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Microenvironment interactions and B-cell receptor signaling in Chronic Lymphocytic Leukemia: implications for disease pathogenesis and treatment

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Abstract

Chronic Lymphocytic Leukemia (CLL) is a malignancy of mature B lymphocytes which are highly dependent on interactions with the tissue microenvironment for their survival and proliferation. Critical components of the microenvironment are monocyte-derived nurselike cells (NLCs), mesenchymal stromal cells, T cells and NK cells, which communicate with CLL cells through a complex network of adhesion molecules, chemokine receptors, tumor necrosis factor (TNF) family members, and soluble factors. (Auto-) antigens and/or autonomous mechanisms activate the B-cell receptor (BCR) and its downstream signaling cascade in secondary lymphatic tissues, playing a central pathogenetic role in CLL. Novel small molecule inhibitors, including the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib and the phosphoinositide-3-kinase delta (PI3Kδ) inhibitor idelalisib, target BCR signaling and have become the most successful new therapeutics in this disease. We here review the cellular and molecular characteristics of CLL cells, and discuss the cellular components and key pathways involved in the cross-talk with their microenvironment. We also highlight the relevant novel treatment strategies, focusing on immunomodulatory agents and BCR signaling inhibitors and how these treatments disrupt CLL-microenvironment interactions.

Keywords

CLL; nurselike cells; stromal cells; BCR; BCR signaling; BCR signaling inhibitors

1. Background

Chronic Lymphocytic Leukemia (CLL) is the most frequent leukemia in the Western World, with an estimated incidence of about 4.5 new cases per 100.000 individuals annually and a

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Conflict of interest disclosure

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median age at diagnosis of 72 years. CLL is characterized by the clonal expansion and accumulation of mature $CD19^+CD5^+$ B lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs. CLL cells are phenotypically similar to antigen-experienced B cells, and express high levels of surface molecules (such as CD23, CD25, CD69 and CD71) that are up-regulated after antigen encounter, and low levels of markers down-regulated following cellular activation, such as CD22, Fc gamma receptor IIb and CD79b [1]. In addition, they express the memory B-cell marker CD27 [2] and show gene expression profiles similar to memory B cells [3]. The cellular origin of CLL is still debated, although transcriptome analyses of CLL and normal B-cell subsets from human blood and spleen revealed that CLL cells carrying unmutated immunoglobulin heavy chain variable region (*IGHV*) genes (U-CLL) derive from unmutated mature $CD5^+$ B cells and CLL cells carrying mutated *IGHV* genes (M-CLL) derive from a distinct, previously unrecognized $CD5^+CD27^+$ post-germinal center B-cell subset [4].

2. Biological and genetic features of CLL cells

CLL has a very heterogeneous clinical course; some patients experience very stable disease without requirement for therapy, while others show more aggressive disease and require early treatment. Clinical and biological prognostic factors have been identified that help to define the risk for disease progression in individual patients and to develop personalized treatment strategies. The most important prognostic factors are the clinical staging systems developed by Rai [5] and Binet [6], serum markers including β2 microglobulin levels [7], thymidine kinase levels [8], and soluble CD23 levels [9], cellular markers including CD38 [10] and ζ chain associated protein kinase 70 (ZAP70) [11, 12], and genetic parameters including the mutational status of *IGHV* genes [10, 13], and cytogenetic aberrations [14].

CD38 is a transmembrane protein that supports B-cell interaction and differentiation through the binding of CD31 [15], a cell-adhesion molecule expressed by cells of the CLL microenvironment, including nurselike cells (NLCs) [16] and T lymphocytes [17]. Patients with high CD38 expression have a faster progression and a shorter life expectancy [10]. ZAP70 is a key signaling molecule in T and NK cells, and is structurally homologous to spleen tyrosine kinase (SYK). ZAP70 enhances BCR signaling [18] and patients whose cells express high levels of ZAP70 protein have a more aggressive disease course [11, 12]. The mutational status of *IGHV* genes has a very strong prognostic significance. U-CLL cases carry BCRs with ≥98% homology with the corresponding germline sequence and show a more aggressive disease and a shorter median survival time compared to M-CLL (<98% homology) [10, 13]. Additional categorization of CLL into “subsets” based on common *IGHV* gene expression and shared BCR structure has been described (reviewed in [19]). There is a significant correlation between selected cytogenetic abnormalities and CLL patients’ survival. In previously untreated CLL patients, frequently found aberrations are 13q deletions (55%), chromosome 12 trisomy (15%), 11q deletions (12%) and 17p deletions (8%) [14, 20]. Patients carrying 13q deletions generally have low-risk disease and a favourable outcome [14]. The deleted region comprises two miRNAs, *miR-15-a* and *mir-16-1*, that are highly expressed in normal $CD5^+$ B cells, where they may act as negative regulators of the anti-apoptotic molecule B-cell lymphoma 2 (BCL2) [14]. A mouse model with a targeted deletion of *miR-15-a* and *mir-16-1* locus has been generated and

recapitulates many features of CLL [21]. 17p and 11q deletions, comprising the p53 and the ataxia telangiectasia mutated (*ATM*) genes, respectively, are predictors of poor clinical outcome [14]. Whole genome/exome sequencing analyses of CLL samples identified additional recurrent mutations (> 5% cases at diagnosis) (reviewed in [22]) affecting *NOTCH1* [23, 24], splicing factor 3B subunit 1 (*SF3B1*) [25, 26], baculoviral IAP repeat containing 3 (*BIRC3*) [27] and myeloid differentiation primary response (*MYD88*) genes [23]. These mutations are generally enriched in CLL patients that have transformed to Richter's syndrome [24], or in progressive/refractory CLL cases [25, 26]. Recent studies [28, 29] have provided insight into the accumulation of subclonal variants in the CLL clone overtime, including *ATM* [28], *TP53* [28, 29], *SF3B1* [29] and *NOTCH1* mutations [29], which depends both on the ability of each mutation to provide survival advantage to the cells in terms of proliferation and/or protection from apoptosis, as well as on the accumulation of selected high-risk mutations after treatment.

