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B cell receptor signaling in chronic lymphocytic leukemia

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Abstract

BCR signaling plays an important pathogenic role in chronic lymphocytic leukemia (CLL) and B cell lymphomas, based on structural restrictions of the BCR, and BCR-dependent survival and growth of the malignant B cells. In CLL and lymphoma subtypes, ligand-independent ("tonic") and ligand-dependent BCR signaling have been characterized, which can involve mutations of BCR pathway components or be triggered by (auto-) antigens present in the tissue microenvironment. In CLL, based on high response rates and durable remissions in early-stage clinical trials, there is rapid clinical development of inhibitors targeting BCR-associated kinases (BTK, PI3Kδ), which will change treatment paradigms in CLL and other B cell malignancies. Here, we discuss the evolution of this field, from BCR-related prognostic markers, to mechanisms of BCR activation, and targeting of BCR-associated kinases, the emerging Achilles' heel in CLL pathogenesis.

Keywords

Chronic lymphocytic leukemia (CLL); B cell receptor (BCR); microenvironment; BTK; SYK; PI3δ

The lymphatic tissue microenvironment stages BCR activation in CLL

CLL cells proliferate in distinct microanatomical tissue sites called "proliferation centers" or "pseudofollicles", a hallmark finding in CLL histopathology[1]. In these areas, CLL cells are in intimate contact with accessory cells, such as monocyte-derived nurselike cells (NLC) [2, 3], T cells[4], and mesenchymal stromal cells[5], which, together with matrix factors, constitute the CLL microenvironment[6]. Proliferation of CLL cells in tissues accounts for daily birth rate of approximately 0.1 to 2% of the entire clone, as demonstrated by deuterated "heavy" water (^2H_2O) labeling in patients[7]. The lymphatic tissues are the apparent principal site of BCR activation for normal and malignant B cells. BCR activation can be induced by antigen or can be ligand-independent ("tonic" BCR signaling), and triggers a cascade of signaling events that normally cause B cell selection, proliferation, differentiation, and antibody production[8]. Thereby, BCR signaling allows for the expansion of selected, foreign antigen-specific B cells, and deletion of unwanted, self-

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reactive B cells[9]. In B cell malignancies, such as CLL[10–13] and diffuse large cell B cell lymphoma (DLBCL)[14], BCR signaling plays a critical role in pathogenesis, even though the mechanisms of BCR stimulation are heterogeneous and to some degree controversial[15–19]. Antigen-dependent and -independent BCR activation are two fundamentally different mechanism of BCR pathway activation which exist in B cell lymphomas. For example, there can be activating mutations in the BCR pathways, such as mutations in the coiled-coil (CC) domain of CARD11 in DLBCL, gastric lymphoma, and primary central nervous system lymphoma, or activating mutations of the immunoreceptor tyrosine-based activation motifs (ITAMs) within the CD79B and CD79A signaling modules of the BCR, as in ABC DLBCL[14, 16]. In CLL, on the other hand, BCR pathway activation does not appear to involve activating pathway mutation and therefore can be viewed as antigen-dependent, resulting from BCR ligation via antigens (auto-antigens and/or microbial antigens) that are present in the microenvironment. Recent studies raise an additional target of BCR recognition, i.e., the binding of an intrinsic IGHV motif[15, 20]. There are several sets of indirect data suggesting the importance of BCR signaling in CLL; the most compelling are comparative gene expression profiling (GEP) data that revealed BCR signaling as the most prominent pathway activated in CLL cells isolated from areas of proliferation within the lymphatic tissues[21]. In this review we discuss mechanisms of BCR activation in CLL and the potential for targeting BCR signaling with novel inhibitors of BCR-associated kinases BTK, SYK, and PI3Kδ.

BCR activation in the CLL: mechanism and relationship with prognostic markers

The BCR is composed of a ligand binding moiety, the antigen-specific surface membrane immunoglobulin (smIg), paired with the signal transduction moiety, Ig-α/Ig-β heterodimers (CD79A, CD79B, see Figure 1). Engagement of the BCR by antigen induces membrane movement and aggregation of BCR components that lead to phosphorylation of ITAMs in the cytoplasmic tails of CD79A and CD79B. The latter is accomplished by Lyn and other Src family kinases (Fyn, Blk), which in turn activate SYK, BTK, and PI3Ks (Figure 1). BCR oligomerization and BCR microcluster growth[22] activate downstream pathways, including calcium mobilization, MAP kinases and RAS activation, activation of phospholipase Cγ, protein kinase C β (PKCβ), and CARD11, causing recruitment of BCL10 and MALT1 into a multiprotein “CBM” complex that activates IκB kinase (IKK), thereby initiating NF-kB signaling[14, 16]. Collectively, these signaling events promote B cell survival and proliferation [16].

Several prognostic markers in CLL, such as IgV_H mutational status[23, 24], ZAP-70[25, 26] and CCL3[27] are associated with (auto)antigen binding and function of the BCR, suggesting a relationship between enhanced BCR signaling and worse prognosis. Also, BCRs in CLL patients are characterized by a biased usage of *IGHV* and *IGLVκ/λ* genes, which differ from those of normal B cells. Oftentimes, specific *IGHVs* partner with specific *IGHD-Js* and specific *IGLVs* with specific *IGLJs*, leading to remarkably similar, “stereotyped” HCDR3s and smIgs[28, 29]. These data support the concept of antigen-driven selection and expansion of CLL clones, and they suggest that recurrent binding of restricted sets of antigenic epitopes are linked to the selection of those normal B-cells clones that enter the CLL pathogenetic process[11, 30–32]. More evidence for the importance of BCR signaling in CLL comes from comparative GEP data that revealed BCR signaling as the most prominent pathway activated in CLL cells isolated from lymphatic tissues[21]. Along the same lines, cells from those patients with the worse outcome (e.g., U-CLL) display GEPs identifying activation of genes downstream of the BCR[33].

