

Chapter 2

The CLL Cell Microenvironment

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Abstract Cross talk between CLL cells and accessory stromal cells in specialized tissue microenvironments, such as the secondary lymphoid organs, favors CLL progression by promoting malignant B cell growth and drug resistance. Disrupting the cross talk between CLL cells and their milieu is an attractive, novel strategy for treating CLL patients. This chapter summarizes current knowledge about cellular and molecular interactions between CLL cells and their supportive tissue microenvironment and the therapeutic targets that are emerging, focusing on the CXCR4–CXCL12 axis and small molecule inhibitors that are targeting the B cell receptor-associated kinases SYK, BTK, and PI3K δ . Clinically relevant aspects of these new therapeutics will be discussed, along with an outlook into future biologically oriented therapeutic strategies. The rapid progress in dissecting the CLL microenvironment and the promising early results of these new targeted treatments in CLL indicate that CLL has become a role model for microenvironment-dependent cancers.

Keywords Chronic lymphocytic leukemia • CLL • Microenvironment • Nurselike cells • Stromal cells • CXCR4 • CXCL12 • B cell receptor • BCR • SYK • BTK • PI3K δ • Chemokines • Chemokine receptors

Introduction to the CLL Microenvironment

CLL cells expand in specialized tissue microenvironments, such as the bone marrow (BM) and secondary lymphoid organs, where CLL cells interact with different populations of accessory cells, such as mesenchymal stromal cells [1]

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and monocyte-derived nurselike cells (NLC) [2–4], as well as T cells [5, 6]. While the vast majority of circulating CLL cells in the blood are resting, nondividing cells, tissue CLL cells proliferate at a relatively high rate, accounting for a daily birth rate of approximately 0.1–1 % of the entire clone, as demonstrated by deuterated water labeling in CLL patients [7]. CLL cell proliferation occurs in microanatomical sites called proliferation centers, also called pseudofollicles, a hallmark histopathology finding in CLL [8, 9]. Conceptually, CLL and other mature B cell malignancies, such as follicular lymphoma (FL), mucosa-associated lymphoid tissue (MALT) lymphomas, and multiple myeloma (MM), are characterized by the expansion of the neoplastic B cells in a friendly, regulated coexistence with the microenvironment [9]. These interactions between CLL cells and the microenvironment resemble the pattern that normal counterpart B cells engage in. Particularly in the secondary lymphatic tissues, CLL cell B cell receptors (BCRs) become activated [10] either by currently still ill-defined microbial or autoantigens [11] or in an antigen-independent fashion [12, 13]. BCR activation then sets in motion a signaling cascade that results in the expansion of the CLL clone, in concert with other signals, such as CD40 ligand (CD154), BAFF and APRIL, and plexin B1 (reviewed in [14, 15]).

Consequently, the proliferative drive for the malignant cells is, at least initially, largely dependent upon external signals from the microenvironment, and the CLL cells undergo apoptosis unless their survival is reinforced by these external stimuli. Early evidence of microenvironment dependency came from the notion that CLL cells normally undergo spontaneous apoptosis in suspension culture unless they are cocultured with accessory stromal cells such as mesenchymal bone marrow stromal cells (BMSC) [2, 16, 17] or monocyte-derived NLC [2, 18]. Microenvironment dependence is also reflected by the difficulty of establishing CLL cell lines in the absence of EBV [19]. CLL–stroma interactions are not targeted by current “conventional” treatments, which may explain why, despite major therapeutic advances, CLL still remains an incurable disease. Based on this concept, CLL and other related mature B cell malignancies are expected to be particularly responsive to microenvironment-directed treatment approaches, and the clinical success of small molecule inhibitors of BCR-associated kinases in CLL patients in early stage clinical trials [20, 21] suggests that the microenvironment dependency of CLL cells may indeed be the “Achilles’ heel” of CLL.

Tissue Microenvironments in the Bone Marrow and Secondary Lymphoid Organs: Cellular Players and Model Systems

The BM and secondary lymphoid organs have entirely different, distinct microenvironments, supporting lymphocyte maturation and differentiation. The BM harbors hematopoietic stem cells (HSC) and fosters the development of mature B cells from committed progenitors. B cell lymphopoiesis in the marrow results in

the generation of B cells with functional antigen (Ag) receptors (BCRs). Mature B cells then migrate to secondary lymphoid organs where they are exposed to Ag within germinal centers (GC) of secondary lymphoid follicles. The microenvironment of GC allows maturing B cells to interact with CD4⁺ T cells for the necessary help upon Ag recognition and with specialized stromal cells (follicular dendritic cells/FDC) for the required quality control following affinity maturation [22, 23]. Each of these finely regulated steps ultimately results in the proliferation, maturation, and differentiation into Ag-specific effector plasma cells and memory B cells [24].

