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## Biology of Chronic Lymphocytic Leukemia in Different Microenvironments: Clinical and Therapeutic Implications

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### Introduction

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world. An often indolent lymphoproliferative disorder, CLL is characterized by the progressive accumulation of monoclonal, small, mature-appearing CD5<sup>+</sup> B-cells in the peripheral blood, bone marrow and secondary lymphoid organs.[1] With the notable exception of allogeneic stem cell transplantation, CLL is currently an incurable disease, despite the fact that good initial responses to chemoimmunotherapy are obtained, which prolong overall survival.[2] Current treatment modalities appear to eradicate malignant CLL cells less efficiently in the bone marrow and lymph nodes than in peripheral blood. Thus, patients who initially achieve a remission will eventually develop recurrent disease. Moreover, the differential response of the disease in different anatomic locations indicates a significant role of the tissue microenvironment in supporting CLL cell survival and enabling them to evade the toxic effects of chemotherapy.

Recent work has demonstrated that the trafficking, survival and proliferation of CLL cells is tightly regulated by the surrounding tissue microenvironment (Figure 1). This conclusion is bolstered by several lines of evidence. When cultured *in vitro*, CLL cells rapidly undergo apoptosis, but they can be temporarily rescued from programmed cell death by contact with stromal cells. Stromal cells are also known to confer a protective effect against chemotherapy-induced apoptosis.[3, 4] Additionally, there are differences in the characteristics of CLL cells in the various tissue compartments. CLL cells in the peripheral blood are arrested in G0/G1 phase of the cell cycle [5] and display features that are consistent with a defect in programmed cell death and prolonged *in vivo* survival [1]. Previously, this observation was thought to suggest that CLL is a malignancy of quiescent non-proliferating cells. However, recent data on telomere length[6] and *in vivo* measurement of CLL cell kinetics demonstrated that CLL cells exhibit a more prominent turnover than

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previously appreciated [7]. Patients with a higher proliferation rate are more likely to have active disease and clinical progression [7, 8]. This apparent discrepancy is due to the fact that proliferation of CLL cells takes place primarily in the secondary lymphoid tissues, although it also occurs to a lesser extent in the bone marrow, in areas with a vaguely nodular architecture termed 'pseudofollicles' or 'proliferation centers' [8, 9].

The significant differences in the properties of the cells in the peripheral blood and lymphoid tissues are, at least in part, explained by antigenic stimulation and close interaction with various accessory cells as well as by exposure to different cytokines, chemokines, and extracellular matrix components (Figure 1). In the last decade there have been major advances in the understanding of the reciprocal interactions between CLL cells and the various microenvironmental compartments. Here, we will discuss the role of the microenvironment in the context of efforts to develop novel therapeutics that target the biology of CLL.

### **CLL cells in the context of the normal immune system**

Normal B cells are programmed to rapidly respond to the environment, while causing little damage to normal tissues. They possess the ability to recognize, process and present foreign antigens to other components of the immune system, and to undergo maturation and eventually secrete antibodies directed at a specific antigen. They can undergo programmed cell death when their role is over. The reciprocal interaction of B cells with the surrounding environment leads to recruitment of cellular elements into specific tissue compartments. Furthermore, B cells migrate to various compartments that regulate their differentiation, proliferation, and survival or apoptosis. This normal immune response is achieved via multiple proteins that are produced by the B cell and the surrounding microenvironmental cells, leading to a well-orchestrated and tightly regulated sequence of events.

It is not surprising that CLL cells, the malignant counterpart of normal B cells, retain the ability to interact with their surrounding environment. However, the finely tuned orchestration and normal compartmentalization of the immune response is altered. The cause of this malignant transformation is most likely a combination of genetic predisposition and environmental triggers, leading to genetic and epigenetic changes resulting in exaggeration of positive signals and attenuation of inhibitory and pro-apoptotic mechanisms.

### **Interplay between tumor biology and the local microenvironment**

Invasion of the primary and second lymphoid tissues by CLL cells disrupts the normal tissue architecture and physiology. The spleen and lymph nodes are diffusely infiltrated by CLL cells, while the bone marrow is involved in an interstitial, nodular and/or diffuse pattern. CLL cells retain the capacity to react to a variety of external stimuli and the tissue microenvironment provides supporting signals that may differ within the various anatomic sites.

CLL cells respond to the surrounding microenvironment *in vivo* as demonstrated by the activation of specific signaling pathways in the tumor cells in the tissue microenvironment resulting in changes in gene expression, cellular activation, proliferation, and apoptotic threshold [8, 10]. In a genome wide microarray study we found that purified CLL cells isolated concomitantly from peripheral blood, bone marrow, and lymph nodes show characteristic gene expression profiles that reflect differential activation of signaling pathways in the various anatomic compartments.[8] In particular, CLL cells in the lymph node upregulated > 100 genes responsive to BCR activation and NF- $\kappa$ B signaling and involved in proliferation. Several studies reported on comparative measurements of activation markers expressed on CLL cells and their proliferation rates in different anatomic

compartments [8, 11–14]. The expression of activation markers such as CD38 and CD69 as well as proliferation is increased in CLL cells in the lymph node and bone marrow compared to circulating cells [8, 11–13]. Likewise, the antiapoptotic regulators BCL-XL, survivin and MCL1 are expressed at higher levels in CLL cells in lymph nodes compared to their counterparts in the peripheral blood [15]. In addition, “apoptotic priming”, which describes the proximity of a cell to the apoptotic threshold, is reduced in bone marrow resident CLL cells [10].

## The microenvironment

### Antigenic stimulation and B-cell receptor signaling

BCR signaling is a crucial component of normal B-cell development and plays an important role in differentiation, survival, proliferation, and antibody secretion. Conclusive experimental evidence established the importance of BCR signaling in the pathogenesis of activated B cell-like diffuse large B-cell lymphoma (ABC-DLBCL) [16, 17]. While the evidence is somewhat more circumstantial, BCR activation is now also emerging as a central stimulus in the pathogenesis of CLL [18–22]. This view is based on several lines of evidence including specific BCR structures indicating a restricted antigen specificity of CLL cells [23, 24], immunophenotypic characteristics shared with antigen-experienced B-cells [25–27], as well as phenotypic characteristics of anergic B-cells [28, 29], the demonstration of ongoing BCR signaling *in vivo* [8], and a correlation of increased BCR activation or reactivity with clinical outcome [30].

Based on the presence or absence of somatic mutations in the immunoglobulin heavy chain variable (*IGHV*) gene expressed by the clonal cells, CLL can be divided into two main subgroups.[31, 32] Expression of a mutated *IGHV* identifies a subtype that follows a stable or slowly progressive course, while expression of an unmutated *IGHV* gene is associated with progressive disease and inferior survival [31, 32]. Additionally, CLL cells use a restricted repertoire of *IGHV* genes, which encode part of the antigen interacting domains of the BCR. Thus, preferential usage of certain *IGHV* genes indicates a role for antigen selection in the development of the disease [23, 24]. Furthermore, some cases express virtually identical BCRs, so-called “stereotyped BCRs”, that recognize shared antigens [33–35]. These antigens remain incompletely defined but in many cases may be the target antigens of so called polyreactive or natural antibodies, including microbial antigens and autoantigens expressed by dying cells [36–38]. In the case of unmutated CLL, it is thought that the particular molecular motifs involved in tumor development are autoantigens; this view is supported by the observation that the majority of CLL clones exhibiting stereotyped BCRs also demonstrate unmutated *IGHV* genes, as well as several studies of soluble Igs demonstrating polyautoactivity in unmutated CLL [39–42]. In contrast, stimulation via foreign antigen is likely to underlie the pathogenesis of mutated CLL, which exhibits less structural restriction of the BCR [41]. It is important to note that at present we have an incomplete understanding of where B-cells encounter antigen. Regardless, the secondary lymphoid tissues are likely to be the major anatomic site for BCR-antigen interaction. Antigens arriving through the lymph flow are sequestered and immobilized by cellular elements in the lymph nodes [43], thus providing an optimal setting for B-cell receptor stimulation. This is consistent with our observation of stronger BCR activation on CLL cells on the lymph nodes as compared to blood or bone marrow [8].

BCR signaling can be broadly divided into two main types; one that is antigen independent or “tonic” [44] and another that is antigen-mediated (Figure 2). “Tonic” signaling is mediated via PI3K $\alpha$  and PI3K $\delta$ , whereas antigen-dependent signaling involves activation of PI3K $\delta$  in addition to several tyrosine kinases and adapter molecules. Antigen-dependent signaling is initiated by the tyrosine kinases LYN and SYK. *In vitro* studies have shown that

BCR engagement on CLL cells triggers an intracellular signaling cascade leading to calcium mobilization, activation of the MEK/ERK, AKT/mTOR, and NF- $\kappa$ B pathways and upregulation of the anti-apoptotic proteins MCL1, BCL-XL and XIAP (Figure 1) [45–49].