3. The CLL microenvironment

CLL cell interactions with the supportive tissue microenvironment play a critical role in disease pathogenesis [30]. CLL cells recirculate between peripheral blood and secondary lymphoid organs, where they proliferate in distinct tissue areas, termed "pseudofollicles", at a daily birth rate of approximately 1–2% of the entire clone, as determined by deuterated water labeling [31]. Homing to tissues is dependent on a tightly regulated interaction between chemokines that are secreted by stromal cells within the tissues, which attract and retain CLL cells to tissues sites via corresponding chemokine receptors, in cooperation with adhesion molecules on the leukemia cells and respective tissue ligands. Over the years, several cellular components of the CLL microenvironment have been described, along with the signaling pathways involved in CLL homing, survival and proliferation, which now provides a rationale for targeting the CLL microenvironment.

3.1 Nurselike cells and mesenchymal stromal cells

NLCs represent a critical component of the CLL microenvironment (Figure 1 and Table 1). NLCs are cells of monocytic origin, which spontaneously differentiate *in vitro* from monocytes in high-density cultures of CLL peripheral blood mononuclear cells [32] and which can be found *in situ* in lymphoid organs from CLL patients [33, 34]. Gene expression profile analyses of CLL cells after CLL-NLC co-culture showed that NLCs activate the BCR and nuclear factor kappa B (NF-κB) signaling pathways in CLL cells [35]; similar gene signatures were identified in CLL cells isolated from lymph nodes of patients [36], demonstrating that NLCs are a valid model for studying the CLL microenvironment. NLCs induce chemotaxis and promote survival of CLL cells through secretion of chemokines C-X-C motif ligand 12 (CXCL12) [32] and CXCL13 [34], and expression of TNF family members B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) [37], and they promote CLL disease progression *in vivo* in mouse models of CLL [38, 39]. NLCs express antigens that can activate the BCR on CLL cells, including vimentin and calreticulin [40]. They also express CD31, the ligand for CD38, which is expressed on CLL cells [16]. The mechanism through which NLCs differentiate *in vitro* remains incompletely defined; a recent study demonstrated that high mobility group box 1 (HMGB1) released by CLL cells

can stimulate NLC differentiation through activation of the receptor for advanced glycation end-product (RAGE)- toll like receptor 9 (TLR9) pathway [41]. Gene expression profile analyses have shown that NLCs exhibit an M2-like phenotype of tumor associated macrophages (TAMs) [42, 43] and it was recently demonstrated that the M2-phenotype skewing is further promoted by nicotinamide phosphorybosiltransferase (NAMPT), the enzyme responsible for nicotinamide adenine dinucleotide (NAD) biosynthesis, which is produced at high levels by CLL cells [44]. Additional studies have shown that monocytes, protect CLL cells from *in vitro* apoptosis by secreting soluble CD14, which activates NF-κB in CLL cells [45], and induce gene expression profile changes in CLL including inflammatory cytokine production [46].

Mesenchymal stromal cells, such as bone marrow stromal cells (BMSCs), are “feeder” layers for normal hematopoietic progenitor cells and contribute to normal bone marrow architecture. Mesenchymal stromal cells are also commonly found in secondary lymphatic tissues of CLL patients [47], where they provide survival and migration signals to CLL cells (Figure 1 and Table 1). CLL cells are protected from spontaneous and drug-induced apoptosis by direct contact with BMSCs [48, 49], and they are able to co-opt and disrupt normal bone marrow architecture [50]. Stromal cells constitutively secrete chemokines, which organize CLL-cell trafficking and tissue homing [51], and provide additional signals that support CLL survival and promote drug resistance. BMSCs induce up-regulation of aggressive disease markers in CLL cells, including ZAP70 and CD38, as well as down-regulation of C-X-C motif receptor 4 (CXCR4) [52]. BMSCs have also been recently shown to down-modulate CD20 expression from the surface of CLL cells [53], with implications for resistance to anti-CD20 antibody treatment. In addition, stromal cells promote glutathione synthesis in CLL cells [54], and induce glycolysis through NOTCH-mediated c-MYC activation, thus promoting cell survival and drug resistance [55]. Not only CLL cells benefit from bone marrow stroma contact, the stromal cells in turn also become activated by CLL cells, with induction of protein kinase C beta II (PKCβII) expression and NF-κB pathway activation [56]. CLL cells are also able to release microvesicles, which are enriched in activated signaling proteins [57] and can activate the AKT pathway in BMSCs [58], supporting the relevance of a bidirectional cross-talk between CLL cells and stromal cells.

3.2 Endothelial cells and follicular dendritic cells

Additional cellular elements in the CLL microenvironment include endothelial cells and follicular dendritic cells (FDCs) (Figure 1 and Table 1), which are essential for tissue homing and CLL retention to tissues. Adhesion to microvascular endothelial cells promotes CLL survival, activation and drug resistance [59–63]. CLL cells bind to β1 and β2 integrins [62] and to BAFF and APRIL on the surface of microvascular endothelial cells [60]. In addition, endothelin 1 (ET-1) engagement on CLL cells by endothelin subtype A receptor (ETAR) on endothelial cells promotes CLL survival and drug resistance, which can be blocked by ETAR inhibition [63]. *In vitro* culture with FDCs rescues CLL cells from spontaneous apoptosis by direct cell contact, dependent on ligation of CD44 on CLL cells and subsequent up-regulation of myeloid cell leukemia 1 (MCL1), a member of the BCL2 family of anti-apoptotic proteins [64]. The CD100/plexinB1 cross-talk also appears to be involved in this context [65]. Reciprocal cross-talk between CLL cells and FDCs via the

CXCR5-CXCL13 and the lymphotoxin beta receptor (LTβR)/lymphotoxin alpha beta (LTαβ) signaling pathways is essential for CLL positioning within lymphoid follicles and for leukemia progression *in vivo* in the EμTCL1 mouse model of CLL [66].