In the BM, stromal “feeder” cells maintain HSC in specialized “niches” which are close to the marrow vasculature (vascular niche) or to the endosteum (osteoblast niche) [25]. The importance of stromal cells for hematopoiesis was initially demonstrated in long-term BM cultures [26] and was utilized by Whitlock and Witte to develop a culture system to study the early stages of B cell maturation [27]. In vitro [28], CLL cells are attracted to BMSC, and the protective effects of BMSC require the close proximity between CLL and the stromal counterparts [2, 17, 28, 29]. The high affinity of CLL cells for stromal cells is exemplified by a striking in vitro phenomenon termed pseudoemperipolesis [28]. Pseudoemperipolesis describes the spontaneous migration of a fraction of CLL cells beneath BMSC, which occurs within a few hours of coculture. In phase contrast microscopy, pseudoemperipolesis is characterized by the dark appearance of lymphocytes that migrated into the same focal plane as the stromal cells. Pseudoemperipolesis describes symbiotic complexes of leukemia cells with their stromal cell component [30, 31]. During this cell interaction, leukemia cells migrate beneath the adherent BMSC or are trapped by membrane projections, but do not become internalized by the stromal cells. Coculture systems of CLL cells with BMSC, typically BMSC cell lines, have been standardized [29] and represent a reliable tool for studying CLL cell activation by BMSC, as well as stroma-mediated drug resistance. Intrinsic qualitative and quantitative abnormalities of CLL patient-derived primary BMSC have recently been characterized [32], as well as the effects of more physiologic hypoxia present in the marrow microenvironment on BMSC function [33]. NLC, on the other hand, activate CLL cells in a different fashion than BMSC, as demonstrated by gene expression profiling (GEP) in vitro [34, 35] and in vivo [10]. Specifically, BMSC induced a GEP pattern with prominent upregulation of the lymphoid proto-oncogene TCL1, paralleled by decreases of TCL1-interacting FOS/JUN [35]. In contrast, NLC induced a GEP response in CLL cells with characteristic induction of genes in the BCR and NFκB pathways [34] that is strikingly similar to the GEP of CLL cells isolated from lymph nodes of CLL patients [10]. Several other genes of potential importance were also differentially upregulated by BMSC (e.g., TNFRSF17, VPREB3, TNFSF10) and NLC (i.e., TNFRSF17, EGR2 and 3, MYCN), but their precise functions in the CLL microenvironment remain to be explored.

NLC owe their name to the similarities with thymic nurse cells that nurture developing thymocytes [2]. In vitro, NLC differentiate from blood monocytes cocultured with CLL cells in high-density culture conditions after 7–14 days [2].

In vivo, NLC can be found in the spleen and lymphoid tissues of CLL patients [3, 36], and the importance of NLC for CLL disease progression was highlighted in recent CLL animal models [37, 38]. NLC attract CLL cells by secreting CXCL12 [2] and CXCL13 [3] and protect CLL cells from spontaneous or drug-induced apoptosis through CXCL12 [2, 39], BAFF, APRIL [39], CD31, plexin-B1 [40], and activation of the BCR-signaling cascade [34]. Collectively, these data indicate that CLL cell cocultures with BMSC and NLC represent validated model systems for studying the impact of different microenvironments on CLL cell biology and for drug testing.