IGHV mutational status

The antigen binding sites of Igs derive from recombination of V, D, J gene segments for the heavy chains and V and J segments for the light chains, which subsequently can be modified by somatic mutation to higher affinity after antigen engagement, with or without T cell help. Based on the degree of somatic hypermutation of the Ig heavy chain variable gene segments (*IGHV*s), patients can be classified as "unmutated" (U-CLL), if they have 98% or more sequence homology with the germline sequence, or "mutated" (M-CLL) cases, if they have less than 98% sequence homology (Table 1)[34]. U-CLL cases have a more unfavorable clinical course and shorter survival, whereas M-CLL typically have slower disease progression and longer survival[23, 24]. These findings supported the concept that CLL may consist of two distinct diseases with different clinical presentation, disease biology, and cellular origin[30, 35]. However, because GEP revealed that both M-CLL and U-CLL B cells have similar, largely overlapping GEPs, albeit highly different from normal B cells[33, 36], a single cellular origin for M-CLL and U-CLL cases seemed necessary. This implies that the differences in clinical behavior between M-CLL and U-CLL are determined by differences in responsiveness to external signals (such as BCR-responsiveness). More specifically, M-CLL cells are thought to be selected and expanded by high-affinity binding to restricted sets of antigenic epitopes (Figure 2) that either occur infrequently because they are on foreign antigens and/or because they induce anergy due to high-affinity binding[30–32]. Consequently, the M-CLL clone remains overall stable or expands at a slower rate. Examples for the restricted reactivity of BCRs from M-CLL are binding to the Fc-tail of IgG ("rheumatoid factors")[37–40], or to β-(1,6)-glucan, an antigenic determinant of yeasts (Figure 2)[41]. In contrast, U-CLL patients express polyreactive low-affinity BCRs that can recognize various environmental and auto-antigens, such as vimentin, myosin}, or rheumatoid factors[37–40, 42–47] present in the tissue microenvironment. BCR binding can occur more frequently in U-CLL, which could lead to increased BCR signaling capacity that is related to higher ZAP-70 expression[48, 49].

ZAP-70

ZAP-70 is a cytoplasmic protein tyrosine kinase (PTK) that initially was identified in T cells and subsequently in activated but not resting normal human B cells[50–52], suggesting that its presence in B lymphocytes is a consequence of cell stimulation. In T lymphocytes, ZAP-70 is intimately involved in T-cell receptor (TCR) signaling; in B lymphocytes, ZAP-70 can have a role in BCR signaling. It is structurally similar to the B-cell signal transduction molecule, SYK. Following TCR activation, ZAP-70 is recruited to ITAMs and becomes activated via tyrosine phosphorylation, leading to downstream launching of survival and proliferation pathways. ZAP-70 is also expressed in a subset of CLL patients[25, 33]. Because ZAP-70 is one of the most prominent genes distinguishing M-CLL from U-CLL by gene expression profiling (GEP)[25, 33] and its detection as a protein in U-CLL cells, it can be used as a surrogate marker for U-CLL[26, 53] and as an important prognostic factor in CLL[25, 48]. Patients with ZAP-70 expression in more than 20% of CLL cells have a relatively shorter median time from diagnosis to initial treatment[53], and ZAP-70 appears to be a stronger risk factor for aggressive CLL than a lack of *IGHV* mutations[53]. ZAP-70 expression in CLL is associated with increased BCR signaling capacity[48], which is not dependent on ZAP-70's tyrosine kinase activity and could be due to adapter protein function in BCR signaling or its ability to interact with c-Cbl[54, 55]. ZAP-70 expression is also associated with greater responsiveness to the chemokines CCL19, CCL21, and CXCL12[56–58], resulting in greater CLL cell migration and activation of survival-associated signaling in ZAP-70^{+} CLL. These findings are similar to findings related to CD38 and U-CLL, suggesting that CD38, U-CLL, and ZAP-70 label CLL clones with a higher capacity for homing to the tissue compartment in response to chemokines[56, 58,

59],where such clones then become stimulated, being particularly responsive to external signals such as those delivered by the BCR.

