
E
Antigen
PIP3
Vav
Rac1/2
p38
F-actin
Akt
GSK3
FoxO
mTorc1

Figure 1. Signaling pathways of the B-cell receptor, its role in B-cell malignancies and targets of inhibition (see facing page). Schematic of the B-cell receptor signaling pathway. **(A)** Antigen engagement initiates receptor clustering and facilitates Lyn-medated phosphorylation of tyrosines within CD79 and CD19. In chronic lymphocytic leukemia, the SFK Lck mediates phosphorylation of CD79. **(B)** Syk binds to phospho-tyrosine residues within the ITAM of CD79 and is activated. Adaptor proteins such as BLNK, LAB, NCK, BCAP and Grb2 associate with phospho-tyrosines outside the ITAM on CD79. Proteins such as BLNK and BCAP are substrates of Syk. **(C)** Phospho-tyrosine residues within BCAP and CD19 attract the regulatory subunit of PI3Kδ leading to the activation of catalytic p110. $PI(4,5)P_2$ is converted to $PI(3,4,5)P_3$ which attracts PH domain containing proteins such as BTK, PLCγ2, Akt and Vav to the plasma membrane. **(D)** Phospho-tyrosine residues within BLNK act as a scaffold for membrane-associated BTK and PLCγ2, facilitating activation of the former to phosphorate and activate the latter. This catalyzes distal signaling pathway activation leading to NF-κB, JNK and ERK activation. **(E)** Further distal signals include activation of Vav leading to cytoskeletal rearrangement, and of Akt leading to activation of mTORC1 and inhibition of FoxO.
CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; ITAM: Immuno-receptor tyrosine-based activation motifs; MCL: Mantle cell lymphoma.

by the proteasome [65,69]. However, the role of c-Cbl appears more complex than mere regulation of protein stability because it is reported to downregulate the BCR signal by binding to BLNK and preventing its association with PLCγ2 [70].

Other inhibitory receptors worth mentioning are those regulated by CD22 and CD32. Upon phosphorylation of their ITIMs by Lyn, PTEN and SHIP-1/2, respectively remove the 3′ and 5′ phosphate of PIP_3 and attenuate signaling downstream of PI3K [71–73]. This regulatory pathway is activated by prolonged exposure to autoantigens and forms the basis of auto-reactive B-cell elimination [74]. Last, the phosphatase SHP-1 has been reported to downregulate the BCR signal by dephosphorylating targets such as SFK, ITAMs, ZAP70, SYK, BLNK and LAB [75].

Further regulation of the BCR signal is provided by negative feedback mechanisms that are independent of phosphatase activity. Following its activation, Btk can become phosphorylated on S180 by PKCβ activated by Btk-catalyzed stimulation of PLCγ2. This disrupts the interaction of the PH domain of Btk with PIP_3 and removes Btk from the plasma membrane [76]. Furthermore, a recent report has demonstrated that Akt can also phosphorylate Btk on S51 and T495 which leads to its sequestration and degradation [77]. Another more distal example of negative feedback inhibition of BCR signaling is found in the regulation of NF-κB by the CBM complex. It is reported that TAK1 can induce both kinase-independent and -dependent degradation of Bcl-10 by respectively coordinating the binding of E3 ubiquitin ligases and activating JNK [78].

This meticulous regulation of the BCR allows it to constantly maintain its initiation, amplification and termination under normal conditions. However, alteration in these pathways has the potential to transform B cells into an array of malignant diseases.

BCR signaling in malignant B cells

• BCR signaling drives B-cell malignancies

It is widely accepted that signals emanating from the BCR play an important role in the initiation and progression of many B-cell malignancies. For example, gene expression profiling of chronic lymphocytic leukemia (CLL) cells isolated from lymphatic tissues found that the BCR pathway was active in these cells to a greater degree than what is found in circulating CLL cells [79]. Furthermore, CLL cells use a restricted repertoire of *IGHV* genes, with many patients expressing virtually identical BCRs, known as sterotyped BCRs [80–83]. Similarly, mantle cell lymphoma (MCL) cells also express sterotyped BCR [84,85], and display constitutive activation of BCR signaling components such as the PI3K pathway [86,87]. Other examples include diffuse large B-cell lymphoma (DLBCL) [88], follicular lymphoma (FL) [89], hairy cell leukemia (HCL) [90,91], Burkitts lymphoma (BL) [92], Waldenström's macroglobulinemia (WM) [93], marginal zone lymphoma (MZL) [94] and acute lymphoblastic leukemia (ALL) [95] cells which have all been shown to display some sort of defect in BCR signaling. However, the malignant cells in each of these diseases display distinct alterations in the BCR signaling pathway, reflecting defects originating from tonic/autonomous and/or chronic engagement of the BCR.

