INVITED PAPER

RECENT ADVANCES IN THE PATHOGENESIS AND TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Abstract

Chronic lymphocytic leukaemia (CLL) is a common lymphoid malignancy characterized by the expansion and progressive accumulation of mature autoreactive B lymphocytes. The disease is clinically heterogeneous and incurable by standard chemotherapy. A major feature of the disease is the marked dependence of the leukaemic cells on various microenvironmental stimuli, which promote leukaemia cell growth, survival, and drug-resistance. Recently, considerable progress has been made in the understanding of the molecular mechanisms that drive CLL. The identification of recurrent genetic lesions using next generation sequencing technology has provided new data on the pathophysiology of the disease and has improved its prognostication. The recognition of the critical role of the B cell receptor (BCR) in driving the disease has resulted in the development of BCR pathway inhibitors that have the potential to completely transform CLL treatment in the near future. Other novel therapeutic agents, such as BCL2 antagonists and chimeric antigen receptor (CAR)-modified T-cells, are also showing great promise in clinical trials. In this review, we summarize some of these recent advances, with a particular focus on the BCR and corresponding pathway inhibitors.

Key words: chronic lymphocytic leukaemia, B-cell receptor, fostamatinib, ibrutinib, idelalisib.

Introduction

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia in adults from Western countries. It has an annual incidence of about 5 new cases per 100.000 individuals and accounts for approximately one third of all leukaemia cases [1, 2]. Interestingly, the disease is considerably less frequent in Asia, where the prevalence is approximately ten times lower. The reasons for this difference are still unknown, but both environmental and genetic factors are believed to play a role. With respect to the latter, it is worth noting that first-degree relatives of patients with CLL have a seven times higher risk of developing the disease [3].

CLL is primarily a disease of the elderly, with median age at diagnosis between 67 and 72 years, and is two times more common in males than females [1]. It is virtually always preceded by monoclonal B cell lymphocytosis (MBL), a condition characterized by the expansion of a small monoclonal CLL-like B-cell population that can be detected in the peripheral blood of about 3.5% of healthy individuals [4, 5]. MBL has the same gender and age predisposition as CLL (reaching a frequency greater than 10% in people > 60 years) and is significantly more common in relatives of CLL patients (15–20%). However, MBL usually does not progress and only a small proportion of cases will develop CLL (~ 1% per year) [6].

CLL is incurable with standard treatment regimens, with the exception of allogeneic stem cell transplantation for which only a small percentage of patients are eligible [7]. There is no evidence that early treatment of asymptomatic patients with standard treatment regimens can prolong survival [8]. For this reason, the traditional approach has been to wait and postpone treatment until the patients develop progressive or symptomatic disease [8]. However, increased understanding of the pathobiology of the disease has led to the development of novel therapeutic approaches that are well tolerated and have curative potential. Here we review some of the recent advances in the pathogenesis and treatment of CLL, focusing on those advances that may bring the goal of cure within reach in the near future.

Pathogenesis

Genetic defects and microenvironmental interactions

The malignant cells in CLL are mature B lymphocytes that co-express the T-cell antigen CD5, the B cell antigens CD19 and CD23, and low levels of surface IgM. A hallmark of these cells is their resistance to apoptosis, which appears to be the main reason for their relentless accumulation. However, the increase in tumour burden is also a consequence of increased proliferation, with typical CLL cell birth rates ranging from 0.1% to 1.8% of the clone per day [9].

The disease, as is the case of other cancers, is driven by a combination of genetic defects and microenvironmental interactions. The most common genetic alteration in CLL is del13q14, which occurs in 50%–60% of cases [10]. This deletion invariably involves the microRNAs miR-15a and miR16-1, which target the expression of several important anti-apoptotic and cell cycle regulatory proteins, including BCL2, CCND1, CCND2, CCND3, CDK4 and CDK6 [11, 12]. However, deletion of other genes located in this region may also contribute to the pathogenesis of the disease [13]. Because 13q14 deletion is found at a similar frequency in MBL and CLL, often as a single lesion, it is believed to represent an early event in the pathogenesis of the disease.

Other common chromosomal abnormallities in CLL are deletion of 11q22 (loss of the

ATM gene), trisomy of chromosome 12 and deletion of 17p13 (loss of the TP53 gene), which are found in 18%, 16% and 7% of the patients at diagnosis, respectively [10]. These chromosomal abnormalities provide important prognostic information, with del11q22 and 17p13 being associated with significantly shorter treatment-free and overall survival compared to patients with a normal karyotype or isolated del13q14 or trisomy chromosome 12 aberrations.

The advent of next generation sequencing together with gene copy number analysis allowed the identification of additional recurrent genetic lesions in CLL. The genes that were found to be most frequently mutated are TP53, NOTCH1, SF3B1, BIRC3 and MYD88 [14]. Mutations in TP53, NOTCH1, SF3B1 and BIRC3 are detected in 5–10% of CLL patients at diagnosis and only 1–3% of individuals with MBL. Mutations in these genes, however, are considerably more prevalent in chemo-refractory patients and patients with Richter's transformation, suggesting that they are acquired or selected during disease progression [14]. The MYD88 gene is altered in a smaller percentage of CLL patients (around 3% of patients at diagnosis) and is more often mutated in patients with indolent disease [15].

In addition to these genetic defects, a number of microenvironmental interactions are believed to contribute to the pathogenesis of the disease. These interactions occur primarily in specialized structures in the lymph nodes and bone marrow, called pseudofollicles or proliferation centres, where CLL cells are in close contact with various microenvironmental elements, including T cells, mesenchymal stromal cells, nurse-like cells and follicular dendritic cells (Figure 1].

