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Review

Chronic Lymphocytic Leukaemia

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Abstract. *Chronic lymphocytic leukaemia remains the most common adult leukaemia in Western countries. In this review we tried to give an overview of the important prognostic markers and the developments in this field. We also reflected about the accumulative character of the disease and the influence of the micro-environment on the CLL cell.*

Chronic lymphocytic leukaemia (CLL) is the most common adult leukaemia in the Western world, with an age-adjusted incidence of 3.5 per 100,000 per year (USA, 1996-2000) and a prevalence of 46,000 individuals in the European Union (five years post diagnosis condition) (1). It is more common in males than in females and it is largely a disease of the elderly, with a median age at diagnosis of 72 years (2, 3). CLL is characterized by the accumulation of mature-looking immunoincompetent lymphocytes that are typically positive for CD5, CD23, CD19 and CD20. Indeed, in 95% of the cases, CLL of the B-cell phenotype is diagnosed, explaining why the term CLL can readily be used instead of B-CLL (B-cell CLL). The accumulation of the clonal cell population occurs in the blood, the bone marrow, the lymph nodes and the spleen. The aetiology of CLL is unknown, but a genetic component offers the best explanation (4). This is illustrated by the higher prevalence of the disease in relatives of a CLL patient, as well as the phenomenon of anticipation, with an increase of severity and earlier age of onset with each generation. The higher frequency of autoimmune disorders in CLL siblings also argues for a genetic involvement in the aetiology of the disease. However, many questions remain unanswered in the genetics of CLL, such as how to explain the different mutations in identical twins suffering from CLL (5-7). There is no established role for environmental factors as inducing or influencing factors. This was illustrated by Japanese migration studies: CLL is not frequent in Japan and Japanese who immigrate to the USA remain at low risk for the disease (6). Exposure to factors such as pesticides, sunlight, ionizing radiation, alkylating agents and chemicals, have all been investigated but no hard evidence has been found (4, 8-10). Viruses such as the Epstein-Barr virus (EBV) and hepatitis C have not been implicated in the pathogenesis of CLL.

The diagnosis of CLL requires the presence in the peripheral blood of more than 5,000 small mature-appearing lymphocytes per microlitre (4). The typical B-CLL phenotype is $CD19^+$, $CD5^+$, $CD23^+$ and $FMC7^-$ with weak expression of surface immunoglobulin (sIg) and weak or a lack of expression of membrane CD79b, the extracellular epitope of the B-cell receptor (BCR) β chain (11, 12). Based on the markers CD5 (+), CD22 (+), CD23 (+), FMC7 (–) and sIg (weak), a 5/5 scoring system was developed to distinguish typical (≥3/5 positive markers) and atypical (<3/5 positive markers) forms of CLL (10). Later, CD79b was implemented as a sixth marker (13).

The distinction between typical and atypical forms of CLL is also made based on morphological criteria of the cells. In typical CLL, >90% of the cells are small to medium sized lymphocytes with a constant and homogeneous volume. The 15% subgroup of atypical CLL cases comprises two subtypes: CLL with >10% prolymphocytes (CLL/PL) and atypical CLL with lymphoplasmacytic differentiation and/or cleaved cells (mixed-cell type).

Classification Systems and Prognostic Factors in B-Cell Chronic Lymphocytic Leukaemia

The classification of CLL, morphological as well as immunophenotypical, is more than just descriptive. Indeed, CLL is a disease with a highly variable course, mainly falling into two subclasses. The first consists of patients in whom the disease has an indolent course with no need for

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Table I. *Rai classification system for chronic lymphocytic leukaemia.*

Risk level	Stage	Clinical features at diagnosis	Median survival time (years)
Low	0	Blood and marrow lymphocytosis	≥10
Intermediate	I	Lymphocytosis and lymphadenopathy	9
	II	Lymphocytosis and splenomegaly or hepatomegaly	7
High	III	Lymphocytosis and anaemia (Hb <11g/dl)	5
	IV	Lymphocytosis and thrombocytopenia (<100,000/μl)	5