3.3 T and NK cells

Interactions between CD40-expressing B cells and CD40 ligand (CD40L) on activated $CD4^+$ T cells are critical in the context of antigen presentation and induction of normal B-cell responses [67]. Similarly, activation of malignant B cells by CD40 ligation promotes survival of CLL cells [68] (Figure 1 and Table 1). In CLL, the overall number of circulating T cells, oligoclonal in both the $CD4^+$ and the $CD8^+$ compartment, is increased, though their functionality appears to be compromised [69]. Increased numbers of effector memory $CD4^+$ cells and terminally differentiated $CD8^+$ lymphocytes associate with a more advanced disease stage [70]. $CD4^+$ and $CD8^+$ cells fail to form functional immune synapses [71, 72], show reduced Rho GTPase mediated T-cell motility [73] and display higher expression of exhaustion markers including programmed cell death protein 1 (PD-1) [70, 74, 75]; accordingly, CLL cells express high levels of PD-1 ligand (PD-L1) [70, 72]. Interference with the PD-1/PD-L1 axis by PD-L1 blocking antibodies prevents CLL development and restores immune effector functions, including those of T cells and macrophages, in the EμTCL1 adoptive transfer model of CLL [76]. T cells from CLL patients additionally show increased expression of the inhibitory receptor cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [77] and increased proliferation when CTLA-4 is blocked with anti-CTLA-4 antibodies [77]. Over the years, several studies have also reported defective NK-cell function. Human leukocyte antigen G (HLA-G) molecule overexpression in the plasma of CLL patients [78] induces NK-cell apoptosis and impairs NK-cell mediated cytotoxicity. Reduced NK-cell cytotoxicity has been associated to low expression levels of the activating receptors natural killer cell p30-related protein (NKp30) [79, 80] and natural killer group 2 member D (NKGD2) [81]; NK cells can also produce soluble BAFF, which interferes with NK-cell mediated CLL cell lysis after rituximab administration [82], and show reduced responses to the activating soluble BCL2-associated athanogene 6 (BAG6) ligand produced by CLL cells [79]. Taken together, these findings indicate that both the T and NK-cell compartments have overall reduced effector activities, which can explain the evasion of CLL cells from immune-mediated destruction.

3.4 Chemokines and adhesion molecules

CLL cell trafficking and homing to tissue microenvironments is tightly regulated, involving activation of chemokine receptors and adhesion molecules on the CLL cells. CLL chemotaxis towards stromal cells is promoted by the chemokine CXCL12 (previously called stromal cell derived factor 1 or SDF-1), which is secreted both by BMSCs [83] and by NLCs [32] (Figure 1 and Table 1). Additionally, NLCs secrete the chemokine CXCL13 [34], which attracts CLL cells through interaction with its receptor, CXCR5 [34]. High levels of $CXCR4^+$ cells have been associated with higher risk of lymphoid organ infiltration and poorer disease outcome [84], as well as higher responsiveness to BCR stimulation [85]. CXCR4 surface expression is stabilized through hyper-phosphorylation mediated by proviral integration site for moloney murine leukemia virus (PIM) kinase [86] and regulated by receptor endocytosis after CXCL12 binding [87]; consequently, CXCR4 surface levels are

low in lymph nodes and bone marrow of CLL patients, where CXCL12 levels are high [88]. CXCR4 is in close proximity to CD38 on the surface of CLL cells, and CD38 synergizes with CXCR4 signaling to promote homing and chemotaxis to CXCL12 [89]. CLL cells expressing high levels of ZAP70 [90], CD38 [15] and very late antigen-4 (VLA-4) integrins [91] show higher chemotaxis towards CXCL12, and a higher degree of extravasation in an *in vitro* model of CLL migration [92]. CXCR4 stimulation is associated to prolonged CLL cell survival *in vitro* [32, 37] and activation of extracellular signal-regulated kinase (ERK) kinase and signal transducer and activator of transcription 3 (STAT-3) signaling [93, 94]. The integrin VLA-4 interacts with CD38 molecule [95], is involved in CLL-cell adhesion to stroma [96] and its expression is associated with inferior clinical outcome of patients [97]. Another layer of complexity is added by chemokines secreted by the CLL cells themselves. Activated CLL cells secrete high levels of the chemokines C-C motif ligand 3 (CCL3) and CCL4 following BCR stimulation or after co-culture with NLCs [35] and higher plasma levels of CCL3 and CCL4 in CLL patients are associated with an inferior clinical outcome [98]. CCL3 and CCL4 presumably recruit T cells and monocyte/macrophages to tissue sites for interactions with CLL cells [99, 100]. In addition, CLL cells activated via CD40 secrete CCL17 and CCL22 [101, 102], which also can recruit T cells [101].

3.5 Angiogenic factors

In the normal bone marrow, balanced expression of pro- and anti-angiogenic factors, in concert with chemokines and cytokines, supports stable tissue maintenance and tissue homeostasis; imbalances between pro- and anti-angiogenic factors result in pathological angiogenesis. There is now more than circumstantial evidence supporting a role for angiogenesis in CLL pathogenesis. Microvessel density is increased in bone marrow biopsies of CLL patients [103] and angiogenic factors are expressed by CLL cells, including vascular endothelial growth factor (VEGF) [104], which is also a negative prognostic indicator [105]. CLL cells express VEGF receptors [106], and high levels of neuropilin-1 (NRP1), a VEGF coreceptor [107]. The levels of two other important angiogenic factors, basic fibroblast growth factor (bFGF) [108] and platelet-derived growth factor (PDGF) [109] are increased in CLL patients and correlate with disease stage and chemotherapy resistance. Interaction of mesenchymal stromal cells with CLL cells increases the production of VEGF and PDGF [110]; in turn, PDGF binding activates the AKT pathway in stromal cells with subsequent secretion of additional VEGF [109]. High levels of angiogenic factors may decrease the stability of the endothelial cell layer, thus allowing neo-angiogenesis and transendothelial migration of CLL cells. Neo-angiogenesis can be targeted with immunomodulatory agents including lenalidomide, which reduces both VEGF and bFGF levels and increases the stability of the endothelium [111].

4. B-cell receptor signaling in CLL

The B-cell receptor is a multimeric complex composed by the antigen-specific surface immunoglobulin (sIg) and the Ig-α/Ig-β hetero-dimers (CD79A, CD79B) (Figure 2). Antigen binding to the sIg induces activation of upstream kinases, including SYK and the Src kinase LYN, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmatic tails of CD79A and CD79B. This, in turn, activates the