What appears constant in many B-cell lymphomas is that IgM is the typical isotype of their BCR, probably due to the bias of this particular configuration of BCR toward stimulating survival and proliferation of B cells [96]. Below we will summarize some of the major aberrantly activated BCR signaling pathways found in different leukemia and lymphomas, and highlight their importance in the initiation, survival and expansion of these malignant cells.

• Chronic lymphocytic leukemia

CLL is a debilitating disease characterised by the gradual accumulation of mature B cells that are resistant to apoptosis. The disease provides an excellent example of the prominent role BCR signaling plays in the pathogenesis of B-cell malignancies **(Figure 1A)**. This role was determined from early studies of BCR structure in CLL cells which showed the genes coding for variable (antigen binding) regions of BCR heavy chain maintained germline sequences in approximately half of patients diagnosed with this disease. These patients with so-called unmutated CLL (UM-CLL) have disease which has less favorable prognosis than patients where these genes have been somatically hypermutated, so called mutated CLL (M-CLL) [97]. Other studies showed that BCRs on CLL cells from different patients could be virtually identical with respect to *IGVH* genes and sequences, suggesting a common antigen or feature of the BCR that is involved in the pathology of this disease [82]. Common antigens targeted by BCR on CLL cells are reported to include epitopes associated with apoptosis and oxidation [98], yeast/fungi cell wall components [99], myosin [100] and vimentin [101], and BCR on CLL cells from UM-CLL patients are both polyreactive and responsive to BCR stimulation. In keeping with their ability to strongly respond to BCR engagement, UM-CLL cells generally have high expression and/or activation levels of many of the components of the BCR signaling pathway such as Syk [102], Lyn, Btk, PLCγ2, PI3Kδ, GAB1, PTPN22 [103], PKCβ and NF-κB. Furthermore, UM-CLL cells also generally express ZAP70 [104–106] and conflicting reports argue on one hand that this kinase mediates the phosphorylation of ITAM motifs and subsequent recruitment of Syk [107], while others have shown that kinase dead ZAP70 can still enhance the BCR signaling response by acting as a scaffold protein [108,109]. Work from this Department has demonstrated that another kinase called Lck displays heterogeneous expression in CLL cells and is able to augment the BCR signaling response [110]. An important feature of BCR signaling in CLL cells that distinguishes it from other B-cell malignancies is that it fails to activate the JNK pathway [111], however, why this is the case requires further investigation. Direct engagement of the BCR is not the only way in which this receptor contributes to disease pathogenesis in CLL. Some BCR heavy chain structures are said to be represented stereotypically on CLL cells, and one study has demonstrated that particular regions, namely the FR2 and HCDR3, can interact to allow autonomous BCR signaling, particularly in CLL cells from UM-CLL patients, irrespective of antigen stimulation [112].

In contrast to UM-CLL, CLL cells from patients with M-CLL express low surface IgM and show higher basal levels of Ca^{2+} and ERK activation consistent with constitutive low level stimulation of BCR [113] resulting in induction of cell anergy [114,115]. Targeting either the ERK or the NF-AT pathway with specific inhibitors is reported to reduce the survival of anergic CLL cells, suggesting that BCR anergy contributes to clonal maintenance in M-CLL anergy [114].

Taken together, these findings in both UM- and M-CLL demonstrate that BCR signaling contributes to proliferation and survival of the malignant clone in CLL. However, the way in which BCR signaling is stimulated, be it through active engagement or through tonic/chronic/autonomous signaling may have impact on disease progression. CLL cells circulate between the peripheral blood (PB) and proliferation centers. Expansion of the malignant clone depends upon its ability to remain within proliferation centers such as the lymph node where there is a microenvironment rich in proliferation and survival signals [113]. BCR engagement on CLL cells induces increased adhesion [116,117] and suppressed expression of the receptor for sphingosine 1-phosphate [118], both which lead to increased lymph node residency. This role of BCR in keeping CLL cells within lymph nodes has recently been exploited therapeutically where drugs targeting Btk (ibrutinib) and PI3Kδ (idelalisib) induce lymphocytosis while reducing both lymphadenopathy and splenomegaly [119,120].