Recently a type of BCR signaling that appears to be unique to CLL cells has been described. Specifically, Duhren-von Minden and colleagues showed that epitopes in the framework region of surface immunoglobulins (sIg) expressed on CLL cells serve as autoantigens, raising the possibility of auto-stimulation of the leukemic cells [50]. Building upon previous work demonstrating that the pre-BCR is able to induce ligand-independent cell-autonomous signaling by binding to an intrinsic pre-BCR glycosylation site [51], the group found that the BCRs of CLL cells constitutively signal even in the absence of any added antigen. Intriguingly, this type of signaling was equally demonstrated for BCRs derived from mutated and unmutated CLLs, and appeared to be cell-autonomous in that it was demonstrable on isolated individual cells [50]. Notably, such autonomous activation was not found with BCRs derived from multiple myeloma or other lymphoma cells. The heavy-chain complementarity-determining region (HCDR3) of the BCR was identified as the crucial interacting unit, since its insertion into a non-autonomously active BCR resulted in autonomously driven signaling. The authors then showed that the HCDR3 interacts with an intrinsic motif in the framework region 2 (FR2) of the sIg's VH region [50]. Mutations in this internal FR2 epitope abrogated any autonomous signaling demonstrating that this epitope acts as an autoantigen binding to CLL BCRs. This finding does not in and of itself negate the important role for the classical model of extrinsic antigen in the pathogenesis of CLL. Further study is warranted to define the respective roles of the two modes of BCR signaling in CLL.

The responsiveness of CLL cells to BCR activation *in vitro* is heterogeneous.[52] IGHV unmutated CLL cells are typically BCR signaling competent whereas IGHV mutated CLL cells respond weakly or not at all to BCR crosslinking induced by anti IgM antibodies [30]. The Zeta-associated protein of 70-Kd (ZAP-70), a transducing signaling kinase downstream of the T-cell receptor, is expressed in most cases of IGHV unmutated CLL but less frequently in IGHV mutated CLL [25, 53–55]. Expression of ZAP-70, akin to IGHV mutation status, serves as a powerful prognostic marker and correlates with a more aggressive disease course [54–57]. ZAP-70 expression is associated with increased BCR signaling *in vitro* [46]. The non-responsiveness to BCR activation in some CLL cells is reminiscent of anergized B-cells and suggests that these CLL cells are chronically stimulated by antigen *in vivo* [29, 30]. Consistent with this view is the low expression of surface IgM in CLL compared to normal B-cells and the recovery of BCR responsiveness after prolonged *in vitro* culture [28]. Furthermore, CLL cells that do not respond to surface IgM crosslinking respond to anti-IgD or anti-CD79a antibodies, indicating that the intracellular signaling pathway is functional [30]. Nevertheless, it has become clear that the presence of ZAP-70 enhances BCR responsiveness [58]. Interestingly, this effect of ZAP-70 is independent of the kinase domain, but requires recruitment of ZAP-70 to the BCR [59, 60]. The demonstration of decreased internalization of an activated BCR in a B-cell line that was engineered to express ZAP-70 provides a link between decreased IgM expression that correlates with absence of ZAP-70 and an anergic phenotype [60]. Thus, one effect of ZAP-70 might be to interfere with anergy by maintaining higher IgM expression.

*In vitro*, BCR crosslinking protects CLL cells from apoptosis primarily through the PI3k/Akt pathway and increased expression of MCL1 [45, 48, 61]. BCR triggering also up-regulates adhesion and costimulatory molecules, and increases CLL cell migration in response to the chemokines CXCL12 and CXCL13 [62]. Moreover, BCR signaling likely plays a central role in promoting CLL cell proliferation. *In vitro* engagement of the BCR in CLL cells induces expression of MYC, cyclin D2 and cyclin-dependent kinase 4 (CDK4).

Interestingly, although BCR activation promotes G1 cell cycle progression, cell division is not induced [49, 63]. Presumably additional co-stimulatory signals such as CD40L and IL4, provided in the tissue microenvironment, are required.

### Cell-cell interactions

Most studies exploring the cellular interactions in CLL have been performed using peripheral blood cells. Experimental methods relying on the investigation of circulating CLL cells in isolation lack the ability to appropriately mimic the complex cellular interactions occurring in the lymphatic niche. In spite of this limitation, many *in vitro* observations have helped to elucidate the *in vivo* crosstalk between CLL cells and non-malignant cells [64, 65]. In the tissue microenvironment, CLL cells reside in close contact with T-lymphocytes, stromal cells, endothelial cells, follicular dendritic cells and macrophages. Interactions between these components regulate CLL cell trafficking, survival and proliferation in a manner that may be partly dependent on direct physical cell-to-cell contact or mediated through the exchange of soluble factors (Figure 1).

### T-cells

The interaction between CLL cells and T cells is an important component of the malignant process. First, T-cells are important for CLL cell proliferation.[66, 67]. This has been directly demonstrated in a xenograft murine model of CLL where activated CD4<sup>+</sup> T-cells were required for CLL cell proliferation [66]. In CLL patients, T-cells, predominantly of the CD4<sup>+</sup> type, often make up a substantial fraction of the lymphoid infiltrate in the bone marrow and lymph nodes [68], where they are located both around and within proliferation centers [11, 69]. CD40, a key regulator of B-cell-T-cell interaction, is stimulated by CD4<sup>+</sup> T-cells expressing CD154 [70], the ligand for CD40, that are preferentially colocalized with CLL cells in pseudofollicular proliferation centers [9]. CLL cells activated *in vitro* via CD40, alone or in combination with IL-4, enter into cell cycle [71, 72] and are rescued from both spontaneous [73] and drug-induced apoptosis [74]. CD40 signaling in CLL cells induces anti-apoptotic molecules such as MCL1, BCL-XL, BFL1 and Survivin [9, 75, 76]. Promotion of CLL cell survival and proliferation by CD40 signaling is mediated through the PI3K/AKT, MEK/ERK [72, 76] and NF- $\kappa$ B pathways [72–74]. Survivin, a member of the family of inhibitor of apoptosis proteins (IAP's), that is preferentially expressed in the large proliferating CLL cells interspersed with T-cells in lymph node pseudofollicles, integrates apoptosis resistance and proliferation [9, 77].

In addition to interactions mediated through direct cell-cell contact, T-cells also secrete soluble factors that may contribute to CLL cell growth and survival. IL-4 inhibits spontaneous and drug induced apoptosis in CLL cells via a mechanism involving BCL-2 upregulation [78]. Interestingly, both IL-2 [79] and TNF $\alpha$  [80, 81] variably induce CLL cell proliferation. Similarly, IFN $\gamma$  [82], IFN $\alpha$  [83] and IL-13 [84] were also shown to support CLL cell survival.

Secondly, CLL cells can modify the cellular immune system to evade immune surveillance. Mechanisms are most likely multi-factorial including production of immune-suppressing cytokines such as tumor growth factor- $\beta$  [85], and IL-10 [86], and expression of reduced levels of adhesion and co-stimulatory molecules [87], as well as increased numbers and altered function of regulatory T-cells [88, 89]. Gene expression profiling of purified T-cells from CLL patients revealed changes in genes involved mainly in cell differentiation, cytoskeleton and vesicle formation, trafficking and cytotoxicity that contribute to decreased immune response [90]. Accordingly, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in CLL show impaired ability to form immunological synapses which is induced by direct cell contact with CLL cells [91] and is mediated through tumoral expression of CD200, CD270, CD274, and CD276 [92].

## Stromal cells

Mesenchymal stromal cells (MSC) are another important cellular component of the tissue microenvironment. Early studies exploring CLL-stromal cell interactions relied upon bone marrow-derived stromal cells [3, 93]. These cells consist of a heterogeneous population of cells that provide structural and functional support for normal hematopoiesis. Later on, other types of human and murine MSCs have been shown to exhibit similar effects on CLL cells [94]. Stromal cells produce and secrete various cytokines, chemokines, proangiogenic factors, and extracellular matrix components, and also express surface receptors that predominantly regulate CLL cell migration and survival. The CLL-MSC crosstalk is bidirectional; thus, tumor cells are not only being supported by stromal cells but also are capable of activating and inducing stromal cell proliferation and secretion of mediators that sustain and intensify the malignant process [95–98]. In the lymphoid tissues of CLL patients, stromal cells are diffusely located throughout the tissue and in perivascular areas where they admix with CLL cells [99]. The stromal cells are highly productive of SDF-1 [100] and are also markedly positive for  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), a marker induced in myofibroblasts that have been activated by tumor stromal specific growth factors [99]. Interestingly, CLL cells cocultured with bone marrow stromal cells are rescued from both spontaneous [3, 93, 101] and drug-induced apoptosis [3, 101], in a mechanism dependent on direct cell-cell contact [3, 4]. Murine fibroblast cell lines have been shown to protect CLL cells from apoptosis by maintaining expression of the antiapoptotic proteins BCL-XL, XIAP and FLIP<sub>L</sub> in the leukemic cells. Furthermore, cell-cell interactions activate the NF- $\kappa$ B pathway in a PI3K-dependent manner [72]. CXCL12, secreted from stromal cells, guides CLL cell migration towards the stromal layer and promotes penetration beneath it, a phenomenon called pseudoemperipolesis [101].