CLL cells receive proliferation and survival signals from these microenvironmental elements through various ligand/receptor interactions, including CD40L/CD40, VCAM-1/VLA-4, BAFF/BAFF-R, APRIL/TACI, CXCL12/CXCR4 and CD31/CD38 [16]. These signals induce leukaemic cell proliferation and further increase leukaemic cell survival by activating the expression of important anti-apoptotic and cell cycle regulatory genes, such as Myc, Bcl-xL, Mcl-1 and Survivin [17– 19].
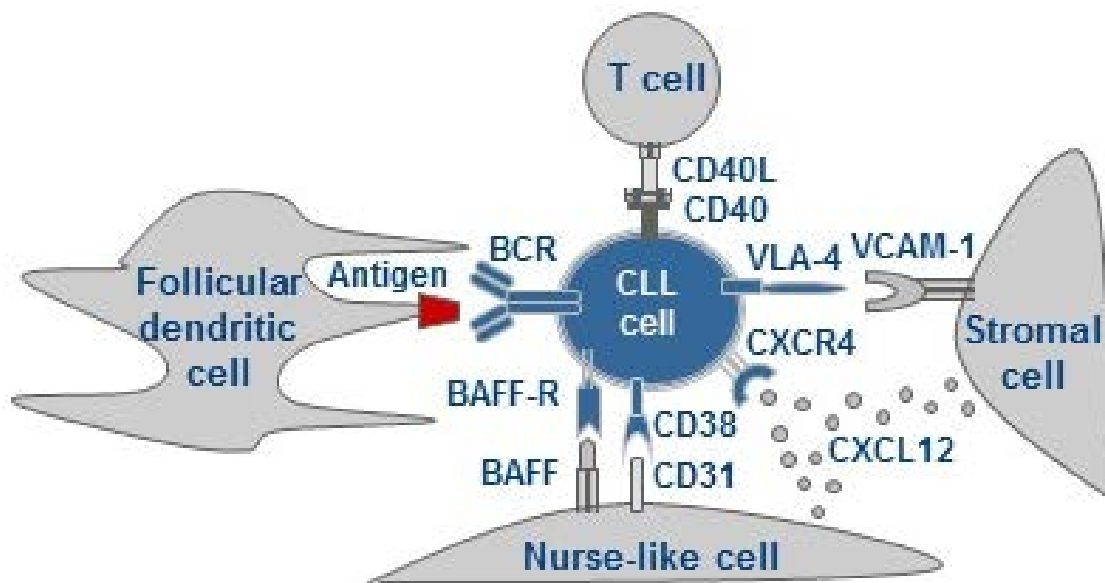
Figure 1 – Microenvironmental interactions implicated in the pathogenesis of CLL. CLL cells interact with CD4+ T cells, stromal cells, nurse-like cells and follicular dendritic cells through various receptors, including CD40, VLA-4, CXCR4, CD38, BAFF-R and the BCR. These interactions promote CLL survival and induce proliferation

Structure and function of the B cell receptor

In addition to signals derived from microenvironmental cellular elements (T cells, stromal cells, nurse-like cells), CLL cells also receive important growth-promoting signals through the B cell receptor (BCR) [20– 22].

The BCR is a signalling complex that is composed of an antigen recognition unit, which is the membrane immunoglobulin (IG) molecule, and a signalling unit that consists of a heterodimer of the proteins CD79a (Igα) and CD79b (Igβ) (Figure 2A).
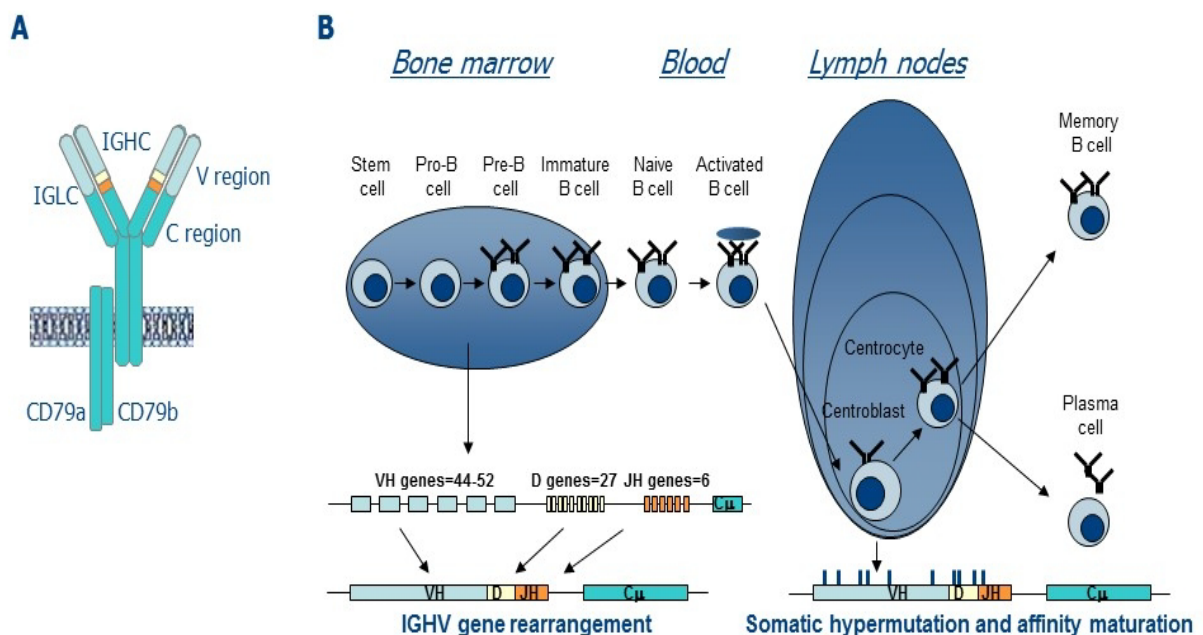