cytoskeleton, including hematopoietic cell-specific LYN substrate-1 (HS1) protein [112, 113] and the related F-actin polymerization, as well as other upstream kinases, including BTK and PI3Ks and downstream pathways, including phospholipase C gamma 2 (PLC-γ2), calcium signaling, PKC, NF-κB signaling, mitogen-activated protein kinases (MAPKs) and nuclear transcription. Activation of phosphatases, including Src homology 2 (SH2) domain containing protein tyrosine phosphatase-1 (SHP1) and SH2 domain containing inositol 5-phosphatases 1/2 (SHIP1/2), and of negative co-receptors (e.g. CD22, CD5) contributes to negative regulation of the BCR signaling response. There is increasing evidence that BCR signaling plays a relevant role in CLL pathogenesis [114, 115]. CLL-BCRs show differential degrees of somatic mutations, which correlate with the clinical prognosis of patients [10,13] and one third of CLL patients express quasi-identical ("stereotyped") BCRs [116], suggesting that common antigens may be relevant to disease pathogenesis across patients subsets. CLL cells show features of mature B cells [3, 4], and most of them express surface immunoglobulins of both IgM and IgD isotypes [117, 118]. Cells expressing high levels of CD38 [10, 117], ZAP70 [119] and carrying unmutated *IGHV* genes [10, 120] are generally more responsive to IgM stimulation. On the other hand, M-CLL cells usually show constitutive phosphorylation of signaling proteins, including ERK kinase [121, 122] and reduced levels of responsiveness to BCR stimulation, generally referred to as "anergy". Despite one single study, which identified mutations in the CD79B gene [123], there is general consensus on the absence of somatic mutation on both *CD79A* and *CD79B*, which may lead to constitutive activation of BCR signaling, a phenomenon observed in the ABC subtype of diffuse large B-cell lymphoma [124]. The role of IgD signaling in CLL is less defined [118, 125, 126]. IgD stimulation can cause CLL-cell apoptosis [125] or survival and plasma cell differentiation [126] and differential responsiveness to IgD stimulation has been linked to clinical outcome [127]. Although the nature of the antigens stimulating CLL-BCRs in patients is still poorly defined, some reports have shown that U-CLL BCRs are polyreactive and mostly recognize autoantigens and other environmental antigens [40, 128–137] including cytoskeletal non-muscle myosin heavy chain IIA (MYHIIA), vimentin, cofilin-1, Fc tail of IgG, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), lipopolysaccharide (LPS), apoptotic cells, oxidized low-density lipoprotein (ox-LDL), lupus-associated ribonuclear protein Smith (Sm), human immunodeficiency virus 1 and hepatitis C viral antigens and bacterial antigens (Figure 3). In contrast, affinity-matured BCRs from M-CLL cases bind to a restricted set of more specific antigens, including β-(1,6)-glucans from yeast and fungi [138] and the Fc tails of rheumatoid factors [131–133, 139] (Figure 3). Binder et al., also demonstrated that recombinant CLL-BCRs from U-CLL patients are able to recognize vimentin and calreticulin proteins exposed on the surface of NLCs and these interactions are responsible for stroma-mediated anti-apoptotic effects [40]. Interestingly, when M-CLL BCR sequences are reverted to their germline equivalent, the CLL-BCRs regain polyreactivity [130]. In addition to antigen-dependent signaling responses, autonomous signaling capacity of CLL-BCRs due to self-recognition of epitopes within the BCR third complementarity-determining region of the heavy chain (HCDR3) has been described [140, 141] and has been recently reported to be involved, together with BCR responses to low-affinity autoantigens, in leukemia development *in vivo* in the EμTCL1 mouse model of CLL [115]. Therefore, both antigen-dependent and antigen-independent/autonomous signaling responses appear to be involved in CLL pathogenesis.

4.1 Targeting the microenvironment: lenalidomide and CXCR4/CXCL12 inhibitors

Lenalidomide is an immunomodulatory agent, which interferes with multiple components of the CLL microenvironment. Lenalidomide only induces mild apoptosis of leukemic cells, but reduces CLL proliferation through a cereblon/p21 dependent mechanism [142] and interferes with NLC-mediated [143] and endothelium-mediated [111] survival support. Lenalidomide has pleiotropic effects on the CLL microenvironment: it increases $CD4^+$ T-mediated antigen presentation, proliferation and activity [144, 145], and enhances NK and $CD4^+$ T-cell mediated anti-tumor immune responses [146, 147]. Lenalidomide restores functional immune synapse formation between T and CLL cells, down-regulates the immunosuppressive axis PD-1/PD-L1, and enhances T-cell motility [71, 73]; it also causes B-cell activation [148], and interferes with the activity and proliferation of T-regulatory cells [149]. Lenalidomide is active alone, in CLL relapsed/refractory patients [150, 151], or as initial treatment for elderly patients [152, 153] or in combination with rituximab [150, 154] and is currently tested in combination with ibrutinib [155].

The CXCR4/CXCL12 signaling axis represents another important therapeutic target in CLL. CXCR4 antagonists have been developed, including peptide CXCR4 antagonists (BKT140), small molecule CXCR4 antagonists (AMD3100, now called plerixafor), and antibodies to CXCR4 (MDX-1338/BMS 93656) [156]. Plerixafor inhibits CXCL12-mediated signaling activation on CLL cells, along with chemotaxis and F-actin polymerization and interferes with CLL-NLC and CLL-BMSC interactions [157]. Mobilization of CLL cells to the peripheral blood was observed in a phase I clinical trial of plerixafor used in combination with rituximab in relapsed CLL patients [158]. CXCL12 targeting has been achieved through the use of RNA oligonucleotides, such as NOX-A12, which inhibit CLL-cell migration *in vitro* and sensitize CLL cells towards cytotoxic agents [159]. NOX-A12 is currently tested in a phase II trial in combination with bendamustine and rituximab in relapsed CLL patients [160].

4.2 Targeting BCR-associated kinases BTK, PI3K, and SYK: ibrutinib, idelalisib, fostamatinib and novel small molecule inhibitors