Table II. *Binet classification system for chronic lymphocytic leukaemia.*

Stage	Clinical features at diagnosis	Median survival time (years)
A	Blood and marrow lymphocytosis and less than three areas of palpable lymphoid involvement	>7-10
B	Same with three or more areas of palpable lymphoid involvement	5-7
C	Same plus anaemia or thrombocytopenia	<2-5

therapy, while the second class of patients suffers from a more aggressive disease, with a rapid need for treatment (14). Recognizing this heterogeneity, Rai and colleagues and Binet and colleagues developed staging systems for assessment of the extent of disease in an individual patient (15-18). Both clinical staging systems divide the patient population into three prognostic subgroups, based on factors reflecting tumour burden. While the system of Rai is most used in the USA, Binet's staging system is more popular in Europe. Table I and II shows the classification system of Rai and Binet, respectively.

These systems remain the cornerstones on which decisions regarding medical follow-up and treatment are built but they fail to predict the course of the disease in patients in whom CLL is diagnosed in early stages (18). Therefore prognostic factors have been established to help make therapeutic decisions. These can be divided into clinical, biological, cytogenetic and IgV_H(-related) ones and will be discussed in the following sections.

Clinical Prognostic Parameters

Clinical parameters associated with poor prognosis are older age, male gender, black race and poor performance status (2, 19).

Biological Prognostic Factors

Biological markers which are accepted as giving valuable prognostic information are the absolute lymphocyte count (cut-off 4,000/μl) (20) and the lymphocyte doubling time (1 year) (21). An atypical lymphocyte morphology and diffuse infiltration pattern in the bone marrow are also both associated with poorer prognosis (22, 23). Biological parameters which are associated with poorer prognosis in Binet group A are high serum levels of β2 microglobulin (24), high serum soluble CD23 (25) and high serum activity of thymidine kinase (26).

CD38, another valuable biological parameter, is a surface molecule that supports B-cell interactions and differentiation. Expression of CD38 on the malignant cells is associated with poor prognosis (27, 28). The more detailed impact of CD38 will be discussed in a further section.

Cytogenetic and Molecular Markers

The development of new techniques, such as fluorescence *in situ* hybridization (FISH), has increased the detection of numerical and structural chromosome abnormalities. These abnormalities are rarely evident at clinical presentation and are therefore manifest examples of clonal evolution (29). The later occurrence during disease progression of cytogenetic abnormalities again indicate that they are not likely involved in the aetiology of CLL. In contrast to other B-cell lymphomas which are often under the influence of chromosomal translocations, B-CLL typically develop deletions.

The most common cytogenetic alteration is deletion 13q14 (51%), followed by deletion 11q22-q23 (17-20%), trisomy 12 (15%) and deletion 17p13 (5%) and 6q21 (6%) (30-33). Complex abnormalities may also be present. Deletion of 13q is associated with a better prognosis. The exact target gene in this deletion remains unknown, but the deleted region contains two micro-RNA genes, *miR-15a* and *miR-16-1* (34, 35). Micro-RNAs are a group of small non coding RNA molecules which can reduce the levels of their target transcripts, as well as the amount of protein encoded by these transcripts (36, 37). Both *miR-15a* and *miR-16-1* reduce the transcription level of the anti apoptotic protein Bcl-2 thereby

promoting apoptosis. Normal non-CLL $CD5^+$ B-cells have high levels of *miR-15a* and *miR-16-1* in comparison to CLL cells. Apparently, *miR-15a* and *miR-16-1* are deleted in the good prognosis 13q14 deleted CLL subtype.

