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Mini-review

Targeting the microenvironment in chronic lymphocytic leukemia offers novel therapeutic options

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ABSTRACT

Chronic lymphocytic leukemia (CLL) cells display features consistent with a defect in apoptosis and exhibit prolonged survival *in vivo*. Survival of these malignant cells is influenced by interactions with non-leukemic cells located in permissive niches in lymphoid organs. Leukemic cells subvert the normal architecture of the lymphoid organs, recruiting stromal cells, dendritic cells and T lymphocytes, all reported as playing active roles in the survival and proliferation of CLL. The same survival-promoting environment also rescues/protects leukemic cells from cytotoxic therapies, giving way to disease relapse.

This review summarizes and discusses current knowledge about the intricate network of soluble and cell-bound signals regulating the life and death of CLL cells in different districts. At the same time, it seeks to hone in on which discrete molecular elements are best suited as targets for treating this still incurable disease.

1. Introduction

Chronic lymphocytic leukemia (CLL), one of the most common types of adult leukemia in Western countries, is characterized by the accumulation of mature $CD5^+$ B cells in the peripheral blood and lymphoid organs [1]. Mainly diagnosed in older adults, CLL is widely heterogeneous in terms of progression, therapeutic response and outcome. Research to identify prognostic biologic markers for CLL has thus been a major priority, and has yielded fruitful results [2]. Today, the early recognition of patients characterized by an aggressive form of the disease is guided by a number of different molecular markers, including the absence of mutations of the IgHV genes [3], the surface expression of CD38 [4] and CD49d [5] and the intracellular presence of ZAP-70 [6]. Cytogenetic abnormalities are also powerful prognosticators, with deletion of 17p and 11q strongly associated with rapid disease progression, short survival and resistance to conventional DNA-damaging chemotherapies [7]. Single gene mutations are rapidly being uncovered by sequencing the coding genome of CLL cases, including NOTCH1 [8], splicing factor 3b subunit 1 (SF3B1) [9,10], baculoviral IAP repeat-containing 3 (BIRC3) [11], exportin 1 (XPO1), myeloid differentiation primary response gene 88 (MYD88) and Kelch-like 6 (KLHL6) [12].

Investigation into the origin and development of this form of leukemia has provided solid evidence in favor of the current view that survival and proliferation of CLL cells depends on the microenvironment [13–15]. The malignant cells are dynamically compartmentalized into different districts, which determine their growth potential and modulate their sensitivity to cytotoxic drugs. It is plausible to assume that when CLL cells are located in the lymphoid organs, they come into contact with the antigen and a cocktail of stimulatory and accessory signals presented by a vast array of cells [16]. The resulting bidirectional interactions would lead to establishment of a progressively abnormal microenvironment that promotes proliferation and survival [17]. These signals may also create intracellular conditions promoting accumulation of novel genetic mutations or expansion of previously existing mutated subclones, both events favoring disease progression. Another important point is that the lymphoid niche provides a shield from the effects of chemotherapy, thus serving as a reservoir from which relapse may occur [18]. In contrast, because they are located farther from the antigen source and accessory signals, circulating CLL cells become increasingly fragile and prone to apoptosis.

The creation of growth-favorable niches thus appears to be critical to the survival of CLL cells [19]. By identifying the molecular links between leukemic cells and the microenvironment, as well as the processes that regulate homing to the lymphoid niche, it may be possible to disrupt the survival advantage conferred to

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Table 1
Novel agents currently in clinical development for CLL targeting key microenvironmental components and signaling pathways.

Agent	Target/mechanism of action	Clinical trial phase
Daratumumab	Anti-CD38	Phase I/II
Natalizumab	Anti-CD49d	Phase I
HCD122	Anti-CD40	Phase I
Bevacizumab	Anti-VEGF	Phase II
Milatuzumab	Anti-CD74	Phase I
BAY61-3606 R406	Syk inhibitors	*In vitro*
PP2 SU6656	Lyn inhibitors	*In vitro*
LY294002 CAL101	PI3K inhibitors	Phase I/II
A-443654	AKT inhibitor	*In vitro*
PCI-32765	BTK inhibitor	Phase II
PF-956980	JAK3 inhibitor	*In vitro*
LC-1	NF-kB inhibitor	*In vitro*
Plerixafor T140	SDF-1/CXCR4 inhibitors	Phase I/II
Lenalidomide	Immunomodulator	Phase I/II
CD40L-encoding oncolytic adenovirus	CD40 ligand gene therapy	*In vitro*
Chimeric antigen receptor (CAR) modified T-cells	Genetic manipulation of autologous T cells	*In vitro*