CCL3 and CCL4

CCL3 and CCL4, previously called Macrophage Inflammatory Proteins-1 alpha and beta (MIP-1α,β) are chemokines of the CC subfamily and inducible in hematopoietic cells involved in adaptive immune responses (macrophages, dendritic cells, B and T lymphocytes). CCL3 is a novel, robust and independent prognostic marker in CLL that can easily and reliably be measured by ELISA. CCL3 plasma concentrations in CLL patients are strongly associated with established prognostic markers and independent prognostic markers for time to treatment[60]. Both CCL3 and CCL 4 are members of a cluster of cytokines associated with worse clinical outcome in CLL[61]. CCL3 signals through the chemokine receptors CCR1 and CCR5, whereas CCL4 signals only through CCR5. CCL3 and CCL4 function as chemo-attractants for monocytes and lymphocytes[62]. Previous studies established that CCL3 is a key response gene upregulated in normal and neoplastic B cells in response to BCR signaling[21, 63–65] and repressed by Bcl-6[66]. CLL cells upregulate and secrete CCL3 and CCL4 in response to BCR stimulation and in co-culture with NLC[65], a model system resembling the lymphatic tissue microenvironment[2, 65]. This BCR- and NLC-dependent induction of CCL3 and CCL4 is sensitive to inhibition of BCR-signaling, using SYK-[67, 68], BTK-[69], or PI3Kδ[70] inhibitors, both *in vitro* and *in vivo*. The exact function of CCL3 in lymphomagenesis remains unclear. Based on the postulated function of B cell-derived CCL3 in normal immune responses, increased CCL3 secretion by CLL cells may cause attraction and homing of accessory cells to the malignant B cells in the tissue microenvironments[65, 71]. It is well recognized that CLL cells in the proliferative compartment are interspersed with T cells[4, 72] and cells of monocyte/macrophage lineage, termed NLC[65]. Conceivably, CLL cell-derived CCL3 may attract these accessory cells, thereby creating a favorable microenvironment which allows CLL cells to interact with T cells and NLC to receive survival- and proliferation-signals. This is supported by *in vitro*[63] and *in vivo*[73, 74] studies which indicated that normal B cell activation within lymphoid tissues results in CCL3 and CCL4 secretion, leading to the recruitment of $CCR5^+$ regulatory T cells for cognate interactions with B cells and antigen presenting cells (APCs) [73, 74]. In ongoing clinical trials with new agents targeting the BCR pathway (SYK-, BTK-, and PI3Kδ-inhibitors), increased levels of CCL3 and CCL4 rapidly normalized after initiation of therapy with the BTK-inhibitor ibrutinib[69] and the PI3Kδ-inhibitor idelalesib[70].

BCR signaling in CLL

Normal B lymphocytes depend on the presence of an intact BCR signal transduction pathway for survival[75], and this is likely due to a combination of “tonic” (antigen-independent) and antigen-induced BCR signaling[75, 76]. These same two types of BCR signaling impact on the survival and growth on malignant B-cell clones in CLL and in other non-Hodgkins lymphomas (NHLs), although the relative contributions of these types of signals in these two classes of B-cell lymphoproliferative disorders is debatable. Two findings favor tonic stimulation being dominant in CLL: [1] unmanipulated primary CLL B cells display phosphorylated Lyn[77], Syk[78], ERK[79] and subunits of NFkB[80]; and [2] changes in IgM glycosylation after surrogate antigen engagement, which retards endocytosis, extends surface membrane display, and might allow interactions with lectins in/on the stroma [47]. However it remains unclear if each of these is a primary event or secondary to ongoing BCR engagement by autoantigens present in the microenvironment or on the cells themselves. Finally, an experimental model using transgenic over-expression of MYC leads to a CLL-like disease that appears independent of antigen[81]. Although this

model generates a disease of $CD5^{-}$ murine B cells that originate in the spleen and subsequently populate lymph nodes and bone marrow, it and related models[81] emphasize the cooperation of tonic and antigen-induced BCR signals and gene overexpression (MYC) in the development of CLL versus NHL. In opposition to tonic signaling being dominant in CLL is the absence[82] or rare presence[83] of mutations in BCR signaling components, which may enhance tonic or antigen-induced BCR-mediated signals, as have been identified in DLBCL[14]. Furthermore, the strong bias in *IGHV* gene use[34] and the association of certain discrete *IGHV*s with specific *IGHD* and *IGHJ* segments[28, 29] ("stereotyped BCRs") in CLL mediate against a global enhancement in BCR signaling that is independent of antigen-BCR engagement and selection.

Regarding the latter, polyreactive BCRs from U-CLL patients can recognize various autoantigens and other environmental or microbial antigens[37–40, 42–47]. For example, CLL BCRs can bind cytoskeletal non-muscle myosin heavy chain IIA and vimentin, as well as the Fc-tail of IgG, ssDNA, or dsDNA, LPS, apoptotic cells, insulin and oxidized LDH. In addition, microbial antigens, such as on bacteria and fungi, can be targeted. For example, Hoogeboom *et al*. recently described a small subset of M-CLL patients expressing *IGHV3–07* with short third complementary determining regions of the IG heavy chain variable domain (HCDR3) sequences (designated "V3–7Sh") with high-affinity for a major antigenic determinant of yeasts and filamentous fungi, β-(1,6)-glucan[41]. Interestingly, β-(1,6)-glucan also promotes the proliferation of V3–7Sh CLL cells, suggesting that BCR stereotypy in CLL results from antigen selection and affinity maturation and that ubiquitous antigens, such as β-(1,6)-glucan and auto antigens, could promote the expansion of certain CLL clones via antigen/pathogen-specific BCR signaling. Thus, (auto)antigen recognition, binding, and subsequent signaling through smIg likely leads to B-cell survival and proliferation in human CLL.

In addition, a recent study demonstrated an interesting and unexpected form of auto-reactive BCR activation in CLL[15]. When CLL BCRs were expressed by retroviral gene transfer in mouse cells lacking endogenous components of the BCR, binding of the HCDR3 to an epitope in the second framework region (FR2) led to induction of Ca^{++} signaling (Figure 2A). This *in vitro* finding could relate also to the presence of phosphorylated Lyn and Syk and subunits of NFkB seen in CLL cells[77, 80, 84], although it does not appear to account for clinical differences between M-CLL and U-CLL, as the same process occurred with BCRs of both CLL subsets[15], or for the lack of CLL cell proliferation in the absence of external BCR stimulation[41]. Along the same lines, Binder *et al*. reported an alternative epitope for CLL BCR self-recognition located in FR3 of Igs[20]. Thus, it appears that recognition of structural Ig determinants may play a key role in BCR signaling in CLL. This feature appears to be characteristic of Igs made by CLL cells and not by tumor cells from other B-cell lymphoproliferative disorders[15], and is consistent with the ability of CLL BCRs to bind Ig components and act like "rheumatoid factors"[37–40]. This also supports the notion that the development of CLL involves the selection of normal B-cell clones with distinct BCR structures that often bind limited types/classes of autoantigenic epitopes[30].