• Diffuse large B-cell lymphoma

BCR signaling in a subset of DLBCL known as activated B-cell like (ABC)-DLBCL shows

traits of active BCR signaling, and gene expression analysis revealed that a large amount of these cells display increased levels of BCR signaling components [121]. Furthermore, studies using internal reflection fluorescence (TIRF) microscopy found that the BCRs on ABC-DLBCL cells form clusters on the cell surface with proteins containing phosphotyrosine localizing underneath [88,122]. Other studies investigating the mechanism of cell survival discovered that these cells depend on anti-apoptotic signals provided by the NF-κB pathway [123], found to be constitutively active in some cases of ABC-DLBCL owing to mutations in CARMA1 [124] **(Figure 1D)** and loss-of-function mutations in negative regulators of NF-κB [125]. However, malignant cells in the majority of ABC-DLBC contain wild-type CARMA1 but still require this protein to maintain viability [126]. In these cases knockdown of BCR signaling units CD79a/b or any of its proximal signaling components (BLNK, Syk, Btk, PLCγ2, PI3K or PKCβ) induces apoptosis [88]. In particular, the malignant clones from approximately 20% of ABC-DLBCL cases bear mutations within the ITAM motifs of CD79a/b **(Figure 1A)** leading to reduced receptor endocytosis and increased surface expression of the BCR [88]. These cells have an enhanced BCR signaling response and are able to induce a greater and more prolonged activation of Akt. In particular, CD79b mutants are more resistant to moderation of the BCR signal by Lyn and consequently this may generate an oncogenic addiction to BCR-induced NF-κB activation [88,127]. Another protein that is frequently mutated in ABC-DLBCL is the adapter protein MYD88 [128] which is responsible for coupling toll-like receptors (TLR) to the NF-κB pathway. An L265P mutation in MYD88 was shown to augment autocrine secretion of the cytokines IL-6 and IL-10 resulting in increased ABC-DLBCL survival [128]. The relevance of such mutations to BCR-induced survival signals was highlighted when one group knocked down MYD88 in concert with CD79a/b, and found that this synergized to induce ABC-DLBCL cell killing [128,129]. Interestingly, this same mutation occurs in approximately 3% of CLL patients [130], and MYD88 has recently been shown to work in concert with the BCR of ZAP70 positive CLL cells to stimulate cell survival and proliferation [131].

• Burkitt's lymphoma

BL is an aggressive malignancy derived from germinal center B cells. A distinctive characteristic of this disease is a chromosomal translocation whereby the proto-oncogene MYC is linked to the heavy chain of BCR [132]. However, this alone is insufficient to cause BL, and studies in mice suggest that MYC works with active PI3K to generate the Burkett's lymphoma phenotype [133]. In contrast to the chronically active BCR signal displayed in ABC-DLBCL, BL cells are resistant to BTK and CARMA1 knockdown, but remain sensitive to a reduction in CD79a and Syk expression [127,132]. BCR ligation on BL cells induces activation of the PI3K pathway, and inhibition of this activation either by directly targeting PI3K or by targeting its downstream target mTORC1 kills these cells [132]. Tonic BCR signals are also present, and are the result of mutations within the transcription factor TCF3 (or E2A) or its negative regulator ID3 [132]. TCF3 normally regulates the expression of the BCR and represses the levels of the phosphatase SHiP1. Thus, mutant forms found in BL lead to increased BCR expression and activation of the PI3K pathway.

• Follicular lymphoma

FL cells are malignant germinal center B cells admixed with nonmalignant cells such as T cells, follicular dentritic cells (FDC), macrophages, fibroblasts and endothelial cells [134,135]. A hallmark of this disease is a t(14:18)(q32:q21) translocation resulting in an overexpression of Bcl-2 and resistance to apoptosis [136]. However, these cells also display high basal levels of active Syk, and resistance to apoptosis is at least partially mediated by PI3K activation [137,138]. It is clear that BCR plays a role in FL disease pathology [139,140], autoreactivity is reported to be a feature of some FL clones [141] and a more recent report has suggested BCR signals can be initiated within malignant cells by interaction of mannosyl residues on surface-expressed BCR with lectins expressed on stromal cells in the tumor microenvironment **(Figure 1A)** [142,143]. Such antigen-independent signaling then drives expansion of the malignant clone. However, this is not the whole story because there also exist subclones that are unresponsive to BCR crosslinking due to elevated phosphatase activity [142]. Further investigation is required to understand the signaling mechanisms present in these latter clones, and how they contribute to disease.