The Importance of BCR Activation in the CLL Microenvironment

BCR activation and signaling have emerged as a key mechanism for CLL cell expansion, even though the precise mechanism of BCR stimulation and the nature of the antigen(s) that activate the BCR remain largely unknown [11, 14, 41]. Direct evidence for the importance of BCR signaling in CLL comes from recent comparative GEP data that revealed BCR signaling as the most prominent pathway activated in CLL cells isolated from lymphatic tissues [10]. BCR activation can be induced either by antigen or be ligand independent (“tonic” BCR signaling) [12], and it activates a cascade of signaling events which normally cause B cell selection, proliferation, differentiation, and antibody production. Thereby, BCR signaling allows for the expansion of selected, specific normal B cells, and hence the deletion of unwanted, self-reactive B cells [42]. Engagement of BCRs by antigen induces phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmatic tails of Ig- α and Ig- β [43] by Lyn and other Src family kinases (Fyn, Blk), which also activate BTK, CD19, and PI3K. These events are associated with BCR oligomerization and BCR microcluster growth, leading to the recruitment and activation of SYK via ITAMs [44]. Upon phosphorylation, SYK, BTK, and PI3K activate downstream signaling pathways, including calcium mobilization and activation of AKT kinase, extracellular signal-related kinase 1/2 (ERK, also called p44/42 mitogen-activated protein kinase/MAPK), and myeloid cell leukemia-1 (Mcl-1) [45, 46]. In CLL cells, BCR activation also causes phosphorylation of SYK, activation of phospholipase C γ 2, and intracellular calcium mobilization and facilitates DNA binding of the calcium–calcineurin-dependent transcription factor NFAT2 [47], as well as MEK1/2-dependent expression of the proto-oncoprotein MYC [48]. The clinically most relevant targets within the BCR-signaling cascade in CLL are BTK, PI3K δ , and SYK.

BCR-Associated Kinases BTK, PI3K δ , and SYK

Bruton's Tyrosine Kinase, BTK

BTK is a non-receptor tyrosine kinase of the Tec kinase family and plays a central role in BCR signaling. BTK is primarily expressed in hematopoietic cells, particularly in B cells, but not in T cells or plasma cells [49]. BTK deficiency due to mutations in BTK is the genetic basis for X-linked agammaglobulinemia (XLA) [50, 51], a primary immunodeficiency characterized by low serum immunoglobulin levels and lack of peripheral B cells. Upon BCR activation, BTK becomes activated by other tyrosine kinases, such as Lyn and SYK, resulting in activation of transcription factors necessary for B cell proliferation and differentiation [52]. In addition to its role in BCR signaling, BTK also is involved in signaling of other receptors related to B cell migration and adhesion, such as the CXCR4 and CXCR5 chemokine receptors and adhesion molecules (integrins) [53–55].

Ibrutinib, previously called PCI-32765, is the first in-human BTK inhibitor which binds specifically and irreversibly to a cysteine residue in the BTK kinase domain and inhibits BTK phosphorylation and its enzymatic activity [56]. Ibrutinib shows encouraging clinical activity in patients with B cell malignancies, particularly in CLL patients [21]. Herman et al. reported about ibrutinib-induced CLL cell apoptosis in the presence of CLL pro-survival factors (CD40L, BAFF, IL-6, IL-4, TNF- α , fibronectin, stromal cell contact) [57]. We recently reported that ibrutinib inhibits CLL cell survival and proliferation, as well as leukemia cell migration towards tissue-homing chemokines (CXCL12, CXCL13) [58]. Ibrutinib also downregulated secretion of BCR-dependent chemokines (CCL3, CCL4) by the CLL cells, both in vitro and in CLL patients receiving therapy with ibrutinib. These data demonstrate that ibrutinib effectively inhibits CLL cell migration and survival, possibly explaining some of the characteristic clinical activity (CLL cell redistribution) of ibrutinib. Along the same lines, de Rooij and colleagues recently reported about ibrutinib's interference with CLL cell chemotaxis and integrin-mediated CLL cell adhesion [59], suggesting that these BCR-independent actions of ibrutinib explain the redistribution of CLL cells from the tissues into the peripheral blood, characteristically seen during the first months of treatment with ibrutinib and other BCR-signaling inhibitors [20, 21, 60].

Phosphoinositide 3'-Kinase Delta, PI3K δ

PI3Ks integrate and transmit signals from different surface molecules, such as the BCR [61], chemokine receptors, and adhesion molecules, thereby regulating cellular functions, such as cell growth, survival, and migration [62]. PI3Ks are divided into three classes (I through III). Class I kinases contain four isoforms designated PI3K α , PI3K β , PI3K γ , and PI3K δ . While the PI3K α and PI3K β isoforms are

ubiquitously expressed and the PI3K γ isoform has a particular role in T cell activation, PI3K δ expression is largely restricted to hematopoietic cells, where it plays a critical role in B cell homeostasis and function [63]. Mice with inactivating PI3K δ mutations have reduced numbers of B1 and marginal zone B cells, low levels of immunoglobulins, poor responses to immunization, defective BCR and CD40 signaling, and can develop inflammatory bowel disease [63, 64]. In CLL cells, PI3K are constitutively activated [65], and unmutated high-risk CLL patients show overexpression of PI3K by quantitative polymerase chain reaction [66]. Furthermore, growth and survival signals from the microenvironment, such as adhesion to stromal cells [67], CXCR4 activation [28], and BCR activation [46], cause PI3K activation in CLL cells.