The two types of autoantigen-mediated BCR signaling in CLL appear to have common and distinct features[17]. For example, both result from autorecognition: one binding non-Ig determinants of exo- or autoantigens that are likely multimeric in nature such as on microbes or apoptotic cells, and the other reacting with mono/oligomeric autoepitopes existing on adjacent or in the same Ig(s). The two signal-initiating events, however, probably differ as well. Reactivity with Ig autoepitopes could occur in all members of the clone on a continuous basis, because the cell carries the targets of smIg binding and consequent BCR activation with it (its smIg). This would in effect be "constitutive" signaling, but not because of potential pathway mutations as can occur in NHL but rather as the result of ongoing BCR

crosslinking. In contrast, reactivity with non-Ig epitopes would likely occur most often and most effectively at sites where these autoantigens exist or are made (e.g., the tissue microenvironment where cellular apoptosis or molecular catabolism occurs). For these reasons, it is unlikely that all members of a CLL clone would be involved in this type of binding and hence stimulation; rather this would occur more readily for those clonal members residing in solid tissues and possibly more often in certain types of solid tissues (e.g., lymph nodes > bone marrow)[21]. Hence this form of CLL cell signaling would be recurrent, albeit intermittent for an individual cell within the clone. Finally, the consequences on CLL cell function of these two types of autoantigen binding might differ. For non-Ig binding events, which could be of higher affinity because of their multimeric nature and could be of longer duration if they occurred in solid tissues, this would more likely lead to both survival and growth signals. For Ig binding, which would be of a more oligo/monomeric nature and continuous, this might lead to a "tolerogenic" signal and anergy, which could nevertheless result in longer cell survival[85].

Thus, CLL cells could receive both continuous and intermittent BCR-mediated signals that would facilitate cell survival and expansion. The relative frequency that the latter occurred would likely be one of the factors determining the birth rates of individual clones, and hence their pathogenicity. The proposed sequence of (initial) antigen-driven CLL B-cell expansion, with clonal selection and evolution of exo-antigen-independent clones that can persist and subsequently expand, is an interesting hypothesis to integrate these two different models[30, 32, 35, 86, 87].

Targeting BCR signaling in CLL

For therapeutic purposes, BCR signaling could be abrogated on different levels, for instance by antigen deprivation, by interference with antigen binding, or by disruption of BCR downstream signaling. The promiscuity of U-CLL BCRs for binding to different antigens, and the ubiquitous presence of many of the BCR ligands in the CLL microenvironment complicate the first two approaches, which at this time are not being pursued. In contrast, targeting of BCR signaling has become a field of major interest and explosive drug development. The lead compounds in this arena in CLL are kinase inhibitors that target the BCR-associated kinases BTK, PI3Kδ, and SYK (Table 2). Development of therapeutic kinase inhibitors has largely focused on ATP-competitive compounds that target the ATP binding site of protein kinases[88]. However, key challenges in the development of such kinase inhibitors have been relatively poor selectivity, and binding site competition due to high concentrations of endogenous ATP substrate. Covalent, irreversible inhibitors, such as the BTK inhibitor ibrutinib, are an alternative, because they exhibit high selectivity, prolonged pharmacodynamics, and potency in overcoming endogenous ATP competition[89]. A largely restricted expression of these enzymes in B cells confers target selectivity that is geared towards CLL B cells, but it is currently unknown how normal B cells are affected by use of these novel agents. Moreover, BTK, PI3Kδ, and SYK are expressed by other hematopoietic cells, albeit generally at lower levels, and therefore non-B cell effects of these kinase inhibitors (which can be beneficial and/or result in side-effects) might occur. Finally, we need to keep in mind that the specificity of a kinase inhibitor is never absolute, and, to make things even more complex, BTK, PI3Kδ, and SYK participate in signaling involved in diverse pathways. This complexity makes data interpretation about mechanism of actions and clinical responses to these drugs difficult. For example, the BCR kinase inhibitor-associated lymphocytosis in CLL due to CLL cell redistribution appears to be a class effect of each of these novel drugs[70, 90, 91] and could be interpreted as a non-BCR "off-target" effect, given that BCR blockade is not expected to result in such dramatic interference with retention/homing mechanisms. Indeed, BTK, PI3Kδ, and SYK are components of chemokine- and integrin receptor-signaling in normal B cells and CLL

cells[69, 92, 93], and hence this phenomenon actually can be considered an “on-target” non-BCR effect.

Bruton’s tyrosine kinase, BTK

BTK is a non-receptor tyrosine kinase of the Tec kinase family that plays a central role in BCR signaling. BTK is primarily expressed in hematopoietic cells, particularly in B cells and not in T cells or plasma cells[94]. BTK is of critical importance for B cell development, as demonstrated by the absence of blood B cells in patients with X-linked agammaglobulinemia (XLA), and reduced mature B cell numbers in the murine counterpart, X-linked immunodeficiency (xid, see Table 2). Both XLA and xid result from deficient BTK function due to BTK mutations[95–98]. Upon BCR activation, BTK becomes activated by other tyrosine kinases, such as Lyn and SYK, resulting in activation of transcription factors necessary for B-cell proliferation and differentiation[99]. In addition to its role in BCR signaling, BTK is involved in signaling of other receptors related to B cell migration and adhesion, such as chemokine receptors (CXCR4 and CXCR5) and adhesion molecules (integrins)[92, 100, 101].