the malignant cells, thereby improving the effects of conventional chemotherapy [2,13,20]. The weaponry of targeted therapies available for CLL patients has grown exponentially in recent years and now includes several novel drugs that interfere with different proliferation/survival circuits. Some have reached clinical trials, with documented benefits in terms of reduced toxicities and duration of responses. They include monoclonal antibodies (mAbs), glucocorticoids, immunomodulatory agents, drugs with specific intracellular molecular targets, vaccines and cellular immunotherapies [2,21]. Even if highly diverse in terms of mechanisms of action, these agents share the ability to disrupt the interactions between malignant, bystander stromal cells and defense systems [13]. This leads to radical changes in the cytokine/chemokine network and cell surface receptors, ultimately reducing external support to the tumor cells [20,22].

This review summarizes what is currently known about the proliferative compartment of CLL, and discusses the various molecular signals involved, in terms of their suitability for therapeutic targeting (see Table 1).

2. Targeting the proliferative compartment of CLL

The CLL microenvironment in lymphoid organs is created and maintained through a dynamic, interactive co-evolution between leukemic and normal bystander cells. The hallmark of this transformation is exemplified by the proliferation center, a focal aggregate of pro-lymphocytes and para-immunoblasts that cluster in pseudofollicular structures [23–25]. These roughly nodular areas without mantles are observed in LN and BM and represent the histopathological hallmark of CLL. Pseudofollicles contain aggregates of $Ki67^+$ proliferating tumor cells which express CD5, but differ from reactive germinal center B cells by being $CD10^-$, $Bcl\text{-}6^-$, and $Bcl\text{-}2^+$ [26]. These $Ki67^+$ cells are surrounded by new vessels [27], sprouting in response to the production of vascular endothelial growth factor (VEGF) by actively proliferating malignant B cells [28,29]. However, pseudofollicles are not simply a collection of proliferating monoclonal B lymphocytes, but rather a sort of melting pot for bilateral interactions with different populations of stromal, dendritic and endothelial cells and T lymphocytes, which are all potential players in the pathogenesis and progression of CLL [30,31].

2.1. Antigen-mediated signals

Several independent pieces of evidence indicate that activation of the BCR signaling pathway plays a central role in sustaining CLL survival and in driving proliferation. First, CLL cells display a genetic profile compatible with that of antigen-activated mature B cells [32]. Second, microarray studies have shown that CLL cells located in lymphoid organs display an up-regulation of genes belonging to the BCR signaling pathway [33]. Third, biochemical studies have demonstrated that CLL cells are characterized by enhanced expression and constitutively active phosphorylation of lyn and syk, two tyrosine kinases belonging to the BCR signaling apparatus [34,35]. Consistently, the effector pathways downstream of the BCR, including PI3K/Akt [36], MAPK [37] and NF-kB [38] appear to be activated in selected subsets of CLL patients. Considered together, all these indications seem to imply chronic antigen exposure *in vivo*, at least in selected districts and in selected patient subsets.

The confirmation of a differential role of the BCR in distinct disease subsets comes from the finding that the clinical course of CLL can be divided depending on the presence or absence of somatic hypermutation in the immunoglobulin variable heavy chain region (IGHV) genes [3,39]. More recent studies have been dedicated to the analysis of specific stereotyped third heavy chain complementary determining regions (HCDR3s), showing a striking association between stereotyped BCRs and clinical behavior. These observations suggest that an antigen-driven process is critical in modulating disease outcome, irrespective of the mutational status in CLL [20,40]. Underscoring the relevance of the BCR pathway in disease development and progression is the evidence of promising clinical activity of several drugs specifically targeting distinct players of the pathway. Inhibitors of the key kinases in this pathway, including SYK/LYN, PI3K-ATK, and BTK, have been found in pre-clinical models to decrease CLL cell viability both directly and indirectly through modulation of the microenvironment [41,42].