• Mantle cell lymphoma

The malignant clone in MCL, like that in CLL, expresses a restricted repertoire of BCR genes, and it is thought that BCR signaling plays an important role in the survival of MCL cells and progression of disease **(Figure 1A)** [144]. A recent report found that BTK was constitutively activated in these cells and BCR crosslinking resulted in sustained activation of Syk. These kinases facilitated BCR-induced survival by mediating the secretion of autocrine factors and inducing adhesion to human bone marrow stromal cells (HMSCs) [145]. Like in CLL, drugs targeting the BCR pathway such as ibrutinib and idelalisib have shown remarkable efficacy also in the treatment of MCL [146].

Targeting BCR signaling pathways

• Proximal BCR signaling pathways

Lyn inhibitors

Lyn is found overexpressed and constitutively active in many B-cell malignancies where it contributes to high basal levels of tyrosine phosphorylated proteins. In CLL, this SFK is thought to contribute to malignant cell survival because experiments using PP2 and SU6656 (compounds which inhibit Lyn **[Figure 1A]**) induce apoptosis of CLL cells [147]. The pro-survival role of Lyn may be mediated by reported ability to deactivate procaspase-8 through phosphorylation-induced dimerization [148], or by downregulating the tumor suppressor activity of PP2A [149]. Another study has suggested that Lyn plays an important role in CLL progression and lymphoid organ infiltration by malignant cells because of its ability to phosphorylate HS1 and regulate cytoskeletal functionality [150]. However, any conclusions drawn from these studies should be approached with caution because the drugs used to inhibit Lyn (PP2, SU6656 and dasatinib) also affect a broad range of SFKs when used at the concentrations reported [147,151]. Attempts to address the potential role of Lyn in CLL cells have used geldanamycin to disrupt Lyn's association with HSP90. Although such treatment results in downregulation of Lyn expression and the induction of CLL cell apoptosis, mechanisms independent of Lyn may be inducing apoptosis because HSP90 regulates the stability of other proteins that may be important to the survival of CLL cells [152]. Nevertheless, a new study by Kim *et al*. demonstrated that Lyn could be a promising target for treatment of bortezomib-resistant mantle cell lymphoma (BTZ-resistant MCL). In this study, treatment of these resistant cells with dasatinib caused inhibition of proliferation likely by preventing CD19 binding with Lyn and the p85 regulatory subunit of PI3K [153]. Similar observations were made when Lyn was depleted with siRNA, and therapeutic potential is strongly suggested by xenograft studies showing that tumor size in the mice injected with BTZ-resistant MCL cells can be significantly reduced by dasatinib treatment [153]. In humans there is at least one case report showing that a CLL patient with co-existing chronic myeloid leukemia could be successfully treated with dasatinib [154], suggesting that general targeting of SFKs may be an effective approach. In this light, specific inhibition of Lyn may be a controversial target in the therapy of B-cell malignancies [155,156] because of its role as a positive and negative regulator of BCR signaling [157,158] through its ability to phosphorylate both ITAMs and ITIMs [155]. Specific inhibition of Lyn may block negative feedback of BCR signaling and allow strong signals, perhaps mediated by other SFKs, to take precedence.

Lck inhibitors

An example of an SFK mediating BCR signaling in B-cell malignancies is Lck. This SFK is classically expressed in T cells where it plays an important role in proximal antigen receptor (TCR) signaling. However, this kinase is also present in B1 B-lymphocytes and to participate in BCR signaling [159]. In CLL cells our work has shown that Lck is an important mediator of BCR signaling **(Figure 1A)**, treatment of CLL cells with either a specific Lck inhibitor or Lck-specific siRNA resulted in inhibition of BCR-induced phosphorylation of IKK, ERK and Akt, and a reduction in BCR-mediated survival [110]. This same study also noted that constitutive activation of Lyn was not affected by the inhibitor compound used, indicating that Lck could be a potential target for treatment of CLL because of its proximal role in initiating BCR signaling. Work in this laboratory as well as in others has shown that CLL cells display heterogeneous Lck expression [160,161], and it is suggested that Lck induces CLL resistance to glucocorticoid-induced apoptosis [162]. Although the level of Lck expression dictates the strength of BCR signaling, it is yet unclear how this relates to disease outcome.