Ibrutinib, previously called PCI-32765, is the first in human BTK inhibitor. The drug binds irreversibly to a cysteine residue (Cys-481) in the BTK kinase domain and inhibits BTK phosphorylation and its enzymatic activity[102]. Pre-clinical activity of ibrutinib in B cell lymphoma was first demonstrated in a canine B-cell lymphoma model[102]. Next, Herman *et al*. reported that ibrutinib induced CLL cell apoptosis in the presence of pro-survival factors (CD40L, BAFF, IL-6, IL-4, TNF-α, fibronectin, stromal cell contact)[103]. We reported that ibrutinib inhibits CLL cell survival and proliferation, as well as leukemia cell migration towards the tissue homing chemokines CXCL12 and CXCL13[69]. We also found that ibrutinib downregulated secretion of BCR-dependent chemokines (CCL3, CCL4) *in vitr*o and in CLL patients receiving ibrutinib. Furthermore, ibrutinib effectively thwarted disease progression in the TCL-1 mouse model of CLL[69]. De Rooij and colleagues reported ibrutinib’s interference with CLL cell chemotaxis and integrin-mediated CLL cell adhesion[93], suggesting that these BCR-independent actions of ibrutinib explain the redistribution of CLL cells from the tissues into the peripheral blood. In DLBCL, ibrutinib demonstrated selective toxicity in cell lines with chronic active BCR signaling[14], down regulating IRF4 and synergizing with lenalidomide in killing of activated B cell-like (ABC) subtype DLBCL cells[104].

The most mature clinical data about effects of ibrutinib on B cell malignancies are available for patients with CLL, MCL, and DLBCL. At a fixed dose of 420 mg orally daily, ibrutinib induces full BTK occupancy[91]. In CLL patients, ibrutinib induces lymphocytosis during the first weeks of therapy, which is variable among patients but directly related to the presence of the drug[91]. This lymphocytosis is due to the re-distribution of CLL cells from the tissue compartments into the peripheral blood[91, 105]; it is asymptomatic, and resolves in most patients during the first months of therapy. Byrd et al. recently reported that single-agent ibrutinib induces an overall response rate (ORR) of 71% in CLL patients with relapsed or refractory disease, and an additional and 15% to 20% of patients had a partial response with lymphocytosis[106]. The response was independent of clinical and genomic risk factors present before treatment. At 26 months, the estimated progression-free survival rate was 75% and the rate of overall survival was 83%. Wang et al. reported single-agent efficacy of ibrutinib in patients with relapsed or refractory in MCL with a complete response rate of 21% and a partial response rate of 47%[107]. The most common treatment-related adverse events were mild or moderate diarrhea, fatigue, and nausea. Grade 3 or higher hematologic events were infrequent and included neutropenia, thrombocytopenia, and anemia. The estimated median response duration was 17.5 months, the estimated median progression-free

survival was 13.9 months, and the estimated rate of overall survival was 58% at 18 months. Phase 3 clinical trials of ibrutinib in CLL and MCL are ongoing.

Phosphoinositide 3'-kinase delta, PI3Kδ

PI3Ks integrate and transmit signals from different surface molecules, such as the BCR[108], chemokine receptors, and adhesion molecules, thereby regulating cellular functions such as cell growth, survival, and migration[109]. PI3Ks are divided into 3 classes (I, II, III). The class I kinases contain four isoforms designated PI3Kα, β, γ, and δ. While the PI3Kα and β isoforms are ubiquitously expressed, the PI3Kγ and δ isoforms are limited to hematopoietic cells, with PI3Kγ having a role in T-cell activation and PI3Kδ playing a critical role in B-cell homeostasis and function[110]. Mice with inactivating PI3Kδ mutations have reduced numbers of B1 and marginal zone B cells, low levels of immunoglobulins, poor responses to immunization, and defective BCR and CD40 signaling; these animals can develop inflammatory bowel disease[110, 111]. In CLL cells, PI3K are constitutively activated[112], and *IGHV* unmutated, high-risk CLL patients show overexpression of PI3K by quantitative polymerase chain reaction[113]. Furthermore, growth and survival signals from the microenvironment, such as adhesion to stromal cells[114] and CXCR4 [115] and BCR [116] engagement, cause PI3K activation in CLL cells.

Idelalesib (GS-1101), previously called CAL-101, is a potent and highly selective PI3Kδ inhibitor that is the first PI3Kδ inhibitor in clinical use[117]. Idelalesib induces apoptosis in B-cell lines and primary cells from patients with different B-cell malignancies, including CLL[118], MCL, and multiple myeloma[117, 119]. Idelalesib also inhibits constitutive and CD40-, TNF-alpha-, fibronectin-, and BCR-induced PI3K activation[117, 118][119]. Idelalesib inhibits CLL cell chemotaxis towards CXCL12 and CXCL13 and migration beneath stromal cells (pseudoemperipolesis)[70]. These *in vitro* results are corroborated by clinical data showing marked reductions in circulating CCL3, CCL4, and CXCL13 levels, paralleled by a surge in lymphocytosis during GS-1101 treatment[70]. Therefore, it appears that GS-1101 has several mechanism of action, directly decreasing cell survival while reducing interactions that retain CLL cells in the tissue microenvironments. The promising early-stage clinical trials of idelalesib are being further pursued in Phase 3 clinical trials in patients with CLL.

