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Chronic Lymphocytic Leukemia – Microenvironment and B Cells

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Abstract

Chronic lymphocytic leukemia (CLL) has been considered as an accumulative disease deriving from defects in apoptosis, but recent studies showed that CLL is a dynamic process in which monoclonal B cells proliferate within pseudofollicular proliferation centers. Microenvironmental interactions are essential for the survival and proliferation of CLL cells. The cell traffic between blood and secondary lymphoid tissues is controlled by tissue-specific chemokines and their specific receptors on B lymphocytes. Interstitial cell migration and adhesion events, predisposed by activational stimuli, determine CLL cell localization. Stimulation through the B cell receptor plays an important role in the expansion of the malignant clone in CLL. B cell receptors become activated either in an antigen-dependent or in an antigen-independent fashion in the secondary lymphatic tissues. However, low expression of the BCR correlates with reduced induction of protein tyrosine kinase activity and defective intracellular calcium mobilization and tyrosine phosphorylation. In contrast to normal B cells, leukemic cells are poor antigen presenting cells. This is due to the fact that leukemic cells have a reduced expression of costimulatory molecules and defects in the formation of immunological synapse with T cells. Increased surface expression of the costimulatory molecules on CLL cells correlates with their proliferation. At present, conventional treatments are not directed to interactions between CLL cells and their microenvironment, which is probably one of the reasons why, despite the significant progress in treatment, the disease still remains incurable. In this regard, identifying key biomarkers of intercellular interactions of neoplastic CLL population in comparison with clinical laboratory abnormalities in CLL enable clarification of essential processes in the development of the disease, and can be the basis for

stratifying patient groups in order to optimize therapeutic approaches, which will make them relevant and promising.

Keywords: Chronic lymphocytic leukemia, Microenvironment, B cell receptor complex, Chemokine receptors, Cell adhesion molecules, Costimulatory receptors

1. Introduction

Chronic lymphocytic leukemia (CLL) is a disease with highly variable clinical manifestation and course ranging from an aggressive, life-threatening disease to an indolent form, which does not require treatment for many years. Current clinical staging systems are accessible and easy to apply; however, they do not allow a sufficiently accurate opportunity for individual assessment of the disease progression. Recently, significant progress has been made in our understanding of CLL pathogenesis. CLL has been considered as an accumulative disease deriving from an inherent defect in apoptosis, but recent studies showed that it is a dynamic process, wherein monoclonal B cells proliferate within pseudofollicular proliferation centers, hallmark of histopathology on this disease.

CLL cells interact with different types of cells, such as mesenchymal stromal cells (MSC), monocyte-derived nurse-like cells (NLC), follicular dendritic cells (FDC), and T cells. Microenvironmental interactions are critical for the survival and proliferation of the leukemic cells. A key role in the pathogenesis of the disease is given to the B cell receptors (BCRs), which in the secondary lymphatic tissues become activated either by antigen-dependent or in an antigen-independent fashion. The proliferative drive for the malignant cells is dependent from external microenvironmental signals, and the CLL cells undergo apoptosis unless their survival is reinforced by these external stimuli.

The cell traffic between the blood and secondary lymphoid tissues is controlled by tissue-specific chemokines and their specific receptors. B cells express CD184 (CXCR4) as a functional receptor, responsible for directing them mainly to the bone marrow (BM) environment. The specific ligand CXCL12 has been shown to have anti-apoptotic effects on CLL cells and protect them from spontaneous and chemotherapy-induced apoptosis in a contact-dependent manner via stromal cells or nurse-like cells. The chemokine receptor CD185 (CXCR5) plays an important role in lymphocyte homing guiding B cells into the B cell zone of secondary lymphoid organs and, together with CD197 (CCR7) (positioning in T cell zones of secondary lymphoid tissues), ensures the close contact between T and B cells and activation. CLL cells secrete chemokines as CCL3, CCL4, and CCL22, and therefore an autocrine way of stimulation on monoclonal B cells is also suggested.

Interstitial cell migration and adhesion events, influenced by activating stimuli, determine CLL cell localization. CD44 has been implicated in cell activation, migration, and tissue retention via binding to its extracellular matrix ligand hyaluronan. CD38, CD49d, MMP9, and CD44 are physically associated in a supramolecular cell surface complex; however, the complex is not

present in normal B cells. Taking into account the known properties of the individual molecules and their functional overlap, it seems that the complex CD38 / CD49d / MMP9 / CD44 plays a role in the migration into the tissues and in pro-survival signaling.

Stimulation through the BCR plays an important role in the selection and expansion of the malignant clone in CLL, and the prognostic impact of the mutational status of immunoglobulin heavy chain variable region (IGHV) genes could be considered as a consequence of the relevance of this process in CLL. Low expression of the BCR correlates with the reduced induction of protein tyrosine kinase activity and defective intracellular calcium mobilization and tyrosine phosphorylation. Although there are individual differences among patients, almost all CLL cases display very low levels of surface IgM and CD79b.

Costimulatory receptors on monoclonal B cells are expressed throughout B-cell development and are implicated in cell survival and differentiation. CD40/CD40L interaction stimulates B cells to proliferate, differentiate, upregulate costimulatory molecules, and increase antigen presentation. In contrast to normal B cells, leukemic cells are poor antigen presenting cells. This is due to the fact that leukemic cells have a reduced expression of costimulatory molecules such as CD80 and CD86 and they have a defect in the formation of immunological synapse with T cells. Surface expression of the costimulatory molecules CD80 and CD86 on CLL cells is increased with their proliferation. CD267 (transmembrane activator calcium modulator and cyclophilin ligand interactor - TACI) was identified as a receptor for B cell-activating factor (BAFF) and A proliferation-inducing ligand (APRIL), two members of the TNF ligand family. Both ligands induce proliferation, activation, and the survival of B cells, therefore the variable expression of CD267, detected in patients with CLL, is probably important for the disease characteristics.

The advances in fundamental understanding of the disease mechanisms in CLL will lead to improved therapies for patients. At present, conventional treatments are not directed to the interactions between CLL cells and their microenvironment, which is probably one of the reasons why, despite the significant progress in treatment, the disease still remains incurable. In this regard, studies on key biomarkers of intercellular interactions of the leukemic population enable clarification of key processes in the development of the disease, and can be the basis for defining a separate risk patient group to optimize therapeutic approaches, which will make them relevant and promising.