2.2. Nurse like cells (NLCs)

NLCs are named after their resemblance to thymic nurse cells, which nurture developing thymocytes by driving their maturation and differentiation in a contact-dependent fashion [43]. When mononuclear cells from the blood of CLL patients are cultured without stromal cells, a constant finding is the outgrowth of an adherent cell population to which CLL lymphocytes are attached. This population actively protects leukemic cells from spontaneous apoptosis *in vitro* [44]. It produces high levels of CXCL12 and expresses distinct molecules, including the lineage marker CD68, BAFF (B cell-activating factor of the tumor necrosis factor family) [45], CD31 and plexin-B1 [46]. Proliferation centers in spleen and lymphoid tissues of CLL patients contain $CD68^+$ myeloid cells that are believed to represent the tissue counterparts of NLCs [13,47,48]. Recruitment of NLC precursors can be actively pursued

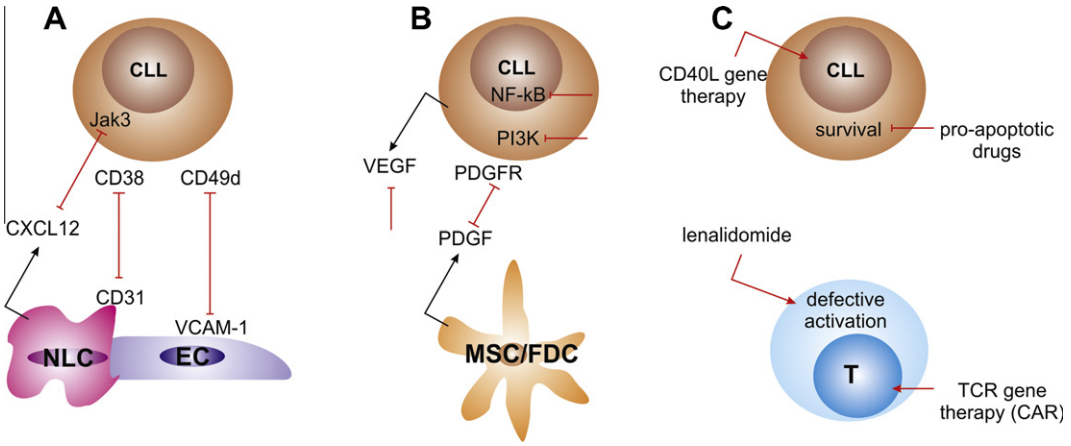

Fig. 1. Receptor-ligand axes operative in the CLL microenvironment. (A) NLCs that can promote CLL cell survival through the production of CXCL12 and through cognate interactions between CD31 and CD38, which are expressed on NLC and CLL cells, respectively. CLL-EC contact via VCAM-1-CD49d interactions may contribute to CLL cell survival. The inhibition of these interactions could be useful as a therapeutic strategy. (B) Interaction of MSC/FDC with CLL cells increases PDGF and VEGF production. Contacts between CLL and stromal cells lead to the activation of NF-κB and PI3K signaling pathways, increasing leukemic cell survival. Several molecules block these pathways, and are being tested in different clinical trials. (C) T lymphocytes from CLL patients are dysfunctional, as they are unable to form an immune synapse. Lenalidomide restores and potentiates functional T cell activity. Moreover, genetic manipulation of autologous T cells to target specific tumor antigens is an area of intense investigation. Creating over-expression of CD40L on CLL cells could lead to efficient antigen presentation, activating apoptotic programs.

by CLL cells through the secretion of CCL3 and CCL4, in turn triggered in response to signals mediated by the B cell receptor [49] and by CD38 [50]. The latter represents the starting point of a consecutive chain of events, with activation of $CD68^+$ macrophages, which secrete tumor necrosis factor-α (TNF-α), in turn up-regulating the expression of vascular cell adhesion protein 1 (VCAM-1) by endothelial cells. The final effect is increased adhesion of $CD49d^+$ CLL cells on endothelial cells, with extended survival of the neoplastic clone [50].

It has been proposed that this circuit may be targeted using anti-CD49d monoclonal antibodies (mAbs) to prevent triggering of the cascade signals. The advantage of this approach would be that Natalizumab (Tysabri™), a humanized antibody specific for the molecule, is already approved for the treatment of multiple sclerosis [51] and Crohn's disease [52] (Fig. 1).