The presence of trisomy 12 has no significant prognostic influence, while deletion of 17p, 11q and 6q21 are associated with a worse median survival time (33). The involded gene in the 17p deletion appears to be *p53* and for 11q it is occasionally *ATM* (ataxia-telangiectasia mutated). *p53* is a tumour suppressor and loss of its protein is involved with resistance to therapy with alkylating agents (such as chlorambucil), purine analogues (such as fludarabine) and rituximab [chimeric anti-CD20 monoclonal antibody (mAb)] (38). *p53*-Mutated B-CLL cells do respond to therapy with alemtuzumab (humanized IgG1 anti-CD52 Ab) (39).

Mutation Status of the IgV_H Genes and Surrogate Markers

Mutation status of the IgV_H genes. During normal B-cell development, B-cells undergo several gene rearrangements leading to affinity maturation of the sIg. The rearrangement occurring during the germinal centre reaction is somatic hypermutation (SHM) and consists of the introduction of point mutations in the gene coding for the variable region of the heavy chain of the sIg. SHM provides a genetic "passport stamp" signifying that the cell has entered the germinal centre (40). To study the origin of B-CLL, the presence of SHMs were evaluated. In contrast with previous reports, Schroeder and Dighiero (41), Fais *et al*. (42) and Oscier *et al*. (43) described the presence of a substantial number of B-CLL cases with mutated IgV_H genes. Oscier *et al*. suggested that the mutated CLLs were associated with chromosomal deletions at 13q14 and the unmutated cases with trisomy 12. Later, two independent groups described the association between SHM and prognosis (27, 44). The median survival of somatically mutated CLL was around 25 years while that of unmutated cases was only 8 years. The prognostic value of Ig rearrangements was confirmed by several other groups (45-47). The cut-off value to identify mutated subtypes is more than 98% homology to germline sequences. However, some controversy exists about this value and some groups propose 95% as cut-off (48-50).

The correlation of the mutation status with prognosis led to the hypothesis that B-CLL actually comprises two different diseases: one originating from a naïve pre-germinal centre B-cell and one arising from a post-germinal centre memory B-cell. To test this hypothesis, Rosenwald *et al*. (51) and Klein *et al*. (52) performed cDNA and oligo nucleotide-based DNA chip microarrays, respectively. Both groups concluded that unmutated (UM) and mutated (M) CLL largely display an overlapping gene expression profile which is more closely related to that of memory B-cells (52). Nevertheless, both groups did identify genes with a discrepant expression between UM and M-CLL. Klein *et al*. identified 23 genes, while Rosenwald *et al*. identified hundreds of discriminating genes. The most differently expressed gene between the CLL subtypes was zeta-associated protein kinase of 70 kDa (*ZAP-70*), a T-cell receptor (TCR)-associated protein tyrosine kinase (PTK) that normally is absent in B-cells.

Surrogate markers for IgV_H gene mutation status. The sequencing of IgV genes is expensive, time consuming and not available in the majority of clinical settings. This made the identification of surrogate markers for the IgV_H mutational status attractive. The microarray studies of Klein *et al*. (52), Rosenwald *et al*. (51) and Vansconcelos *et al*. (53) offered interesting information to address this problem and the first surrogate marker resulting from the microarray studies was ZAP-70, as mentioned before (51-53). Other possible surrogate markers for the IgV_H mutational status are CD38, and a combination of lipoprotein lipase (LPL) and the metalloproteinase ADAM29 (27, 53).

Zeta-associated protein of 70 kDa (ZAP-70). ZAP-70 is a useful surrogate marker for the IgV_H mutation status. It is a PTK with a molecular weight of 70 kDa, belonging to the Syk family of PTKs. This family contains the PTKs Syk (72 kDa) and ZAP-70 which show 50% homology. Whereas Syk is ubiquitously expressed in a broad variety of cells, the expression of ZAP-70 is restricted to T-cells and natural killer (NK) cells (54). However, the CLL microarray studies demonstrated its presence in B-CLL and knock-out studies have identified a role for ZAP-70 in developing and activated B-lymphocytes (55-57).