2. Microenvironment: Cells and interactions

Malignant B lymphocytes develop in the specific tissue microenvironment of the lymph node and eventually in the bone marrow (BM), where they interact with various cell populations - mesenchymal stromal cells (MSC), monocyte-derived nurse-like cells (NLC), follicular dendritic cells (FDC), and T cells. Unlike the non-dividing cells circulating in the peripheral blood (PB), tissue B cells proliferate (0.1–1% of the clone per day) [1]. This process occurs in pseudofollicular proliferation centers [2, 3]. The interaction of the leukemic cells with the microenvironment is similar to the model of normal B-cell engagement. Secondary lymphoid

tissues are the place, where the B cell receptor (BCR) of monoclonal cells is activated [4] by microbial antigens, autoantigens [5], or independently of antigens [6, 7]. Activation triggers a signaling cascade facilitated by additional stimulatory signals, which results in the clone growth.

2.1. T lymphocytes

The interaction between monoclonal B lymphocytes and T lymphocytes is an important component of the malignant process. T cells are extremely important for malignant proliferation [8]. This was proven in a mouse xenograft model of CLL, in which activated CD41+ T cells proved to be a necessary condition for CLL cell proliferation. In patients suffering from this disease, T cells, mainly CD41+, often constitute a significant fraction of the lymphoid infiltrate in bone marrow and lymph nodes [9], located within and around proliferation centers [10, 11]. One of the factors mediating the migration to the particular location is the expression of the CD197 (CCR7) chemokine receptor on T cells, which facilitates recirculation through secondary lymphoid tissues, following the gradient of its specific ligands CCL19 and CCL21 secreted in the T-cell zone of the lymph node [12]. The CD40 receptor, a key modulator of the interaction between B- and T-lymphocytes, is stimulated by CD41+ T cells, which express its ligand CD154 (CD40L) [13] and are co-localized with leukemic cells in the proliferation centers [14]. In addition to the interactions through a direct intercellular contact, T lymphocytes secrete soluble active molecules, interleukins, cytokines, and chemokines, which stimulate proliferation and inhibit apoptosis of monoclonal B cells. Interleukin-4 (IL-4) inhibits spontaneous and drug-induced apoptosis through a mechanism causing hyperproduction of Bcl-2 [15]. The concurrent action of IL-2 [16] and Tumor Necrosis Factor- α (TNF- α) [17, 18] induces malignant proliferation. Interferon- γ (IFN γ) [19], IFN α [20], and IL-13 [21] have a similar effect. Leukemic cells also change the cellular component of the immune system by the production of immunosuppressive cytokines Tumor Growth Factor- β (TGF- β) [22] and IL-10 [23] and the expression of low levels of the adhesion and costimulatory molecules [24], thus leading to the increase and dysfunction of regulatory T lymphocytes [25, 26]. The gene expression profile (GEP) of T cells from patients with CLL shows multiple changes in the genes participating mostly in cellular differentiation, cytoskeleton, traffic, and cytotoxicity, which leads to an inhibited immune response [27].

2.2. Mesenchymal Stromal Cells (MSCs)

MSCs are another major cellular component of tissue microenvironment. These cells are a heterogeneous population, performing structural and functional interactions in normal hemopoiesis [28]. Stromal cells secrete cytokines, chemokines, proangiogenic factors, and extracellular matrix components. They express surface receptors, which mainly regulate migration and facilitate the survival of CLL lymphocytes. The interaction of the leukemic cells with MSC is in both directions. Not only monoclonal B cells migrate and are activated under the influence of MSC, but vice versa, they also activate them and induce stromal-cell proliferation and the secretion of mediators that increase the intensity of the malignant process [29, 30, 31, 32]. In the lymphoid tissues of patients with CLL, stromal cells are diffusely distributed within the entire tissue and in the perivascular spaces, where they are mixed with leukemic

cells [33]. CLL lymphocytes cultured with bone-marrow stromal cells avoid both the spontaneous and drug-induced apoptosis [34, 35], through a mechanism dependent on direct intercellular contact [34]. CXCL12 secreted by the stromal cells directs the migration of the leukemic cells, which express the CXCR4 (CD184) receptor, through the stroma, and facilitate the penetration beneath it (a phenomenon called pseudoemperipoiesis) [35]. Surface receptors and extracellular matrix elements induce anti-apoptotic stimuli in CLL lymphocytes, which are in contact with the stroma. Adhesion of tumor cells to stromal cells is mediated by the $\beta 1$ and $\beta 2$ integrins [36]. MSCs express Vascular Cell Adhesion Molecule 1 (VCAM-1) [37]. Attachment of $\alpha 4\beta 1$ integrin (CD49d/CD29) to VCAM-1 or to the extracellular matrix component fibronectin protects CLL cells from a spontaneous or Fludarabine-induced apoptosis [36, 38] by means of PI3K/AKT signals and the increase of BCL-XL [39]. The interaction of the leukemic cell with the stroma also includes Matrix metalloproteinase-9 (MMP-9), Vascular Endothelial Growth Factor (VEGF), and endothelial cells. MMP-9 is the main matrix metalloproteinase produced by monoclonal B cells; it enhances extravasation and infiltration in lymphoid tissue by means of the proteolytic degradation of basal membranes and extracellular matrix components [40]. Regardless of its proteolytic activity, MMP-9 partially mediates anti-apoptotic signals in the leukemic clone when cultured with bone-marrow stromal cells [41]. The attachment of MMP-9 to $\alpha 4\beta 1$ and CD44v in CLL lymphocytes leads to the activation of the Lck/Yes novel tyrosine kinase (LYN) and the Signal Transducer and Activator of Transcription 3 (STAT3) [39]. The expression of MMP-9 from leukemic cells is regulated by the $\alpha 4\beta 1$ integrins and CXCL12 [40]. Monoclonal B lymphocytes in the bone marrow and lymph nodes express elevated levels of surface MMP-9, which shows the activation of tumor cells in tissues microenvironment [39].