Syk inhibitors

Syk plays an important role in both autoimmune diseases and hematological malignancies [163]. Initial small molecule inhibitors targeting Syk were designed for the treatment of inflammatory diseases [164]; however, preclinical studies suggested that Syk could also be a promising target for treatment of B-cell malignancies [102,165–167]. For example, Syk has been shown to be activated in 44% of DLBCL patient samples and responsible for regulating pathological BCR signaling [168], making Syk a valid potential target for the treatment of DLBCL. Syk was also shown to be constitutively active in peripheral B cells from a large number of CLL patients, and inhibition of this kinase is resulted in the induction of apoptosis through a mechanism involving downregulation of Mcl-1 protein levels [102].

Fostamatinib is an orally available prodrug of the active drug R406, and is a relatively selective Syk inhibitor [169]. Treatment of CLL cells with fostamatinib resulted in a reduction in cell migration, adhesion and a moderate induction of apoptosis, achieved by an inhibition of both BCR and integrin signaling [102,165,170]. *In vivo* studies in CLL and non-Hodgkin lymphoma mouse models supported the therapeutic potential of Syk inhibition in B-cell malignancies [167,171]. In the clinic, 55% of CLL patients treated with fostamatinib achieved partial response compared to 22% of DLBCL, 11% of MCL and 10% in FL patients [172].

Cerdulatinib is a dual Syk/JAK kinase inhibitor that showed activity in both ABC- and GCB-types of DLBCL. This compound induced apoptosis in DLBCL as well as G1/S cell cycle arrest [173]. There are more selective Syk inhibitors currently under devolvement, namely entospletinib, PRT318 and P505–15. These compounds have shown encouraging results in preclinical studies in CLL [174] and in DLBCL [168].

BTK inhibitors

BTK is a nonreceptor protein tyrosine kinase that belongs to TEC family kinases and plays a crucial role in BCR signaling [175]. Loss of function mutations within BTK result in X-linked agammaglobulinemia, a disorder characterized by the absence of mature B lymphocytes and immunodeficiency through lack of antibodies [176]. BTK is an important mediator of BCR-induced calcium flux, NF-κB activation and B-cell proliferation, making it a central player in B-cell physiology [175]. With respect to B-cell malignancies, BTK knockdown studies in ABC-DLBCL cell lines have demonstrated this kinase is essential for tumor growth and cell survival [88]. Signaling through BTK is also essential for the survival and proliferation of the malignant B cells in Waldenström macroglobulinemia (WM), a lymphoplasmacytic lymphoma, where mutation of MYD88 leads to induction of these signals [177]. Furthermore, BTK plays a key role in the malignant behavior of CLL and MCL cells where chronic antigen-stimulation of the BCR facilitates disease progression [144,178,179]. Taken collectively, these studies have been used to justify targeting BTK as a valid therapeutic option to treat B-cell malignancies [180].

Ibrutinib, a first-in-class inhibitor of BTK, is an orally available small molecule that covalently binds to Cys-481 and irreversibly blocks the kinase activity of BTK **(Figure 1D)**. The effect of this blockade is observable also in CLL cells isolated from patients being administered this compound, these cells exhibit reduced levels of PLC-γ and ERK phosphorylation, of expression of genes induced by BCR and NF-κB pathways [181]. Studies of *in vitro* models that resemble the tumor microenvironment and of *in vivo* xenografts have shown that ibrutinib inhibits cell proliferation, survival and migration of CLL cells [182,183]. Ibrutinib has pleiotropic inhibitory effects allowing it to attenuate signaling induced by the BCR, CD40, BAFF, TLR and chemokine receptors. This indicates that the therapeutic effect of ibrutinib is likely due to regulation of microenvironmental influences rather than to direct induction of apoptosis. Ibrutinib is also shown to inhibit secretion of the chemokines CCL3 and CCL4 from CLL cells, and reduced serum levels of these chemokines is observed in CLL patients treated with this agent [116,182,183]. An interesting feature of most CLL and MCL patients treated with ibrutinib is that they show transient lymphocytosis due to egress of malignant cells from the protective microenvironment in the lymph nodes [119,184–186], and this is likely due to inhibition of chemokine- and BCR-induced integrin α4β1-mediated adhesion [116,117,187,188].