Figure 2 – BCR expression during B cell development and maturation. A) Structure of the BCR. The immunoglobulin heavy and light chains are indicated with IGHC and IGLC, respectively. The V regions are depicted with light blue and the C regions with green color. B) B cells develop from pluripotent stem cells in the bone marrow, then exit into the blood and continue to undergo maturation in secondary lymphoid tissues, such

The IG molecule is composed of two identical heavy chains (HC) and two identical light chains (LC). Each of these chains can be subdivided into a variable (V) and a constant (C) region. The V region is the part of the molecule that binds to the antigen, while the C region has effector functions. Each V region is comprised of four areas of relatively limited diversity, known as the framework regions (FRs), and three areas with considerable sequence diversity, known as complementarity determining regions (CDRs). The CDRs contain the amino acid residues that directly contact the antigen and thus confer the specificity of the IG molecule.

The genes that encode the IG HCs and LCs are generated through a series of molecular events that occur during early stages of B cell maturation. The first event is the random juxtaposition of one each of multiple HC variable (VH), diversity (D) and joining (JH) gene segments, resulting in the assembly of a gene that encodes the V region of the IG HC molecule (Figure 2B). A similar event subsequently occurs at the level of the VL and JL gene segments, giving rise to the gene that encodes the V region of the IG LC molecule.

The random assembly of multiple VH/D/JH and VL/JL elements allows for the production of a huge variety of combinations and corresponding molecular structures, providing considerable diversity to the structure of the antigen-binding portion of the BCR. Just considering the combinatorial events of the IG HC and LC gene loci, there are greater than 1.6×10^6 possible VH/D/JH and VL/JL combinations. In addition, random trimming or addition of nucleotides at the VH-D, D-JH or VL-JL junctions results in even greater diversity in BCR structure.

In later phases of B cell ontogeny, diversity is further increased by the somatic hypermutation (SHM) and class-switch recombination (CSR) processes. These processes are induced by antigen encounter and occur in the germinal centres of the secondary lymphoid organs. SHM is characterized by the introduction of mutations within the rearranged IG genes that increase the affinity of the IG molecule for

antigen, whereas CSR leads to replacement of the IGHC constant region gene, switching antibody production from IgM to IgG, IgE or IgA. SHM and CSR have been estimated to increase the potential for variation by 10^3–10^6 fold. Hence, the B-cell repertoire altogether can comprise up to 10^{12} different specificities [23]. This number by far exceeds the number of B cells in the human body, which in turn suggests that the probability that two independent B-cell clones would carry exactly the same BCR by chance alone is virtually negligible.

Evidence for a role of the B cell receptor in CLL pathogenesis

A role for the BCR in the pathogenesis of CLL was first postulated in the early 1990-ies, when several groups published studies showing that CLL cells frequently express BCRs that are encoded by a relatively restricted repertoire of VH, D and JH gene segments [24–26]. Subsequent studies showed that these structurally similar HCs are frequently paired with identical LCs, resulting in the expression of BCRs with similar or virtually identical antigen-binding sites [27–29]. As already mentioned, these significant structural similarities cannot be explained by chance alone, suggesting that they are the result of selection of leukaemic cells with particular antigen-binding properties. Importantly, expression of structurally similar, so-called "stereotyped" BCRs, has been observed in approximately one third of CLL patients, suggesting that a BCR-dependent mechanism selects and drives the expansion of the malignant clones in a substantial proportion of cases [30].

A second important finding obtained from the early immunogenetic studies was the observation that the leukaemic BCRs in approximately 40–50% of the cases are encoded by unmutated IGHV genes, whereas these genes are mutated in the remaining cases [31]. Subsequent studies showed that CLL patients with unmutated IGHV genes (U-CLL) have a more aggressive clinical course and shorter survival than patients with mutated IGHV genes (M-CLL) [32, 33]. This strong association between a BCR-related feature (i.e., IGHV mutational

status) and clinical course suggested that the BCR pathway is involved not only during CLL development but also during disease progression. Further support for this possibility came from gene expression profiling studies, which showed that freshly isolated CLL B cells express high levels of genes that are induced by BCR engagement [34, 35]. These BCR target genes were especially enriched in CLL cells isolated from major sites of antigen encounter, such as lymph nodes, and were found to be expressed at higher levels in U-CLL than M-CLL cells [34, 35]. Altogether, these data suggested that CLL cells are continuously exposed to antigen *in vivo* and indicated that the two prognostic subsets differ either with respect to the nature of the antigens that they recognize or with respect to their capacity to propagate the antigenic stimuli [21].

BCR signals generated in CLL cells

CLL cells receive two types of signals from their BCRs. The first type is triggered by binding to external antigen, which results in aggregation of neighbouring BCRs and co-receptors, and subsequent assembly of a signalling complex that further propagates the signal to the interior of the cell (Figure 3A) [36]. In normal B cells this signal can induce a variety of responses, including proliferation, survival, differentiation, anergy or apoptosis. The final outcome is influenced by several factors, such as the nature of the antigen, the availability of costimulatory signals and the stage of B cell differentiation. In CLL cells crosslinking of the BCR with anti-IgM antibodies, which are used to mimic external antigen, can lead to either increased survival or induction of apoptosis [20, 21]. The outcome is primarily determined by the nature of the stimulus, with sustained receptor engagement by immobilized anti-IgM usually being associated with increased leukemic cell survival [37, 38].