Several small molecule BCR signaling inhibitors, mainly targeting BTK, PI3K and SYK kinases, have been generated and have shown excellent clinical activity (Figure 4 and Table 2). BTK is a non-receptor tyrosine kinase of the Tec family, and is rapidly phosphorylated by both LYN and SYK kinases upon BCR engagement, resulting in the activation of PLCγ2, AKT and ERK kinases, and NF-κB signaling [161]. BTK mutations in humans are associated to X-linked agammaglobulinemia, a primary immunodeficiency characterized by the absence of peripheral blood B cells and decreased levels of serum immunoglobulins [161]. In addition, BTK is also involved in the regulation of migration and adhesion via CXCR4/CXCR5 and integrin signaling [162]. Ibrutinib is an orally bioavailable inhibitor which was approved in 2014 for the treatment of mantle cell lymphoma and CLL; it blocks BTK kinase activity by forming a covalent bond with cysteine-481 residue [163, 164]. Ibrutinib is capable of overcoming pro-survival signals derived from the CLL microenvironment *in vitro*, including those from NLC- contact, CD40 ligation, BAFF, fibronectin (FN), IL-6, IL-4, TNFα [165] and BCR stimulation [166]. Ibrutinib inhibits CLL-cell proliferation [166], chemotaxis towards CXCL12 and CXCL13 [166, 167],

integrin-mediated adhesion [167], CLL-cell release of exosomes [168] and of CCL3 and CCL4 chemokines, *in vitro* and in CLL patients receiving ibrutinib therapy [166]. Ibrutinib has been tested either alone [169, 170], or in combination with chemo-immunotherapy, including rituximab [171] or bendamustine and rituximab, or fludarabine cyclophosphamide and rituximab [172]. High rates of durable remissions in patients with relapsed refractory disease, including patients with high-risk genetic lesions (e.g. del17p and del11q) [169, 171, 173, 174], as well as in previously untreated older patients (>65 years) have been reported [173, 175]. Early lymphocytosis and organomegaly reduction followed by lymphocyte count normalization are typical effects of ibrutinib treatment [176, 177, 178], linked to CLL-cell inhibition of proliferation and induction of cell death *in vivo*. Mutations in BTK and PLCγ2 were identified through whole-exome sequencing of peripheral blood samples from patients experiencing relapse after ibrutinib treatment, including a cysteine-to-serine mutation at position 481 in BTK (C481S) leading to a protein product with reduced kinase activity, that is only partially inhibited by ibrutinib [179, 180], and three putative gain-of-function mutations in PLCγ2, including arginine-to-tryptophan at position 665 (R665W), leucine to phenylalanine at position 845 (L845F) and serine to tyrosine at position 707 (S707Y) [179]. In addition to ibrutinib, novel small molecules inhibitors of BTK kinase have been tested and are currently under early clinical development, including ACP-196 and ONO-4059 [181].

PI3Ks are divided into 3 classes (I through III) and class I is further composed by four different isoforms (α,β,γ,δ). PI3Ks are responsible for the activation of AKT kinase along the BCR signaling pathway, as well as they exert effects on cell metabolism, migration, proliferation, and survival [182]. The predominant form expressed by hematopoietic cells is PI3Kδ, which plays a critical role in B-cell homeostasis and function. Idelalisib is a PI3Kδ inhibitor, which was approved by the FDA in 2014 for the treatment of previously treated CLL patients when used in combination with rituximab. Idelalisib is a highly selective PI3Kδ inhibitor [183], which antagonizes CLL-survival signals coming from the microenvironment, such as NLC-contact [184], CD40 ligation, TNFα, fibronectin and BCR stimulation [165, 183]. Idelalisib reduces CLL-cell chemotaxis towards CXCL12 and CXCL13 [184], CCL3 and CCL4 release by CLL cells *in vitro* and in patients receiving idelalisib therapy [184], and synergizes with ibrutinib in reducing CLL adhesion to vascular cell-adhesion molecule-1 (VCAM-1) and fibronectin [185]. Idelalisib has been tested as single agent [186, 187], or in combination strategies [188–191]. Similar to patients receiving ibrutinib, idelalisib induces early lymphocytosis followed by lymphocyte count normalization. Additional PI3K inhibitors are currently under development, including duvelisib, also called IPI-145, a potent PI3K γ–δ inhibitor, which antagonizes BCR and microenvironment interactions *in vitro* [192], even in cells carrying the BTK C481S mutation [193], and has been tested either alone [194, 195] or in combination with anti-CD20 antibodies. Additional small molecule inhibitors in early clinical development include the pan-PI3K inhibitor pilaralisib, also called SAR245408 [196], the PI3K β,δ inhibitor GS-9820, and the PI3Kδ inhibitors ACP-319 and TGR-1202 [197, 198].

SYK kinase belongs to the SYK/ZAP70 family of non-receptor kinases, and activates signaling pathways downstream of the BCR, chemokine and integrin receptors, suggesting

that SYK participates in tissue homing and retention of activated B cells [199]. Fostamatinib (FosD, R788) is an orally available inhibitor of SYK, which induced partial responses in relapsed CLL patients in phase I/II study [200], but further development of this drug focused on rheumatoid arthritis [201]. Additional SYK-specific inhibitors are under development, including GS-9973, which has been tested alone [202] or in combination with idelalisib, and PRT-2070.

5. Conclusions and perspective

A plethora of cellular and molecular components shape the CLL microenvironment, and the mechanisms involved in CLL proliferation and survival have been progressively unraveled. The CLL microenvironment has gained extensive attention during the last few years, thanks to the introduction of small molecule inhibitors, which target the CLL-microenvironment cross-talk. The BCR signaling pathway is central to CLL activation and likely to be triggered by antigens expressed in the tissue microenvironment. Inhibitors targeting BCR-associated kinases, including ibrutinib and idelalisib, have changed the landscape of treatment for CLL patients, inducing durable remissions in relapsed/refractory patients, including those carrying unfavorable genetic alterations (e.g. del17p, del11q). Recently, point mutations in one of the drug targets, BTK kinase, and activating mutations in closely related BCR pathway molecules (i.e. PLCγ2) have been linked to resistance [179]. Randomized trials comparing new drugs and/or their combinations with standard chemo-immunotherapy regimens are ongoing and will allow a better definition of optimal treatment strategies. The complexity of the cross-talk between CLL cells and their microenvironment, as well as the mechanisms of drug resistance and treatment failure still need to be better defined and are currently investigated.