Syk and ZAP-70 are important in BCR and TCR signalling respectively. In normal B-cell receptor (BCR) signalling (Figure 1A), activation of the BCR by ligand binding or crosslinking leads to phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of CD79a and CD79b by a member of the Src family of PTKs, Lyn. ITAM phosphorylation creates the docking sites for the recruitment and activation of Syk (58, 59). Further signal transduction occurs *via* phosphorylation of SLP65/BLNK (SH2 domain-containing leukocyte protein of 65 kDa/B-cell LiNKer protein) and phospholipase Cγ (PLCγ), leading to cellular proliferation and suvival, or to apoptosis, depending on co-signals received by the cell and the stage of cellular differentiation (60, 61). Recently, Contri *et al*. showed the apparent up-regulation of Lyn in CLL cells in comparison to normal B-cells and its different subcellular localization in these cells (61). These findings might explain the increased basal tyrosine protein phosphorylation and the low responsiveness to BCR ligation in CLL. They also showed that inhibitors of Lyn remarkably reduced the survival of the CLL

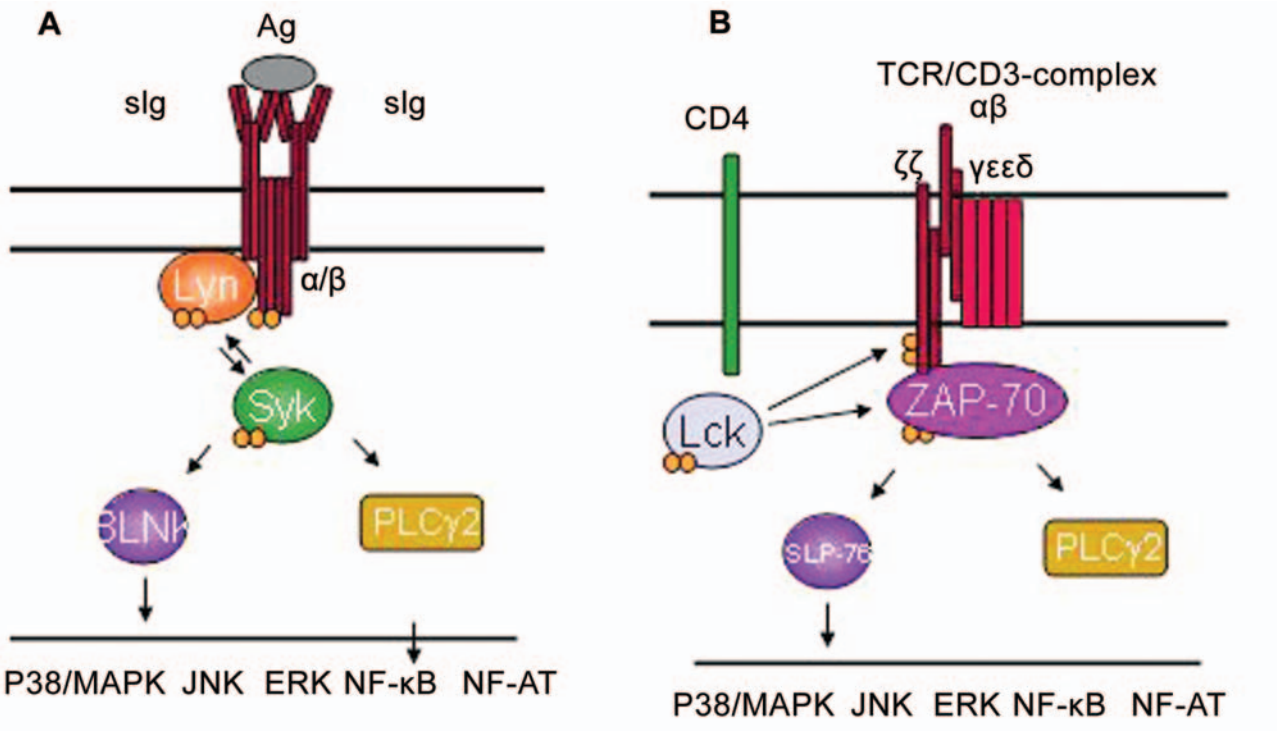

Figure 1. *A. B-cell receptor (BCR) stimulation, for example by cross-linking of two closely located sIgs, leads to phosphorylation of the ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tails of CD79a and CD79b. This phosphorylation is performed by Lyn, which is a protein tyrosine kinase (PTK) of the Src family of PTKs. The phosphorylated ITAMs create the docking sites for the recruitment and activation of Syk, which is phosphorylated by Lyn and by itself (auto-phosphorylation) and thus activated. Further signal transduction occurs via phosphorylation of SLP-65/BLNK (SH2 domain-containing leukocyte protein of 65 kDa/B-cell LiNKer protein) and PLCγ (phospholipase Cγ), leading to cellular proliferation and suvival, or to apoptosis, depending on co-signals received by the cell and the stage of cellular differentiation. B. Activation of the T-cell receptor (TCR) is followed by phosphorylation of the ITAMs (located in the cytoplasmic tails of the CD3 and ζ subunits of the TCR) by the Src family PTK Lck. ZAP-70 is then recruited to the phosphorylated ITAMs via its SH2 domain and becomes activated. In turn, it is able to enhance Lck and ζ phosphorylation and it also phosphorylates intermediates such as SLP-76 and PLCγ, leading to calcium mobilization and activation of downstream pathways which include MAPK and PI3 kinase. These pathways activate transcription factors such as NFκB, AP-1 and NFAT, leading to cell proliferation and differentiation. [Adapted from Orchard et al., 2005 (64)].*