GS-1101, previously called CAL-101, is a potent and highly selective PI3K δ inhibitor and represents the first and currently the only PI3K δ inhibitor in clinical use [68]. GS-1101 induces apoptosis in B cell lines and primary cells from patients with different B cell malignancies, including CLL [69], mantle cell lymphoma, and multiple myeloma [68, 70]. GS-1101 also inhibits constitutive and CD40-, TNF-alpha-, fibronectin-, and BCR-induced PI3K activation [68–70]. In patients receiving GS1101 therapy there is an initial redistribution of CLL cells from the tissues into the blood, along with a rapid lymph node size reduction and a transient lymphocytosis during the first weeks of treatment [71], which is not explained by inhibition of pro-survival signaling. GS-1101 inhibits CLL cell chemotaxis towards CXCL12 and CXCL13 and migration beneath stromal cells (pseudoemperipolesis) [72]. These in vitro results are corroborated by clinical data showing marked reductions in circulating CCL3, CCL4, and CXCL13 levels, paralleled by a surge in lymphocytosis during GS-1101 treatment [72]. Therefore, it appears that GS-1101 has several mechanisms of action, directly decreasing cell survival while reducing interactions that retain CLL cells in the tissue microenvironments.

Spleen Tyrosine Kinase, SYK

SYK belongs to the SYK/ZAP-70 family of non-receptor kinases and activates signaling pathways downstream of the BCR. SYK-deficient mice have severely defective B lymphopoiesis [73, 74], with a block at the pro-B to pre-B transition, consistent with a key role for SYK in pre-B cell receptor signaling. Moreover, in vivo studies recently demonstrated that SYK is critical for survival and maintenance of mature normal and malignant B cells [73, 75]. Besides their role in immune responses, SYK activation also modulates cell adhesion and chemotaxis of normal cells, such as B cells [76, 77], suggesting that SYK participates in tissue homing and retention of activated B cells.

R788 (fostamatinib disodium, FosD) is the only SYK inhibitor in clinical use to date. Fostamatinib, the clinically used oral formulation, is a prodrug that rapidly converts in vivo into the bioactive form called R406 [78, 79]. Previous studies established that R406 is a relatively selective SYK inhibitor, although R406 also

displayed activity against other kinases including Flt3, Jak, and Lck [79]. After encouraging results in a phase I/II study in patients with relapsed B cell lymphomas, particularly in patients with CLL, where the objective response rate was 55 % [20], further development of this drug is focused on rheumatoid arthritis (RA) [80]. As such, there is at this time only one ongoing clinical trial of fostamatinib in patients with diffuse large cell B cell lymphoma (DLBCL, NCT01499303). Alternative SYK-specific inhibitors are under development and have demonstrated promising preclinical activity in CLL models [81]. Importantly, similarities in clinical response pattern of CLL patients to treatment with SYK, BTK, or PI3K δ inhibitors (transient lymphocytosis due to redistribution, rapid lymph node shrinkage) suggest overlapping functions of these kinases in BCR signaling, CLL cell migration, and homing [60]. The transient lymphocytosis caused by these new agents has complicated response assessment in these patients, given that progressive lymphocytosis could be interpreted as progressive disease (PD). However, given that CLL patients on these drugs typically show clear signs of response (reduced lymph node sizes, normalization of hemoglobin and platelet counts, resolution of constitutional symptoms) even before stabilization and then resolution of lymphocytosis, this lymphocytosis in the absence of other signs of PD should not be confused with true PD. This interpretation was supported by a group of CLL experts [82], and CLL response criteria may need to be formally revised when these new agents become more widely used.