2.3. Follicular dendritic cells

Follicular dendritic cells (FDC) are accessory cells in normal germinal centers, where they catch and retain antigen–antibody complexes on their cellular surfaces and present antigens to the B cells. After binding to immune complexes, normal B lymphocytes in the germinal center differentiate to memory B lymphocytes or plasma cells [42]. Normally, FDC are localized in secondary lymphoid tissues but not in the bone marrow [43], while in CLL these cells are localized in lymph node pseudofollicles [44, 45] and in nodular bone marrow infiltrates [46]. They secrete important anti-apoptotic and growth factors including BAFF, IL-15, and express adhesion molecules Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Adhesion Molecule-1 (ICAM-1), Plexin B1, and CD44 [42, 44]. The effects of FDC on malignant B lymphocytes have been studied on a follicular dendritic cell line (HK). There is evidence that HK cells facilitate the survival of B cells, protect them from a spontaneous or drug-induced apoptosis via the direct intercellular contact mechanism [47].

2.4. Tissue associated macrophages

Tissue associated macrophages have been studied in an in vitro model - NLC. In case of continuous culturing of peripheral blood mononuclear cells of CLL patients, large, rounded, rarely binuclear, CD68 expressing cells grow [48, 49]. NLC take this name since they contribute to the growth and survival of the CLL cells. Cells of a similar phenotype can be found in vivo

[49] in the secondary lymphoid organs of patients with CLL, but their number in the tissues is generally low [33]. NLC differentiate from monocytes as this differentiation depends on intercellular contacts with CLL lymphocytes. When monocytes from healthy individuals are co-cultured with leukemic cells, they differentiate to NLC; however normal B cells are unable to induce this differentiation [49]. NLC secrete chemokines (CXCL12 and CXCL13) and growth factors such as BAFF and APRIL, hold leukemic cells within tissues, and facilitate their survival and proliferation [50, 51, 52]. Malignant cells also express BAFF, APRIL, and their receptors, however, at a significantly lower rate [53, 54, 55], B-cell maturation antigen (BCMA) and CD267 (TACI). Two receptors connect APRIL–BCMA and TACI. Ligands BAFF and/or APRIL lower the spontaneous and drug-induced apoptosis of CLL cells [53, 54, 55]. In recent years, it has been proven experimentally that BAFF in cooperation with MYC protein, that plays a role in cell cycle progression, apoptosis and cellular transformation, causes the development of lymphocytic proliferation in mice that is similar to CLL [56]. MYC and its target genes are highly expressed in CLL cells in lymph nodes [4], which is stimulated by BAFF [56] and BCR activation in vitro [57].

2.5. Monoclonal B cells

The cell traffic between the blood and secondary lymphoid tissues is controlled by tissue-specific chemokines and their specific receptors on lymphocytes. The chemokine receptors expressed on neoplastic CLL cells drive migration to proliferation centers (Figure 1; Table 1).

3. Chemokine receptors on monoclonal B cells

3.1. CD184 (CXCR4) expression

The CD184 receptor binds CXCL12 (stromal cell-derived factor-1/SDF-1), a ligand characterized as a pre-B cell growth factor [58]. It is highly expressed on the peripheral blood leukemic cells surface, facilitates the chemotactic migration through vascular endothelium to bone marrow stromal cells following the CXCL12 gradient [35, 48, 55, 58, 59]. CD184 surface expression is regulated by the ligand CXCL12 through receptor endocytosis occurring as a result of activation [58]. This feature can be used to distinguish tissue leukemic cells expressing low levels of CD184 from blood CLL cells, which express high levels of CD184 [4, 58]. Proliferating CLL lymphocytes from bone marrow and lymphoid tissue show significantly lower levels of CXCR4 and CXCR5, compared to the non-proliferating ones [60, 61]. The BCR signals also decrease the expression of CD184 [62, 63]. As BCR signals may increase via Zap-70 [64], monoclonal B lymphocytes expressing Zap-70 have increased chemotaxis and survival as a response to CXCL12 compared to Zap-70 negative leukemic cells [65], the result of increased BCR signals. Leukemic cells expressing CD38 also show increased chemotaxis [66] compared to CXCL12, which can be inhibited by blocking with anti-CD38 monoclonal antibodies [67]. The signals conducted by CD184 induce mobilization of calcium, activation of PI3Ks [58], p44/42 MAPKs [48], and serine phosphorylation of STAT3 [35]. The signals conducted by CD184 can be inhibited by selective isomorphous inhibitors - the PI3K inhibitors [68] and the spleen tyrosine kinase (SYK) inhibitors [62], thus resulting in impaired migration of both the

normal B cells and the leukemic cells. According to some research teams, the levels of CXCR4 and its ligand CXCL12 correlate to well-established clinical parameters such as the Rai staging system, while other researchers do not observe correlation with the clinical stage of the disease or with the type of bone marrow infiltration, instead they report correlation with the lymphocyte count [69, 70]. It should be considered that CD184 is a constitutionally expressed receptor of B lymphocytes, which could explain the contradictory findings of the different research teams [71].

3.2. CD181 and CD182 expression

CD181 (CXCR1) and CD182 (CXCR2) have a common ligand - CXCL8. Monoclonal B cells secrete CXCL8 and express its receptors, which implies both the autocrine and paracrine stimulation of tumor cell through CXCL8. This stimulation leads to increased expression of anti-apoptotic protein Bcl-2 and survival of the clone [72].

3.3. CD183 expression

The chemokine receptor CD183 (CXCR3) interacts with three ligands (CXCL9, CXCL10, and CXCL11) [73]. CD183 is expressed on the surface of malignant cells as a functional receptor facilitating a directed drive and invasion [70, 73]. Leukemic cells express CXCL9, which constitutes proof of the autocrine way of a neoplastic growth stimulation, in addition to the paracrine way. Studies show correlation between the low expression of CD183 in the advanced stage of the disease (Rai III and Rai IV), non-mutated IGHV status, high CD38 expression, and the shortened general survival span of the patients. It is suggested that the lower levels of CD183 could be used as an independent adverse prognostic factor [74]. Nevertheless, the significance of CXCR3 remains disputable.