2.3. Mesenchimal stem cells (MSCs)

MSC strongly affect the development and progression of various cancers [53]. Interaction of MSC with CLL cells increases the production of VEGF and platelet-derived growth factor (PDGF), concomitantly decreasing thrombospondin-1 [54]. PDGF binding to its receptor leads to activation of MSC via Akt and the subsequent secretion of VEGF [55]. Taken together, these steps are indicative of an angiogenic switch, associated with disease progression [56] (Fig. 1), providing the molecular rationale for clinical testing of inhibitors of VEGF receptor tyrosine kinase [57].

2.4. Follicular dendritic cells (FDCs)

FDC are closely associated with CLL cells in the early phase of bone marrow (BM) involvement as well as in the lymph nodes (LNs). *In vitro* culture with FDC rescues leukemic cells from spontaneous apoptosis by direct cell contact, dependent on ligation of CD44 and on up-regulation of Mcl-1, a member of the Bcl2 family [58]. The CD100/plexinB1 crosstalk also appears to be operative in this context [59].

The signals specific for stromal cells are still ill defined. Independent groups have demonstrated that the phosphatidylinositol 3-kinase (PI3K) pathway is induced by contacts between CLL and stromal cells and provides a significant survival advantage to leukemic cells in culture on a variety of stromal cell types [60,61]. Indirect evidence in line with these data suggests that CLL cells display increased PI3K activity and reduced activity of the degradative enzyme phosphatase and tensin homologue (PTEN) [62]. PI3K is a target for therapy in cancer and inhibitors are now available for the different isoforms of PI3K. The PI3Kδ isoform is of high interest because its expression is restricted to hematopoietic cells, where it plays a critical role in B cell homeostasis and functions [63]. CAL101 is a potent and highly selective inhibitor that promotes apoptosis of CLL cells through inhibition of PI3K signaling and Akt activation in response to a number of extracellular signals [64]. Clinical trials are actively recruiting patients to be treated with CAL101, either as a single agent or in combination with conventional chemotherapy and rituximab [65] (Fig. 1).

The mechanisms for survival are only partially understood: PI3K may contribute to nuclear factor-κB (NF-κB)-mediated transcriptional induction of the pro-survival factor BCL-XL [19]. Other effects include the inhibition of migration caused by CXCL12 [66]. The relevance of the NF-κB pathway for CLL progression is further confirmed by data on the expression of the NF-κB subunit Rel A as a biomarker of disease progression in CLL [38]. Furthermore, the pathway is actively modulated as a consequence of interactions with endothelial cells [67]. Several drugs effective for CLL patients, including lenalidomide, operate by blocking NF-κB activation. LC-1 is one of these and has reached clinical trials, in view of the results obtained *in vitro*, which show dramatic induction of apoptosis [68].

2.5. T lymphocytes

Most malignancies are associated with decreased numbers of circulating T cells. In contrast, T lymphocytes are significantly elevated in CLL, even if their TCR repertoire is contracted with oligoclonal and monoclonal subsets [69–72]. One study suggested that higher T lymphocyte numbers are associated with a poor clinical outcome [73], while others have shown a relative increase in central and effector memory T cells in cases that lack somatic mutations in IgHV genes [74]. It is still unclear whether increased numbers of T lymphocytes in the periphery are paralleled by a similar increase in LN. Proliferation centers contain activated $CD4^+$ T cells adjacent to leukemic cells, likely indicating adhesion and bi-

directional signals [75]. CLL cells secrete CCL22, CCL3 and CCL4, which are involved in T cell recruitment to the LN. This may suggest that leukemic cells themselves play an active role in the accumulation of T lymphocytes. On the other hand, migration in response to CXCL12, CCL21 and CCL19 of T cells from CLL patients is partially defective, as compared to T cells from healthy adults despite similar CXCR4 and CCR7 expression. This is particularly evident when considering T cells from ZAP-70$^-$/CD38$^-$ CLL patients. Since T cells in proliferation centers may help CLL cells to survive and proliferate, the low migratory response towards CXCL12 in T cells from ZAP-70$^-$ CLL patients is believed to favor the indolent clinical course of the disease in these patients [76].

