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The importance of B cell receptor isotypes and stereotypes in Chronic Lymphocytic Leukemia

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Abstract

B cell receptor (BCR) signaling is a central pathway promoting the survival and proliferation of normal and malignant B cells. Chronic lymphocytic leukemia (CLL) arises from mature B cells, expressing functional BCRs, mainly of IgM and IgD isotypes. Importantly, 30% of CLL patients express quasi-identical BCRs, so-called “stereotyped” receptors, indicating the existence of common antigenic determinants, which may drive disease initiation and favor its progression. Although the antigenic specificity of IgM and IgD receptors is identical, there are distinct isotype-specific responses after IgM and IgD triggering. Here, we discuss the most important steps of normal B cell development, and highlight the importance of BCR signaling for CLL pathogenesis, with a focus on differences between IgM and IgD isotype signaling. We also highlight the main characteristics of CLL patient subsets, based on BCR stereotypy, and describe subset-specific BCR function and antigen binding characteristics. Finally, we outline the key biologic and clinical responses to kinase inhibitor therapy, targeting the BCR-associated Bruton tyrosine kinase (BTK), phosphoinositide-3-kinase (PI3K), and spleen tyrosine kinase (SYK) in patients with CLL.

The B cell receptor (BCR) during B cell development

B lymphocytes develop from hematopoietic stem cells through a continuum of developmental stages that originate within the primary lymphoid tissues (i.e. fetal liver and fetal/adult marrow), with later stages of maturation occurring in secondary lymphoid organs, including the lymph nodes and the spleen (Figure 1).[1] One of the first essential steps towards maturation of a normal B cell is the successful rearrangement of immunoglobulin (Ig) heavy chain (IGH) gene segments (V, D and J segments), during a process named VDJ recombination (Figure 2), which occurs in progenitor (pro)-B cells, and leads to precursor (pre)-B-cell development.[2] During this process, a highly diverse repertoire of antigen-

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binding HCDR3 regions[3] is generated, a key determinant for the antigen specificity of the developing BCR. Pre-B cells express an immature BCR, termed pre-B cell receptor (pre-BCR), which is composed of fully rearranged heavy chains and "surrogate" light chains. During this stage of differentiation, the rearrangement of the immunoglobulin light (IGL) chain V and J gene segments takes place, allowing for the expression of a complete BCR on the surface of immature B cells, expressing heavy chains of the M isotype (i.e. IgM). The complete BCR molecule includes two heavy chains and two light chains, which associate with two Igα/Igβ subunits (i.e. CD79a and CD79b), which are necessary for signal transduction and indispensable for B cell survival.[4] VDJ recombination is an error-prone process, which generates a high number of BCRs (up to $3*10^{11}$), including some with reactivity towards self-antigens. These autoreactive B cells normally are negatively selected and undergo apoptosis, while cells expressing non self-reactive BCRs may proceed further in development, and acquire expression of surface IgDs (i.e. immunoglobulins carrying heavy chains of the D isotype), with the same specificity as IgM, resulting in mature B cells expressing both, IgM and IgD.[5] After antigen encounter in the periphery, B-cell activation and differentiation in secondary lymphoid tissues (i.e. lymph nodes, spleen) occurs in specialized structures, named germinal centers (GC), where B cell clonal expansion and somatic hypermutation (SHM) of the variable regions of both heavy and light chain genes takes place (Figure 1). While most of the somatic mutations introduced by SHM reduce the affinity of the BCR for the stimulating antigen and result in cellular apoptosis, in a minority of cases antigen affinity increases, and such B cells are positively selected for further differentiation into memory B cells or antibody-secreting plasma cells.[5] Affinity selection occurs after direct recognition of antigens exposed on the surface of follicular dendritic cells (FDC),[6] a cellular component of GCs, and positive selection of BCRs with the highest affinity for foreign antigens devoids the IgM^+ memory B cell pool from autoreactive B cells, which would otherwise increase the risk for autoimmunity.[7] In addition to the SHM process, B cells can diversify their receptors during a process named class switch recombination (CSR), which also occurs within the GCs, allowing the generation of BCRs that carry heavy chains of different isotypes than IgM and IgD. The immunoglobulin heavy chains constant regions μ (IgM) and δ (IgD), are substituted by either γ, ε, or α heavy chains, generating IgG, IgE and IgA isotypes, which are characteristically involved in responses to viruses and bacteria (IgG), parasites (IgE), and mucosal microbes (IgA) (for a more complete review of IgG, IgE and IgA isotype functions please refer to [8]).