cells, while apoptosis-inducing drugs diminished the activity and amount of Lyn in the cells (61).

The role of ZAP-70 and its position in TCR signalling is largely comparable to that of Syk in B-cells (Figure 1B). Activation of the TCR is followed by phosphorylation of the ITAMs (located in the cytoplasmic tails of the CD3 and ζ subunits of the TCR) by the Src family PTKs Lck and Fyn. ZAP-70 is then recruited to the phosphorylated ITAMs *via* its SH2 domain and becomes activated. In turn, it is able to enhance Lck and ζ phosphorylation and it also phosphorylates intermediates such as LAT, SLP-76, PLCγ and Vav-1, leading to calcium mobilization and activation of downstream pathways which include MAPK and PI3 kinase. These pathways activate transcription factors such as NF-κB, AP-1 and NFAT, leading to cell proliferation and differentiation (62-64).

The importance and significance of ZAP-70 as a surrogate prognostic marker for IgV_H mutational status has been studied extensively (65-68). Wiestner *et al*. (66) correlated the IgV_H mutational status with the *ZAP-70* mRNA expression level *via* quantitative real-time PCR. Other research groups used flow cytometry. Depending on the technique and antibody that were used, the concordance of the ZAP-70 expression and the IgV_H mutational status ranged from 77 to 90% . The 90% concordance experiments were performed with an indirect staining technique using the antibody against clone 2F3.2, while in the 77% experiment, a direct Alexa-488-labelled antibody against clone 1E7.2 was used. The latter publication was able to illustrate that ZAP-70 might be a more valuable predictor of the need for treatment (68). Generally, it is accepted that direct staining with Alexa-488-labelled anti-ZAP-70 (clone 1E7.2) should be the preferred method (69). The setting of the cut-off point however remains an issue (70). Some groups prefer the left margin of the NK- and T-cell populations, others use the right margin of the ZAP-70-negative $CD5^+$ cell population, and a third way is to use an isotype control (69, 71-74).

Most investigators choose to report the mean fluorescence intensity (MFI) instead of the percentage of ZAP-70$^+$ cells. However, this complicates the comparison between results from different laboratories.