Both cell surface receptors and extracellular matrix elements were reported to be responsible for the enhanced survival of CLL cells that are in contact with the stroma layer. Adherence of CLL cells to stromal cells is mediated simultaneously through integrins  $\beta$ 1 and  $\beta$ 2 [93]. MSC highly express vascular cell adhesion molecule 1 (VCAM-1) [102]. Binding of  $\alpha$ 4 $\beta$ 1 integrin (CD49d/CD29, or VLA-4) to either VCAM-1 or to the extracellular matrix component fibronectin rescues CLL cells from both spontaneous apoptosis and fludarabine induced apoptosis [93, 103], through PI3K/AKT signaling and BCL-X<sub>L</sub> upregulation [104].

Another aspect of CLL cell interaction with the stroma layer involves metalloproteinase-9 (MMP9), vascular endothelial growth factor (VEGF) and endothelial cells. MMP-9 is the major MMP produced by CLL cells that promotes their extravasation and lymphoid tissue infiltration through proteolytic degradation of basement membranes and extracellular matrix components [105]. Independently of its proteolytic activity, MMP-9 also partially mediates CLL cell survival in bone marrow derived stromal cell coculture [106]. Binding of MMP9 to  $\alpha$ 4 $\beta$ 1 and CD44v in CLL cells results in LYN and STAT3 activation and induction of MCL1 [104]. Expression of MMP9 in CLL cells is regulated through  $\alpha$ 4 $\beta$ 1 integrins and CXCL12 [105]. In this respect, CLL cells in the bone marrow and lymph nodes acquire and express higher levels of surface MMP-9 than that which could be attributed to tumor cell activation in tissue microenvironment or derived from their adjacent accessory cells [104]. The pro-angiogenic molecule VEGF also decreases spontaneous or drug-induced apoptosis of CLL cells, through upregulation of MCL1, XIAP, and STAT3 signaling [107, 108]. In coculture of CLL cell with bone marrow derived stromal cells, vast amounts of VEGF appear to be secreted from the stromal cells and VEGF blockade results in decreased CLL cell survival [109]. CLL cells cocultured with human vascular endothelial cells are also protected from apoptosis in a mechanism involving NF- $\kappa$ B mediated upregulation of BCL2, MCL1, and BCL-XL [110]. Endothelial cells further increase expression of CD38 and CD49 in CLL cells in a NF- $\kappa$ B dependent mechanism [110]. In addition, activation of CD44 on

CLL cells by extracellular matrix components such as hyaluronic acid can promote CLL cell survival through activation of the PI3K pathway [111].

### **Follicular dendritic cells**

The literature exploring the role of follicular dendritic cells (FDC) in CLL is relatively limited. FDC are accessory cells within normal germinal centers that retain intact antigen-antibody complexes on their cell surface and present these antigens to B-cells [112]. Normal germinal center B-cells that bind to the immune complexes survive and differentiate into either memory B-cells or plasma cells [112]. FDC are normally detected in secondary lymphoid tissue but not in the bone marrow [113]. In CLL, FDC are seen in the lymph nodes, pseudofollicles [69, 114, 115], and in bone marrows of patients with nodular involvement.[116] FDC secretes several important prosurvival factors and growth factors (e.g. BAFF and IL-15) and express other important adhesion molecules such as VCAM-1, ICAM-1, plexin B1 and CD44 [69, 112]. The effect of FDC on CLL cells was studied using a FDC cell line (HK cells); the HK cells were shown to rescue CLL cells from spontaneous and drug induced apoptosis in a manner that was dependent on direct cell-cell contact and associated with an increase in MCL1 [117].

### **Tissue macrophages, monocytes, and Nurse-like cells**

An intriguing example of the distinct ability of CLL cells to affect normal cellular elements, leading to the loss of normal compartmentalization and spatial control of the immune response, is the recently described phenomenon of the interaction between CLL cells and “nurse like cells” (NLC). NLCs are an in vitro model thought to represent a counterpart of tissue associated macrophages in vivo. In long term cultures of peripheral blood mononuclear cells from CLL patients, large, round, occasionally bi-nucleated CD68 expressing cells grow out [118, 119]. Because CLL cells surround these cells and gain a survival advantage, they were termed “nurse like cells” (NLCs). Cells with similar phenotype are also detected in vivo in secondary lymphoid tissues of CLL patients [119], thus strengthening their biological relevance; yet, their numbers in the tissues are probably low [99]. NLCs actually differentiate from monocytes and their differentiation is dependent on cell-cell contact with CLL cells [119]. Monocytes obtained from normal donors cocultured with purified CLL cells also differentiate into NLCs [119] but normal B-cells do not induce differentiation of monocytes into NLCs [119]. Co-culturing CLL cells with NLCs protects CLL cells from spontaneous and drug induced apoptosis [101, 118] in a mechanism that is partially mediated through an increase in MCL1 expression [120]. NLCs produce and secrete chemokines and growth factors including CXCL12 and CXCL13 as well as B-cell activating factor of tumor necrosis factor family (BAFF) and a proliferation-inducing ligand (APRIL), which attract CLL cells into the tissue compartment and support their survival and proliferation.

BAFF and APRIL are TNF superfamily members that are important for B cell differentiation and survival [121–123]. CLL cells themselves also express BAFF and APRIL and their receptors [124, 125]. However, NLCs express higher levels of BAFF and APRIL than CLL cells [120]. BAFF binds to BAFF receptor (BAFF-R), B-cell maturation antigen (BCMA), transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI), while APRIL binds only to the latter two receptors. BAFF and/or APRIL decrease both spontaneous and drug-induced apoptosis of CLL cells [120, 124, 125]. Co-culturing CLL cells with NLCs in the presence of a decoy receptor that binds both BAFF and APRIL partially abolishes the protective effect of the NLCs on the viability of the CLL cells [120]. Recently, it has been shown that BAFF in cooperation with MYC can lead to the development of a CLL-like lymphoproliferation in mice [126]. Notably, MYC and its target genes are up-regulated in CLL cells in the lymph node [8] and MYC is upregulated by

BAFF [126] and by BCR engagement in vitro [127]. Interestingly, CLL cells that highly express c-MYC are more susceptible to apoptosis and can be rescued by BAFF [126].

Additional interactions between CLL cells and accessory cells in the tissue microenvironment involve the ligation of CD38 to CD31 and of CD100 to Plexin B1. CD38 levels on the surface of CLL cells are variable and high CD38 expression is a poor prognostic factor in CLL [31]. In proliferation centers, CD38 is upregulated in CLL cells exposed to activated T-cells expressing CD40L [11]. CD31, the ligand for CD38, is expressed on endothelial cells and NLCs. CD31 induces proliferation and prolongs the survival of CD38<sup>+</sup> CLL cells [128]. CD100, a transmembrane protein that belongs to the semaphorin family, is expressed on CLL cells [129]. The high affinity receptor for CD100, Plexin B1, is expressed on bone marrow stromal cells, follicular dendritic cells, nurse like cells and activated T-cells [129]. It has been demonstrated that engagement of CD100 by PlexinB1 increases CLL cell proliferation and prolongs survival [128, 129].

### Trafficking and homing of CLL cells into lymphoid tissues

Chemokines consist of two major subgroups; one group of homeostatic chemokines is constitutively produced and secreted within the tissue microenvironment and serves to maintain physiological trafficking. The second group includes inflammatory chemokines which are primarily induced in inflamed tissues to recruit effector cells [130]. Serum levels of some of the chemokines or their cognate receptors are highly elevated in CLL and a more effective chemotactic response is a characteristic of more aggressive subtypes of CLL cells. The responsiveness of circulating CLL cells to chemokine stimulation might facilitate the trafficking, homing and invasion of the leukemic cells into the “nourishing” tissue microenvironment.