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Abbreviations

APRIL	a proliferation-inducing ligand
ATM	ataxia telangiectasia mutated
BAFF	B-cell activating factor
BAFFR	BAFF receptor
BAG6	BCL2-associated athanogene 6
BCMA	B-cell maturation antigen
BCL2	B-cell lymphoma 2
BCR	B cell receptor
bFGF	basic fibroblast growth factor

BIRC3	baculoviral IAP repeat containing 3
BMSC	bone marrow stromal cell
BTK	Bruton's tyrosine kinase
CCL	C-C motif ligand
CD40L	CD40 ligand
CLL	Chronic Lymphocytic Leukemia
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
dsDNA	double-stranded DNA
ERK	extracellular signal-regulated kinase
ET-1	endothelin 1
ETAR	endothelin subtype A receptor
FDC	follicular dendritic cell
FN	fibronectin
HCDR3	third complementarity-determining region of the heavy chain
HLA-G	human leukocyte antigen G
HMGB1	high mobility group box 1
HS1	hematopoietic cell-specific LYN substrate-1
IGHV	immunoglobulin heavy chain variable region
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
LPS	lipopolysaccharide
LTαβ	lymphotoxin alpha beta
LTβR	lymphotoxin beta receptor
MAPK	mitogen-activated protein kinase
MCL1	myeloid cell leukemia 1
M-CLL	mutated *IGHV*-gene carrying CLL
MYD88	myeloid differentiation primary response
MYHIIA	non-muscle myosin heavy chain IIA
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphorybosiltransferase

NF-κB	nuclear factor kappa B
NKGD2	natural killer group 2 member D
NKp30	natural killer cell p30-related protein
NLC	nurselike cells
NRP1	neuropilin-1
ox-LDL	oxidized low-density lipoprotein
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PD-L1	PD-1 ligand
PI3K	phosphoinositide-3-kinase
PIM	proviral integration site for moloney murine leukemia virus
PKC	protein kinase C
PLC-γ2	phospholipase C gamma 2
RAGE	receptor for advanced glycation end-product
SDF-1	stromal cell derived factor 1
SF3B1	splicing factor 3B subunit 1
SH2	Src homology 2
SHP1	SH2 domain containing protein tyrosine phosphatase-1
SHIP1/2	SH2 domain containing inositol 5-phosphatases 1/2
sIg	surface immunoglobulin
Sm	lupus-associated ribonuclear protein Smith
ssDNA	single-stranded DNA
STAT-3	signal transducer and activator of transcription 3
SYK	spleen tyrosine kinase
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TAM	tumor associated macrophage
TLR	toll like receptor
TNF	tumor necrosis factor
U-CLL	unmutated *IGHV*-gene carrying CLL
VCAM-1	vascular cell-adhesion molecule-1
VEGF	vascular endothelial growth factor

VLA-4	very late antigen-4
ZAP70	ζ chain associated protein kinase 70

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Highlights

- CLL cells are dependent on interactions with their microenvironment for survival
- Nurselike cells, T and stromal cells are key components of the CLL microenvironment
- B-cell receptor signaling has a central pathogenetic role in CLL
- BCR signaling inhibitors are the most successful new therapeutics for CLL

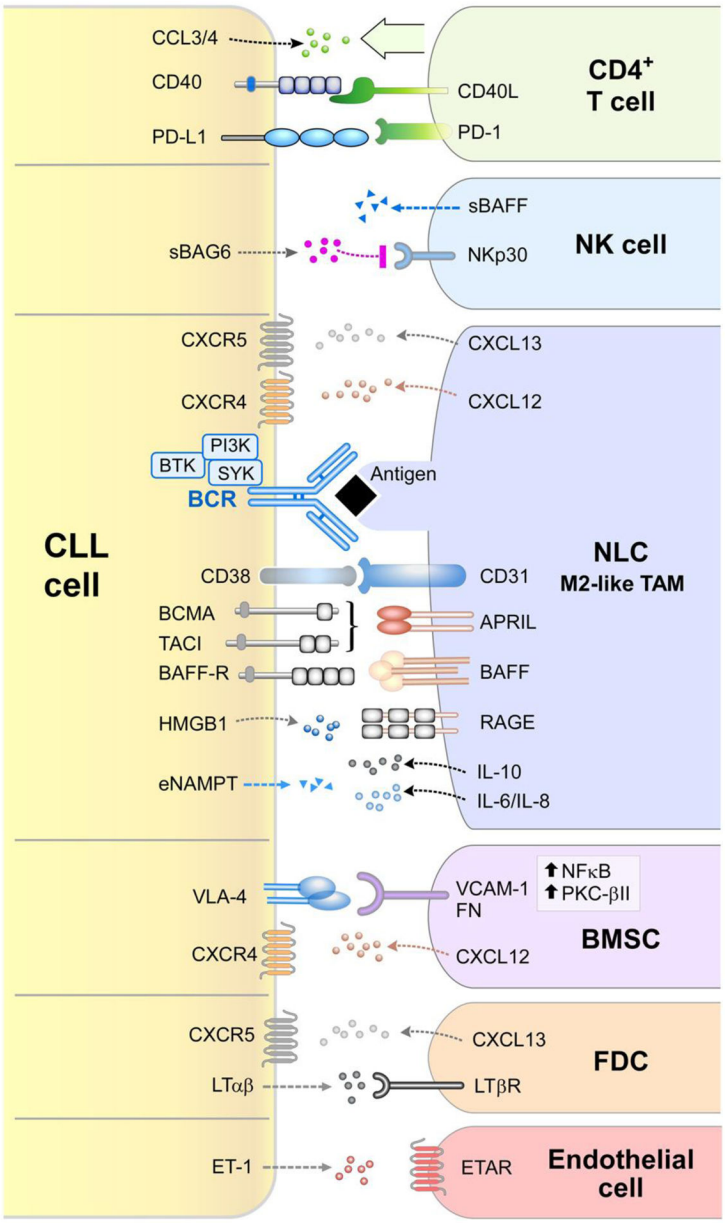

Figure 1. Cellular and molecular components of the CLL microenvironment

Contact between CLL cells and nurselike cells (NLCs) is established and maintained by chemokine receptors and adhesion molecules expressed on CLL cells and corresponding ligands on NLCs, which show phenotypic features similar to M2-like TAMs [42, 43]. BCR signaling is activated in CLL cells after co-culture with NLCs [35], possibly by direct recognition of CLL-BCR ligands expressed by NLCs [40]. Pro-survival pathways activated by the NLC-CLL interaction include the CD38-CD31 axis [15, 16] and the TNF family members APRIL and BAFF, which interact with corresponding receptors BCMA, TACI and BAFF-R [37]. Extracellular release of eNAMPT by CLL cells further promotes M2-skewing of TAMs, with associated release of tumor promoting (i.e. IL-6, IL-8) and immunosuppressive (i.e. IL-10) cytokines [44]. Differentiation of NLCs is promoted by