IgM and IgD isotypes: structural and functional diversity in normal B cells

Mature B cells express 20,000–150,000 IgM molecules and 250,000–300,000 IgD molecules.[9] Early studies in IgM[10] or IgD[11, 12] knockout mice demonstrated highly interchangeable functions of the two receptors in B cell development, affinity maturation and CSR. Several later studies, however, supported the existence of structural and functional diversity in IgM and IgD isotypes in normal B cells. The main structural difference between IgD and IgM is the IgD-specific "hinge" region, an extended peptide sequence located between the Fab (i.e. antigen-binding region) and Fc portion (i.e. tail region of the BCR that interacts with the cell membrane). This hinge region, present in IgD and absent in IgM, favors flexibility of the Fab region of IgD, permitting binding to polyvalent antigens.[13] In

line with this finding, IgD but not IgM receptors have been associated with prolonged signaling activation in normal B cells.[14] The distinct properties of IgM and IgD receptors may also be related to their spatial organization on the plasma membrane and distinct interactions with positive and negative signaling regulators. IgMs are indeed largely monomeric, whereas IgD can frequently be found in large clusters (also named “protein islands”),[15, 16] which may at least in part explain the differential threshold for activation of the two isotypes. IgD, but not IgM, was also recently described to be located in close proximity to the chemokine receptor CXCR4, facilitating transduction of CXCR4 activation signals to downstream effectors.[17] This nanoscale organization of BCRs is determined by the actin cytoskeleton,[9] which regulates the size and lifetime of receptor aggregations[18] (for a more complete review on cytoskeletal regulation of BCR aggregation please refer to [19]).

In the context of self-antigen stimulation, IgM, but not IgD, can be down-regulated following prolonged antigenic stimulation,[20] demonstrating that IgM is the principal isotype that can become “anergic”, a state in which B cells become unresponsive to antigen. More recent work demonstrated that IgD is less sensitive than IgM to endogenous antigens, possibly maintaining the quiescence of B cells in the context of autoantibody stimulation, and limiting autoreactivity of cells carrying anergized IgMs.[21,22] BCR-desensitization associated to anergy is frequently associated to constitutive increase in basal intracellular Ca^{2+} levels, together with an overall reduced responsiveness in terms of phospho-protein activation following BCR stimulation.[23,24] A summary of the structural and functional differences of IgM and IgD isotype receptors is provided in Table 1.

BCR signaling in Chronic Lymphocytic Leukemia (CLL)

Several lines of evidence support the hypothesis that CLL is a BCR-dependent malignancy. First, the mutational status of IGHV genes shows significant variability among patients with CLL, which in turn correlates with the clinical outcome. Specifically, unmutated CLL (U-CLL), defined as cases in which the CLL BCRs have 98% or more identity with the germline IGHV sequence, is typically associated with a more aggressive clinically behavior, [25–27] whereas mutated CLL (M-CLL) cases carrying BCRs with less than 98% IGHV identity, characteristically present with more indolent disease (Figure 3). Second, around 30% of CLL patients express a largely skewed immunoglobulin repertoire, with virtually identical BCRs, the so-called “stereotyped” receptors.[28–33] Third, the BCR signaling pathway is the central pathway activated in the lymph node microenvironment of CLL patients, the primary site of CLL cell proliferation in so-called proliferation centers or pseudofollicles.[34] While most CLL cells express BCRs of both, IgM and IgD isotypes (Table 1), a smaller proportion of cases, around 5–10%, express isotype-switched IgGs.[35, 36] The cell of origin for U and M-CLL is also distinct,[37] with U-CLL deriving from pre-germinal center $CD5^+$ naïve B cells, while M-CLL originating from post-germinal center $CD5^+CD27^+$ memory B cells (Figure 1), further distinguishing these subgroups of patients.

Responsiveness of CLL cells to IgM stimulation differs substantially among samples from different patients; CLL cells from U-CLL patients typically have higher responsiveness to IgM, which promotes CLL cell survival and proliferation, and a more clinical aggressive phenotype than M-CLL cases.[25–27, 38, 39] M-CLL show characteristic features of anergized

B cells resulting from prolonged antigen engagement, including reduced surface IgM levels, baseline activation of Ca^{2+} signaling, and constitutive ERK phosphorylation. [39–41] These features result in diminished IgM responsiveness, which can be restored by IgD ligation, or can spontaneously recover *in vitro* in the absence of stimulatory ligands.[39, 41] In contrast, U-CLL cells express higher sIgM levels,[27, 39] and also tend to express higher levels of the ZAP70 signaling adaptor[42, 43] which may further facilitate increased signaling responsiveness. IgM stimulation results in marked down-regulation of the chemokine receptor CXCR4;[44] vice versa, IgM signaling is modulated by TLR[45] and IL-4 receptor activation,[46, 47] demonstrating cross-talk between the BCR and other signaling pathways in CLL cells. The functional importance of the IgD isotype in CLL remains less defined. IgD signaling can be induced in all CLL samples, without significant differences between U-CLL and M-CLL, [27, 38, 39] and, in contrast to normal B cells,[14] IgD responses appear to be more short-lived,[48] and unable to induce c-MYC protein expression or cell-cycle entry,[49] most likely because of rapid and more pronounced IgD internalization following stimulation. [48] IgM and IgD isotype features in CLL cells are summarized in Table 1. The antigen-driven pathogenicity of the CLL-BCRs has been suggested by a number of studies characterizing several self-antigens for the CLL-BCRs, in particular for U-CLL, including proteins exposed on the cell surface during apoptosis (e.g. myosin heavy chain IIA),[50–52] lipoproteins,[53] cytoskeletal proteins (e.g. vimentin),[36] and microbial proteins (e.g. LPS).[53] Largely autoreactive BCRs derived from pairing of virus-specific heavy-chains with a restricted number of light chains have also been recently described as pathogenic in the Eμ-TCL1 mouse model of CLL,[54] confirming the importance of autoantigenic interactions in leukemia development also *in vivo* in mice. M-CLL derived BCR are less poly-reactive than U-CLL, and possess higher specificity and affinity for antigens, such as fungal antigens, with some cases carrying BCRs displaying rheumatoid factor activity.[55, 56,57]