An example of a chemokine that critically regulates CLL cell migration is CXCL12, a homeostatic chemokine that plays a critical role in normal trafficking and homing. CXCL12 is constitutively secreted at high levels by stromal cells and in vitro by NLCs [118]. CXCR4 (CD184), the receptor for CXCL12, is highly expressed on circulating CLL cells [131–133], which migrate more efficiently towards CXCL12 than normal B-lymphocytes. [131, 132]. CLL cells expressing CD38 and/or ZAP70 show stronger intracellular signaling and better chemotaxis in response to CXCL12 than cells with no CD38 or ZAP70 expression [133–135].

CXCL13 is another homeostatic chemokine which along with its cognate receptor, CXCR5, plays a central role in the recruitment of B-cells into the B-cell zone of secondary lymphoid organs [136]. CXCL13 is constitutively secreted by stromal cells in the B-cell areas of the secondary lymphoid tissues [137]. CXCL13 is expressed in vitro by NLCs as well as in vivo in the CD68<sup>+</sup> macrophages present in CLL lymph nodes [112, 137]. Serum CXCL13 levels are higher in CLL patients compared to healthy individuals [137] and CXCR5 is also highly expressed in CLL cells [137, 138].

CCL19 and CCL21 are chemokines that regulate the recruitment of lymphocytes into the T-cell zone areas of the secondary lymphoid tissues through ligation to their cognate receptor CCR7 [139]. CCL19 and CCL21 are detected in the stroma and in the high endothelial venules (HEV) of lymph nodes in CLL, and the latter are an important route of lymphocyte entry into secondary lymphoid tissue [140]. Circulating CLL cells express high levels of CCR7 [133, 141] which are higher in ZAP-70<sup>+</sup> CLL cells and in patients with prominent lymphadenopathy [133, 140]. CLL cells that are ZAP70<sup>+</sup> or from patients with marked lymphadenopathy migrate more efficiently towards these chemokines [133, 140, 142].



CLL cells are not only capable of responding to cellular elements in the tissue microenvironment but also actively recruit cells from the microenvironment to their immediate vicinity. This involves CLL cell secretion of chemokines such as CCL17, CCL22, CCL3 and CCL4. CCL22 and CCL17 are T-cell attracting chemokines induced in CD40 activated CLL cells in the lymph nodes and bone marrow [70]. Thus, CLL cells can attract CD4<sup>+</sup> T cells to the bone marrow and lymph nodes that augment tumor cell proliferation and survival and further induce release of CCL22, creating a positive feedback loop that further promotes the malignant process [70].

CCL3 and CCL4 are pro-inflammatory chemokines crucial for the response to infection, the mediation of inflammation and the recruitment of monocytes, T-cells from the blood into the tissue compartments [143]. CLL cells secrete these two chemokines in response to activation of the BCR and CD38 as well as during coculture with NLCs. Expression of CCL3 and CCL4 in CLL cells during coculture with NLCs positively correlates with ZAP-70 positivity [144]. CLL3 and CCL4 are also overexpressed in CD38<sup>+</sup>CD49d<sup>+</sup> CLL cells more than those that are CD38<sup>-</sup>/CD49d<sup>-</sup> [145]. Both CCR1 and CCR5, which are the cognate receptors for CCL3 and CCL4, are expressed on monocytes and macrophages and induce their migration. [145]. Consistently, higher numbers of tumor infiltrating CD68<sup>+</sup> macrophages were detected in the bone marrows of patients with CD38<sup>+</sup>CD49d<sup>+</sup> CLL [145]. CCL3 may also indirectly protect CLL cells from apoptosis via induction of VCAM-1 (CD106) in endothelial cells [145]. Plasma levels of CCL3 and CCL4 are elevated in CLL patients compared to healthy individuals [144] and high CCL3 levels correlate with poor prognosis in CLL [146].

## Signaling pathways activated in the tissue microenvironment

Given that CLL cells respond *in vitro* to a wide variety of external stimuli (Figure 1), it is a great challenge to determine which signaling pathways are the most relevant *in vivo*. Clearly, engagement of the BCR, via either autonomous or extrinsic mechanisms provides a signal. The PI3K/AKT, MAPK/ERK, NF- $\kappa$ B, WNT, JAK/STAT and NOTCH signaling pathways have been reported to mediate survival and/or proliferation of CLL *in vitro*. In particular the PI3K/AKT, MEK/ERK and NF- $\kappa$ B pathways have been shown to be activated in the tissue microenvironment. Because the composition of the microenvironment can vary between tissues different signaling pathways may be engaged in different locations. This may be in particular the case for the lymph nodes and for the so-called proliferation centers. The activity of many intracellular signals appears to be stronger and/or more sustained in patients with more aggressive subtypes of the disease and correlates with enhanced tumor proliferation and more rapid disease progression [8, 147]. In particular, PI3K/AKT signaling regulates CLL cell survival and trafficking [148]. The PI3K/AKT pathway mediates the chemotactic response of CLL cells towards CXCL12 [131, 132, 135], CXCL13 [137], CCL19, and CCL21 [133, 140, 149] and promotes CLL cell survival in response to a variety of external stimuli including BCR activation [45], CD40L [72], VCAM-1 [104], CCL19, CCL21 and many others. The inhibition of apoptosis in CLL cells through PI3K/AKT signaling is partially dependent on activation of NF- $\kappa$ B and upregulation of anti-apoptotic genes (e.g. BCL-XL and BFL-1). The classical NF- $\kappa$ B pathway has been shown to be activated in CLL cells in the lymph node more than in those derived from peripheral blood. A variety of *in vitro* stimuli induce NF $\kappa$ B activity in CLL cells including engagement of the BCR [48] or CD40 [72–74], exposure to cytokines such as BAFF or APRIL [120] and coculture with stromal cells [72] or endothelial cells [110]. Compared with normal B cells, CLL cells overexpress anti-apoptotic proteins such as BCL-2 and MCL1 [150, 151] and the resistance to apoptosis is further enhanced in the lymphoid tissues via upregulation of BCL-2 family molecules including MCL1 BCL-XL and Survivin [10, 15]. MCL1 expression in CLL cells is commonly regulated through PI3K/AKT signaling [45]. BCL-XL can be upregulated in CLL cells through BCR [45], and CD40 signaling [75] or through

VCAM-1 [104], stromal cells [72] and endothelial cells [110]. The MAPK/ERK pathway also transmits pro-survival signals in CLL cells, as demonstrated in response to stimulation with CXCL12, CXCL13, CCL19 and CCL21. In addition, the MEK/ERK pathway is an important regulator of cell cycle progression and proliferation. MEK1/2 activity is important for MYC expression and S-phase entry of CLL cells. MEK/ERK mediates MYC expression in response to engagement of the BCR and Toll-like receptor 9 (induced in vitro by CpG-ODN) and with BAFF stimulation. Accordingly, both phosphorylated ERK and MYC are mostly expressed in large proliferating CLL cells confined to the proliferation centers within the lymph nodes [127]. MYC can contribute to genomic instability by selecting cells with defective DNA damage response, and its expression may be a driver of clonal evolution [152].

Cell proliferation is regulated by D-type cyclins that bind to CDK4 and CDK6, resulting in the phosphorylation of the retinoblastoma protein and the G1-S phase transition of the cell cycle. Cyclin D2 is overexpressed in CLL cells [153], especially in cells residing in the LN [8]. IgM ligation induces cyclin D2 and CDK4 in CLL cells. [63] The down-regulation of the cell cycle inhibitor p27 and progression of CLL cells into S phase are probably dependent on additional costimulatory signals such as CD40 ligand and IL-4 that are provided mainly by T-helper lymphocytes in the proliferation centers of the lymph node [154]. Accordingly, within CLL cells in the proliferation centers cyclin D2 is highly expressed and p27 is down regulated [155]. Cyclin D2 expression is regulated either directly through NF- $\kappa$ B or indirectly by c-Myc. Consistent with this finding is the observation that NF- $\kappa$ B activity is increased in the LN and particularly enhanced in CLL cells within the proliferation centers [8, 155].