Spleen tyrosine kinase, SYK

SYK belongs to the SYK/ZAP-70 family of non-receptor kinases, and activates signaling pathways downstream of the BCR. SYK-deficient mice have severely defective B lymphopoiesis[120, 121], with a block at the pro-B to pre-B transition, consistent with a key role for Syk in pre-B-cell receptor signaling. Moreover, *in vivo* studies recently demonstrated that SYK is critical for survival and maintenance of mature normal and malignant B cells[120, 122]. Besides their role in immune responses, SYK activation also modulates cell adhesion and chemotaxis of normal cells, including B cells[123, 124], suggesting that SYK participates in tissue homing and retention of activated B lymphocytes.

R788 (fostamatinib disodium, FosD) is the only SYK inhibitor in clinical use to date. Fostamatinib, the clinically used oral formulation, is a prodrug that rapidly converts *in vivo* into the bioactive form called R406[125, 126]. Previous studies established that R406 is a relatively selective SYK inhibitor, although R406 has some activity on other kinases including Flt3, Jak, and Lck[126]. After encouraging results in a Phase I/II study in patients with relapsed B cell lymphomas, particularly in patients with CLL where the objective response rate was 55%[90], further development of this drug was focused on rheumatoid arthritis (RA)[127]. As such, there is at this time only one ongoing clinical trial of

fostamatinib in patients with DLBCL. Alternative SYK-specific inhibitors are under development and have demonstrated promising pre-clinical activity in CLL models[68]. Importantly, similarities in clinical response patterns of CLL patients to treatment with a SYK-, BTK-, or PI3Kδ inhibitors (transient lymphocytosis and rapid lymph node shrinkage) suggest overlapping functions of these kinases in CLL by inhibiting BCR signaling, and cell migration and homing[105].

The transient lymphocytosis caused by these new agents has complicated response assessment in these patients, given that progressive lymphocytosis in isolation is formally interpreted as progressive disease (PD). However, given that CLL patients on these drugs typically show other signs of response (reduced lymph node sizes, normalization of hemoglobin and platelet counts, resolution of constitutional symptoms) even before stabilization and then resolution of lymphocytosis, this lymphocytosis in the absence of other signs of PD should not be confused with true PD. This interpretation was supported by a group of CLL experts[128], and CLL response criteria may need to be formally revised when these new agents become more widely used.

Concluding remarks

After decades of exciting, fruitful research about BCR structure and signaling and their relation to prognosis, the CLL field recently transformed due to the success of early-stage clinical trials with kinase inhibitors designed to target BCR signaling kinases (BTK-, PI3Kδ-, and SYK-inhibitors). In addition, a series of research projects have shed new light onto the mechanisms of BCR activation in CLL. These discoveries will impact CLL therapy in a broad sense, with an expected major push for these new, more targeted therapeutic agents, along with a deeper understanding about the role of BCR signaling in CLL pathophysiology. As with any major discovery, these new concepts challenge us with new questions. For example, the clinical response to this class of kinase inhibitors in CLL initially is puzzling, given that it is characterized by an early resolution of enlarged lymph nodes and organs (i.e. spleen), accompanied by lymphocytosis due to redistribution of tissue-resident CLL cells into the blood during the first few months of therapy. This unexpected finding points away from the BCR and toward functions of these same kinases in pathways outside the BCR. At the same time, and more in line with BCR signaling inhibition, these kinase inhibitors also thwart CLL cell proliferation and possibly survival, although we are only beginning to learn about the relative contribution of each of these functional aspects of BCR-associated kinases for CLL cells. Collectively, BCR activation and signaling in CLL has become one of the most exciting translational fields in biomedicine and, in a broader sense, a role model for translational cancer research. The clinical findings now need to be taken back to the bench in order to better define the critical mechanism(s) of action of this fascinating new class of drugs.

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Highlights

In CLL, the BCR is activated by antigens in the tissue microenvironment

CLL BCRs can recognize autoantigens, microbial antigens, intrinsic IGHV motifs

BCR activation promotes CLL cell maintenance and expansion

Inhibitors of the BCR-associated kinases BTK and PI3Kδ are in Phase 3 clinical trials in CLL and MCL