Mechanism of CLL Cell Migration and Adhesion

Normal B cell trafficking and function largely depend upon interactions between B cells and accessory stromal cells [83, 84]. For example, stromal cells in secondary lymphatic tissues constitutively express chemokines such as CXCL12 and CXCL13 that provide guidance for B cell positioning within distinct lymph node compartments [83, 85–87]. According to Springer's multistep paradigm [88], lymphocyte trafficking and homing require the cooperation between chemokine receptors and adhesion molecules, such as integrins, CD44, and L-selectins, which are expressed on normal and malignant lymphocytes. Lymphocytes actively enter and home within tissue microenvironments, such as the secondary lymphatic tissues, where stromal cell networks provide guidance cues by secreting chemokines, establishing chemokine gradients, and expressing ligands for lymphocyte adhesion molecules. Coordinated lymphocyte entry, migration, and territoriality are essential during immune surveillance and induction of specific immune responses [85, 89–91]. In B cell lymphomas/leukemias, the neoplastic B cells largely retain the capacity of their normal counterparts for trafficking and homing, as demonstrated in CLL and B cell acute lymphoblastic leukemia (ALL), both *in vitro* [3, 28] and *in vivo* [92].

The term chemokines initially was coined in 1992 as a short form of “chemotactic cytokines.” Currently, the human chemokine system includes more than

40 chemokines and 18 chemokine receptors [93]. Chemokines are small secreted proteins that are released either constitutively or in response to stimulation and cause migration of cells towards a gradient of the chemokine (chemotaxis). The two main subfamilies of chemokines, CXC and CC chemokines, are distinguished based upon two conserved cysteine residues, which are separated by either an intervening or adjacent amino acid, accounting for CXC or CC chemokines, respectively [93]. Chemokines bind to chemokine receptors, which belong to the large family of seven transmembrane domain G-protein-coupled cell surface receptors (GPCRs). Following activation, the intracellular domains cause dissociation of G-proteins, which are composed of three distinct subunits (α -, β -, γ -heterotrimers). This leads to formation of the second messengers inositol triphosphate (IP3) and diacylglycerol (DAG), resulting in cytoplasmatic calcium mobilization and activation of multiple downstream signaling cascades, such as the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/MAPK (also called ERK 1/2) signaling pathways.

T and B lymphocytes express receptors for various chemokines, and their expression and function is modulated during lymphocyte differentiation and activation [94]. Circulating lymphocytes interact transiently and reversibly with vascular endothelium through adhesion molecules (selectins, integrins) in a process called rolling. Chemokines displayed on the luminal endothelial surface activate chemokine receptors on the rolling cells, which triggers integrin activation [88]. This results in the arrest, firm adhesion, and transendothelial migration into tissues where chemokine gradients guide localization and retention of the cells [95]. These steps are collectively referred to as “homing” and are essential for normal development of the organism, organization and function of the immune system, and tissue replacement.

Chemokine Receptors on CLL Cells

CXCR4 (CD184)

CXCR4 is expressed at high levels on the surface of peripheral blood CLL cells [28, 96–98] and mediates CLL cell chemotaxis, migration across vascular endothelium, actin polymerization, and migration beneath and underneath CXCL12-secreting BMSC [28, 96, 99, 100]. CXCL12 also has a pro-survival effect on CLL cells [2, 39, 101], which is not surprising, given that CXCL12 initially was characterized as pre-B cell growth-stimulating factor (PBSF) [102]. CXCR4 surface expression is regulated by its ligand CXCL12 (previously called stromal cell-derived factor-1/SDF-1) via receptor endocytosis [28], with downregulation of surface CXCR4 on tissue CLL cells by CXCL12 present at high levels in the tissues. This characteristic can be used to distinguish tissue (lymphatic tissue- and marrow-derived) from blood CLL cells, which express low or high CXCR4 levels,

respectively [10, 28]. Proliferating, Ki-67⁺ CLL cells from marrow and lymphatic tissue displayed significantly lower levels of CXCR4 and CXCR5 than nonproliferating CLL cells [103]. In vivo deuterium (²H) labeling of CLL cells revealed that patients with higher CXCR4 expression on their CLL cells had delayed appearance of newly produced CD38⁺ cells in the blood and increased risk for lymphoid organ infiltration and poor outcome [104]. These ²H studies also revealed intraclonal heterogeneity of CXCR4 expression, with an enrichment of CLL cells expressing lower CXCR4 surface levels in the CD38⁺/CD5^{bright} fraction, along with increased ²H incorporation [104]. These in vivo data indicate that lower blood CXCR4 surface levels label a fraction of CLL cells that has recently exited the tissues into the blood.