## Models of the CLL microenvironment

Modeling tumor-host interactions is an area of intense investigation. Such models are of particular interest given the fact that tissue resident CLL cells are not readily available. Currently, the most widely utilized *in vivo* model for CLL is the transgenic TCL1 mouse, in which the human *TCL1* gene is expressed under the control of the immunoglobulin heavy chain variable region promoter and enhancer. [159] TCL1 is an oncogene commonly activated in mature T-cell lymphomas that enhances AKT signaling. Onset of disease is late in life and the tumor cells in TCL1 transgenic mice are relatively large lymphoid cells, expressing unmutated IGHV genes [159]. There is evidence for a role of BCR signaling in this model and a dysregulation of the T-cell compartment similar to what has been described in human CLL [160]. The TCL1 transgenic model has also been used successfully to study novel therapeutic approaches (discussed below). In contrast to the transgenic model, New Zealand Black (NZB) mice early in life spontaneously develop autoimmunity and B-cell hyperactivity, while a CLL-like disease manifests later in life. The late-onset clonal disease is of the IGHV unmutated type that is also ZAP70 positive. [156] NZB mice were found to harbor a point mutation in the 3'-flanking sequence of the pre-miR-16-1, which results in decreased levels of miR-16 in lymphoid tissues [157]. This is reminiscent of the most common chromosomal lesion in human CLL; a deletion of the 13q14 chromosomal region containing the *mir-15a/16-1* and *DLEU2* genes [158]. Recently Klein and colleagues showed that the deletion of the 13q14-minimal deleted region (MDR) harboring the *DLEU2/miR-15a/16-1* cluster in mice results in development of a condition that resembles human CLL [161]. The leukemic cells of these mice express unmutated IGHV genes and some of them even present with BCRs showing stereotypical antigen binding regions [161]. Other transgenic mice models of CLL include (NZB $\times$ NZW)F1 mice programmed to express IL5 [162], mice overexpressing both BCL2 and a tumor necrosis factor receptor-associated factor [163] and *myc/Baff* transgenic mice [126].

A complementary approach has been to xenograft the Mec-1 cell line[165] or primary CLL cells[66,164] into immune-compromised mice. Recently, Bagnara et al. reported that peripheral blood mononuclear cells (PBMCs) from CLL patients xenografted into NOD/scid/ $\gamma c^{\text{null}}$  (NSG) mice localized and proliferated primarily in the murine spleen. These investigators found that proliferation of CLL cells *in vivo* was dependent on co-engrafted human T-cells.[66] Furthermore, by comparing CLL cells isolated from spleens of xenografted mice to CLL cells from human blood and LN, Sun et al showed that the murine spleen microenvironment supports CLL cell proliferation and activation to a similar degree as the human lymph node, which notably includes activation of BCR and NF- $\kappa$ B signaling in the xenografted cells. The model was then used to test the *in vivo* effects of ibrutinib, a Bruton's tyrosine kinase inhibitor in clinical development. Ibrutinib inhibited BCR and NF- $\kappa$ B signaling induced by the microenvironment, decreased proliferation, induced apoptosis, and reduced the tumor burden *in vivo*. [167] Thus, these data indicate that the spleen of xenografted NSG mice can sufficiently model the role of the human microenvironment on CLL cells to make this a valid model for investigations of tumor microenvironment interactions and the evaluation of possible novel treatment approaches.

## Targeting the microenvironment in CLL

The increasing appreciation of the role of the microenvironment in supporting CLL cell proliferation and survival as well as its contribution to chemoresistance has informed novel therapeutic approaches. Accordingly, a major effort is underway to find efficient ways to “chemically dissect” CLL cells from the microenvironmental signals, by either blocking extracellular triggers or abrogating intracellular signaling (Figure 3). In recent years, different compounds have been developed that are able to antagonize surface receptors or cytokines including small molecules that target signaling kinases and anti-apoptotic proteins. Clinically, impressive responses characterized by diminished lymphadenopathy and splenomegaly have been observed with some of these novel agents.

### CXCR4 receptor antagonists

CLL cells in the peripheral blood express high levels of the surface receptor CXCR4, which signals for chemotaxis, polymerization of actin, and migration through the vascular endothelium [131]. Blockade of the CXCR4-CXCL12 axis using CXCR4 receptor antagonists such as plerixafor (AMD3100) or T140 can efficiently antagonize CXCL12-mediated signaling and chemotaxis as well as stroma mediated protection from both spontaneous and drug induced apoptosis [101, 169]. The clinical use of plerixafor in CLL is investigated in combination with rituximab. Preliminary results showed that this agent induces dose-dependent mobilization of CLL cells to the peripheral blood [170].

### Targeting BAFF and APRIL

As previously mentioned, BAFF and APRIL signaling through their cognate receptors BAFF-R, TACI and BCMA are important for normal B-cell survival and may play a role in CLL. Atacicept is a recombinant soluble form of the extracellular binding domain of TACI. By acting as a molecular decoy, it neutralizes the effects of BAFF and APRIL, blocking the activation of TACI, BCMA, and the BAFF-receptor. A phase Ib study of Atacicept demonstrated that intravenous doses of up to 27 mg/kg were well tolerated in 21 patients with refractory or relapsed CLL with one PR (ORR 5%) in the highest dose cohort [171].

### Targeting BCR signaling

The pivotal role of BCR signaling in CLL pathogenesis points to this pathway as an ideal target for novel anti-CLL therapy (Figure 3). Small-molecule drugs targeting SYK, Bruton's tyrosine kinase (BTK), or PI3K isoform p110delta (PI3K $\delta$ ) show impressive results in

patients with relapsed/refractory as well as in treatment naïve CLL (reviewed in [20, 21]). In the first few weeks of treatment with these agents responses typically manifest with a substantial regression in lymphadenopathy that is frequently paralleled by a transient lymphocytosis [172–174]. The early increase in the circulating lymphocytes is assumed to reflect redistribution of the CLL cells from the lymphatic tissues into the circulation, as a consequence of disruption of mechanisms involved in migration and retention of the tumor cells in their protective tissue microenvironments [175, 176]. Continued treatment over the course of months results in gradual decrease in the lymphocytosis and a deepening of responses with a high rate of remission achieved with increasing duration of treatment [172]. The clinical responses seen with these kinase inhibitors are attributed to the combined effects of direct cell cytotoxicity, inhibition of survival pathways, and impairment of CLL cell trafficking and tissue retention.

### LYN inhibitors

LYN, a SRC family non-receptor tyrosine kinase, plays an important role in initiation as well as in the termination of BCR signaling. This dual role of LYN is due to its propagation of the BCR signal via phosphorylation of SYK and its concurrent activation of inhibitory phosphatases that terminate the response. Dasatinib, which was originally approved for treatment of chronic myeloid leukemia, is an oral kinase inhibitor primarily targeting ABL and SRC kinases but it also inhibits other kinases, including BTK [177]. In preclinical studies dasatinib has been shown to induce apoptosis of CLL cells that was associated with reduction in MCL1 and BCL-XL expression [178–180]. In a phase II trial in 15 patients with relapsed or refractory CLL, treatment with dasatinib (140mg/daily) achieved an overall response of 20% with a progression-free survival of 7.5 months. The major adverse reaction of the treatment was myelosuppression [181].

### SYK inhibitors

SYK is a key protein kinase of proximal BCR signal transduction that is also involved in B-cell migration and adhesion independently of BCR activity [182]. Following BCR engagement, SYK is phosphorylated by LYN and amplifies the BCR signal through activation of downstream signaling pathways. In peripheral blood CLL cells SYK phosphorylation on the activating Y352 residue has been demonstrated [183]. In addition, CLL cells in the lymph node show increased phosphorylation of SYK compared to peripheral blood CLL cells, indicating activation of the kinase in the tissue microenvironment [8].

To date several SYK inhibitors have been studied in CLL *in vitro* and *in vivo* including fostamatinib (R788, the oral pro-drug of R406, the active metabolite), PRT318 and P505-15. In preclinical studies, treatment with SYK inhibitors resulted in inhibition of BCR activation, moderate apoptosis of CLL cells, reduced basal kinase activity of SYK, AKT, and ERK, and decreased MCL1 levels [183]. Furthermore, SYK inhibitors have been shown to antagonize exogenous pro-survival signals provided by stromal cell or NLC coculture [62, 183, 184], secretion of BCR regulated chemokines CCL3 and CCL4 [62, 183, 184], and migration towards CXCL12 and CXCL13 [62, 184]. In an E $\mu$ -TCL1 transgenic mouse model, treatment with fostamatinib inhibited BCR signaling, reduced the proliferation and survival of the leukemic clone and extended the life of the treated mice [185].

Fostamatinib was the first SYK inhibitor introduced into clinical study [174]. In a phase 1/2 trial in patients with relapsed/refractory B-cell malignancy, fostamatinib was shown to be well tolerated with the most common adverse reactions being myelosuppression, fatigue and diarrhea. The highest overall response rate was achieved in patients with CLL/SLL (55%, 6 of 11, with a median progression free survival of 6.4 months), as compared to only 10–22%

in the other NHLs [174]. The on-target effect of fostamatinib in CLL has been demonstrated by downregulation of BCR regulated target genes in tumor cells of CLL patients on fostamatinib [186]. Furthermore, fostamatinib inhibited CLL cell activation and proliferation. However, there was no correlation between the degree of inhibition of BCR signaling and clinical response suggesting that pathways bypassing BCR activation might play a role in shaping the response to such kinase inhibitors [186]. Fostamatinib is being tested in late stage clinical trials for rheumatoid arthritis [187] and in patients with diffuse large B cell lymphoma. Some novel SYK inhibitors have shown promising pre-clinical activity, and may have increased potency and specificity [184].