Another important issue to consider when using ZAP-70 for risk assessment is its stability over time. Shankey *et al*. (69) reported the decrease of ZAP-70 of 17% over 24 hours in cells stored in preservative-free full heparin blood. Chen *et al*. (75) reported ZAP-70 loss in a B-CLL patient after treatment and Poulain *et al*. (76) very recently reported ZAP-70 decrease after corticoid therapy. It is also important to consider the higher ZAP-70 levels in B-CLL cells of bone marrow (77) and lymph node origin (78).

Other IgV_H surrogates. Analysis of CD38 expression was useful in distinguishing B-cells at various stages of differentiation from naïve through memory cells (79). Therefore, in the search for surrogates for the mutation status, CD38 was examined. Damle *et al*. studied flow cytometric CD38 expression and mutational status and found discordant results in 15% of patients with UM CLL (27). The cut-off value they used for CD38 was ≥30% positivity. Despite these results, later studies showed more discrepant results (30%) (80, 81). Moreover, CD38 can change during the course of the disease, not only as a result of treatment, but also because of disease progression, or as a reaction to a concurrent illness. Ghia *et al*. observed bimodal expression values in 27.8% of 148 patients and determined that this population was prone to CD38 increase over time (82). Because of all these findings, the threshold level for CD38 positivity remains a matter of debate. CD38 has recently been described as a marker of newly formed CLL cells emigrating from lymph node areas (83).

Microarray studies suggest LPL and ADAM29 may be IgV_H mutation surrogates (27, 53). LPL plays a central role in lipid metabolism and is undetectable in normal B and T lymphocytes (84, 85). Oppezzo *et al*. speculated that LPL could play a role in lipid raft formation or stabilization, an important biological process in B-cell activation (86, 87). ADAM29 transcripts are highly restricted to the testis but its precise functions, in testis as well as in CLL cells, remain unknown (88, 89). Oppezzo *et al*. identified the correlation of the LPL/ADAM29 ratio with the IgV_H mutational status with a discordance of only 8%, which was slightly lower than that for ZAP-70 (15%) in their series (86). However, Nückel *et al*. found only 6% of patients with an LPL/ADAM29 ratio <1 (90), which stood in sharp contrast with the 60% of Oppezzo *et al*. This discrepancy was explained by technical differences, showing the difficulties that occur when using the LPL/ADAM29 ratio as a prognostic marker. Heintel *et al*. only considered LPL and found a concordance between IgV_H mutational status and *LPL* mRNA levels >10, of 84% (91). Van Bockstaele *et al*. found a positive predicitve value of 83% and a negative predictive value of 78% (92), when using LPL as a predictive marker for IgV_H mutation status.

Impact and significance of the prognostic factors. Several prognostic factors have been evaluated in prospective studies such as the CALGB 9712 and the German and British CLL4 trial (93-95). These trials have resulted in enough information on how the newer prognostic markers ought to be used. Only in the 5% of patients who have 17p deletions should this be used to direct therapy (70). This deletion is so closely associated with failure to respond to standard therapy and early relapse that such patients should be treated with non standard agents such as alemtuzumab, high-dose steroids or flavopiridol. Ideally, this should be done in clinical trials.

The prognostic markers 11q deletion and IgV_H mutational status are proposed to be useful in stratifying patients for future clinical trials. For the prognostic markers CD38 and ZAP-70, further standardization is needed before they can be fully implemented.

Role of the B-Cell Receptor and B-Cell Receptor Signalling in CLL

BCR signalling is far less efficient in CLL cells than in normal B-lymphocytes and whether Syk or ZAP-70 is the responsible PTK remains unclear. More than 10 years ago, Latour *et al*. observed that in CLL, Syk have a 100-fold greater intrinsic activity than ZAP-70 (96). However, Chen *et al*. have since reported that ZAP-70$^+$ CLL cells establish far more developed BCR signalling than their ZAP-70$^-$ counterparts, thereby broadening the function of ZAP-70 from prognostic surrogate towards a functionally important factor in CLL (97). ZAP-70$^+$ CLL cells are more likely to respond to IgM crosslinking, with increased tyrosine phosphorylation and calcium flux than ZAP-70$^-$ cells (98). However, Gobessi *et al*. found that BCR-mediated activation of ZAP-70 is very inefficient in CLL, with a preserved capacity of ZAP-70 to recruit downstream signalling molecules in response to Ag-receptor stimulation (99). An incorrect spatial orientation of ZAP-70 or a greater capacity of Syk to associate with phosphorylated ITAMs have both been mentioned as possible explanations for this observation (96, 99-101).