A significant number of T cells in proliferation centers express CD40L (CD154), a member of the TNF superfamily that mediates interactions with CD40$^+$ CLL cells, rescuing them from apoptosis [77]. This effect is mediated by up-regulation of the pro-survival protein survivin [78], repression of BCL2 and induction of BCL-XL and BCL2A1 [79]. This anti-apoptotic mechanism can be therapeutically modulated using lucatumumab (HCD122), an anti-CD40 humanized monoclonal antibody that blocks interaction of CD40L with CD40 and also mediates antibody-dependent cell-mediated cytotoxicity (ADCC) [80]. Moreover HCD122, inhibits CD40L-induced activation of signaling pathways, proliferation, survival, and secretion of cytokines [81]. This antibody is currently in phase I clinical trials [80].

T lymphocytes from CLL patients are dysfunctional in that they are unable to form a fully effective immune synapse [82]. This yet uncharacterized molecular defect is driven, at least in part, by interactions with the malignant cells and is reversed by lenalidomide [83,84]. This agent has a wide range of immunomodulatory activities, including stimulation of T cells through CD28, enhancement of the expression of cytokines (including IL-2 and IFN-γ), repression of regulatory T cells with concomitant induction of Th17, and increase of NK- and of antibody-dependent cytotoxicities [85]. In addition, lenalidomide also shows growth inhibitory and pro-apoptotic properties [86]. It is highly effective when used as a single agent [87] and clinical trials are under way to determine whether combining the drug with more established agents might be effective (Fig. 1).

A plausible explanation for the hypo-responsiveness of the T cell compartment of CLL patients lies in the inefficient antigen presentation effected by neoplastic cells. This is partly due to the low expression of CD40L, resulting in diminished co-stimulation via CD40. Ligation of CD40 on CLL cells induces phenotypic and biochemical changes that facilitate CLL cell-T cell interactions and enhance the sensitivity of CLL cells to clearance by adaptive and innate immune-effector mechanisms. Some groups have tried to prime T cells by over-expressing CD40L on CLL cells, to increase antigen presentation by leukemic cells. Surface expression of CD40L on CLL cells after gene therapy treatment promotes expression of costimulatory molecules including CD40, CD80, and CD86 on neighboring bystander CLL cells, thereby making them better costimulants for T-cell activation [88]. Gene therapy with CD40L may be effective if administered in combination with rituximab, which sensitizes CLL cells to mAb-induced cell death (Fig. 1). Lenalidomide appears promising also in this context as it promotes expression of functional CD40L on CLL cells [89].

Reprogramming of autologous T cells to target specific tumor antigens is a second an area of intense investigation and promising results. The most successful strategy so far involves the use of an antibody-derived antigen-binding moiety fused with an internal signaling domain such as CD3ζ to form a chimeric antigen receptor (CAR) [90]. CARs have theoretical advantages over other T-cell-based therapies. They use the patient's own cells, which avoids the risk of graft-versus-host disease. They can be created quickly, and the same chimeric antigen receptor can be used for multiple patients. Preliminary results from an ongoing trial suggest that low doses of autologous T cells infected with a CD19-targeted CAR infused into a patient induce tumor lysis syndrome followed by persistent clinical response, highlighting the potency of this therapy [91] (Fig. 1).

2.6. Nucleotide-mediated signals

Considerable evidence indicates that an immune response is not solely determined by antigenic stimulation, but rather that complex interactions among the endocrine, nervous and immune systems are at the basis of immune homeostasis [92]. As a first example, extracellular nucleotides [such as adenosine triphosphate (ATP)] and nucleosides (such as adenosine), together with the enzymes involved in their metabolism and purinergic receptors, constitute a network of signals that may shift the balance from survival to apoptosis. Our lab has shown that CLL cells nestled in the LN proliferation centers activate an adenosinergic axis, which involves the ectoenzymes CD39 and CD73, causing the accumulation of the end product adenosine. An adenosine-rich environment creates local conditions that protect CLL cells from spontaneous or drug-induced apoptosis and that inhibit chemotaxis [93] (Fig. 2). It is plausible to assume that targeting the adenosinergic axis might have a considerable therapeutic impact on the control of CLL progression and/or on potentiating the effects of chemotherapy. One way to achieve this could be via blocking of CD73, an approach proposed for solid tumors [94]. Alternatively, the use of antagonists of the A2A receptor, which could limit the increase in cytoplasmic cAMP levels associated with anti-apoptosis and chemoresistance, may be envisioned. There are several specific antagonists of the A2A receptor, one of which is in clinical trials for Parkinson's disease [95] (Fig. 2).