The second type of signal occurs in the absence of an external ligand and has been termed cell-autonomous BCR signal (Figure 3B). This signal appears to be responsible for the observed increase in the basal activity of several signalling molecules that are located immediately downstream of the BCR, such as the kinases LYN, SYK, PI3K, BTK, and PKC [39– 44]. Inhibition or RNAi-mediated knockdown of these signalling molecules induces apoptosis in CLL cells, suggesting that the cell autonomous BCR signal increases leukaemic cell survival.
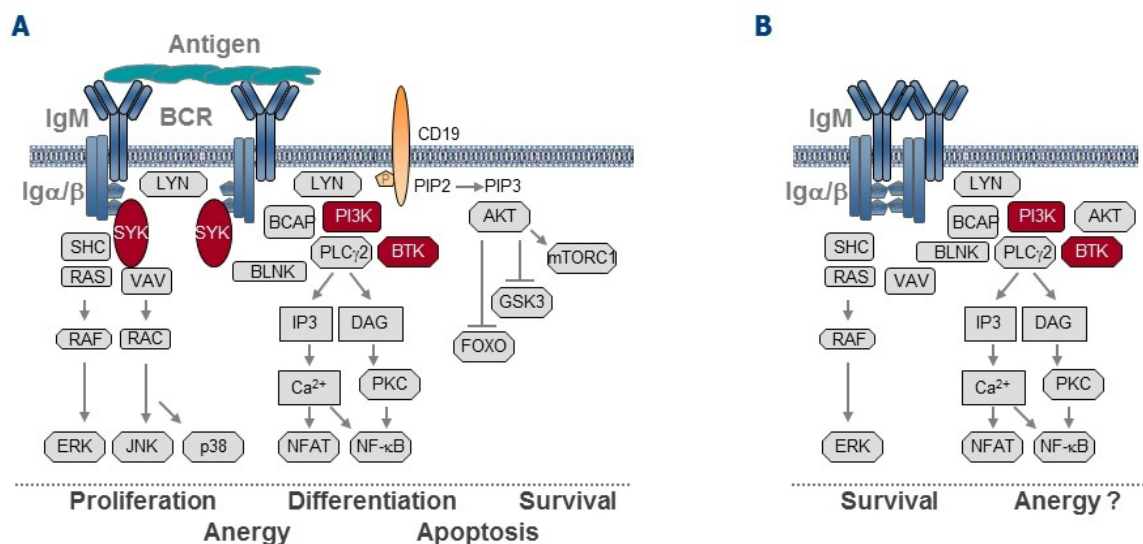

Figure 3 – BCR signals generated in CLL B cells.
A) Aggregation of the BCR with external antigen induces the phosphorylation of the ITAMs within the cytoplasmic tails of the Ig-α/Ig-β heterodimer. Phosphorylation of the ITAMs is mediated by LYN or other members of the SRC family kinases. SYK is subsequently recruited to the phosphorylated ITAMs and activated by a multistep process that involves phosphorylation by SRC family kinases and trans-autophosphorylation. SYK further propagates the BCR signal by phosphorylating and recruiting several signalling intermediates, such as BLNK, BCAP, VAV, SHC and PI3K. Recruitment of PI3K leads to the production of phosphatidylinositol-3, 4, 5-triphosphate (PIP3) and subsequent BTK and AKT activation. SYK and BTK then activate PLCγ2, which produces the second messengers

inositol-1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG). These second messengers induce the release of intracellular Ca^{2+} and activate PKC, which then activate the transcription factors NFAT and NF-κB. Other molecules that are activated downstream of SYK are the mitogen-activated protein kinases ERK, JNK and p38MAPK. The cellular outcome in response to BCR engagement depends on the relative activity of the above described signalling molecules, and is influenced by the nature of the antigen, the availability of co-stimulatory signals and the stage of B cell differentiation. The main therapeutic targets along the BCR pathway in CLL B cells are indicated with white letters and red background.

B) Intermolecular BCR-BCR interactions generate a 2nd type of signal in CLL cells. These interactions occur because of the unique capacity of CLL CDR3 regions to bind to internal IG epitopes. The downstream signalling pathways that transduce this BCR signal have still not been fully characterized, but many of the same molecules that transduce the signal induced by external antigen appear to be involved. The main cellular consequence of this signal appears to be increased leukaemic cell survival, but certain features of anergy that are typical of CLL cells could also be caused by this signal

The molecular mechanism that generates the cell autonomous BCR signal was only recently revealed [45]. This signal is triggered by inter- or intra-molecular interactions between BCRs expressed on the same cell, which interact because of the apparently unique capacity of the leukaemic CDR3 regions to bind to internal IG motifs located in the FR2 and FR3 regions [45, 46]. Such interactions appear to be a common feature of CLL BCRs, as they have been detected with all of the CLL-derived BCRs that were investigated so far [45].

The external antigens that trigger the leukemic BCRs *in vivo* have still not been fully characterized, but a number of candidate molecules were recently identified. This particularly refers to U-CLL, where the malignant cells typically express polyreactive BCRs that bind with low affinity to autoantigens generated during apoptosis or oxidation, such as nonmuscle myosin heavy chain IIA, vimentin, filamin B, cofilin-1, dsDNA, Sm, or oxidized lipoproteins [47–51]. In M-CLL, the antigens that are recognized by the leukaemic BCRs are still largely unknown, except for a few cases that were recently reported to express BCRs that bind with high-affinity to the fungal antigen β-(1, 6)-glucan or the Fc portion of human IgG [52– 54].