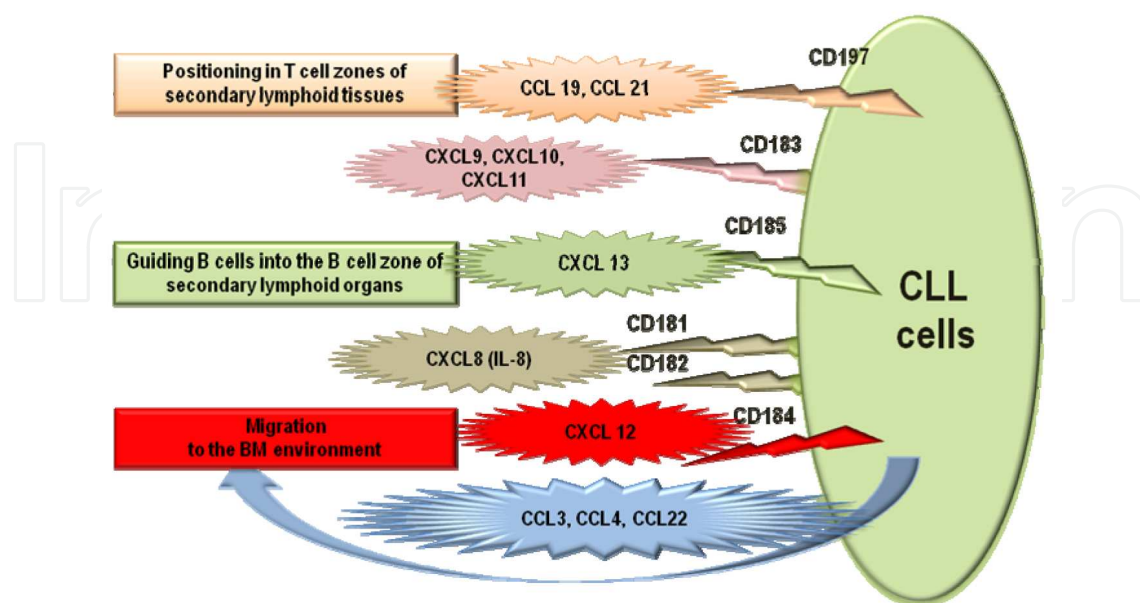


Figure 1. Chemokine receptors and chemokines involved in monoclonal B cell activation, migration, and proliferation

3.4. CD197 expression

CD197 (CCR7) is the main receptor responsible for the traffic of dendritic cells, the B- and T-lymphocytes through the high endothelial venules and their localization in the T cell zones of the secondary lymphoid organs. Its ligands - CCL19 and CCL21 are expressed in the T cell zone of secondary mucosa-associated tissues and high endothelial venules but not in the B cell zones, sinuses, and peripheral blood. CD197 is expressed mainly by naïve T lymphocytes but can be detected at certain levels also on B lymphocytes. The role of the receptor for the development of the secondary lymphoid organs has also been proven [12]. The expression of CD197 on leukemic B cells is significantly higher in patients with lymphadenopathy, compared to patients with organomegaly. The CD197-CCL21 binding activates intracellular signaling pathways, which lead to elevated expression of MMP-9 and infiltration of leukemic cells through basal membranes [12]. The increased expression of CD197 on Zap-70+ CLL cells enhances the response of CD197 to ligands [64], and the CD5 phosphorylation additionally stimulates its surface expression [75]. The binding of both CD197 and CD185 to the respective ligands elevates the expression of the partially expressed gene 10 (PEG10) in CD23+ CD5+ CLL cells. The increased synthesis of protein stabilizes caspase-3 and caspase-8 (which normally are pro-apoptotic proteins), protects them from degradation, thus leading to suppression of the TNF- α induced apoptosis [76, 77]. A correlation of CD197 with the Rai staging system has been found, which shows the need of further studies on the possible prognostic significance of this receptor [71].

| RECEPTOR | LIGAND | CLL EXPRESSION | FUNCTION |
|--------------------------------|--------------------------|--|---|
| CD181 (CXCR1) CD182 (CXCR2) | CXCL8 (IL-8) | Aberrant or activation expression | Inhibition of apoptosis by up-regulation of Bcl-2 |
| CD183 (CXCR3) | CXCL9, CXCL10, CXCL11 | Low to moderate continuous expression | Th1 response, inflammation, integrin activation, chemotaxis migration |
| CD184 (CXCR4) | CXCL12 | High, low in receptor endocytosis as a result of interaction with CXCL12 | Contact-dependent inhibition of apoptosis. Migration and homing |
| CD185 (CXCR5) | CXCL13 | High | Migration, inhibition of apoptosis |
| CD197 (CCR7) | CCL19, CCL21 | Moderate to high, correlates with lymphadenopathy | Inhibition of apoptosis |

Table 1. Chemokine receptors and corresponding chemokines

3.5. Chemokines secreted by B cells

Activated monoclonal lymphocytes secrete the CCL3, CCL4 [59], and CCL22 [13] chemokines that participate in the adaptive immune response and are chemo-attractants for T lymphocytes

and monocytes (Table 2). CCL3 and CCL4 are normally secreted by B cells after the activation through BCR and CD40–CD40L interaction [59, 62]. CCL3 secreted from tumor cells induces T cell traffic to the activated CD38⁺/Ki-67⁺ leukemic lymphocytes for the purpose of enabling intercellular interactions, which enhance proliferation [62]. Similarly to CCL3 and CCL4, CCL22 participates in the process of attraction of T cells in tissues. It acts as a secondary signal to T lymphocytes; its secretion from malignant cells starts after attachment of CD40 to CD40L [13]. The monoclonal population in peripheral blood does not secrete CCL22; secretion is observed in lymph nodes and bone marrow, probably also as a result of the attachment to CD40. In classifying CLL patients on the basis of their response to CD40 ligation, it was found that the patients who did not show a response have less time until disease progression and a possibility for proliferation of B cells in conditions of lower stromal stimuli [78] (Figure 1).