### BTK inhibitors

BTK is a member of the TEC family of kinases that is critical for BCR signaling [188]. Mutations in BTK result in X-linked agammaglobulinemia, an inherited disorder manifesting with profound decrease in antibody production and severe defect in B-cell development [189]. Notably, BTK mRNA and protein expression levels are increased in CLL cells [190]. Ibrutinib (PCI-32765) is an orally administered irreversible and specific inhibitor of BTK that induces modest apoptosis in CLL cells irrespective of IGHV mutational status or interphase cytogenetics and overcomes pro-survival and proliferation signals provided by various tissue microenvironmental elements (such as CD40L, BAFF, IL-4, IL-6 and TNF $\alpha$ , fibronectin and stromal cells coculture and CpG oligonucleotide) [176, 190]. Ibrutinib abrogates CLL cell signaling, migration, and adhesion in response to tissue homing chemokines (such as CXCL12, CXCL13 and CCL19) and abrogates integrin  $\alpha$ 4 $\beta$ 1-mediated adhesion to fibronectin and VCAM-1 [175, 176, 190]. Both *in vitro* and *in vivo* ibrutinib has been reported to inhibit CLL cell secretion of CLL3 and CCL4 [176]. At the molecular level, this agent inhibits BTK tyrosine phosphorylation following BCR or CD40 stimulation and abrogates activation of downstream signaling pathways including ERK, PI3K, and NF- $\kappa$ B in CLL cells [190]. Treatment with ibrutinib in the TCL1 mice model of CLL resulted in inhibition of disease progression [176].

A phase I open-label dose-escalation study evaluated the efficacy and tolerability of ibrutinib in patients with relapsed or refractory B-cell NHL and B-cell CLL [191]. Dose escalation proceeded to 12.5 mg/kg without dose-limiting side effects and with pharmacodynamic evidence for complete inhibition of BTK. 60% of all patients achieved an OR, with 16% achieving a CR; 16 patients with CLL/SLL were evaluated, with an objective response reported in 11 of these patients (69%). Notably, the observed responses were of marked duration, as the median progression-free survival for all patients reported at the time of data cutoff was 13.6 months. A subsequent phase 1b/2 study of ibrutinib in CLL patients who were either i) greater than 65 years of age and previously untreated or ii) diagnosed with relapsed or refractory disease, further demonstrated that ibrutinib was well tolerated with the most common side effects including diarrhea, fatigue and nausea. ORR was 71% for treatment naïve patients, 67% for relapsed or refractory patients, and 50% for high risk patients [172]. The estimated PFS at 26 months was 75% for the relapsed/refractory cohort and 96% for treatment naïve patients demonstrating a remarkable duration of response.

### Targeting the PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR signaling pathway is a critical intracellular signaling cascade controlling cell survival and proliferation in both malignant and non-malignant cells. PI3K is a pivotal “hub” connecting multiple extracellular signals to cellular responses. PI3K acts to convert phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub> (PIP<sub>2</sub>)) to PI(3,4,5)P<sub>3</sub> (PIP<sub>3</sub>) which in turn forms a functional signaling complex with BTK and AKT. Several isoforms of PI3K have been characterized; the  $\delta$  isoform of PI3K is selectively expressed in hematopoietic cells and functions to relay BCR, BAFF, CD30, and TLR signaling. CLL

cells express the PI3K $\delta$  and display increased PI3K activity [192]. Given the selective expression of PI3K $\delta$  - in contrast to the more pervasive  $\alpha$  and  $\beta$  isoforms - specific inhibition of the  $\delta$  isoform is not expected to be toxic to normal tissues. Many PI3K inhibitors are currently being investigated in both pre-clinical and clinical settings. ON 01910.Na (Rigosertib), a multikinase phosphoinositide 3-kinase (PI3K) inhibitor in phase III trials for myelodysplastic syndrome, has demonstrated promising pre-clinical *in vitro* activity, inducing apoptosis in CLL cells that are cultured in contact with stromal cells via a dual mechanism of action involving both PI3K/AKT inhibition and induction of oxidative stress [193].

### GS-1101 (CAL-101)

GS-1101 is an orally available highly selective PI3K-delta inhibitor that induces apoptosis in CLL cells [194]. The cytotoxic effect of GS-1101 is maintained despite the presence of various microenvironmental components that normally support malignant CLL cells (including stromal cells, NLC, fibronectin, CD145 or TNF $\alpha$  BAFF and BCR stimulation). GS-1101 also inhibits the secretion of both anti-apoptotic and pro-inflammatory cytokines [192, 195]. GS-1101 sensitizes CLL cells to drug-induced apoptosis in the presence of stromal coculture [195] and inhibits CLL cell chemotaxis toward CXCL12 and CXCL13 [195]. The drug abrogates constitutive PI3K signaling in CLL cells as well as AKT and/or ERK activation by anti-IgM, soluble CD40 ligand and chemokines [195, 196]. In CLL patients treated with GS-1101 the serum levels of CCL3, CCL4, and CXCL13 were markedly reduced.[195]. A phase 1 study in 54 patients with previously treated CLL demonstrated the acceptable safety and promising clinical activity of GS-1101, with 26% of patients achieving an OR[173]. A >50% reduction in lymphadenopathy was observed in 80% of patients. Adverse events grade 3 or higher were minimal and included pneumonia and neutropenia, observed in less than a quarter of patients. Phase 2/3 clinical trials of GS-1101 are currently underway.

### AKT Inhibitors

The serine/threonine kinase AKT is a key nodal regulator of cellular survival known to phosphorylate several cellular substrates including caspase 8[197], caspase 9[198], BAD [199], mTor [200], and the Forkhead family of transcription factors.[201] Furthermore, AKT activation is associated with resistance to chemotherapy. Early phase clinical trials are underway investigating the use of AKT inhibitors in several malignancies including CML, although experience with these agents in CLL is limited. MK-2206, an orally active allosteric AKT inhibitor has been shown to enhance the antitumor efficacy of other chemotherapeutic agents *in vitro* in several malignancies, although clinical use in CLL has not been reported [202]. Perifosine, another oral inhibitor of AKT, is undergoing phase II evaluation in patients with refractory and relapsed leukemia [203]. Pending the outcome of these and other clinical trials, further investigation is warranted to determine the utility of the use of AKT inhibitors in CLL.

### mTOR inhibitors

Mammalian target of rapamycin (mTOR), a serine/threonine kinase of the PI3K/AKT/mTOR signaling network, is involved in cell growth, metabolism and proliferation and is commonly activated in B-cell neoplasms. Rapamycin (sirolimus, rapamune, Wyeth) is an immunosuppressive drug used to prevent rejection in organ transplantation. The drug profoundly blocks B-cell receptor mediated proliferation [204]. Preclinical studies in CLL have shown that rapamycin or its analog RAD001 block cell cycle progression by interfering with expression of critical cell cycle molecules [205]. Everolimus (RAD001, afinitor, Novartis), an orally available derivative of sirolimus, was evaluated in a phase II pilot trial in previously treated patients with CLL. The study was stopped early because of increased

toxicity, although the drug showed modest clinical activity [206]. In another phase 2 study, everolimus (10mg/day) administered to patients with recurrent/refractory CLL achieved partial remissions in 18% of patients [207]. In a subset of these patients, treatment with everolimus was accompanied by an increase in lymphocytosis in parallel to reduction in lymphadenopathy.

### Targeting the RAF/MEK/ERK signaling pathway

Sorafenib (BAY43-9006; nexavar) is an oral small molecule multi-kinase inhibitor approved for the treatment of advanced renal cell carcinoma [208] and unresectable hepatocellular carcinoma [209]. Sorafenib is a potent RAF serine/threonine kinase inhibitor targeting the RAF/MEK/ERK pathway and also inhibits other receptor tyrosine kinases involved in tumor progression and angiogenesis [210]. Sorafenib induces CLL cell death that is mediated via caspase activation and a decrease in MCL1 [211–213]. It overcomes apoptosis protection induced by NLC or stromal cell coculture and stromal-mediated chemoresistance [211–214]. The drug has been shown to block RAF/MEK/ERK signaling and the chemotaxis response induced by CXCL12 in CLL cells [211, 214]. It has also been shown to inhibit RAF and ERK activation by NLC or stromal cells [213, 214] and to interfere with VEGFR/STAT3 signaling induced by stromal cell coculture [213]. Sorafenib also abrogates BCR mediated signaling and survival in CLL cells. Interestingly, CLL cells derived from the lymph nodes are more sensitive to sorafenib than the cells found in the peripheral blood [214]. The clinical efficacy and tolerability of sorafenib in relapsed CLL is currently being evaluated in a phase II clinical trial.