To further understand the role of the BCR pathway in the pathogenesis of CLL, we recently conducted a study in which we investigated the capacity of different antigen-BCR and BCR-BCR interactions to induce leukaemia in a well-established *in vivo* animal model of CLL [55]. The model that we used are Eμ-TCL1 transgenic mice, which are predisposed to develop CD5+/IgM+ B cell leukaemias because of targeted overexpression of the TCL1 oncogene in the B cell compartment [56]. These mice were bred with transgenic mice expressing various transgenic BCRs, including BCRs with cell-autonomous activity and BCRs specific for different foreign or self-antigens. The leukaemias that developed in these animals uniformly expressed BCRs with cell-autonomous activity, which in most cases were also capable of reacting with low-affinity apoptosis-associated autoantigens. In contrast, leukaemias did not develop from B cells expressing high-affinity BCRs, regardless whether the antigens were provided as foreign- or self-antigens. Altogether, these data suggest that two types of BCR interactions co-operate in the pathogenesis of CLL: cell-autonomous BCR-BCR interactions and BCR interactions with low-affinity apoptosis-associated autoantigens. Since both types of interactions are essentially interactions with endogenous antigenic determinants, these data also suggest that CLL can be considered an autoantigen-driven disease [55, 57].

CLL development and evolution

The data presented in the previous sections suggest that the pathogenesis of CLL is a multistep process, consisting of several distinct phases that are characterized by the acquisition of specific genetic defects, which co-operate with various microenvironmental signals in driving the expansion of the leukaemic clones (Figure 4). The primary genetic defect most likely resides at the level of the haematopoietic stem cell and represents either an acquired genetic lesion or an inherited genetic predisposition. This defect could be responsible for the positive selection and expansion of B cells that express low-affinity autoreactive BCRs with autonomous signaling capacity, analogous to

the role of the TCL1 oncogene in the transgenic model described above. Evidence for the existence of such an early genetic lesion comes from a recent study by Kikushige *et al*, who transplanted haematopoietic stem cells (HSC) isolated from the bone marrow of patients with CLL into immuno-deficient NSG mice [58]. These transplants led to the outgrowth of oligoclonal CD5 + B cell populations, suggesting that the propensity to generate clonal B cells is already present at the HSC stage in patients with CLL. Importantly, the expanded B cell clones expressed IG HC genes that are typical of human CLL, further suggesting that selection of BCRs with particular structural properties is one of the earliest events in the pathogenesis of the disease.

In the subsequent phase, acquisition of genetic lesions such as del13q14 would result in the outgrowth of individual clones with features of MBL. Such monoclonal expansions could be further propelled by interactions with various tissue elements, such as T cells, stromal cells and nurse-like cells. The role of the BCR in this setting could be to facilitate some of these interactions, particularly with T cells, by providing antigen for peptide processing and presentation. This possibility is further supported by a recent study showing that CLL patients have T helper cells specific for endogenous CLL antigens, which are capable of activating autologous CLL cells *in vitro* and inducing their expansion in a mouse xenograft model *in vivo* [59].
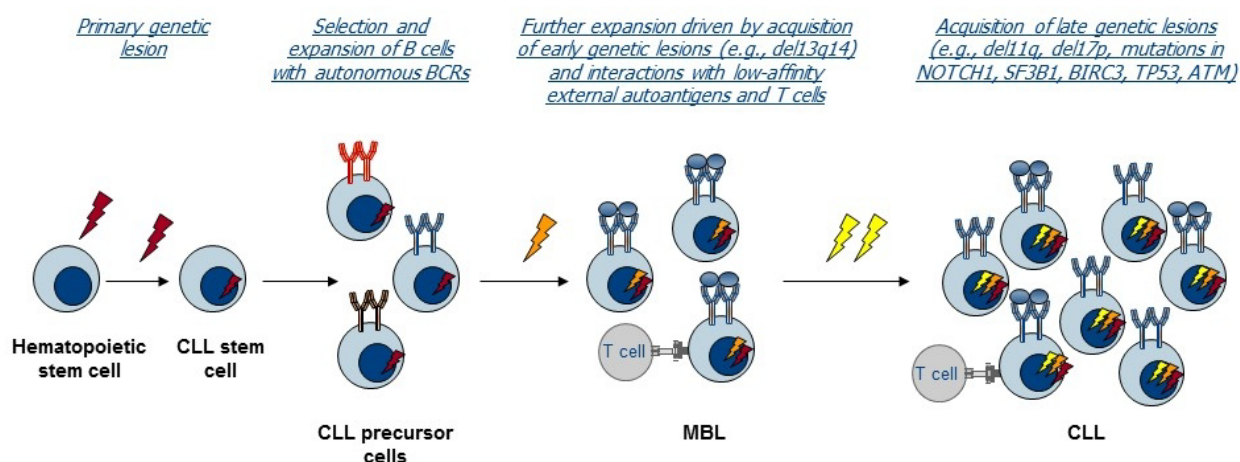

Figure 4 – Clonal evolution of CLL. Schematic depiction of CLL development and evolution. Genetic lesions are indicated by lightning symbols

Progression to CLL likely depends on acquisition of genetic lesions that are rare in MBL but frequent in CLL, such as mutations in NOTCH1, SF3B1, BIRC3, TP53 and ATM [60, 61]. Acquisition of such genetic lesions is more likely to occur in cells that are more actively dividing, which could explain why disease progression is more rapid in CLL patients whose leukaemic cells show greater responsiveness to BCR and other microenvironmental stimuli [62].