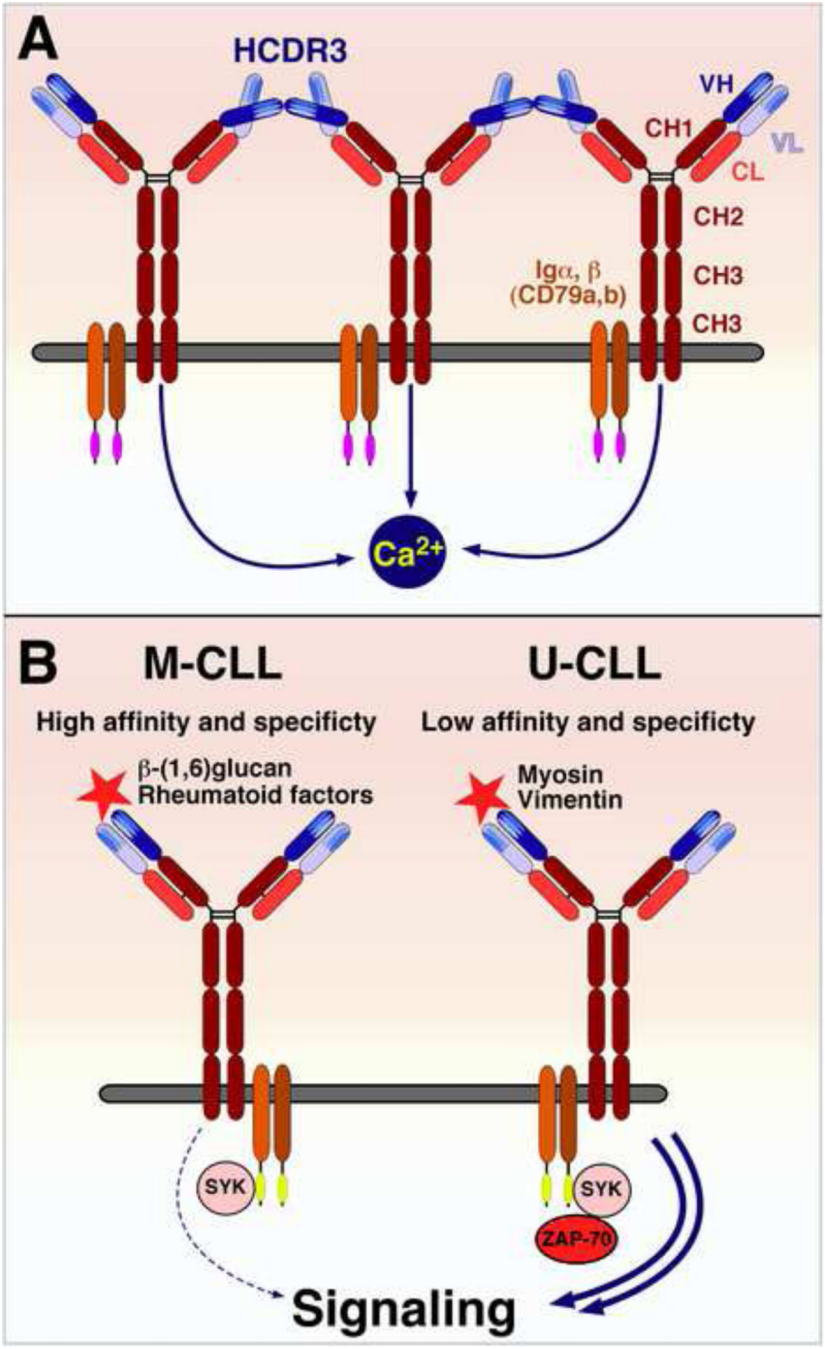

Figure 1. BCR components and upstream signaling

The BCR consists of the antigen-binding heavy chains IgH (VH and CH1-4) and light chains IgL (VL and CL) that are noncovalently coupled to the Ig-α (CD79A) and Ig-β (CD79B) signaling subunits. Antigen encounter and BCR clustering promotes tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAMs) by Src-family kinases LYN, FYN, and BLK. Phosphorylated ITAMs then recruit SYK through interactions with its SH2 domains. SYK activation triggers activation of a signaling cascade that engages BTK, PI3Ks (including PI3Kδ), NF-κB, PI3K, NF-AT, MAP kinase, and RAS signaling pathways, leading to cell survival and proliferation. Upstream BCR signaling

kinases can be targeted by the small molecule SYK inhibitor fostamatinib [90], the BTK inhibitor ibrutinib[91], and the PI3Kδ inhibitor idelalisib[117].