At a functional level, CLL-BCR engagement activates a complex cascade of intracellular signaling molecules, including upstream kinases LYN, SYK, BTK and PI3K, which transduce signals to calcium signaling modulators (e.g. PLCγ2), cytoskeletal activators (e.g. HS1 protein),[58] and to downstream effectors, including AKT and ERK kinases, the NF-κB pathway, nuclear transcription factors, but also anti-apoptotic proteins of the BCL2 family, such as MCL1 [59, 60]. Nuclear transcription results in numerous outcomes, including production and secretion of CCL3 and CCL4 chemokines,[61, 62] two important chemoattractants for lymphocytes and monocytes/macrophages (Figure 4). IgM stimulation induces more prolonged signaling resulting in secretion of higher levels of CCL3 and CCL4; such effect cannot be appreciated following IgD signaling, which is largely restricted to early cytoskeletal activation (e.g. HS1 phosphorylation).[48] BCR signaling duration and intensity is tightly regulated by several mechanisms including receptor endocytosis, positive (e.g. CD19) and negative (e.g. CD5, CD22) co-receptor signaling, and activation of phosphatases, which fine-tune the functional outcome of the response.[63] CLL-BCRs can also signal in the absence of external antigen, in an autonomous fashion, by interactions between individual BCRs through recognition of conserved epitopes within specific regions of the immunoglobulin heavy and light chains.[64, 65] The autonomous signaling activity of the CLL-BCRs has also been defined indispensable for leukemia development and

progression in the Eμ-TCL1 mouse model of CLL,[66] increasing the complexity of BCR activation mechanisms involved in disease pathogenesis.

CLL- BCR stereotyped subsets

Dissecting the functional and molecular features of subsets of CLL patients with unique "stereotyped" BCR has provided valuable insight into the role of BCR signaling in CLL.[28–33] BCR "stereotypy" refers to highly restricted and sometimes identical variable heavy complementarity determining region 3 (VH-CDR3) sequences among different CLL patients, a characteristic that can be detected in approximately 30–35% of CLL cases, almost two thirds being U-CLL.[28–33] In the most recent analysis of a series of 21,123 IGHV sequences from CLL patients,[67] 23 " "major" (i.e. most populated) subsets were identified and represented 12% of all CLLs. {Agathangelidis, 2012 #20844;Agathangelidis, 2016 #20975}HCDR3 stereotypy between geographically distant and unrelated patients implies that CLL ontogeny is not stochastic, but rather related to common antigenic determinants. Stereotypy extends to shared somatic mutations, similar genetic and epigenetic profile of the leukemic clones, similar antigen-binding properties and functional responses through the BCR and other immune receptors, and also to similar clinical outcomes (Table 2).

Two paradigmatic stereotyped subsets associated with poor clinical outcome are subset #1 and #2. **Subset #1** exhibits an aberrant and distinctive gene-expression profile signature with several differentially expressed transcripts involved in the regulation of apoptosis, cell proliferation, oxidative processes and BCR signaling.[68] Additionally, subset #1 is enriched for *NOTCH1*[69, 70] and *NFKBIE* (i.e. gene encoding for IκBε, a negative NF-κB regulator) aberrations.[71] In CLL cells from subset #1, BCR cross-linking by anti-IgM results in significantly higher rates of cell proliferation when compared to non-subset #1 cases using the same IGHV genes but heterogeneous HCDR3s.[68] However, Bergh et al showed that triggering subset #1 leukemic cells with one of their putative antigens, namely oxidized low-density lipoprotein (oxLDL),[53] induced BCR clustering and internalization but did not result in intracellular signal transduction.[72] In a proportion of these cases, TLR9 stimulation could bypass BCR silencing, inducing cell cycle entry, and suggesting that interaction with oxLDL alone is not sufficient to drive cellular proliferation of subset #1 cells.[72] Stereotyped **subset #2** cases express either mutated (60%) or unmutated (40%) BCRs encoded by the IGHV3–21/IGVL3–21 gene pair, but is uniformly aggressive independently of SHM status.[28, 29, 73–75] Similarities among subset #2 cases include a distinctive pattern of SHM for M-CLL cases, and a remarkable high frequency of *SF3B1* mutations.[33, 69, 70, 76] Importantly, IGHV3–21 expressing CLL cells show the highest levels of signaling responsiveness when compared to non-IGHV3–21 CLL of both U-CLL and M-CLL.[27] Interestingly, IGLV3–21 was recently shown to have independent poor prognostic significance, irrespective of its association with stereotyped subset #2,[77] and to be associated with high levels of MYC target gene expression and low CXCR4 surface expression, implying an ongoing, and possibly cell-autonomous signaling activity, in line with the intramolecular recognition properties of IGLV3–21 described by Minici et al,[65] as discussed in more detail at the end of this section.