B cell antigen receptor (BCR) signaling results in down-modulation of CXCR4 [105, 106], along with enhanced chemotaxis towards CXCL12 and CXCL13, at least in our hands [105]. This may explain why ZAP-70⁺ CLL cells display increased chemotaxis and survival in response to CXCL12 when compared to ZAP-70-negative CLL cells [101], given that ZAP-70 expression is associated with a higher responsiveness to BCR stimulation [107]. CD38⁺ CLL cells also display higher levels of chemotaxis [108], and CD38 activation enhanced chemotaxis towards CXCL12, whereas a blocking anti-CD38 mAb inhibited chemotaxis [109]. CXCR4 signaling in CLL cells is pertussis toxin sensitive and induces calcium mobilization, activation of PI3 kinases [28] and p44/42 MAP kinases [2], and serine phosphorylation of signal transducer and activator of transcription 3 (STAT3) [110]. CXCR4 signaling can be inhibited by isoform-selective PI3 kinase inhibitors [111], including CAL-101 [112], and inhibitors of SYK [105] and BTK [58], leading to impaired CLL cell migration.

Other Chemokine Receptors in CLL (CXCR3, CXCR5, CCR7)

CXCR3 (CD182) is the receptor for the CXC chemokines CXCL9, CXCL10, and CXCL11. These interferon-gamma (IFN γ)-induced chemokines are secreted at sites of inflammation and function in a paracrine or autocrine fashion [113]. CXCR3 is expressed on subsets of normal B and T cells [114]. CXCR3 is consistently expressed on CLL and splenic marginal zone lymphoma B cells, but not on normal CD5⁺ B cells, and more inconsistently on neoplastic B cells from patients with other B cell lymphomas [115, 116]. CXCR3 expression levels on CLL cells are variable, and low CXCR3 expression was associated with advanced stages (Rai III/IV), diffuse marrow infiltration, other risk factors, and poor survival in one study [117], although the functional role of CXCR3 expression in CLL remains unclear.

CXCR5 (CD 185) is the receptor for CXCL13, a chemokine that regulates lymphocyte homing and positioning within lymph follicles [118]. CXCR5 is expressed by mature B cells, a small subset of T cells, and skin-derived dendritic cells (reviewed in [119]). CXCR5 gene-deleted mice display defective formation of

primary follicles and germinal centers in the spleen and Peyer's patches and lack inguinal lymph nodes [120]. Subsequently, the ligand for CXCR5 was identified and termed B cell-attracting chemokine 1 (BCA-1) [121] and now is designated CXCL13. CXCL13 is constitutively secreted by stromal cells in B cell areas of secondary lymphoid tissues (follicles), where B cells encounter antigen and differentiate [118, 122]. CXCR5 induces the recruitment of circulating naïve B cells to follicles [118, 122] and is also responsible for the microanatomic positioning within the germinal center (GC) [84, 86, 91, 123]. In addition, it has been suggested that the primordial function of CXCL13 may be the recruitment of primitive B cells to body cavities for T-independent responses, prior to its involvement in the complex lymphocyte positioning during T-dependent antibody responses [124]. CLL cells express high levels of CXCR5 [3, 97, 100, 116, 125]. CXCR5 expression levels are similar on CLL B cells and normal, CD5⁺ B cells and higher when compared to normal, CD5-negative B cells, T_{FH} cells, or neoplastic B cells from other B cell neoplasias [3]. Stimulation of CLL cells with CXCL13 induces actin polymerization, CXCR5 endocytosis, chemotaxis [100], and prolonged activation of MAPK (ERK 1/2). In CLL, CXCR5 signals through G_i proteins, PI3 kinases, and the p44/42 MAPK pathway [3]. CXCL13 mRNA and protein is expressed by NLC in vitro and in vivo [3]. These data suggest that CXCR5 plays a role in CLL cell positioning and cognate interactions between CLL- and CXCL13-secreting stromal cells, such as NLC in lymphoid tissues.