HMGB1-RAGE) interactions [41]. NLCs attract CLL cells by secreting CXCL12 [32, 83, 87, 89] and CXCL13 chemokines [34, 66], which interact with their cognate receptors CXCR4 and CXCR5, which are expressed at high levels on CLL cells. BCR stimulation induces CCL3 and CCL4 chemokine secretion [35], which recruit T cells and monocytes to tissue microenvironments. The CD40/CD40L axis favors survival and proliferation of CLL cells [68, 101, 102], and interaction of PD-L1 ligand with PD-1, which is expressed at high levels on the surface of T cells from CLL patients, favors immune evasion of CLL cells from T-cell cytotoxicity [70, 72, 76]. Several factors contribute to reduced NK-cell cytotoxicity, including low expression of NK-cell activating receptors, such as NKp30 [79, 80], soluble BAFF release by NK cells [82], and soluble BAG6 release by CLL cells [77]. Adhesion to bone marrow stromal cells (BMSCs) is mediated by VCAM-1 or FN interaction with VLA-4 integrins [96], and chemotaxis towards BMSCs involves the CXCR4-CXCL12 axis [85]. Cross-talk between CLL cells and follicular dendritic cells (FDCs) through the CXCR5-CXCL13 and LTαβ-LTβR axis is essential for CLL positioning within lymphoid follicles and leukemia progression *in vivo* [66]. CLL cells additionally secrete ET-1, which interacts with ETAR receptor on endothelial cells and promotes survival and drug resistance [63].

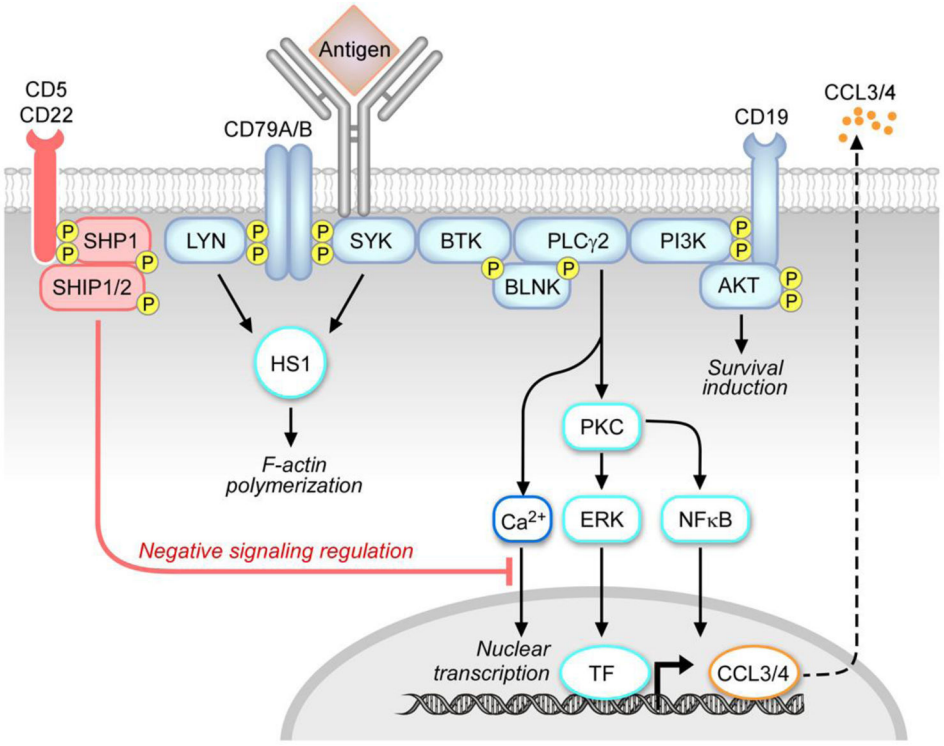

Figure 2. The BCR signaling pathway

BCR triggering by an antigen induces activation of early kinases, including LYN and SYK [199], which then transduce the signal to cytoskeletal activators, including HS1 protein [112, 113], and to other early effectors of the signaling response, including BTK kinase [161]. Through the BLNK adaptor, BTK activates PLCγ2, and subsequent downstream responses, including calcium signaling (Ca^{2+}), PKC, NFκB and ERK kinase [121, 122], and nuclear transcription factors (TF). The positive co-receptor CD19 contributes to the activation of the PI3K-AKT pathway and to survival induction [182]. The signaling response ultimately promotes activation of nuclear transcription, including CCL3 and CCL4 chemokine genes, which are then produced and secreted [35]. The signaling response is tightly modulated by negative coreceptors (e.g. CD22, CD5) and phosphatases, including SHP1 and SHIP1/2.

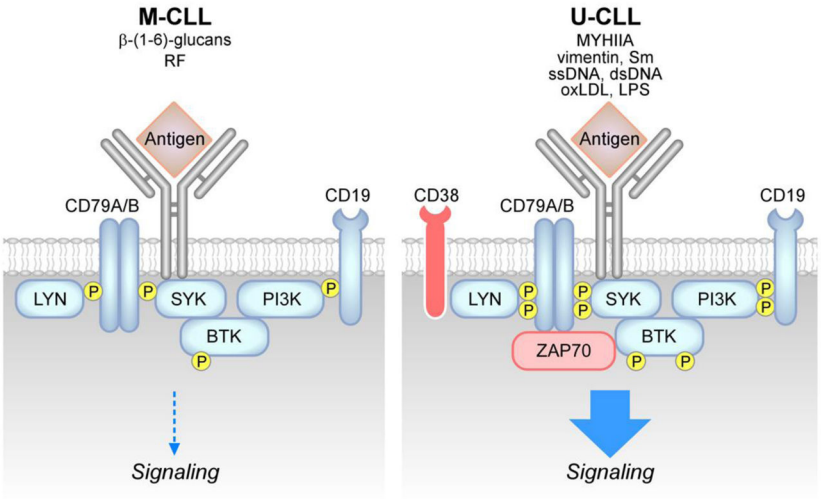

Figure 3. Differences between M-CLL and U-CLL signaling pathways

M-CLL cells show constitutive phosphorylation of signaling proteins and reduced activation of the signaling response after BCR triggering by external antigens [121, 122], including β-(1,6)-glucans [138] and rheumatoid factors (RF) [131–133, 139]. U-CLL cells express BCRs specific for autoantigens, including non-muscle myosin heavy chain IIA (MYHIIA), vimentin, lupus associated ribonuclear protein Smith (Sm), single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), oxidized low-density lipoprotein (oxLDL) as well as microbial antigens, including lipo-polysaccaride (LPS) [40, 128–137]. U-CLL are generally highly responsive to antigenic stimulation [10, 120], as well as those expressing high levels of CD38 [10, 117] and ZAP70 [119].