Treatment

Chemoimmunotherapy

The alkylating agent chlorambucil was the standard first line treatment for CLL until phase 3 studies demonstrated an improved overall response rate (ORR) and prolonged progression-free survival (PFS) in patients treated with the purine analogue fludarabine [63]. Subsequent studies showed that the ORR and PFS can be further improved by combining fludarabine with cyclophosphamide [64]. A major step forward was the addition of the monoclonal anti-CD20 antibody rituximab to the fludarabine/cyclophosphamide combination, resulting in an even more effective regimen. This FCR regimen was the first to show an improvement in overall survival (OS) and has since become standard first line therapy for CLL [65]. However, the FCR regimen is associated with significant myelosuppression and a high rate of early and late infections, which is why it is considered unsuitable for most elderly patients

and patients with co-morbidities [66]. Considering that these categories account for > 70% of patients that require treatment, this means that effective therapeutic regimens are still lacking for the majority of patients with CLL.

In recent years a number of less toxic regimens were explored in elderly CLL patients or patients with co-morbidities. These include reduced-intensity FCR and combinations of chlorambucil or bendamustine with the anti-CD20 monoclonal antibodies (mAb) rituximab or obinituzumab [67– 73]. These regimens showed improved tolerability, but efficacy appeared to be generally reduced when compared to FCR. Among these regimens, greatest activity was observed for the combination of obinituzumab and chlorambucil, which showed increased response rates and prolonged PFS in comparison to the combination of rituximab and chlorambucil or chlorambucil alone [73]. The obinituzumab and chlorambucil combination also showed improved OS compared to chlorambucil alone, which is the first time that a treatment has shown an overall survival benefit in elderly CLL patients or patients with comorbidities.

Despite these improvements, it is worth noting that there is no evidence that any of the current chemoimmunotherapy protocols has curative potential. For these reasons the focus on CLL research in recent years has shifted towards novel targeted therapies, such as BCR pathway inhibitors, BCL2 antagonists and chimeric antigen receptor (CAR)-modified T cells. It is hoped that these novel therapies may change treatment paradigms in the near future and bring the goal of cure closer to reality.

BCR pathway inhibitors

Interest in BCR pathway inhibitors as potential therapeutic agents in CLL was triggered by accumulating evidences that the BCR pathway plays a major role in the development of the disease [21]. The first compounds that were tested were inhibitors of the kinase SYK, such as piceatannol [41], R406/R788 (also known as fostamatinib) [41, 74], SYKII [75] and BAY 61-3606 [76]. These compounds were found to induce moderate apoptosis in unstimulated primary CLL cells and to block survival signals induced by sustained engagement of the BCR with immobilized anti-IgM antibodies, which

together suggested that they are capable of targeting both the cell autonomous BCR signal as well as the BCR signal generated by binding to external antigen [77]. This possibility was further corroborated by *in vivo* experiments in the Eμ-TCL1 transgenic mouse model of CLL, which showed reduced proliferation and survival of the leukaemic B cells and prolonged survival of animals treated with fostamatinib [74].

Fostamatinib was also evaluated in a phase 1/2 trial of patients with relapsed or refractory B cell malignancies [78]. The highest response rate was observed in CLL/SLL, where 6 of the 11 treated patients (55%) achieved a partial response (PR). All responding CLL/SLL patients exhibited a transient initial lymphocytosis that occurred in parallel with a reduction in lymphadenopathy. This phenomenon was subsequently shown to be caused by mobilization of CLL cells from the tissues into the blood and has since been observed with all other BCR pathway inhibitors. The reason for this mobilization is because BCR pathway inhibitors can also block signals downstream of chemokine receptors and/or integrins, which are required for the migration and adhesion of the leukemic cells to stromal elements [79].

Because of corporate decisions, no other clinical studies with fostamatinib in CLL were subsequently conducted. However, a more selective SYK inhibitor called GS-9973 has been developed more recently and is currently being investigated in a phase 2 clinical trial of CLL [80, 81]. Preliminary results from this study suggest that this SYK inhibitor also has substantial activity in patients with CLL [81].

Another important target along the BCR pathway in CLL B cells is PI3Kδ [82]. CLL cells generally express high levels of active PI3Kδ [42, 43], and sustained activation of this pathway has been shown to be required for their proliferation and survival [37, 83]. PI3Kδ, in addition to transducing signals from the BCR, also plays an important role in transducing co-stimulatory signals that originate from other receptors, such as BAFF, CD40, and Toll-like receptors. These signals can be blocked by a recently developed selective PI3Kδ inhibitor called idelalisib (also known as CAL-101 or GS-1101), which also induces moderate apoptosis in unstimulated CLL cells [43, 84, 85].

Idelalisib was tested in a phase 1 study of patients with relapsed/refractory CLL, including patients with del17p13 [86]. The drug was well tolerated and demonstrated considerable activity in these patients [86]. A phase III study comparing idelalisib and rituximab against rituximab and placebo in relapsed CLL patients with significant co-morbidities was more recently conducted and showed a significantly improved overall response rate and an overall survival benefit for the addition of idelalisib [87]. Based on these results, idelalisib was recently registered in the USA and EU for previously treated patients with CLL.