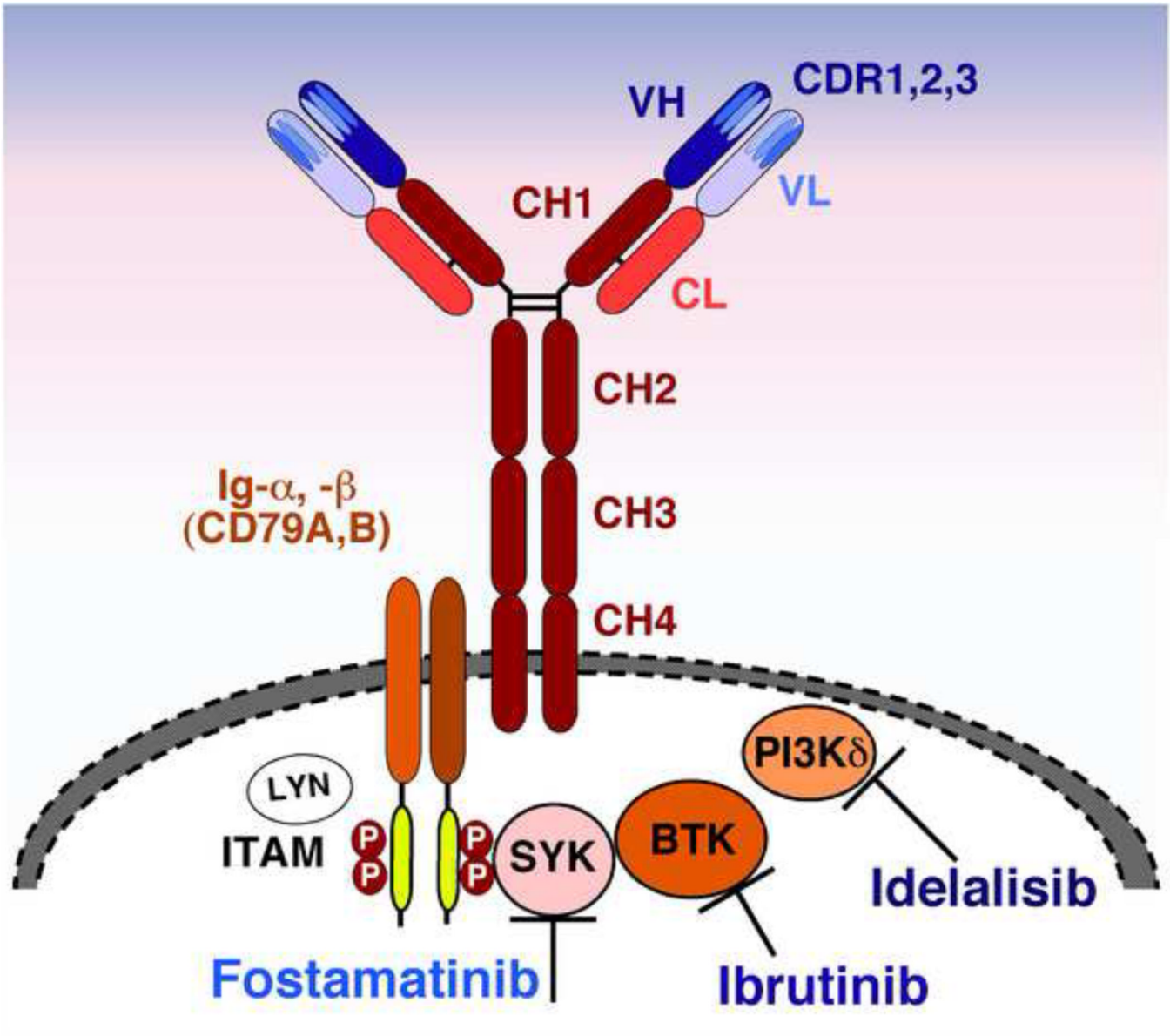

Figure 2. Mechanism of BCR activation in CLL

(A) BCR signaling can be activated in an exo-antigen-independent fashion, for example by interactions between the third complementary determining region of the IG heavy chain variable domain (HCDR3) with an epitope in the second framework region (FR2) of either the same smIg or an adjacent smIg. The latter would lead to calcium signaling[15]. (B) Exo-antigen-dependent activation of the BCR in CLL can occur in an antigen-restricted, specific and high-affinity fashion in M-CLL, or in a less restricted, low-affinity fashion (U-CLL). Examples of CLL BCR ligands are given next to each of the red stars, which represent the BCR ligand. BCR signaling in U-CLL is further amplified in the presence of ZAP-70.

Table 1

BCR-related risk factors in CLL

	Cut off	Method of detection	Pros and cons	Prognostic value in CLL
IGHV mutational status	98% deviation from germline sequence	PCR amplification and sequencing	Does not change over the course of the disease, expensive, time-consuming	"unmutated" (U-CLL, ≥ 98% sequence homology = shorter PFS and OS; "mutated" cases (M-CLL, <98% sequence homology) = longer PFS and OS
CD38	Threshold is controversial (5%, 7%, 20% and 30% cut-offs)	FACS	Relatively inexpensive, now part of routine immunophenotyping, CD38 expression may vary over time	Not standardized and validated in prospective trials
ZAP-70	Threshold is controversial, 20% positive CLL cells by FACS is commonly used	Western blotting, quantitative RT-PCR, immunohisto chemistry, and FACS	Relatively inexpensive, stable over time, difficult to standardize (quantification of cytoplasmic staining)	Not standardized and validated in prospective trials
CCL3, CCL4 (MIP-1 α,β plasma levels	10 pg/mL threshold for CCL3; 60 pg/mL threshold for CCL4	ELISA	Inexpensive, reliable and reproducible. Levels can change over time and can be influenced by non-CLL related events, such as inflammation	High levels correlate with shorter PFS, CCL3 levels rapidly normalize during therapy with inhibitors targeting BCR-associated kinases (BTK[69], PI3Kδ[70]), not validated in prospective trials

Table 2

Expression and function of BCR-associated kinase

Kinase	Structure	Gene deletion/mutation	Activating receptors in B cells	Function in CLL	Inhibitor(s)
Spleen tyrosine kinase (SYK)	Non-receptor tyrosine kinase that is structurally related to ZAP-70. Contains a kinase domain and two N-terminal SH2 domains that bind p-Tyr residues in ITAMs	In mice: severe defect of B lymphopoiesis, with a block at the pro-B to pre-B transition; blood-lymphatic shunts[130]	BCR, integrins[131]	CLL cell survival and migration via BCR- and chemokine receptor -signaling[67, 68]	Fostamatinib (R406/R788)[127], Portola compounds (P142-76 and P505-15)[68]
Bruton's tyrosine kinase (BTK)	Non-receptor tyrosine kinase, member of the Tec family of kinases	In humans: X-linked agammaglobuline mia (XLA, Bruton's agammaglobuline mia), normal pre-B cells in the marrow which fail to mature and enter the blood; in mice: X-linked immunodeficiency (xid)	BCR, integrins, chemokine receptors[93]	CLL cell survival, proliferation[69,104], and migration[69,94], BCR signaling, chemokine secretion (CCL3, CCL4) [69]	Ibrutinib (PCI-32765)[103], Avila compounds (AVL-292)
PI3Kδ	Class IA PI3K, activity controlled by associated p85α, p85β, p55α, or p50α subunits	In mice: lack of B1 cells and marginal zone B cells and deficient antibody responses,	BCR, integrins, chemokine receptors	CLL cell survival and migration[70, 119], chemokine secretion (CCL3, CCL4)	Idelalisib (GS-1101)[118], Infinity compounds (IPI-145)