The CCR7 (CD197) receptor has two ligands, CCL19 and CCL21. CCL19 and CCL21 are constitutively expressed by reticular cells, high endothelial venules (HEVs), and dendritic cells (DC) and play a role in lymph node homing of naïve and regulatory T cells and DC [126]. Moreover, the CCR7–CCL19/CCL21 axis is involved in organizing the architecture and function of the thymus. CCR7 is expressed by DCs, thymocytes during defined stages of their development, and B and T cell subpopulations. CCR7 is also expressed by various neoplastic cells, and CCR7 expression correlates with lymph node metastasis in solid tumors [127], including malignant melanoma and colorectal and prostate cancer. In sharp contrast to CXCR5-deficient mice, which show reduced peritoneal B-1 and B-2 B cells, CCR7 deficiency results in a massive accumulation of T cells and B-2 B cells in the peritoneal and pleural cavities, caused by an impaired egress of CCR7-deficient lymphocytes from body cavities [128]. CLL cells express CCR7 and migrate across vascular endothelium in response to CCL19 and CCL21 [99, 125]. Moreover, expression levels of CCR7 correlated with lymphadenopathy [99, 125] and expression of ZAP-70 and CD38 [101]. CCL19- and CCL21-induced migration and actin polymerization of ZAP-70⁺/CD38⁺ CLL cells were higher when compared to CLL cells lacking ZAP-70 and CD38 [101]. Moreover, CCL21 significantly increased B-CLL metalloproteinase-9 (MMP-9) production in MAP kinase (ERK1/2-)-dependent fashion [129], suggesting cross talk between these pathways during trafficking and tissue homing. CCR7 signaling for chemotaxis in response to CCL19 and CCL21 involves PI3 kinases and the Rho kinase [130]. Anti-CCR7 mAbs recently were shown to cause complement-dependent cytotoxicity against CLL cells and therefore were proposed as a potential therapeutic [131]. Overall,

these data support the concept that CCR7 plays an important role in trafficking and homing of CLL cells to the lymphatic tissues.

Chemokines Secreted by CLL Cells: CCL3, CCL4, and CCL22

CCL3 and CCL4 are chemoattractants for monocytes and lymphocytes [132]. CCL3 expression in normal B cells is induced by BCR triggering and CD40 ligand [133–135] and repressed by Bcl-6 [136]. Activated CLL cells express and secrete CCL3/4 [34, 137, 138] in response to BCR stimulation and in coculture with NLC [34]. This BCR- and NLC-dependent induction of CCL3/4 is sensitive to inhibition of BCR signaling, using, for example, a SYK inhibitor [34, 105]. CLL patients display elevated CCL3/4 plasma levels [34], and plasma levels of CCL3 were strongly associated with established prognostic markers and time to treatment. A multivariable analysis revealed that CCL3, advanced clinical stage, poor risk cytogenetics, and CD38 expression were independent prognostic markers in a cohort of 351 CLL patients [139]. The function of CCL3/4 in CLL remains poorly defined, but based upon the function of B cell-derived CCL3/4 in normal immune responses, increased CCL3/4 secretion by CLL cells may induce trafficking and homing of accessory cells, particularly of T cells and monocytes, to CLL cells in the tissue microenvironments [34, 140].

Regulatory T cells (T_{reg}), identified by expression of the transcription factor FoxP3, typically express the chemokine receptor CCR4 and migrate towards the ligands for CCR4, called CCL22 and CCL17. It was proposed that CCL17 and/or CCL22 secretion could be responsible for an accumulation of FoxP3⁺ T_{reg} cells in the tumor microenvironment, which might suppress local immune responses and favor tumor progression in diseases such as breast cancer or Hodgkin's disease [141, 142]. CLL cells obtained from the tissues, but not from the blood, express CCL22 and variable levels of CCL17 mRNA. After CD40 ligation, CCL22 and CCL17 mRNA became induced in blood CLL cells, and CCL22 protein was released into CLL cell supernatants, which in turn attracted CCR4⁺ T cells. Conceivably, by attracting T cells and other immune cells, CLL cell-derived chemokines foster the coevolution of CLL cells and their supportive microenvironment, actively creating a favorable microenvironment in which CLL cells interact with T cells and other accessory cells that deliver survival and proliferation signals.

VLA-4 (CD49d) Adhesion Molecules in CLL

Integrins are a superfamily of heterodimeric glycoproteins, consisting of various α - (1 through 11) and β - (1 through 6) subunits, whose function is to mediate cell–cell and cell–matrix adhesion in various cell types. The term “integrin” was first proposed in 1986 to describe membrane complexes involved in the transmembrane