### Targeting anti-apoptotic proteins

The resistance of CLL cells to apoptosis is related to high expression of BCL-2 family anti-apoptotic proteins. The overexpression of these antiapoptotic proteins in CLL is endogenous as well as extrinsic and is in part regulated by signals derived from the tissue microenvironment [151]. Therefore, in recent years, several therapeutic strategies have been developed to target anti-apoptotic proteins in CLL, including antisense BCL-2 oligonucleotide, BH3 mimetics and others.

Oblimersen sodium is a synthetic BCL-2 antisense oligonucleotide that induces a decrease in BCL-2 mRNA and protein levels and apoptosis in CLL cells [215]. Oblimersen sodium when combined with different cytotoxic drugs increases CLL cell apoptosis. In a phase I/II trial, oblimersen sodium as a single agent, showed minimal activity in patients with relapsed/refractory CLL. Dosing was limited by development of a cytokine release syndrome. At the dosage of 3 mg/kg/d, two (8%) of 26 patients achieved a partial response [216]. A phase III study in patients with relapsed/refractory CLL showed that addition of oblimersen to fludarabine plus cyclophosphamide (FC) increased the CR/nPR rate compared to FC alone [217]. Accordingly, CR/nPR was achieved in 20 (17%) of 120 patients in the oblimersen group and eight (7%) of 121 patients in the FC-only group [217]. The combination of oblimersen-FC further resulted in increased survival in subsets of patients who achieved a least a partial response and in those who had fludarabine sensitive disease [217, 218].

Obatoclax mesylate (GX15-070) is a small-molecule pan-BCL-2 antagonist. This compound belongs to a class of BH3 mimetic agents that inhibit the activity of the anti-apoptotic BCL-2 members that antagonize the proapoptotic proteins BAX and BAK [219]. The BH3-only proteins BAX and BAK are directly sequestered and repressed by the antiapoptotic BCL-2 proteins. BH3 mimetic drugs BAX and BAK induce their release allowing them to oligomerize and trigger apoptosis via the formation of pores in the outer mitochondrial membrane. In a phase I trial, administration of obatoclax to heavily pretreated patients with

advanced CLL resulted in a PR in one out of 26 patients. The major toxicities were neurologic including somnolence, ataxia and euphoria [220].

### BH3 mimetics

The BH3 mimetics, ABT-737 and its orally active analog navitoclax, inhibit BCL-2, BCL-XL and BCL-W. In pre-clinical studies ABT-737 has been shown to induce a rapid and potent proapoptotic activity in CLL cells independently of the common clinical and prognostic parameters in CLL [221, 222]. Addition of cytotoxic agents sensitizes CLL cells *in vitro* to ABT-737 [222]. In a phase I study in 29 relapsed/refractory CLL patients, navitoclax dosed at 100mg/d achieved durable partial responses in 35% of patients [223]. Navitoclax was also active in high-risk patients with fludarabine refractory disease, bulky lymphadenopathy and deletion of 17p [223]. The major dose-limiting toxicity was thrombocytopenia related to inhibition of BCL-XL [223].

AT-101 (gossypol isomer) is a small-molecule pan-BCL-2 antagonist. In preclinical studies, AT101 was shown to both induce CLL cell apoptosis and to overcome resistance mediated by stromal cell coculture, while sparing normal stromal cells [224]. A phase I trial of AT-101 in treatment naïve CLL patients with high risk disease demonstrated that the drug was well-tolerated [225]. Furthermore, 5 of 6 patients in this trial exhibited a decrease in lymphocyte count, while all patients demonstrated a reduction in lymphadenopathy.

### XIAP inhibitors

X-linked inhibitor of apoptosis (XIAP) inhibits the proteolytic activity of caspase-3 via direct binding, is highly expressed in CLL cells, and plays an important role in TRAIL-induced apoptosis [226]. Since CLL cells have previously exhibited resistance to TRAIL-based treatments, novel inhibitors of XIAP have been developed in the hope of overcoming TRAIL-resistance in CLL [227]. One such novel small molecule inhibitor, compound A (CA), has been shown to render tumor cells from patients with 17p deletion, IGVH unmutated type CLL susceptible to TRAIL *in vitro*.

### Immunomodulatory drugs

Lenalidomide, a derivative of thalidomide, is an immunomodulatory agent that has significant activity in 5q- myelodysplastic syndrome, multiple myeloma and other B-cell malignancies. In relapsed or refractory CLL, intermittent lenalidomide at 25 mg/d (3 weeks on/1 week off drug) was associated with acceptable toxicity, and achieved an overall response rate of 47%, with 9% of patients demonstrating a complete remission [228]. Another trial of relapsed/refractory CLL patients treated with daily lenalidomide at a dose of 10mg produced an overall response rate of 32%, with 7% CRs[229]. In a treatment-naïve setting, elderly patients with CLL treated with lenalidomide achieved an OR rate of 65%, including 10% CRs and an additional 5% CRs with residual cytopenias [230]. The activity of lenalidomide is irrespective of unfavorable genomic abnormalities such as unmutated IGHV or fludarabine-refractory disease status. However, at least 6 to 9 months of treatment may be needed to achieve the best possible response [229]. The most common toxicity of lenalidomine is myelosuppression [228, 229]. Lenalidomide is frequently associated with a cytokine release syndrome and tumor flare reaction (observed in 30–58% of patients) or tumor lysis syndrome[228, 231, 232]. Tumor flare is a unique immune mediated response to lenalidomide therapy characterized by painful lymph node enlargement that may be accompanied by fever and/or bone pain. It is generally managed with corticosteroids, while in more severe cases it may necessitate narcotics and hospitalization [229, 231]. Tumor flare may be predictive of clinical response to treatment with lenalidomide [233]. The optimal dosing schedule of lenalidomide is not well defined; and a dose of 25 mg once daily in relapsed CLL may be associated with unacceptable toxicity in some patients [231].



Furthermore, lenalidomide may be associated with an increased frequency of venous thromboembolism that may be related to endothelial dysfunction in the context of inflammatory cytokine secretion. Continuous low dose lenalidomide (10mg) is generally well tolerated and effective [229]. Importantly, the treatment is accompanied by increased serum immunoglobulin levels and typically reduces CCL3 and CCL4 plasma levels, which might indicate an inhibitory effect on intracellular signaling in CLL cells [230].

The mechanism of action of lenalidomide is not completely understood. In contrast to thalidomide, lenalidomide has weak antiangiogenic effects [229]. Lenalidomide induces transient immune activation including expression of costimulatory molecules such as CD40, CD80, and CD86 on CLL cells [231, 232], which may be mediated through PI3K $\delta$  [194]. It also increases serum levels of interleukin IL-6, IL-10, IL-2R, IFN $\gamma$ , and TNF $\alpha$  [229, 232]. Lenalidomide in part restores T-cell function by enhancing the formation of immunological synapses and promoting intracellular signaling [91, 160, 234]. In lymph node biopsies from CLL patients treated with lenalidomide a shift towards a Th-1 type immune reaction with production of IFN $\gamma$  was documented, suggesting that lenalidomide might be able to restore anti-tumor immunity and immune surveillance *in vivo* [235]. Furthermore, lenalidomide enhances NK cell activity [236] and improves antibody-mediated cellular cytotoxicity directed by rituximab [237].

## Summary and Outlook

Interactions of CLL cells with the surrounding microenvironment play a central role in the pathogenesis and the progression of the disease. The nature of these interactions is dependent on the properties of the CLL cell itself, but also likely depends upon properties of the specific patient's microenvironment, which may be shaped by the baseline expression levels of cytokines, the composition of various T cell subsets, stromal cell populations, and responses to antigenic stimulation. Major progress in the last decade has led to a much better understanding of both direct and indirect cellular interactions involved in CLL oncogenesis, and to the development of multiple new and promising therapies (Figure 3). Although there is still much to learn, it seems very possible that within a few years the improved understanding of key aspects of tumor biology and the development of novel therapeutic approaches will combine to change the natural history of CLL.