Another example illustrating the importance of the connections between metabolism and the immune system is nicotinamide, the main precursor of NAD$^+$. We reported that treatment of CLL cells with nicotinamide triggers a rapid and robust activation of the apoptotic program and blocks proliferative responses. These effects are mediated by a functional loop that involves SIRT1 as the key player. SIRT1 is the main member of the sirtuin family and inactivates p53 by deacetylating a critical lysine residue. According to this model, nicotinamide blocks SIRT1, resulting in a net increase of active p53. These effects are even more apparent when CLL cells are treated with chemotherapeutic agents, known to activate the p53 pathway (Fig. 2).

For these reasons, the combination of DNA-damaging chemotherapeutics and nicotinamide should yield optimal apoptotic responses [96]. An alternative possibility would be to combine nicotinamide with traditional histone deacetylase inhibitors, resulting in a synergistic antileukemic activity [97] (Fig. 2).

3. Targeting the homing process

A growing body of evidence indicates that malignant B cells exploit physiological mechanisms of tissue-specific lymphocyte migration to access supportive microenvironmental niches [98]. Not merely a passive event, re-circulation to and from lymphoid organs is tightly controlled by expression of a number of molecular sensors that guide leukemic cells out of the vessels and into the LN [15]. This complex process, known as homing, can be broken down into three basic steps. The first involves the initiation of motility programs, usually driven by the binding of chemokines to their specific receptors. Within minutes after binding, lymphocytes polarize with extensive modifications in the organization of the cytoskeleton. During the second phase, lymphocytes adhere to the endothelial barrier and negotiate crossing, an event lasting