| CHEMOKINE | RECEPTOR | CLL EXPRESSION | SUPPOSED FUNCTIONS |
|-----------|---------------|---|---|
| CCL3 | CCR1, CCR5 | After activation by BCR, higher in Zap-70+ CLL-cells, SYK-dependent | In case of inflammation, activation of polymorphonuclear leukocytes and B cells |
| CCL4 | CCR5 | | Supports T cells in T-B cell interactions |
| CCL22 | CCR4 | After CD40 ligation | Supports regulatory T cells |

Table 2. Chemokine receptors and corresponding chemokines secreted from CLL cell [72]

4. Cell adhesion molecules on monoclonal B cells

Adhesion molecules facilitate the migration of leukemic cells to proliferation centers in bone marrow and secondary lymphoid tissues [79]; some of them show anti-apoptotic activity and a relation to drug resistance by binding to their receptors, which are expressed from bone-marrow stromal cells. The profile of CLL cells in regards to adhesion molecules has been the subject of studies in recent years since it determines the capacity of leukemic lymphocytes for response to chemokines and migration to regions, where antigens and additional activation stimuli influence B lymphocytes (Figure 2).

4.1. CD38 expression

The CD38 receptor performs and modulates a series of intracellular signals initiated by the cells of microenvironment. The percentage of CD38⁺ cells in the CLL clone is an indicator of the current level of cell activation; the cells with a higher expression of CD38 are better receivers of activation signals and hence the more aggressive portion of the malignant lymphocytes. Studies on patients with CLL using the incorporation in vivo of deuterium (²H) in the form of heavy water (²H₂O) in a cellular DNA have proved higher proliferation rate of CD38⁺

lymphocytes in comparison to CD38⁻ cells [1]. CD38⁺ B lymphocytes react more effectively in binding to surface immunoglobulins (sIg), with Zap-70 also participating in this process. The ligand of CD38–CD31 is expressed on vascular endothelium cells and facilitates cell adhesion. The aggressiveness of CD38⁺ lymphocytes is mediated by their ability to migrate and interact with the cellular microenvironment. CD38 and Zap-70 are functionally related and define the cells with a higher migration potential [66]. In most of CLL patients, the proportion of CD38⁺ cells does not exceed a threshold of 30%, which defines the clone as positive. Disease progression is more common in the smaller proportion of patients with a CD38 expression defined as positive [80]. The higher proliferative potential of these lymphocytes suggests novel genomic disorders and clonal evolution [81, 82, 83, 84], which is supported by the increased incidence of the observed 11q and 17p deletions [85].

4.2. CD49d expression

The level of CD49d (VLA-4) positive B lymphocytes has been recognized as an independent prognostic factor in CLL. CD49d is α -integrin subunit (α 4), which together with CD29 (β 1 subunit) forms the α 4 β 1 integrin binding fibronectin and VCAM-1. Similarly to other integrins, α 4 β 1 mediates the adhesion of cells to the extracellular matrix, the first step of cell migration. CD49d and CD38 participate in the formation of a large macromolecular complex that includes CD49d, CD38, CD44v, and MMP-9 and has been observed in CLL cells without somatic mutations [86]. The physical and functional binding between CD49d/CD29 and CD38 has been proven in CLL [87].

In recent years, CD49d adhesion molecule facilitating both the intercellular contact and the adhesion to extracellular matrix has been established as a new prognostic marker. A multicenter study on 2,972 patients with CLL validated the prognostic significance of CD49d as an independent flow cytometric prognostic marker in terms of overall survival and time-to-treatment start, as defined over a threshold of 30% [88].

4.3. CD44 expression

The CD44 protein family comprises a large group of transmembrane glycoproteins obtained through alternative splicing and post-translational modification. The considerable degree of heterogeneity in molecular structure predetermines various essential functions. CD44 mediates interactions between cells and the extracellular matrix, delivers signals acting as a co-receptor for tyrosine kinases localized in cell membranes or by binding to intracellular signaling molecules and activating intracellular signaling pathways. The expression of different CD44 isoforms depends on the cell type, stage of activation, and differentiation. The participation of CD44 in the development and progression of hematological neoplasms is associated with the increase of apoptotic resistance, invasiveness, regulation of bone-marrow infiltration, and mobilization of leukemic cells in peripheral blood [89]. The surface levels of CD44v in CLL are associated with advanced clinical stage, resistance to therapy, and decreased survival [90]. Unlike other cooperating adhesion molecules such as CD11a, CD49d, CD54, CD58, and CD62L, the expression of CD44 and CD11c correlates with the splenic presentation of the disease [91]. Soluble CD44 is associated with shortened progression free survival [92],

while soluble CD44s and CD44v6 are associated with lymphadenopathy, advanced clinical stage, and resistance to chemotherapy [93]. The results of an in vitro study of the anti-apoptotic effects of CD44 in CLL cells showed protective action of cultured CLL cells with HK-cells against spontaneous apoptosis in increased levels of Mcl-1 (a member of the anti-apoptotic Bcl-2 family). In this study, the blocking of CD44 by monoclonal antibodies leads to a decrease of Mcl-1 and suppression of the protective effect of HK-cells, demonstrating that the anti-apoptotic mechanism is CD44-dependent [47]. The formation of macromolecular complexes from CD44v, CD49d, and MMP-9 on the cells of CLL, but not on normal B lymphocytes, as well as increased secretion of MMP-9 under the action of CD44 antibodies, reveals the molecular mechanism of regulation of invasion [94].

4.4. CD54 expression

CD54 is a glycoprotein that is expressed on the surface of vascular endothelium cells, macrophages, and lymphocytes. It binds type CD11a/CD18 and type CD11b/CD18 integrins. In case of cytokine stimulation by IL-1 and TNF- α , the concentrations of CD54 increase significantly [95]. Upon activation, cells connect to the endothelium via CD54–CD11a/CD18 and penetrate tissues [96]. A significant reduction of CD54 expression levels has been demonstrated in CLL patients compared to a control group of healthy subjects [97]. β 1 and β 2 integrins of the malignant B cell, acting simultaneously, mediate the connection of CD54 to bone-marrow stromal cells and protect them from apoptosis, while normal B lymphocytes cannot be protected by stromal cells due to the loss of this adhesion connection [36].