Other exemplary subsets for their internal biological and clinical homogeneity are stereotyped subsets #4 and subset #8, both expressing γ-switched BCRs (i.e. IgG isotypes). On the one side, **subset #4** is defined by the expression of mutated IGHV4–34/IGKV2–30 BCRs, with long and positively charged VH CDR3s,[28, 29] reminiscent of pathogenic anti-DNA antibodies,[78] and SHM patterns suggestive of edited autoreactive antibodies.[33, 79] The ongoing SHM results in intraclonal diversification of the BCR and implies ongoing interaction with antigen(s).[80, 81] Subset #4 patients are relatively young at diagnosis and experience an indolent clinical course.[30] Subset #4 CLL cells show biochemical and functional features of anergy, including constitutive ERK1/2 activation and lack of responsiveness after BCR cross-linking in terms of MAPK signaling activation and intracellular Ca^{2+} release.[82] Interestingly, anergy could be reversed in this subset by ligation of TLR1/2 and subsequent activation of the miR-17~92 cluster, a regulator of MAPK expression. Stimulation through TLR1/2 resulted in a distinct gene and miRNA expression profiles that were clearly distinct from those of CLL cells from non–subset #4 CLL cases, suggesting a subset-specific regulation of the anergic state.[82] The indolent behavior of other stereotyped subsets, such as subset #148 (stereotyped BCRs carrying mutated IGHV2–5) suggests that anergy and reduced BCR signaling responsiveness may also characterize these subsets,[30] albeit biochemical characterization of anergic features is yet to be performed.

Subset #8 (IGHV4–39/IGKV1(D)-39) patients, at the opposite side of the clinical spectrum, are characterized by a high risk for developing Richter's transformation, and by presence of distinct genetic aberrations (i.e. high frequency of trisomy 12 and *NOTCH1* mutations).[28, 30, 69, 70, 76, 83, 84] CLL cells from subset #8 patients display robust BCR pathway activation upon antigen binding, even when compared to cells from stereotyped subsets #1 and #2, along with an extremely promiscuous binding to both microbial and auto-antigens (e.g. Sm, dsDNA, CpG, LPS).[36] Chu and colleagues demonstrated that several CLL BCRs mostly of the U-CLL subtype, including subset #8 and **subset #6** (unmutated IGHV1–69/IGKV3–20 stereotyped BCRs), bind to apoptotic cells with exposed non-muscle myosin heavy chain IIA (MYHIIA)[51] and such binding significantly correlates with poor patient survival.[50] U-CLL-like stereotyped Ig sequences, mostly utilizing the IGHV1–69 gene have also been identified in naïve B cells from healthy donors[85, 86], demonstrating that an early selection of restricted BCR Igs with properties that resemble natural antibodies generated to fight common pathogens or to clear apoptotic debris,[87, 88] may occur even in normal individuals. The identification of such Ig sequences in the normal B cell repertoire suggests that certain B cell subsets carrying discrete BCR Igs escape immune tolerance possibly due to low autoreactivity. Auto-antigenic stimulation may also take place in the lymph node microenvironment, where calreticulin can be found on the surface of macrophages (i.e. nurselike cells)[89, 90], which in turn may trigger BCR signaling, especially in CLL cells from subsets #1 and #8.[90] Despite these subset-specific characteristics and binding activities, several studies using mimetic epitopes have revealed a largely shared epitopic reactivity of CLL-BCRs, demonstrating that common antigenic structures can be recognized even amongst unrelated CLL clonotypes.[91–94] BCR-BCR interactions driving autonomous signaling have instead been recently shown to have subset-specific epitopes, binding kinetics and affinity.[65] Stronger affinities and longer binding half-lives associate with indolent cases (e.g. subset #4) and weaker, short-lived contacts with progressive ones (e.g. subset #2), again

linking the quality of the BCR signal to the distinct clinical outcomes.[65] Of note, BCR homotypic interactions are not an intrinsic property of the germline clonotypic BCR-Ig, but are acquired through specific Ig affinity maturation. In particular, a subset #2 unifying SHM of the residue corresponding to the splice site between the variable and the constant Ig lambda domain, leads to subset #2 BCR self-recognition. In subset #4, CSR to IgG introduces the binding epitope, thus providing also a structural explanation for the exclusive usage of IgG isotypes by the cases assigned to this stereotyped subset.[29, 33, 65, 95,96] Of note, light-chain mediated binding to the bacterial protein L and to the actin-binding protein cofilin was described for subset #2 recombinant Igs, independent of the subset #2 heavy chain, suggesting that binding to these antigens cannot account for the non-stochastic pairing of subset #2 heavy and lambda light chain.[97] In the structural analysis by Minici et al. the subset #2 BCR homotypic interactions are also largely mediated through the subset #2 IgL. Importantly, however, the subset #2 heavy chain with the characteristically short VH-CDR3{Agathangelidis, 2012 #13724;Agathangelidis, 2012 #13724}[28, 29] facilitates the spatial proximity between the two BCRs, while establishing one direct hydrogen bond with the epitope on the light chain,[65] implying that BCR homotypic interactions may indeed account for the biased pairing of subset #2 heavy and lambda light Ig chain. Regarding subset #4 antigen binding activity, recent BCR specificity studies using a variety of antigenic targets revealed the importance of the autoantigen-mediated selection. In particular, unlike most CLL Igs that bind apoptotic cells, subset #4 BCR Igs recognize elements on viable human memory B cells and this binding necessitates the distinctive immunogenetic characteristics of subset #4, such as the specific SHM and the CSR to IgG. [52, 98, 99] All these evidences demonstrate that both antigenic and BCR-autonomous interactions influence the non-stochastic pairing of the heavy and light chains of stereotyped BCRs, suggesting a fine regulation of subset-specific Ig features.