In contrast, Gobessi and colleagues did observe stronger and prolonged BCR-induced phosphorylation of the positive regulatory residues in Syk in Burkitt's lymphoma B cell line (BJAB) B-cells transfected with ZAP-70, which was consistent with the findings of Chen *et al*. (97, 98). One possible explanation is that ZAP-70 might function as an adaptor protein that facilitates the recruitment of other signalling molecules to the BCR. A second possibility is that catalytically inactive ZAP-70 could decrease activation of

negative regulators of BCR signalling or inhibit events that terminate the signalling response (99).

Besides the functional impact of the BCR in CLL cell signalling, the epitopes recognized by the BCR are also of interest, especially to gain insight into the origin of the disease. Therefore, the usage of IgV_H genes was studied in the hope of finding a role for Ag selection in disease development. The primary structure of the Ag-binding site of the BCR is determined by the DNA sequence of its variable (V) regions, comprised of recombined VH, D and JH segments in the heavy (H) chains, and VL and JL segments in the light (L) chains. Normally, these recombination events occur at random. In CLL however, the frequencies at which specific VH, D and JH gene segments are used are different from what would be expected from random assortment. Some gene segments are much more expressed. The VH genes most commonly used are 1-69 (especially in UM CLL) and 4-34 (especially in M CLL) (29). Moreover, the CLL cases that use the most common V genes in their V(D)J rearrangements tend to use specific D and JH segments, thereby creating characteristic third complementary determining regions (CDR3s) (29). These observations are the clearest examples of selection for specific BCR structures among CLL precursors and suggest that the Abs expressed by at least some CLL cases are highly selected for binding to some unknown self or environmental Ag(s) (102).

CLL: Accumulation or Proliferation?

The accumulation of mature B-cells that have escaped programmed cell death and have undergone cell cycle arrest in the G_0/G_1 phase is one of the hallmarks of CLL (14). CLL cells have a low proliferative activity, and until recently, the hypothesis that an *in vivo* defective apoptosis, among others by up-regulated Bcl-2 levels, accounts for accumulation of B-cells and is responsible for the disease was generally accepted. However, more metabolically active CLL cells are described in CLL clones (12). Moreover, immunofluorescence and flow cytometric analyses indicated that all CLL cells, regardless of their prognostic subclassification, express surface membrane molecules characteristic of activated B-cells (103, 104). In support of this, microarray experiments revealed that the gene expression profile of CLL cells resembles that of BCR-stimulated normal B-cells (51, 52, 103). Telomere lengths of CLL cells are shorter than those of age-matched normal B-cells, thereby suggesting that the CLL cells have passed more rounds of proliferation than their normal counterparts (105); UM CLL cells have much shorter telomeres than M CLL cells, implying they have undergone many more replications. Finally, although there is a virtual absence of cycling cells in the peripheral blood of CLL patients, ill-defined areas of proliferation are seen in the bone marrow and affected lymph nodes (106). These areas are called pseudofollicles, contain larger cells with less condensed nuclear chromatin and display the "activated cell cycle" marker Ki-67 (107). These sites may be places of clonal growth and will be further discussed.

Because all the arguments mentioned above lead to indirect conclusions according to the proliferative capacities of the CLL cells, *in vivo* experiments were conducted. Several tracers to measure cellular proliferation can be used, but the best approach was that where patients drank heavy water (^2H_2O) for several weeks (108, 109). In these patients, [^{2}H]glucose is produced metabolically and the ^{2}H is incorporated into the DNA of the dividing cells. DNA is afterwards extracted from the blood cells and the presence of ^{2}H in the DNA is detected by mass spectrometry (110). Messmer and colleagues (2005) applied this approach in CLL patients and found cell generation rates in the range 0.1-1.75% of the entire CLL clone per day (111). Considering a CLL clone to contain 10^{12}-10^{14} cells, an extensive number of 10^9 to 10^{12} cells are thus produced each day.