Another BCR signalling molecule that has received considerable attention as a potential therapeutic target in CLL is Bruton's tyrosine kinase (BTK). This kinase has a relatively restricted expression, mainly confined to B lymphocytes and myeloid cells, suggesting that its inhibition should produce relatively few side effects. Downregulation of BTK by RNA interference induces apoptosis in human CLL cells *in vitro*, and genetic inactivation of BTK delays leukemia development in the Eμ-TCL1 transgenic mouse model of CLL *in vivo*, providing direct evidence that BTK is a relevant therapeutic target in CLL [88].

A selective, orally available inhibitor of BTK called Ibrutinib has been developed. This drug irreversibly inhibits BTK by covalently binding to its cysteine-481 residue. Preclinical studies showed that ibrutinib induces moderate apoptosis in primary CLL cells and effectively abrogates survival signals provided to CLL cells through various microenvironmental stimuli, including the BCR, integrins and stromal cells [44].

A phase 1 study demonstrated significant activity of ibrutinib in patients with various relapsed or refractory B cell malignancies, including CLL [89]. A subsequent phase 1/2 study involving only patients with relapsed or refractory CLL showed an ORR of 71% [90]. This high response rate was independent of any of the investigated clinical and genomic risk factors, including advanced-stage, number of previous therapies and del17p13. Treatment was well tolerated with only mild side effects, among which most common were transient diarrhoea, fatigue and upper respiratory infection. More recently, a phase 3 study was conducted in relapsed or refractory CLL/SLL that compared ibrutinib to the anti-CD20 antibody ofatumumab [91]. This trial showed significantly improved response rate, progression-free survival and overall survival for the ibrutinib arm. Based on the high response rates observed in these clinical trials, ibrutinib was approved during 2014 in the USA and EU for second line treatment of CLL, including patients with 17p deletion.

Ibrutinib was also evaluated in a phase 2 study of elderly (> 65 years) previously untreated patients with CLL [92]. ORR was 71% and 13% of the patients achieved a complete response. Treatment was well tolerated and adverse events were minimal, suggesting that ibrutinib could also be an appropriate therapeutic option for this group of patients.

A recent phase 2 study evaluated ibrutinib in combination with rituximab in CLL patients with high-risk cytogenetic abnormalities (del17p, TP53 mutation, or deletion 11q) or a short PFS after first-line chemoimmunotherapy [93]. Treatment was well tolerated and ORR was very high (95%), suggesting that the addition of anti-CD20 mAbs to ibrutinib may further improve clinical responses.

BCL2 antagonists

Overexpression of the antiapoptotic protein BCL2 is one of the main reasons for the prolonged survival and increased apoptosis resistance of CLL cells. The reason for BCL2 overexpression in CLL is still not completely understood, but in patients with del13q14 this appears to be due to deletion of microRNAs miR-15a and miR-16, which normally act to downmodulate BCL2 expression [94].

Drugs that can inhibit BCL2 have recently been developed. These drugs induce apoptosis of primary human CLL cells *in vitro*. The BH3-mimetic ABT-263 was the first such drug to be tested in the clinic. It showed very promising activity in a phase 1 study of patients with relapsed/refractory CLL [95]. However, its therapeutic use was limited by dose-limiting thrombocytopenias caused by inhibition of the BCL2-related antiapoptotic protein BCL-xL.

To overcome this problem, ABT-263 was reengineered to create the more BCL2-selective compound ABT-199, which does not inhibit

BCL-xL [96]. Preliminary results from an on-going phase I clinical trial showed an OR rate of 84% and a CR rate of 20%, with 4 of the 56 enrolled patients having a minimal residual disease negative CR [97]. Importantly, patients with high-risk CLL showed similar efficacy with a response rate of 82% in del(17p) and 78% in fludarabine-refractory disease. Major side effects were tumour lysis syndrome, diarrhoea and neutropenia. These preliminary data suggest that ABT-263 could be another highly effective novel therapeutic agent in CLL.

Chimeric Antigen Receptors (CARs)

Recent clinical studies investigating the use of chimeric antigen receptor (CAR)-modified T cells to treat CLL and other B cell malignancies have generated enormous excitement. CARs are chimeric molecules that consist of an antigen recognition domain derived from an antibody specific for a tumour antigen and a T-cell activation domain (Figure 5). The T-cell activation domain is usually composed of the CD3ζ chain of the T-cell receptor complex and a co-stimulatory domain from the co-receptors CD137, CD28, or both.
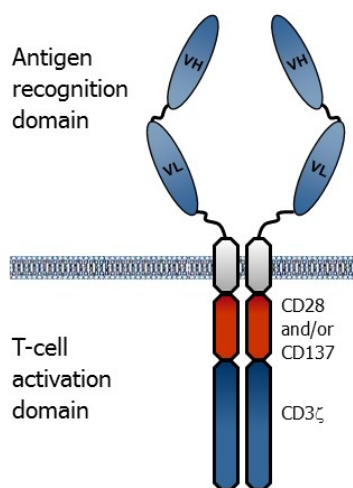

Figure 5 – Structure of chimeric antigen receptors. The antigen recognition domain is composed of a single-chain Fv antibody fragment. The T-cell activation domain is composed of the CD3ζ chain associated with a CD28 and/or CD137 co-stimulatory domain(s)

CAR molecules can be introduced into autologous T cells using lentiviral or retroviral vectors, or through electroporation of RNA or a transposon-based system. These modified T cells are then expanded *ex vivo* by exposure to anti-CD3/CD28 beads and infused back into the patient, where they lyse the cells that express the tumour antigen [98].