BCR signaling inhibitors for CLL treatment

The management of patients has fundamentally changed since the introduction of small molecule inhibitors targeting BCR signaling-related kinases SYK,[100] BTK,[101] and PI3K [102](Figure 4). Durable responses, even in heavily pretreated patients, and/or patients carrying unfavorable cytogenetic risk features [i.e. *del*(17p), *del*(11q)], are common and led to the FDA and EMA approval of the BTK inhibitor ibrutinib[101, 103] and the PI3Kδ inhibitor idelalisib[102, 104], the latter typically used in combination with the anti-CD20 monoclonal antibody rituximab. A common mechanism of action of these drugs involves the rapid redistribution of CLL cells from the lymphatic tissues into the peripheral blood, which correlates with rapid resolution of lymphadenopathy within the first weeks of treatment,[105] together with abrogated leukemia proliferation and accelerated CLL cell death.[106] BTK and PI3K kinases participate not only in CLL survival- and proliferation-related BCR signaling, but also in signaling of receptors related to cell migration, adhesion and tissue homing, including chemokine receptor and adhesion molecule signaling. Accordingly, preclinical studies using BTK and PI3Kδ inhibitors demonstrated inhibition of integrin and chemokine receptor signaling,[104, 107–111] along with BCR signaling blockade.

Ibrutinib disrupts pro-survival signals from nurselike cells (NLC),[107] CD40 ligation, TLR9, BAFF, fibronectin, IL-6, IL-4, TNFα,[110] chemotaxis towards CXCL12 and CXCL13,[107, 108]

integrin-mediated adhesion,[108] and CCL3 and CCL4 chemokine production, *in vitro* and in CLL patients receiving ibrutinib therapy.[107] CLL cells carrying unmutated IGHV genes generally show higher dependence on BTK and BCR signaling for survival, which presumably explains the higher sensitivity of CLL cells with unmutated IGHV to ibrutinib treatment *in vitro*[112] and *in vivo* in patients receiving ibrutinib therapy.[101, 113] Ibrutinib as single agent,[101, 114, 115] or in combination with rituximab,[116, 117] fludarabine, cyclophosphamide and rituximab (FCR),[116] or bendamustine and rituximab (BR),[118] induces durable remissions in previously treated [101, 119–121] or untreated patients.[103,119, 122] Ibrutinib was also shown to promote graft-versus-leukemia (GvL) effects in CLL patients following hematopoietic cell transplantation (HCT),[123] and improve expansion of CD19-directed CAR T cells. [124] This outcome may be related to the effects of ibrutinib on T cells, including increased T cell receptor (TCR) repertoire diversity,[125] promotion of Th1 instead of Th2 $CD4^+$ T cell responses,[126] and downregulation of the immunosuppressive molecules PD-1 and CTLA-4.[127] Five-year follow up of phase II studies of single-agent ibrutinib therapy recently reported high rates of progression free survival (92%[128] to not reached[113]) in treatment naïve patients and 44%[128] to 64.8% in R/R CLL,[113] reemphasizing the remarkable efficacy of this agent. Despite this efficacy and tolerability, resistance to ibrutinib has been described, and is commonly associated with point mutations at the ibrutinib-binding site within BTK (C481S), or with activating mutations of the BCR signaling molecule PLCγ2,[129, 130] but also with clonal evolution [131, 132] and emergence of mutations in BCR-independent proteins, such as EP300 and MLL2, which are implicated in chromatin and histone regulation.[131] *In vitro*, *NFKBIE* mutations have also been associated with reduced responses to ibrutinib treatment.[71] An interesting mode to circumvent BTK (C481S) mutations has been proposed, and involves miRNA-mediated targeting of BTK total protein, which can be achieved through HDAC inhibition.[133] Inhibitors of non-BCR related pathways, including nuclear export [134] and the para-caspase MALT1[135] together with novel small molecule inhibitors with comparable blocking activities against wild-type and C418S-mutant BTK, namely ARQ531[136] and REDX08608,[137] are also currently tested in preclinical settings, with encouraging results. In addition to ibrutinib, novel small molecule BTK kinase inhibitors with higher selectivity towards BTK kinase, and less cross-reactivity with other Tec kinase family members, are currently under clinical development, including acalabrutinib,[138,139] GS-4059,[140] and BGB-3111.[141] Whether these agents will provide greater responses and/or less side effects than ibrutinib remains to be evaluated.