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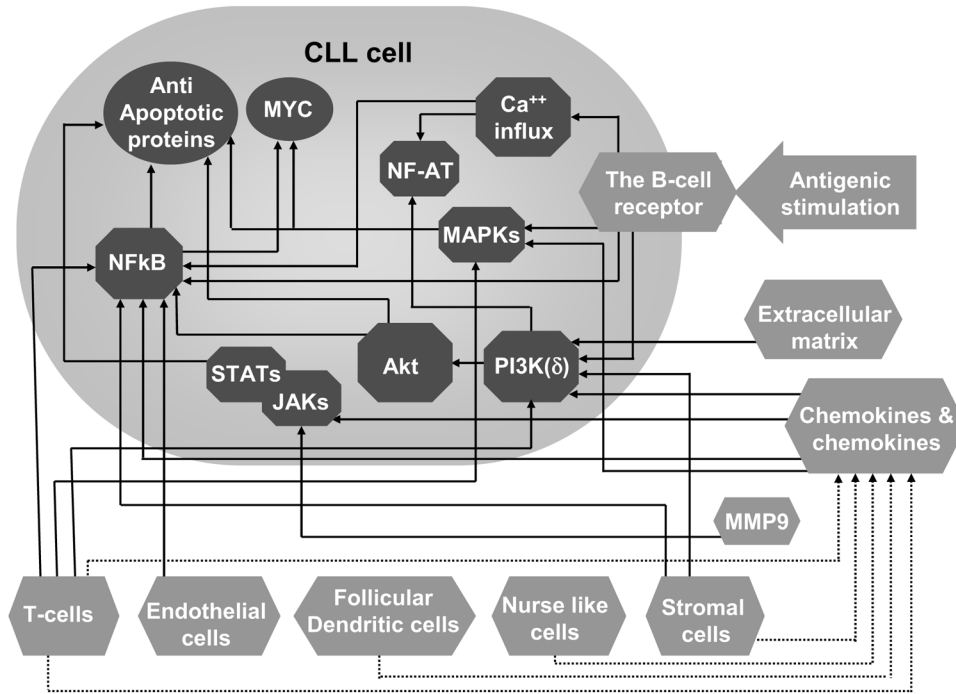
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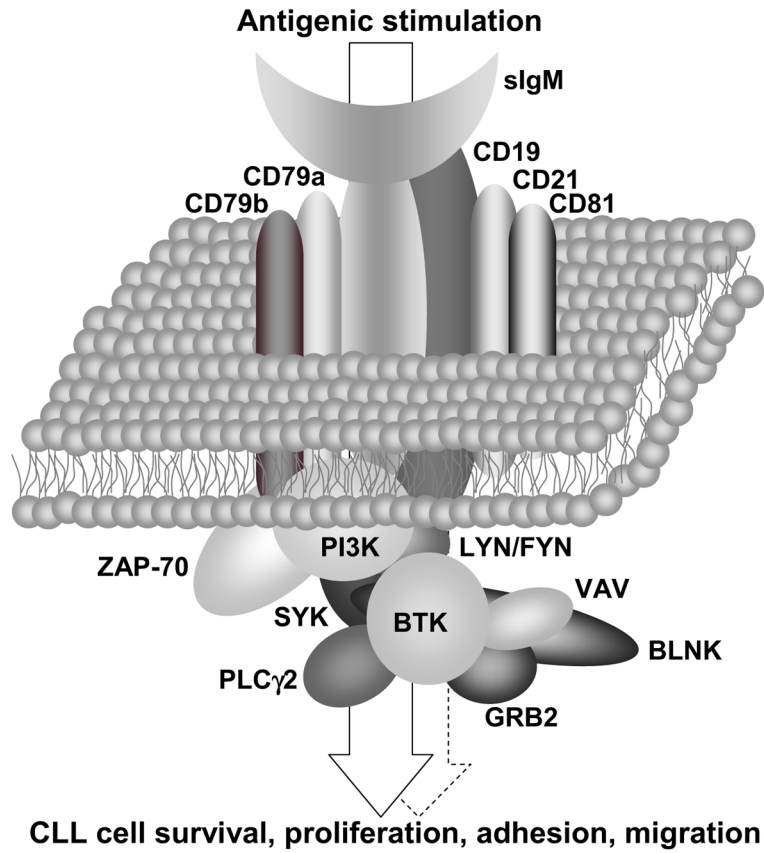
### Key Points

- CLL is characterized by the accumulation of mature monoclonal B cells in the peripheral blood, bone marrow, spleen and lymph nodes.
- Signals from the B cell receptor (BCR) and the tissue microenvironment converge on several key intracellular signaling pathways including the PI3K/AKT, MAPK/ERK, and NF- $\kappa$ B pathways and promote leukemic cell proliferation, survival, and resistance to chemotherapy.
- Tissue sites provide a supportive microenvironment composed of T-cells, stromal cells, cytokines, chemokines and extracellular matrix components.
- The lymph node is a pivotal site of CLL cell activation and proliferation through antigenic stimulation.
- The dependence of CLL cells on signals from the BCR and tissue microenvironment presents opportunities for targeted therapy. Inhibitors of BCR signaling and therapeutic approaches to “chemically dissect” CLL cells from the supportive microenvironment have shown encouraging clinical results.

**The microenvironmental signalosome in CLL**

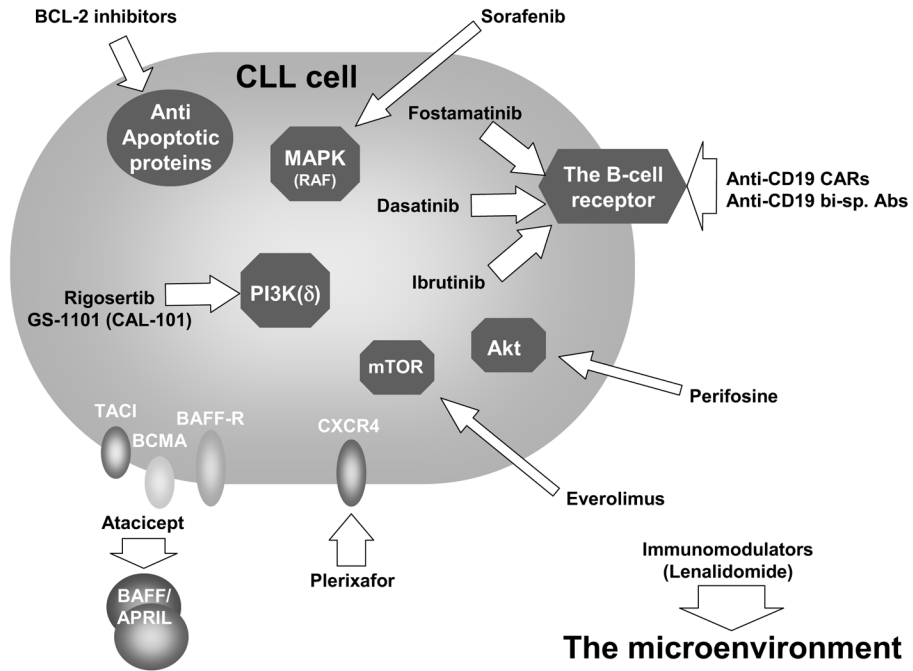


**Figure 1.** The CLL microenvironmental signalosome: the convergence of microenvironmental-induced signaling responses into biochemical pathways within CLL cells. Microenvironmental elements (■) including cells (e.g. T-cells, nurse-like cells), the extracellular matrix (ECM) and enzymes (e.g. MMP9) stimulate CLL cells either directly (arrows) or *via* mediators such as cytokines and chemokines (dashed arrows). These extracellular triggers converge into an array of intracellular biochemical responses (●), resulting in the up-regulation of MYC and anti-apoptotic proteins (●), as well as additional cellular responses.



**Figure 2.** The B-cell receptor – a signaling complex that delivers microenvironmental-derived information into the CLL cell. The sIgM serves as the backbone of the BCR, and is associated with other transmembrane molecules (e.g. CD19, CD21). The transmembrane components of the BCR associate with a variety of enzymes (e.g. SYK, BTK) and scaffold proteins (e.g. BLNK) to form a signaling complex. This complex translates extracellular cues, predominantly antigenic stimulation, into CLL cellular responses including survival, proliferation, adhesion and migration (arrow). Tonic or cell-autonomous activation (dashed arrow) does not require extracellular stimuli.





**Figure 3.** Therapeutic targeting of microenvironment-induced signaling in CLL. Current and experimental CLL therapeutics (arrows) target the various components of the microenvironment-CLL milieu and its associated signaling network. Thus the BCR and its associated components are targeted by antibodies (anti-CD19) or small molecules (e.g. SYK (e.g. fostamatinib) or BTK inhibitors (e.g. ibrutinib)). Small molecules are also utilized to inhibit mTOR, Akt, PI3K(δ) and the MAPK cascades. Extracellular inhibitors such as plerixafor or atacicept can block the association of SDF-1 or BAFF/APRIL, respectively, with their receptors on the CLL cell. Both the microenvironment (e.g. the immune system) and the outcome of its signaling responses in the CLL cells (e.g. upregulation of BCL-2) are avenues for therapeutic targeting.