Autologous CAR-modified T cells specific for the B cell antigen CD19 have been tested in several B cell malignancies, including CLL, ALL and DLBCL [99, 100, 101]. Profound and durable responses were observed across all tested B cell malignancies. Initial data in CLL were particularly encouraging, with the first treated patient achieving a sustained CR [99]. However, subsequent results have been more heterogeneous, with a follow-up trial in 14 CLL patients resulting in 3 CRs, 5 PRs and no response in 6 patients [102]. A number of clinical trials investigating different tumour antigens and various CAR designs, transduction strategies, T-cell culture conditions, and lymphodepleting strategies are currently ongoing in CLL and other B cell malignancies to further optimize this promising novel therapeutic approach.

Combination Therapies

The novel therapeutic agents discussed above have in common several important advantages with respect to standard chemotherapy, including the capacity to induce durable responses in a significant proportion of patients, the absence of myelosuppression and the relatively mild toxicity. However, with the exception of CAR-modified T cells, these novel therapeutic agents produce very few CRs, suggesting that they will be unable to cure the disease on their own. Whereas therapy with non-curative intent aimed at keeping the disease under control could be an appropriate therapeutic goal for elderly patients with CLL, this certainly is not the case for younger patients with the disease. In addition, prolonged treatment with these novel agents would be expected to result in the development of resistance, and acquired resistance to ibrutinib has already been reported [103]. Furthermore, the enormous costs of prolonged treatment with these drugs and the unavailability of data to predict whether the sustained partial responses induced by continuous therapy with BCR pathway inhibitors will be sufficient to reduce the risk of Richter transformation represent additional matters of concern. For all these reasons, rationally designed combinations with curative potential will have to be developed.

One possible rational approach that was recently tested in the clinic is to combine these novel agents with rituximab, which has shown improved quality and sustainability of responses when combined with other treatments in CLL [104]. However, recent clinical trials of rituximab with idelalisib or ibrutinib failed to show an improvement in the percentage of CRs (0% and 8%, respectively), despite the impressive overall response rates (81% and 95%, respectively) [87, 93]. It is important to note, however, that a recent preclinical study showed that all BCR pathway inhibitors, including ibrutinib, idelalisib and fostamatinib, downregulate the expression of CD20, resulting in reduced complement-dependent and antibody-dependent cellular cytotoxicity (ADCC) [105]. Moreover, ibrutinib was also found to antagonize ADCC by direct inhibition of ITK, which is a BTK homologue that is expressed in natural killer cells and is ultimately responsible for triggering the ADCC effect [106]. Together, these data suggest that modifications in the drug administration schedules will have to be considered in future studies investigating combinations of BCR pathway inhibitors with anti-CD20 mAbs to avoid interactions that can cause an antagonizing effect.

A second rationally designed strategy based on mechanistic synergy would be to combine BCR pathway inhibitors with BCL2 antagonists. This combination still awaits to be tested in the clinic, but has shown considerable synergy in preclinical studies of DLBCL and would be expected to be at least as effective in CLL [107].

Lastly, a large, high-throughput combination screen of a library of nearly 500 approved and investigational drugs showed synergy of ibrutinib with other BCR pathway inhibitors, including idelalisib and the SYK inhibitor PRT-060318 [107]. Combinations of BCR pathway inhibitors are interesting not only because they could potentially induce more durable responses, but also because they may prevent the emergence of resistant clones by simultaneously acting on multiple targets along the same pathway.

In summary, the considerable progress made in recent years in the understanding of the molecular mechanisms that drive CLL has resulted in the development of novel, highly active targeted therapies with the potential to provide durable disease control in most patients with CLL. The main question now is how to best combine these novel agents to further improve patient outcomes. Direct comparisons between the different therapeutic combinations will help resolve this issue and may lead to the identification of therapeutic regimens capable of curing the disease in the majority of CLL patients in the near future.

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Р е з и м е

**НОВИ ДОСТИГНУВАЊА
ВО ПАТОГЕНЕЗАТА И ТРЕТМАНОТ
НА ХРОНИЧНАТА ЛИМФОЦИТНА
ЛЕУКЕМИЈА**

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Хроничната лимфоцитна леукемија (HLL)
е честа лимфоидна малигна болест карактеризи-
рана со експанзија и прогресивна акумулација
на зрели автореактивни Б лимфоцити. Болеста е
клинички хетерогена и неизлечива со стан-
дардна хемотерапија. Главна карактеристика на
болеста е значителната зависност на леукемич-
ните клетки на различни микроколински дразби,
кои го забрзуваат растот, го продолжуваат пре-
живувањето и ја зголемуваат резистентноста на
лекови на леукемичните клетки. Неодамна, зна-
чителен напредок е направен во разбирањето на
молекуларните механизми кои се причина за
настанувањето на HLL. Идентификацијата на
рекурентни генетски лезии со користење на
„следна генерација секвенционирање" обезбеди
нови податоци за патофизиологијата на болеста
и овозможи попрецизно утврдување на прогно-
зата. Сознанието за критичната улога на Б кле-
точниот рецептор (BCR) во настанувањето на
болеста резултираше со развој на BCR-инхиби-
тори за кои се очекува целосно да го трансфор-
мираат третманот на HLL во блиска иднина.
Други нови терапевтски агенси, како што се
BCL2-антагонистите и модифицираните Т-клетки
со химерички антиген рецептор (CAR), исто така
многу ветуваат во клиничките испитувања. Во
овој преглед се сумираат некои од овие нео-
дамнешни достигнувања, со посебен осврт на Б
клеточниот рецептор и неговите инхибитори.

Клучни зборови: хронична лимфоцитна леукемија, Б
клеточен рецептор, фостаматиниб, ибрутиниб, идеалисиб.