The PI3Kδ inhibitor idelalisib has been tested as single agent,[142] in combination with rituximab[102, 143] or with rituximab and/or bendamustine.[144] Similar to patients receiving ibrutinib, idelalisib induces early lymphocytosis followed by lymphocyte count normalization. Also similar to ibrutinib, idelalisib effectively antagonizes CLL-survival signals coming from the microenvironment, [104, 110] reduces CLL-cell chemotaxis,[109] and CCL3 and CCL4 release by CLL cells *in vitro* and *in vivo* in patients receiving idelalisib therapy.[109] Additional PI3K inhibitors have been tested in preclinical and early clinical studies, including duvelisib, also called IPI-145, a PI3K γ/δ inhibitor,[145, 146] the pan-PI3K inhibitor pilaralisib, also called SAR245408,[147] the PI3K β,δ inhibitor GS-9820, [148] and the PI3Kδ inhibitors ACP-319[149] and TGR-1202. [150, 151]

SYK kinase inhibition has also been explored, with promising results obtained in relapsed/refractory CLL patients after treatment with fostamatinib.[100, 152] The drug was then further developed for the treatment of rheumatoid arthritis, and other novel SYK inhibitors are currently tested, including GS-9973[153,154] and PRT-2070.

The remarkable clinical effectiveness of BCR signaling inhibitors underscores the importance of B cell receptor signaling and of BCR-associated kinases in the proliferation and homing of CLL cells, in particular at the level of the lymph node microenvironment, making this class of agent the treatment of choice for CLL patients with a wide variety of clinical presentations, biological characteristics and response to prior therapies.

Conclusions

A large number of studies has highlighted the importance of BCR signaling in CLL pathogenesis. The complexity of BCR signaling in CLL subsets is further increased by the existence of isotype-specific functions for IgM and IgD, and of stereotype-specific antigen binding and signaling properties. The therapeutic landscape has remarkably changed since the introduction of small molecule inhibitors targeting BCR-associated kinases, which abrogate CLL cell proliferation and induce durable remissions, even in high-risk and refractory CLL patients. Nonetheless, resistances to these novel agents can emerge, primarily in high-risk patients, and can be challenging in patients receiving long-term therapy with these drugs. It is therefore essential to identify and target additional pathways, which contribute to CLL survival and proliferation in the presence of continuous therapy with these agents, and especially in cases with resistance mutations in BCR signaling molecules. Studies to characterize the mutational landscape driving resistance to BCR signaling inhibitors,[129, 131, 132] and randomized clinical trials utilizing these drugs in combination with newer agents (e.g. the BCL2 inhibitor venetoclax) are underway and will allow a better refinement of individualized treatment strategies for CLL patients.

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Abbreviations used in this article

BCR	B-cell receptor
CLL	Chronic Lymphocytic Leukemia
Ig	Immunoglobulin
U-CLL	unmutated-CLL

M-CLL mutated-CLL

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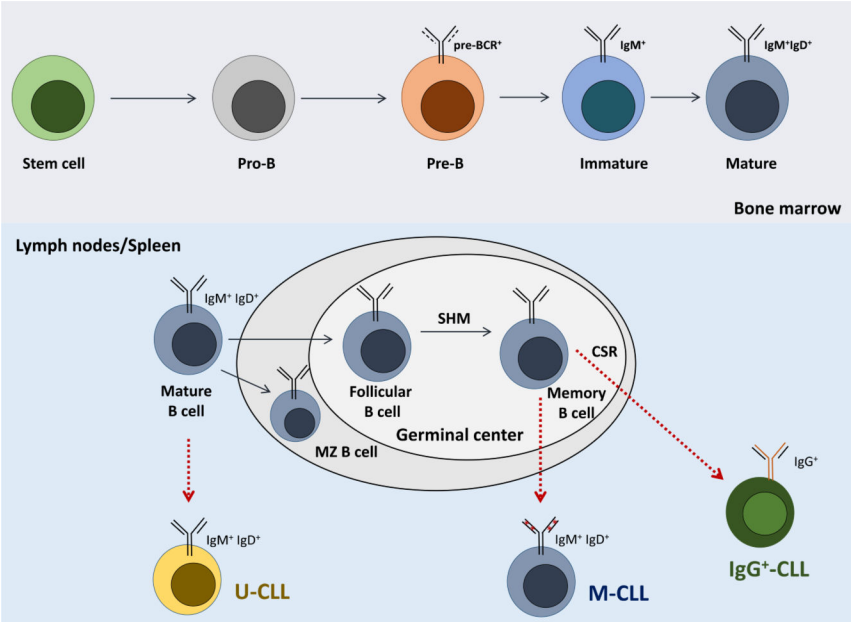

Figure 1. B cell receptor maturation during B cell development and antigen responses.

B cells undergo a series of maturation steps in the bone marrow, that lead to the generation of mature B cells, which express IgM and IgD isotype receptors on their surface. B cells then continue their maturation in secondary lymphoid organs, including the lymph nodes and the spleen, where, after antigen encounter, the BCRs are further diversified through somatic hypermutaion (SHM) and class switch recombination (CSR). CLL arises from distinct precursors, with U-CLL deriving from naïve B cells expressing unmutated immunoglobulins, while M-CLL deriving from memory B cells which have undergone SHM. A smaller, IgG-expressing subset, can arise from memory B cells which have undergone CSR.