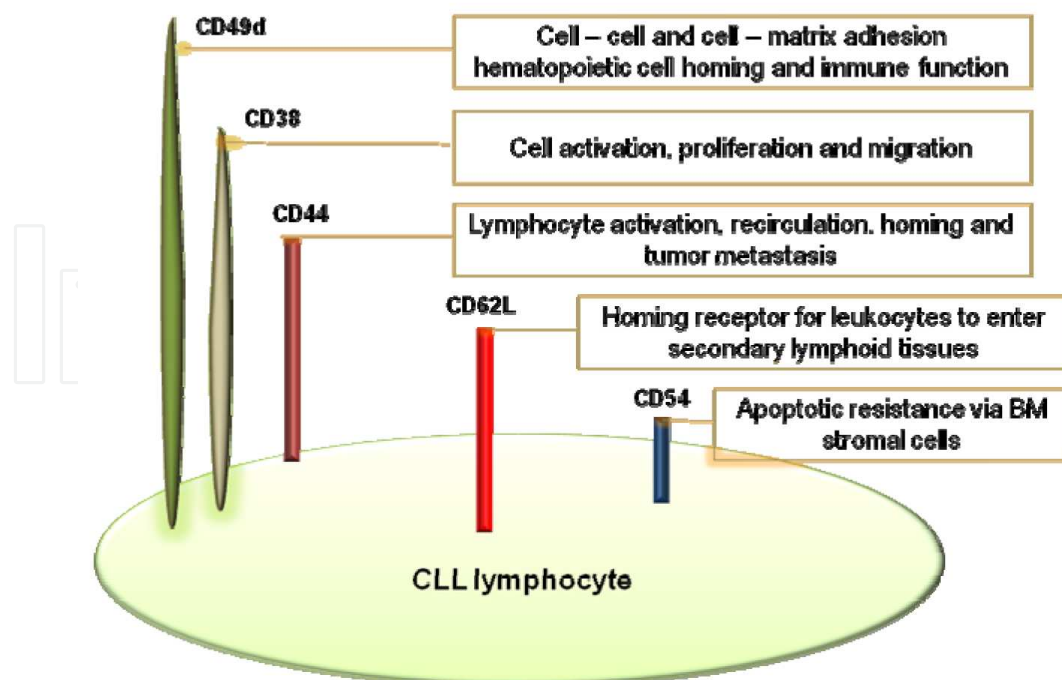


Figure 2. Adhesion molecules and their main functions directly relevant to CLL

4.5. CD62L expression

In the process of lymphocyte homing, B lymphocytes adhere and then enter sinusoidal endothelium before invasion in efferent lymph nodes, after which they leave the lymph nodes. CD62L (L-selectin), a member of the selectin family of adhesion molecules, plays an important role in the traffic and homing of lymphocytes to lymph nodes [98]. CD62L is considered the main adhesion molecule connecting B cell to sinusoidal endothelial cells through interaction with mannose receptor [99]; therefore it is believed that CD62L participates in mechanisms supporting the departure of lymphocytes from lymph nodes. In vitro studies of CLL cells demonstrated that stimulation through BCR causes a decrease of CD62L, in particular the expression on cells of patients with adverse prognostic factors and at risk of disease progression. The decreased level of surface CD62L is due to disaffiliation from the cell membrane and leads to an increase of its plasma levels [63]. A similar process is also seen in B cells activated by formyl peptides and phorbol 12-myristate 13-acetate [100, 101]. Functionally, leukemic lymphocytes, in which the expression of CD62L and CD184 decreases in a response to BCR signals, show reduction of migration to CXCL12 and adhesion to endothelial cells of the lymph node [63]. The adhesion molecule CD62L is the main factor for B cell departure from lymphatic tissue via connection with lymphatic endothelium [63]. After antigen activation, B lymphocytes lose surface expression of CD62L and do not adhere to the endothelium in lymph nodes [99, 102]. These cells remain in a close contact with the microenvironment and with the antigen stimulation, resulting in proliferation and lymph node enlargement. On the contrary, CLL cells, which do not respond to BCR stimuli, exit lymphoid organs quickly and recirculate like normal B lymphocytes, due to their ability to recognize a specific antigen [103]. In an in vitro study of CLL lymphocytes, increased expression of CD62L was associated with prolonged survival of the malignant cells. Since anti-apoptotic signals from the stromal cells were CD62L-dependent, the inhibition of CD62L by antibodies decreased the survival of the tumor cells. The study also showed over-expression of CD62L on malignant B lymphocytes localized in the proliferation centers of lymph nodes and bone marrow [104]. The immunophenotypic analysis of CLL cells in the process of culturing has demonstrated a change in the surface markers participating in intercellular contacts and conducting anti-apoptotic signals. Blockage of the activation and homing receptor CD62L induces cell death, equivalent to currently used chemotherapeutics [105].

5. B cell receptor complex

B cell receptor complex (BCRC) is a multimeric complex composed of a surface immunoglobulin homodimer and a non-covalently bound heterodimer $Ig\alpha/Ig\beta$ (CD79a/CD79b). The signal pathway of BCRC supports cell proliferation and induces the production of antibodies in normal B lymphocytes. Binding to a specific antigen activates BCRC, which delivers a signal to kinases (spleen tyrosine kinase (SYK) and LYN), which phosphorylate and activate $Ig\alpha/Ig\beta$ [106]. This phosphorylation step triggers a cascade of intracellular signals including activation of Bruton tyrosine kinase (BTK) and Phosphatidylinositol 3-kinase (PI3K), which induce the mobilization and activation of further kinases (protein kinase C- β and mitogen-activated

protein kinase - ERK) [107]. The activation of this cascade facilitates anti-apoptotic events and the proliferation of B cells through the enhancement of transcription factors such as the nuclear factor- κ B (NF- κ B) [108]. This signal pathway plays an important role in the pathogenesis of CLL, which has been supported by the following facts:

- Mutation status of BCR sequences is the best prognostic marker for disease progression [109].
- About 20% of untreated patients express very similar, sometimes identical, antigen receptors.
- Signals of BCRC play a significant role in trafficking, homing, and interactions in microenvironment [110].