Clinical trials in relapse or refractory CLL patients have demonstrated high frequency of durable remission even in patients with high risk CLL (e.g., mutated TP53, del17p and del11q) [189]. Ibrutinib has been approved by the US FDA for the treatment of relapsed or

refractory MCL and CLL [184] and for WM [190]. However, resistance to ibrutinib in MCL and CLL has been described. One of the mechanisms of ibrutinib resistance is cysteine-to-serine mutation at C481 in the binding site of BTK [191,192] as well as gain-of-function mutations within PLCγ2 [193]. Many other reversible and irreversible BTK inhibitors are still under development to overcome such resistance to improve selectivity and tolerability [180]. Moreover, combination studies are ongoing in order to achieve better and more durable responses to ibrutinib [190].

PI3K inhibitors

PI3K plays an important role in mediating signaling through the BCR, adhesion molecules and chemokine receptors and is therefore key to B-cell survival, migration and proliferation [194]. There are four catalytic isoforms of PI3K: p110α, p110β, p110γ and p110δ. With respect to B cells, the δ isoform is particularly important because PI3Kδ deletion or mutation in mice results in lack of B1 lymphocytes, lower numbers of mature B cells and impaired antibody production [195]. Importantly, PI3Kδ expression is limited to lymphocytes and particular subsets of myeloid lineage cells such as mast cells and neutrophils, and this makes compounds that specifically target this isoform, particularly attractive because the profile of toxicities will be less than what could be expected from pan PI3K inhibitors or inhibitors which target the more ubiquitously expressed α and β isoforms of this protein. In CLL, PI3Kδ has been shown to be constitutively active in the malignant cells [196], and its inhibition reduces malignant cell viability, making targeting of this PI3K isoform using small molecular inhibitors a potential treatment approach [197]. Idelalisib (formerly GS-1101 or CAL-101) is a highly selective inhibitor of PI3Kδ [197] which has an efficacy that has allowed fast-track approval for the treatment of relapsed and refractory CLL in USA **(Figure 1C)** [198]. Idelalisib is reported to inhibit the prosurvival effect of BCR stimulation in CLL cells [199], and its cytotoxic effect is dose- and time-dependent being mediated by induction of the caspase pathway regardless of p53 or *IGHV* mutation status. Idelalisib treatment also leads to blocking several microenvironmental signals such as CD40L, TNF-α, BAFF, ET1, fibronectin adhesion and nurse-like and stromal cell contact [199–201], likely by also affecting cells such as NK and T cells which produce cytokines known to promote CLL cells survival and proliferation such as TNF-α, CD40L, IL-6 and IFN-γ [200,202,203]. Clinically, CLL patients treated with Idelalisib exhibit lymphocytosis due to redistribution of CLL cells from lymphoid tissues to peripheral blood [204,205]. This redistribution may be caused by reduction in PI3K-mediated cell adhesion and migration [120,199], or by re-expression of the receptor for sphingosine-1 phosphate and induction of lymphocyte egress from lymph nodes/proliferation centers as has been recently suggested [118]. In terms of adverse drug reactions idelalisib has been linked to a characteristic set including, most notably, diarrhea/colitis, increased liver transaminases, skin rash and pneumonitis. Very little is currently known about the biological basis of these adverse drug reactions because they affect only a subset of patients. Thus, more research is needed so that patients at risk of developing these adverse drug reactions can be identified, and methodologies to prevent them introduced.

Future perspective

BCR pathway inhibitors have had remarkable success in the treatment of B-cell malignancies, particularly for patients displaying resistance to conventional chemotherapeutic agents. What is more, these small molecule inhibitors offer a less toxic alternative to conventional chemotherapy and only minor side effects are reported with their use. However, long-term treatment with these molecules may prove more toxic than previously thought. For example, US prescribing information for idelalisib contains a black box risk for serious/fatal diarrhea [198,206]. Moreover, drug resistance of the type observed with the usage of ibrutinib is also a problem [191–193]. To combat the problems associated with toxicity, new-generation drugs against PI3Kδ, such as duvalisib, and BTK, such as acalabrutinib, are either in development or have been introduced into the clinic [207,208]. Furthermore, new targets can be developed within this pathway, and our work has suggested Lck as one target for the treatment of CLL [110]. E3 ubiquitin ligases are also potential targets, recent research has begun to reveal roles for these proteins in B lymphomagenesis [209–211]. Clearly, investigation into BCR signaling and compounds which inhibit this pathway requires ongoing investigation, and it is likely that any inhibitors used will be employed within combinatorial therapeutic approaches in order to increase their efficacy.

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• of interest; •• of considerable interest

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