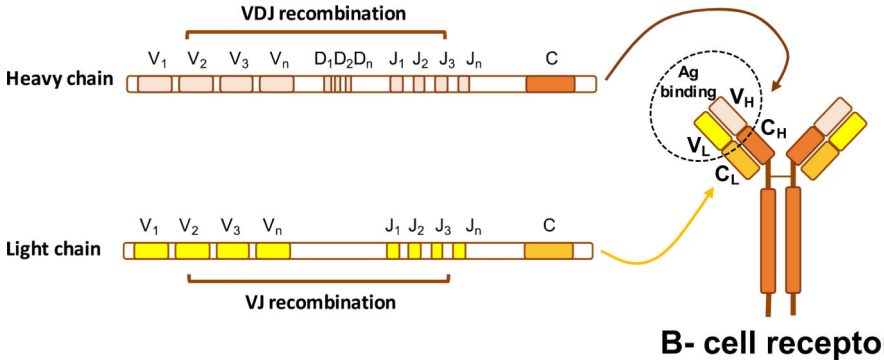

Figure 2. The VDJ recombination process.

Recombination of VDJ genetic regions of the heavy chain and VJ regions of the light chain at the pro- and pre-B stage allows generation of the variable regions of the heavy and light chain of the mature BCR. The high variability of the BCRs is in part due to the large number of V, D, and J gene regions of both Ig chains (e.g. the heavy chain includes 51V, 27D, and 6J genes). The complete BCR is composed by two variable heavy (V_H) and two variable light (V_L) chains, responsible for antigen binding, as well as two constant heavy (C_H) and two constant light (C_L) chains, involved in effector functions.

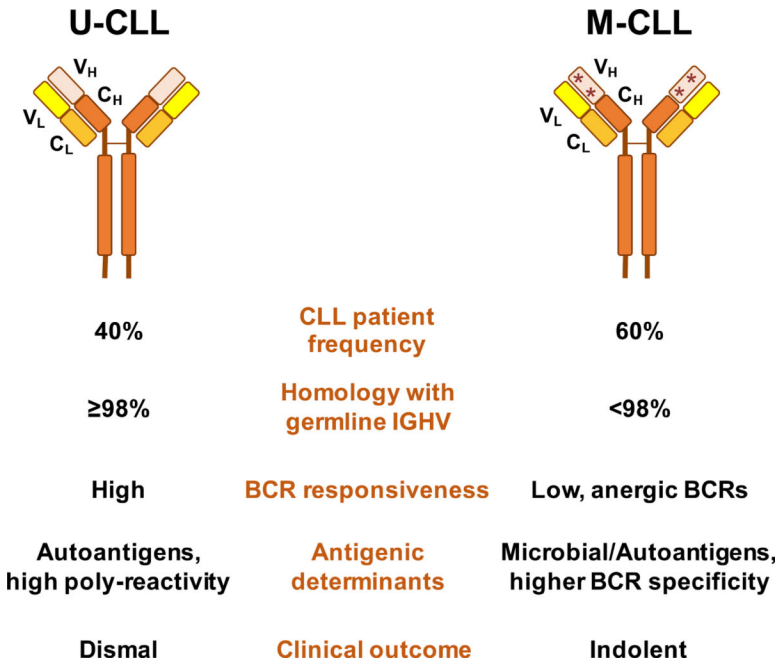

Figure 3. Characteristics of U-CLL and M-CLL patient subsets.

CLL patients can be categorized into two main subsets (U-CLL, M-CLL), characterized by a different degree of somatic hypermutations, BCR responsiveness, antigenic determinants and clinical outcome.