There is a 10-fold decrease in the expression of membrane immunoglobulins [111] due to a critical defect in the formation of the BCR structure [112]. As a result, the stimulation of monoclonal B cells through BCR is impaired. Low expression of BCRC correlates with reduced induction of tyrosine kinase activity and affected intracellular mobilization of calcium and phosphorylation of tyrosine [113]. This low expression is a distinguishing feature of the disease, since similar expression is not found in other mature B cell lymphoproliferative diseases. What causes of this phenomenon is still unclear and is addressed in one study [114], while currently there is a consensus that no genetic defects are present in the components of BCRC [115]. Except in rare cases, surface IgM and CD79b are expressed on CLL cells in very low levels, compared to normal B lymphocytes, but the levels of mRNA and intracellular synthesis of the BCRC components are normal [112]. The correct binding is very important for proteins that consist of multiple subunits. This process takes place in the endoplasmic reticulum, where proteins are modified (split of signal peptides, N-glycosylation, formation of disulfide bridges). In case of failure of the maturation process sequence, various system control mechanisms are activated. They prevent the production of non-functional proteins. A study showed that a complete IgM is not transported to the membrane of the CLL cell [116, 117]. Monoclonal B lymphocytes have a phenotype, which is similar to naïve B cells from the mantle zone, they express CD5 and IgM/IgD and during a normal maturation process they should have expressed non-mutated immunoglobulin genes [118]. However, CLL cells in 50–70% of patients have somatic mutations of the IGHV genes [119], therefore they should have completed the stage of maturation in the lymph follicle. The nature of the leukemic clone is still not established [120, 121], and the absence or presence of somatic mutations is associated with the functioning of single IGHV genes [122].

6. Costimulatory receptors on monoclonal B cells

Cells proliferate in lymph nodes and bone marrow, where under the influence of microenvironment stimuli, they interact with stromal cells and T lymphocytes, resulting in apoptosis impairment and an increase of proliferation. The gene expression profile of CLL cells corresponds to that of activated B lymphocytes [4].

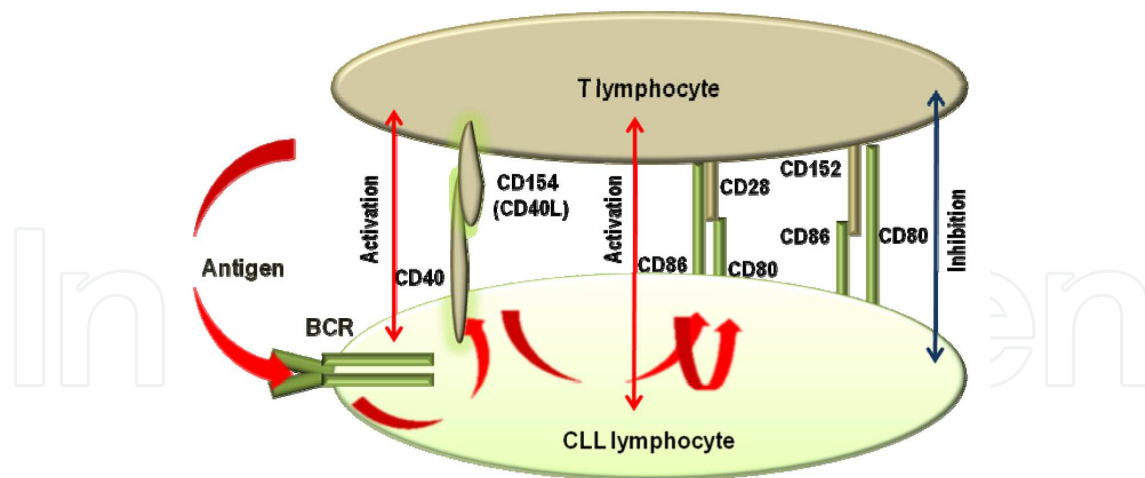


Figure 3. In case of binding of an antigen to the BCR, intracellular signals activate and increase the expression of CD40; the binding to the ligand leads to activation and increased expression of CD86. This in turn leads to activation and de novo expression of CD80. Both regulatory molecules have common ligands CD28 and CD152; these interactions modulate the proliferation of B and T cells

6.1. CD40 expression

CD40 is a molecule belonging to the tumor necrosis factor receptors (TNFR) family. It is expressed during B cell development and plays a major role in cell survival and differentiation [123, 124]. Its physiological ligand CD40L (CD154) is also a member of the TNFR family [125]. Interactions between CD40/CD40L stimulate the proliferation and differentiation of B cells, dendritic cells, and monocytes and enhance expression of costimulatory molecules and the antigenic presentation [126, 127]. After antigen recognition, the T cell receptor (TCR) induces increased CD154 expression on CD4⁺ T cells, enhances signals through CD40, and increases the expression of CD80/CD86 on antigen-presenting cells (Figure 3). This system enhances activation of T cells, their differentiation, and modulates humoral immune response [128]. The congenital deficit of CD154 in X-linked Hyper-IgM Syndrome leads to frequent bacterial infections due to impaired switching of immunoglobulin classes [129] similar to the immune disorders in CLL patients. Leukemic B lymphocytes express variably functional surface CD154, while CD4⁺ T cells in CLL patients do not express surface CD154 after CD3 ligation [130, 131]. Binding of CD40 induces expression of CD95, a receptor for apoptotic signals, but paradoxically it conducts a strong NF- κ B mediated signal for the survival of leukemic cells in vitro [132]. CD40 activation of malignant cells reduces Fludarabine-induced apoptosis in vitro [133]. In patients with CLL, CD40 activation of B lymphocytes increases the expression of B7 molecules and these cells present alloantigens significantly better than non-stimulated CLL-cells (Figure 3) [24]. Surface expression of costimulatory molecules increases as the process is accompanied by an impaired T cell response to alloantigens and tumor antigens in many other B cell lymphoproliferative diseases [134, 135, 136]. In a study of antigen-presenting capacity of CD40-activated CLL lymphocytes and dendritic cells stimulated by apoptotic bodies of CLL cells, both kinds of antigen-presenting types generated specific T cells, proliferating in response to non-stimulated CLL lymphocytes. T cells isolated from patients with CLL

recognize and produce allogenic stimulated and non-stimulated CLL lymphocytes, which shows that the cytotoxic T cells of the patients are functionally intact [137]. CLL lymphocytes activated through CD40 in the presence of IL-4 and INF- γ increase the expression of CD80 and Major histocompatibility complex-II (MHC-II) molecules. Antigen presentation of alloantigen from leukemic cells is comparable to that of normal CD40-activated B cells, as it increases in the presence of IL-4 and INF- γ [138].