Together, all these observations suggest the presence of a proliferating CLL cell population, as source of the accumulating cells.

Microenvironmental Factors Influencing the CLL Cell

In contrast with their long-living capacities in the human body, CLL cells tend to undergo rapid apoptotic cell death when incubated *in vitro* (112). This has raised the hypothesis of pro-survival environmental factors existing *in vivo*, supporting the CLL cells in their suvival and growth. Indeed, interactions with stromal cells (113), or nurse-like cells (114), or interactions between CD38 and its ligand CD31 (115) rescue CLL cells from apoptosis *in vitro* and probably do the same *in vivo* (18). The growth of CLL cells is supported by activated T-cells or other cells expressing CD40 ligand (107), while clonal expansion is facilitated by cytokines, such as interleukin-4 (IL-4) and vascular endothelial growth factor (VEGF) (116, 117), and chemokines such as stromal cell derived factor-1 (SDF-1) (118).

These examples illustrate the mechanisms of the micro environment which facilitate CLL cell survival as well as proliferation. The proliferative compartment is represented by pseudofollicles, which are nodular areas without mantles, present in lymph nodes and bone marrow of CLL patients. These follicles contain cells of the CLL clone, as well as bystander nontumour cells such as T-cells and follicular dendritic cells (FDCs). The T-cells are recruited to the pseudofollicles *via* their CD40 ligand (CD40L) and the interaction with the CLL cells leads to the production, by both cell types, of the cytokines IL-4, interferon α and interferon γ (119, 120). Another group of important bystander cells are the nurse-like cells. *In vitro* studies observed the differentiation of these cells from the non-CLL cells present in the peripheral

blood. Nurse-like cells express SDF-1, a CXC chemokine constitutively secreted by bone marrow stromal cells (121, 122) that binds to CXCR4, a chemokine receptor consistently overexpressed by CLL cells (123, 124). *Via* the interaction of SDF-1 with CXCR4, the CLL cells are protected against apoptosis and are able to migrate between bone marrow stromal cells to infiltrate into the bone marrow. In the bone marrow, the CLL cells are protected against apoptosis *via* interaction with the integrins β1 and β2. The integrin α4β1 allows the interaction between the CLL cell and the activated endothelium (116). The expression of CD100 on the CLL cell allows the interaction, *via* Plexin-B1, with bone marrow stromal cells, activated T-cells and follicular dendritic cells. $CD100^+$ CLL cells exposed *in vitro* to Plexin-B1 increase their proliferative activity and have an extended lifespan (125). Richardson *et al*. described a higher expression of CCR7 on $ZAP\text{-}70^+$ cells leading to an increased responsiveness to the ligands, the chemokines CCL19 and CCL21 (126). They correlate these findings with the more progressive nature of the $ZAP\text{-}70^+$ disease.

Summary and reflections

Chronic lymphocytic leukaemia is still the most prevalent form of leukaemia in Western countries. Because the disease still has no cure, it remains of great interest and research groups are trying to unravel its pathogenesis. Several factors seem to have relevant impact on the behaviour of the CLL cell. The microenvironment, as well as the balance between pro- and antiapoptotic factors, influences the pool of malignant cells. Next to these, the activity of the CLL cell as well, as reflected by its signalling through the BCR, and the phosphorylation status of several PTKs, such as Syk and ZAP-70, are important.

In addition to these characteristics of the CLL cell, we have also tried to give an overview of the prognostic factors in use for classifying CLL patients. Calculating the risk profile of an individual patient remains, for the moment, of great importance in therapeutic decision-making, but many questions remain to be solved.

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