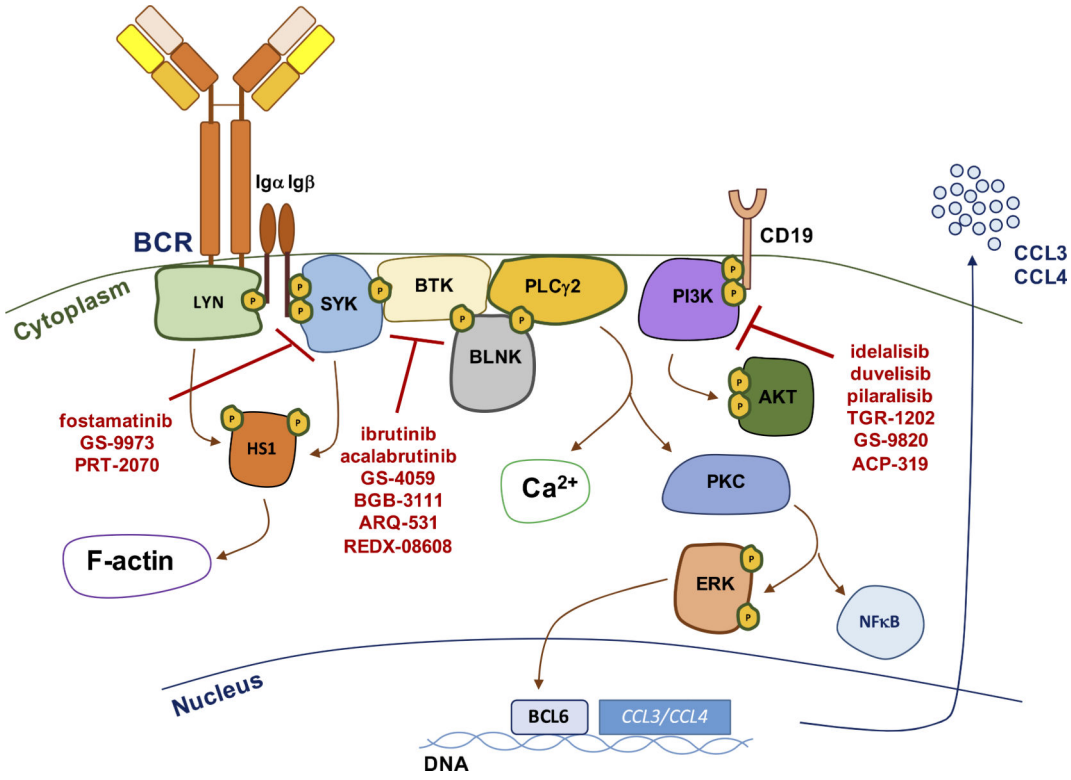

Figure 4. The BCR signaling pathway and its targeted inhibition.

Schematic representation of the main activation events in the BCR signaling pathway. BCR signaling activation is initiated by upstream kinases including SYK, BTK and PI3K, which can be targeted by novel small molecule kinase inhibitors, including the SYK inhibitors fostamatinib, GS-9973, and PRT-2070, the BTK kinase inhibitors ibrutinib, acalabrutinib, GS-4059, BGB-3111, ARQ-531 and REDX-08608 and the PI3K inhibitors idelalisib, duvelisib, pilaralisib, TGR-1202, GS-9820, and ACP-319.

Table 1.

IgM and IgD isotype expression and function in normal B cells and CLL cells.

BCR isotype	Expression in normal B cells	Function in normal B cells	Expression in CLL cells	Function in CLL cells
IgM	Expressed at the immature B-cell stage after productive VDJ recombination [5]; secreted or membrane-bound	Lower threshold for B-cell activation[13]; down-modulated *in vivo* following chronic antigen exposure [20]	Higher levels on U-CLL, as compared to M-CLL [27, 38, 39]	Survival and proliferation[49, 59, 60], cell cycle entry[49], long-lived signaling activation [49]; cross-talk with CXCR4[44], TLR signaling [45] and IL-4 [46]; higher IgM responsiveness in U-CLL [27, 38, 39]
IgD	Co-expressed with IgM in mature B-cells[5]; secreted or membrane-bound; contains a hinge region necessary to polyvalent antigen binding [13]	Higher threshold for B-cell activation[13]; lower association with the CD19 co-receptor [16]	Co-expressed with IgM[27, 38, 39]	Short-lived signaling and cytoskeletal activation, rapid internalization [48, 49]

Table 2.

Biological and molecular features of the most common stereotyped subsets

Stereotyped subset	Subset #1	Subset #2	Subset #4	Subset #8
Frequency	~2,4% [28, 67]	~2.8% [28, 67]	~1% [28, 67]	~0.5 % [28]
IGHV/IGVL gene identity	Clan I IGHV genes/IGHD6–19 /IGHJ4/IGKV1[D]-39 [28, 29, 32]	IGHV3–21/ IGHJ6/ IGLV3–21 [28, 73]	γ-switched IGHV4–34/ IGHJ6/ IGKV2–30 [28, 29]	γ-switched IGHV4–39/ IGHD6–13/ IGHJ5/ IGKV1[D]-39 [28, 29, 83]
IGHV mutational status	Unmutated [28, 29]	Mutated (60%) Unmutated (40%) [28, 29, 74]	Mutated [28, 29, 33]	Unmutated [28, 29, 83]
BCR signaling properties	functional BCR signaling [36, 68]	functional BCR signaling [36]	anergic BCRs [82]	functional BCR signaling [36]
Predicted antigens	Vimentin; Calreticulin; MEACs; healthy Hep-2; apoptotic RAMOS; apoptotic Jurkat; oxidation markers; dsDNA; Insulin/LPS [36,50, 52, 89, 90]	cofilin-1; stomach chief cells; pancreatic exocrine glands [53]	intact anti-I/i motif; viable human memory B cells [33, 52,99]	MEACs; healthy Hep-2; apoptotic RAMOS; apoptotic Jurkat oxidation markers; extractable nuclear antigens; dsDNA microbial antigens/TLR ligands, calreticulin [36,50, 52, 90]
Genetic lesions in treatment-naïve CLL	deletion of 11q, 17p, *NOTCH1, NFKBIE* mutations [68, 70, 71, 76]	deletion of 13q, *SF3B1* mutations [69, 70,75, 76]	deletion of 13q [69, 70,75]	trisomy 12, *NOTCH1* mutations [69,70, 84]
Clinical course/ Risk of transformation	Aggressive (median TTFT of 1.6 yrs)[30]	Aggressive (median TTFT of 1.9 yrs)[30]	Indolent (median TTFT of 11 yrs)[30]	Aggressive (median TTFT of 1.5 yrs),[30] increased risk of Richter's transformation[84]