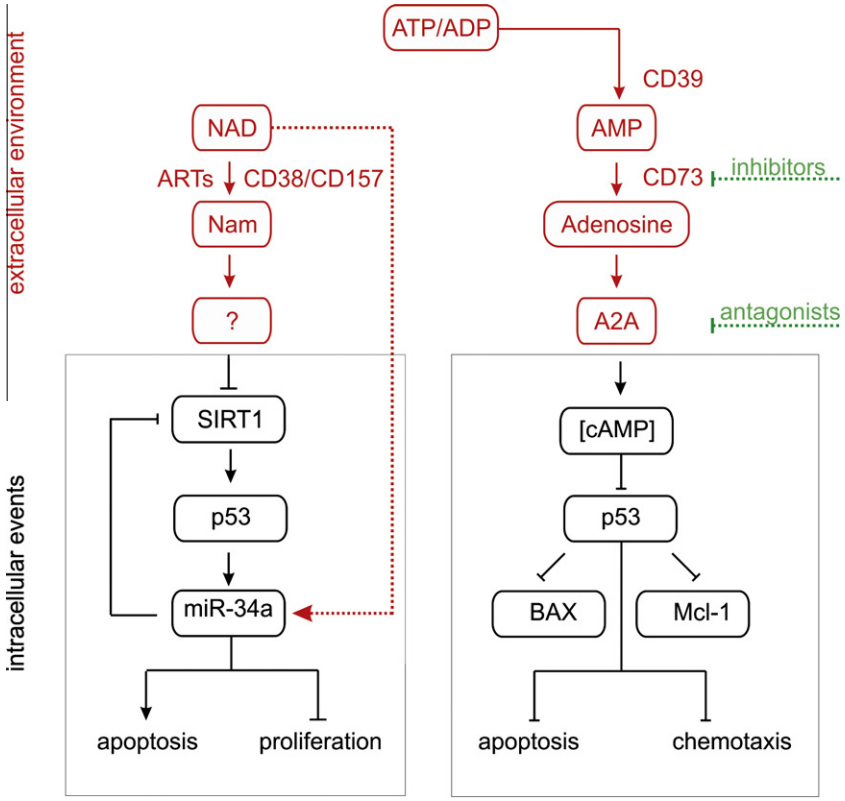

Fig. 2. Pathways regulated by extracellular nucleotides favor accumulation of leukemic cells in specific environments. Increased levels of extracellular NAD result in accumulation of the end product nicotinamide (Nam), a powerful inhibitor of the SIRT1 enzyme. Nicotinamide inhibiting SIRT1, leads to the activation of the p53 network, which results in inhibition of proliferation and induction of apoptosis. Instead, increased levels of ATP activate an adenosinergic axis modulated by the CD39 and CD73 ectoenzymes. Extracellular adenosine binds to specific A2A receptors, driving an intracellular pathway dependent on cAMP, which results in inhibition of chemotaxis and apoptosis.

minutes to hours and mediated mainly by integrins and their ligands. The last phase is characterized by the production and secretion of matrix metalloproteases (MMPs) that allow lymphocytes to move within tissues and to reach their final destination [99]. The molecular players driving this process also appear to be compartmentalized within the same membrane domains, termed invadosomes, which suggests a sequential and inter-regulated phenomenon [100].

3.1. Chemokine signals

The extremely fine homing of CLL cells to and within the BM is mediated by the chemokine receptor CXCR4 [101]. Functional responses to CXCL12 are marked by the activation of a signaling cascade that converges on ERK1/2. Rapid and transient Ca^{2+} fluxes lead to actin polymerization within minutes after chemokine administration [44]. Patients with an aggressive form of CLL have been found to display heightened responses to CXCL12, both in terms of short term signaling and of the ability to migrate *in vitro* [102]. Our group has found that CD38 acts as a facilitator of CXCR4 signaling by enhancing and prolonging activation of the ERK1/2 kinase *in vitro* [103]. Experiments performed in immunocompromised mice have shown that $CD38^+$ CLL clones home more efficiently to the BM and the LN than their counterpart and that antibodies against CD38 significantly block these phenomena, trapping CLL cells in the blood [104].

CXCR4 antagonists, such as Plerixafor (AMD3100) and T140 analogs, can disrupt adhesive tumor/stroma interactions and mobilize leukemic cells from their protective stromal microenvironment, making them more accessible to conventional drugs. Therefore, targeting the CXCR4/CXCL12 axis is an attractive therapeutic approach that is being explored in ongoing clinical trials in leukemia patients [13,105].

Chemokine receptors such as CXCR4, CXCR5 and CX3CR1 regulate more complex phenomena, by activating signals related to cell growth and relying on the activation of MAP kinases and STAT3. These pathways may be pharmacologically targeted using specific inhibitors, including the Jak3 inhibitor PF-956980 [106].

3.2. Adhesion molecules

CLL cells express several integrins, members of the Ig superfamily that play important roles in the regulation of cell behavior either through direct activation of signaling pathways important for cell growth survival or by modulating responses to growth factors.

Besides controlling homing and residence in the lymphoid organs, as well as adhesion and activation of B lymphocytes [107], integrins also promote survival of CLL cells [67]. The expression of α4 integrin (or CD49d) was reported as an independent marker for patients with a more aggressive, bulky form of the disease [108]. Engagement of CD49d/CD29 (α4β1 integrin) is followed by activation of the PI3K pathway with production of MMP-9 [109]. The result is increased migration, an acquired feature potentially favorable to clinical outcome. Our group has shown that the presence of CD38 on the CLL cell membrane significantly enhances CD49d-mediated adhesion by inducing a more complex distribution of F-actin filaments and a marked phosphorylation of the kinase Vav-1 [110]. $CD38^+$/$CD49d^+$ CLL clones adherent to recombinant V-CAM-1 are also more resistant to apoptosis than

$CD38^-/CD49d^+$ clones. As observed with CXCR4, the functional link between CD38 and CD49d relies on their physical association, as inferred by co-localization and co-immunoprecipitation experiments, pointing to the existence of a large supra-molecular complex. The complex is dynamic and the association appears to be strengthened when CLL cells are left to adhere on recombinant VCAM-1 [110]. These data provide further support of the proposal to use anti-CD49d antibodies in the therapy of CLL (Fig. 1).