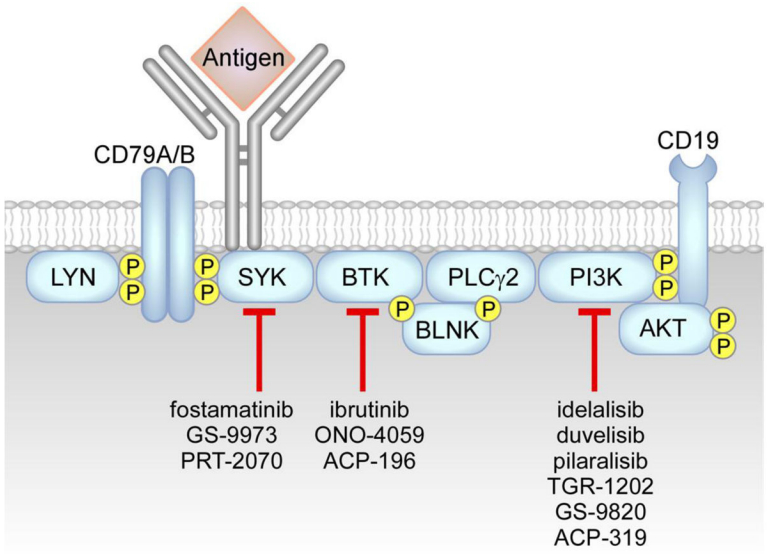

Figure 4. BCR signaling inhibitors

Interference with the BCR signaling axis can be obtained with inhibitors of SYK kinase, including fostamatinib [200], GS-9973 [202], and PRT-2070, of BTK kinase, including ibrutinib [163–180], ACP-196 and ONO-4059 [181], and of PI3K kinases, including idelalisib (δ inhibitor) [165, 183], duvelisib (also called IPI-145, γ,δ inhibitor) [192], pilaralisib (also called SAR245408, pan-PI3K inhibitor) [196], GS-9820 (β,δ inhibitor), TGR-1202 (δ inhibitor) [197], and ACP-319 (δ inhibitor).

Table 1

Signaling axes in the CLL microenvironment: cellular interactions and functions.

Receptor	Ligand	CLL interaction partner	Function	References
BCR	Antigen	NLC	BCR signaling activation, chemotaxis, CCL3 and CCL4 chemokine secretion, survival	[35, 40]
CXCR4	CXCL12	NLC BMSC	Chemotaxis	[32, 83, 87, 89]
CXCR5	CXCL13	NLC FDC	Chemotaxis, CLL positioning within lymphoid follicles	[34, 66]
BAFFR	BAFF	NLC	Survival	[37]
BCMA/TACI	APRIL	NLC	Survival	[37]
CD31	CD38	NLC	Adhesion, survival, proliferation	[15, 16]
RAGE	HMGB1	NLC	NLC differentiation	[41]
LTβR	LTαβ	FDC	CXCL13 release by FDC	[66]
VCAM-1	VLA-4	BMSC	Adhesion	[96]
PD-1	PD-L1	$CD4^+$ T $CD8^+$ T	T-cell dysfunction, impaired immune synapse formation	[70, 72, 76]
CD40	CD40L	$CD4^+$ T	Survival, CCL17 and CCL22 chemokine secretion	[68, 101, 102]
ETAR	ET-1	Endothelium	Survival, drug resistance	[63]

Table 2

Current therapeutic strategies using BCR signaling inhibitors

Inhibitor	Target	Phase of study	Treatment	Patients	Reference/clinicaltrial.gov identifier
Ibrutinib	BTK	III	Monotherapy vs. Ofatumumab (anti-CD20)	R/R	[170]
		II	Monotherapy	R/R and untreated >65yrs	[173]
		II	Monotherapy	R/R	[174]
		II	+ R	R/R and untreated > 65 yrs	[171]
		Ib-II	Monotherapy	R/R	[169]
		Ib-II	Monotherapy	untreated >65yrs	[175]
		Ib	+ BR or FCR	R/R	[203]
		I	+ Lenalidomide	R/R	[155]
ACP-196	BTK	II	Monotherapy	R/R or untreated	NCT02337829
		Ib-II	+Pembrolizumab (anti-PD1)	untreated	NCT02362035
		I	+Obinutuzumab (anti-CD20)	R/R or untreated >65yrs	NCT02296918
ONO-4059	BTK	I	Monotherapy	R/R	[181]
Idelalisib	PI3Kδ	III	+R vs. placebo + R	R/R	[191]
		II	+ R	untreated >65yrs	[190]
		II	Monotherapy	untreated >65yrs	[187]
		I	Monotherapy	R/R	[186]
		I	+ B, BR, F, Chl or ChlR	R/R	[189]
Duvelisib (IPI-145)	PI3Kγ,δ	III	Monotherapy vs. Ofatumumab	R/R	NCT02004522
		I	Monotherapy	R/R	[194]
		I	Monotherapy	R/R previously treated with ibrutinib	[195]
		I	+Obinutuzumab	R/R to prior BTK inhibitor therapy	NCT02292225
Pilaralisib (SAR245408)	pan-PI3K	I	Monotherapy	R/R	[196]
GS-9820	PI3Kβ,δ	I	Monotherapy	R/R	NCT01705847
TGR-1202	PI3Kδ	I	Monotherapy	R/R	[198]
		I	+ Obinutuzumab and Chl	untreated	NCT02100852
		I	+ Ublituximab (anti-CD20) and/or ibrutinib	R/R	[197]
		I-Ib	+ ibrutinib	R/R	NCT02268851

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Inhibitor	Target	Phase of study	Treatment	Patients	Reference/clinicaltrial.gov identifier
ACP-319	PI3Kδ	I	+ ACP-196	R/R	NCT02157324
Fostamatinib	SYK	II	Monotherapy	R/R	[200]
GS-9973	SYK	II	Monotherapy	R/R	[202]
		II	+ idelalisib	R/R	NCT01796470
PRT-2070	SYK	Pending phase I	n.a.	n.a.	n.a.

R/R: relapsed/refractory; F: Fludarabine; C: Cyclophosphamide; R: Rituximab; B: Bendamustine; Chl: Chlorambucil; n.a.: not applicable.