association between fibronectin as part of the extracellular matrix (ECM) and the actin cytoskeleton [143]. Integrins are categorized into subfamilies, with members sharing a common β -subunit pairing with a unique α -subunit. β_1 -integrins are very late activation (VLA) antigens that have the same β_1 -subunit but various α -chains ($\alpha 1$ through 6) [144]. The $\alpha_4\beta_1$ -integrin VLA-4 (CD49d) is a receptor for fibronectin (FN) and vascular cell adhesion molecule-1 (VCAM-1/CD106). VLA-4 is expressed on lymphocytes, monocytes, and most other hematopoietic cells (except for neutrophils); VLA-4 is involved in both cell–cell and cell–ECM adhesions and plays a role in lymphocyte trafficking and homing as part of immune surveillance [94], the trafficking and homing of other hematopoietic cells, and inflammation. Integrins are highly versatile adhesion molecules; their adhesiveness can rapidly be regulated by the cells on which they are expressed: for example, by chemokine receptor activation [88]. VLA-4 mediates lymphocyte adhesion to the VCAM1, also known as CD106, which is expressed on cytokine-activated endothelium. VCAM1 mediates leukocyte–endothelial cell adhesion and may play a role in the development of atherosclerosis and rheumatoid arthritis. VLA-4 also binds fibronectin, an ECM component expressed on MSC [145], by interacting with at least three fibronectin sites: CS-1 and REDV in the IIICS region and H1 in the HepII region [146]. VLA-4 plays a particularly important role for interactions between normal and malignant hematopoietic cells and the marrow microenvironment. VLA-4 integrins cooperate with chemokine receptors in CLL cell adhesion to stromal cells [28, 147], they cooperate with CD38 [148], and their function can be inhibited by the BTK inhibitor ibrutinib [59]. Moreover, VLA-4 expression on CLL cells has prognostic impact [149, 150], indicating the relevance of these interactions in vivo. Collectively, these studies indicate that VLA-4 integrins play a key role for the adhesion of CLL and other leukemia cells to stromal cells and ECM, and they provide a rationale to further explore and target this molecule in CLL.

Therapeutic Targeting of Chemokines and Their Receptors in CLL

The CXCR4–CXCL12 Axis

CXCR4 antagonists initially were developed as new drugs for the treatment of HIV-1 infection (reviewed in [151]), where CXCR4 functions as a co-receptor for HIV-1 entry into T cells. However, their use in HIV-1 was abandoned because of lack of oral bioavailability and low efficacy. CXCR4 antagonists inhibit CLL cell activation by CXCL12 on functional and signaling levels, and they reverse, at least in parts, stromal cell-mediated drug resistance [110]. Several classes of CXCR4 antagonists are in clinical development, such as small modified peptide CXCR4

antagonists (BKT140), small molecule CXCR4 antagonists (AMD3100, now called plerixafor), and antibodies to CXCR4 (MDX-1338/BMS 93656). Plerixafor, a bicyclam, is a specific small molecule antagonist of CXCL12, inhibiting CXCL12-mediated calcium mobilization, chemotaxis, and GTP binding, and it does not cross-react with other chemokine receptors [152]. Plerixafor causes the mobilization of various hematopoietic cells, including CD34-positive HSC, to the blood [153, 154] and was approved by the FDA for administration together with G-CSF for mobilization of HSC in lymphoma and multiple myeloma patients. BKT140 is a high-affinity inverse CXCR4 agonist, which finished phase I/II testing in multiple myeloma patients that undergo stem cell mobilization.

The preclinical data of plerixafor and BTK140 were the basis for a recent clinical trial in relapsed CLL patients, in which patients were treated with rituximab in combination with plerixafor. The goal of this proof-of-principle trial was to determine whether leukemia cells can be mobilized from the tissues, using a CXCR4 antagonist, and then targeted outside of the protective tissues. Data from this trial demonstrated a plerixafor dose-dependent mobilization of CLL cells from the tissues into the blood [155].

Conclusions and Perspective

During the coming years increasing emphasis will be placed towards targeting the microenvironment in CLL and other cancers. Clinically, inhibitors of BCR-associated kinases (SYK, BTK, and PI3K δ) represent the most advanced therapeutics for targeting the microenvironment in CLL, and when compared to other B cell malignancies, these agents display the highest clinical activity in CLL [20, 21]. The current enthusiasm for these novel agents is justified based on the clinical activity and the lack of major side effects, such as myelosuppression, but the precise mechanism of action, the potential benefit of combinations with conventional agents, usefulness of biomarkers such as CCL3 [58, 72, 139], durability of responses, and potential resistance mechanism remain to be explored. Despite these open questions, we can expect a paradigm shift towards kinase inhibitor-based treatment in CLL, which hopefully will benefit large numbers of CLL patients in the near future and which will set a new standard for other diseases to follow.

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