Another adhesion molecule that might be involved in CLL survival is CD44. CD44 isoforms, encoded by a single highly conserved gene, are a family of transmembrane receptors for hyaluronic acid, a major component of the extracellular matrix, and are also involved in selected adhesion functions and in delivering bidirectional (outside to inside and vice versa) signals [111].

CD44 is also an integral component of the CD74 receptor complex, which binds migration inhibiting factor (MIF) [112,113]. While CD74 is sufficient for binding soluble MIF, CD44 is necessary for transmitting the subsequent signals [114]. Initially thought to function mainly as an invariant HLA Class II chaperone, CD74 was later shown to be directly involved in the maturation of B cells through a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its coactivator TAFII [51]. This circuit is also operative in CLL cells [52].

Milatuzumab (Immunomedics) is a novel humanized mAb that targets CD74. This mAb induces rapid internalization into $CD74^+$ cancer cells and elicits significant anti-tumor effects in xenograft models of various lymphoid malignancies in mice. It can be used as a single agent or in combination with chemotherapy or other mAbs, such as rituximab [115]. So far, treatment with milatuzumab appears to be free of severe adverse effects in humans, and initial data indicate that it may be safely administered with other agents. Incorporation of milatuzumab into liposomes further enhances its therapeutic potential in CLL [116]. Preliminary experience indicates that milatuzumab may be used as a single agent in CLL patients, whose functional status makes them ineligible for other more aggressive forms of treatment. Milatuzumab might also be useful in combination with low doses of fludarabine: blocking the CD74 pathway may overcome the protective effect exerted by fibronectin via VLA-4 [117].

3.3. Matrix metalloproteases (MMPs)

MMPs are proteolytic proenzymes involved in degradation of the extracellular matrix during the early steps of tumorigenesis [118], and also plays a role in the late stages of tumor progression, invasion, and metastasis [119]. MMP-9 is the dominant MMP produced by B-CLL cells and contributes to their tissue infiltration [109]. MMP-9 expression correlates with advanced clinical stages of the disease [120]. Its engagement induces an intracellular signaling pathway, which includes Lyn activation, STAT3 phosphorylation, and Mcl-1 up-regulation and prevents B-CLL apoptosis [121]. CD38, CD49d, MMP9 and CD44 were recently reported as components of a supramolecular surface complex of physically associated molecules [122]. A wide body of evidence indicates that CD38 is the link between the discrete steps of the homing process. Expanding on this view, it is tempting to speculate that it might be more effective to target CD38 than to target individual steps. Daratumumab (GenMab), a human anti-human CD38 mAb, entered a phase I/II clinical trial for patients with multiple myeloma and CLL. The mAb induces potent Ab-dependent cellular and complement-dependent cytotoxicities. These properties are apparently unaffected by the presence of BM stromal cells, suggesting that the mAb will be effective in the LN or BM niche. Daratumumab has also been shown to induce potent cytotoxic effects *in vitro* and *in vivo,* while functional effects triggered by the binding portion of the antibody molecule still need to be identified. Recent data also indicate a clear synergy between lenalidomide and daratumumab-dependent cell-mediated cytotoxicity, opening the way to the design of combination therapies [123]. What remains to be analyzed is whether daratumumab influences the adhesive properties of CD38, hence reducing homing of leukemic cells to the lymphoid organs [124].

4. Conclusions

Purine analog-based combination chemotherapy or chemoimmunotherapy is considered to be a highly effective first-line therapeutic option. However, a major problem in the treatment of CLL is that the promising response rates observed in recent years are flanked by a number of patients with high-risk disease that relapse and become chemoresistant. Another major limit is that, for some patients, conventional chemotherapy is unlikely to work or is contraindicated due to comorbidity. The host microenvironment and the resulting interplay between the genetic background and environmental influences thus play a crucial role in disease progression, as well as in resistance to treatment. By targeting selected microenvironmental interactions and/or events mediated by the immune system in CLL, it may be possible to disrupt the shielding of malignant cells derived from those interactions, and also to create strong synergies with conventional therapeutics and overcome resistance mechanisms. New technologies and approaches, such as the animal models recently developed and tested, may soon prove essential for studying the integrated effects of the microenvironment. The development of these or related strategies, together or in combination, is expected to improve the outcome and quality of life of CLL patients.

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