6.2. CD80 and CD86 expression

CD80 and CD86 receptors belong to the B7 family, which is one of the most important secondary signaling mechanisms for maintaining the balance between adequate immune response, immunosuppression, and autoimmunity [139].

The expression of CD80 and CD86 is restricted to antigen-presenting cells, as both molecules play different roles in immune modulation due to different interactions with their ligands CD28 and CD152, respectively. The interaction of CD80 with CD152 has a higher affinity than the interaction of CD86 with CD152, while CD28 connects with CD86 more effectively than with CD80 [140]. Low expression of CD86 is registered on non-activated B cells, dendritic cells, and macrophages, in case of activation the levels of CD86 increase and CD80 is expressed de novo [141, 142]. These interactions form a costimulatory-co-inhibitory system that regulates immune responses (Figure 3). Mice with a CD80 and CD86 deficit are characterized with disorders in both the humoral and cell immune response [143]. Furthermore, in case of CD28 deficit, the receptor immune response to foreign antigens, infectious pathogens, and transplants is ineffective [144]. The interaction of CD80 and CD86 with its receptor CD28 generates costimulatory signals leading to a productive activation, expansion, differentiation, survival of the B- and T-cells and an effective antibody-mediated and cell immune response. This immune response is balanced by signals received through interactions with CD152. Mice with CD152 deficit develop an autoimmune phenotype and die rapidly due to multi-organ destruction [145]. The lack of signals from CD80 and CD86 precludes the development of autoimmune deficit in models of triple deficit of CD80, CD86, and CD152 [146]. The role of B7 in antitumor immunity is confirmed through a model system, where cytotoxic T lymphocytes remove mouse tumors, in which transfection of CD80 and CD86 was performed. Unlike solid tumors, both molecules are expressed in a number of hematologic diseases [147, 148]. For instance, cells from a follicular lymphoma increase their expression of CD80 and CD86 as well as other costimulatory and adhesion molecules after in vitro activation [149]. Malignant Reed Sternberg cells of classical Hodgkin's lymphoma also express high levels of CD80 and CD86 [150]. Expression in multiple myeloma is variable and correlates with prognosis [151]. In CLL, the expression levels are low in non-stimulated malignant lymphocytes and increase after stimulation [148, 152].

6.3. CD267 (TACI) expression

The CD267 receptor binds to two ligands - BAFF and APRIL, thus inducing activation, proliferation, and survival of B cells [52, 153, 154, 155]. CD267 is expressed on subsets of B lymphocytes and activated T cells [156] and the expression varies in different B cell subpopu-

lations, the strongest being on marginal-zone B cells and memory CD27+ B lymphocytes [157, 158, 159], and increases after stimulation [160, 161]. In human B cells, BAFF and APRIL induce IgG and IgA immunoglobulin classes switch in the presence of IL-10 or TGF- β [162]. After the discovery of the mutations in TNFRSF13b, the regulatory role of expression of CD267 in final B cell differentiation and binding to Common variable immunodeficiency (CVID) in humans was established [163, 164]. The binding of BAFF to CD267 stimulates NF- κ B activation in B lymphoma cells in vitro, while soluble forms of CD267 inhibit this induction as well as the production of IgM by B lymphocytes [165]. CD267 (TACI) was initially defined as a receptor interacting with Calcium modulating ligand (CAML) meaning that it conducts signals through both NF- κ B and NFAT/AP-1. The binding of BAFF and APRIL to CD267 can also conduct negative regulatory signals for B cell maturation and activation. In a study on the expression of CD267 in CLL, a significant decrease of the levels compared to healthy control indicators was found [166], which raises the question of the significance of CD267 in the pathogenesis of CLL. The observed lymphocytic proliferation and development of autoimmune diseases in mice with CD267 deficit suggests that this receptor could conduct pro-apoptotic signals in activated B cells [167, 168]. In humans, the higher is the expression of CD267 on monoclonal B lymphocytes the higher is the percentage of apoptotic leukemic cells [166], therefore it was presumed that the decreased receptor expression reduces the negative regulatory signal to B cells. The authors also found inverse correlation between CD267 and Bcl-2 expression of the leukemic cells and a positive correlation between TACI and the expression of prostate apoptosis response-4 (PAR-4), which is a unique pro-apoptotic protein, selectively inducing apoptosis in tumor cells [169]. Based on the observation of increased numbers of circulating and splenal B lymphocytes in TACI-/- deficit mice [167, 170], it can be speculated that CD267 is an inhibitor of B cell proliferation [167]. Similarly, a study of 62 untreated patients with CLL showed inverse correlation between the amount of CD5+ B cells, leukocyte count, and the expression of CD267. The study also showed significantly lower expression of the receptor in Rai stages III and IV patients, as well as in Zap-70+ and CD38+ positive patients, compared to Zap-70 and CD38- negative [166]. Further studies on the expression of CD267 and the clinical course of the disease are needed to establish the precise role of this receptor in the progression of CLL.

7. Conclusion

The phenotypic profile of malignant lymphocytes is of pathological and biological significance. It reflects the relationship between B cells, the tumor microenvironment, and the importance of intercellular interactions. Variations in the expression profile of CLL patients reflect different mutations and impaired regulatory mechanisms. The existence of a complex network of antiapoptotic and prosurvival molecules, including cell adhesion, proinflammatory, angiogenic, and proto-oncogenic molecules, is responsible for supporting the infiltrating malignant cells and for the maintenance of the neoplastic tissue in CLL. Many prosurvival signaling pathways potentially sustaining CLL cell maintenance interact with one another. Thus, it appears that developing new classes of drugs affecting simultaneously various signaling pathways, and therefore abrogating signaling redundancy-associated chemoresistance to classical drugs, is feasible.

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