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Microenvironment Interactions in Chronic Lymphocytic Leukemia: A Delicate Equilibrium Linking the Quiescent and the Proliferative Pool

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the commonest form of leukemia in Europe and North America, and mainly, though not exclusively, affects older individuals. It has a very variable course, with survival ranging from months to decades [1]. It is a neoplastic disorder, characterized by progressive accumulation of monoclonal B lymphocytes, expressing CD5 and CD23 molecules and low amounts of surface membrane Ig and CD79b molecules [2]. About one-third of patients never requires treatment, has a long survival and dies of causes unrelated to CLL; in another third an initial indolent phase is followed by progression of the disease; the remaining third of patients has aggressive disease at the onset and requires early treatment [3]

Accumulation of mature B-cells that have escaped programmed cell death and undergone cell-cycle arrest in the G0/G1 phase is the hallmark of CLL [4]. In this leukemia elevated levels of the cyclin negative regulator p27^{Kip1} protein are found in a majority of patients [5]. Given the key role of this protein in cell cycle progression, the over-expression of p27^{Kip1} could account for the accumulation of CLL B-cells in early phases of the cell cycle. Furthermore, it has been postulated that the survival advantage of CLL lymphocytes is also due to aberrant over-expression of antiapoptotic Bcl-2 family proteins in general [6] and Bcl-2 and Mcl-1 proteins in particular [7]. Other members of the Bcl-2 family, such as anti-apoptotic proteins BCL-XL and BAG1 are overexpressed in CLL B-cells whereas proapoptotic proteins, such as BAX and BCL-XS, are underexpressed [4]. These antiapoptotic proteins sequester pro-apoptotic counterparts and a balance between both determines the fate of a cell. Additionally, the most consistent cytogenetic lesion in CLL is chromosomal deletions of 13q14, resulting in loss of microRNAs, miR-15a and miR-16-1. Expression of these microRNAs has been founded inversely correlated to Bcl-2 expression and thus, suggested that translocation 13q14 is associated to survival of CLL B-cells [8]. These observations establish anti-apoptotic Bcl-2 family proteins as key survival factors for CLL [9].

High expression of cyclin cell cycle negative regulator p27^{Kip1}, antiapoptotic molecules such as Bcl-2 or Mcl-1, and a characteristic non activated phenotype of CLL B- lymphocytes (low surface immunoglobulin (Ig) expression and absence of activated lymphocyte molecules) led to the assumption that CLL disease is a leukemia resulting from accumulation rather than from proliferation. However this traditional view that CLL is a disease deriving from an inherent defect in apoptosis has being called into question [10]. Recent studies suggest that CLL is a dynamic process, comprising leukemic cells that multiply and die at measurable rates. Furthermore, since CLL cells do not appear to be inherently immortal, patient's compromise does not occur from passive accumulation, but from active generation of subclones that over time develop dangerous genetic abnormalities which further change the birth/death ratios [10,11].

These observations have turned the attention towards the occurrence of different subpopulations inside the tumoral clone. It is clear that most, if not all, proliferative events occur in tissues where leukemic cells are able to exploit microenvironment interactions in order to avoid apoptosis and acquire tumoral growing conditions [12]. This concept is supported by reports showing that, despite their monoclonal origin, there are different subpopulations within clonal CLL B-cells [13,14 and 15].

These works which underline the presence of a proliferative B-cell subset within the tumoral clone, furnish new strength to the hypothesis that the microenvironment plays a central role in the maintenance and progression of this disease. Thus, upregulation of antiapoptotic proteins such as Survivin [16], Mcl-1, Bcl-2, as well as specific chemokines and cytokines in CLL (reviewed in [17]), like CCL2 [18], CCL3/CCL4 [19,20] CXCR4-CXCL12 [21], and IL-4 [22] among others, support a process of activation and reinforcement of the malignant cells by the microenvironment. These key interactions provide survival signals to the leukemic cells leading to the progression and treatment resistance of the tumoral clone. Therefore, the development and design of therapeutic agents with the goal of disrupting the crosstalk between malignant B cells and their microenvironment is an attractive novel strategy in the treatment of CLL, a heterogeneous disease that as yet remains incurable.

In this chapter we will compile the available evidence related to the main B-cell/microenvironment interactions responsible to maintain a CLL proliferative subset. We will discuss the present knowledge about the proliferative B-cell subsets and how they are preserved within the tumoral CLL clone.

2. Role of the microenvironment in CLL-B cell survival

All the physiological processes during which B-cells encounter their antigen (Ag) occur in specific anatomical sites so-called "specialized microenvironments". Germinal centers are the typical immunological picture of these activation places. In this environment B-cell stimulation is totally dependent on complex supportive interactions with both Ag-specific and Ag-non-specific accessory populations. T cells and a variety of different types of adherent cells, generally defined as 'stromal cells', are the main elements of this microenvironment.

In CLL disease, the proliferating compartment is represented by focal aggregates of proliferating prolymphocytes and para-immunoblasts that give rise to the called pseudo-follicles or proliferation centres [23]. Pseudo-follicles are the histological CLL hallmark in lymph nodes (LN), splenic white pulp and bone marrow (BM) where they appear as

vaguely nodular areas never surrounded by a mantle zone. These areas are usually infiltrated with an important number of CLL B-cells that after interaction with T-cells and/or stromal/follicular dendritic cells, are able to express the proliferation marker Ki-67 and the progression disease molecules such as CD38 [24] and CD49d [25].

The general observation that CLL B-cells rapidly die by apoptosis after culture in the absence of accessory cells strongly indicate that CLL B-cells maintain their capacity to respond to selected external stimuli that confer to leukemic cells a growth advantage and an extended survival. Furthermore, numerous *in-vitro* evidence indicate a predominant role of the microenvironment in CLL cell survival [26].

T lymphocytes, the bone marrow stromal cells, and the follicular dendritic cells are involved in the natural history of the disease and appear to be major players in delivering key signals for the proliferation of tumoral clone and disease progression [27]. The exposure of malignant cell subclones to microenvironmental stimuli results in increased proliferation, a prerequisite for the occurrence of new genetic abnormalities that lead to the development of a more aggressive disease.

The pattern of tissue infiltration by CLL cells may be variable. More frequently, malignant cells are seen only or predominantly in the peripheral blood (PB) and the BM. In some instances a vast LN involvement is observed together with a modest PB involvement. These clinical observations point to the existence of mechanisms that selectively control the trafficking and homing of malignant lymphocytes to distinct microenvironments. One such mechanism might be accounted by chemokines and chemokine receptors. Recent data indicate that CLL cells may express specific sets of chemokine receptors and/or respond to specific chemokines produced by microenvironmental elements that selectively attract individual cells to explicit anatomical sites [17].

Chemokines constitute a growing family of chemotactic cytokines that are generally involved in leukocyte migration. According to a current classification based on their function, they are subdivided into three different groups: (1) the homeostatic chemokines regulating lymphocyte migration and homing processes under physiological conditions, (2) the inducible chemokines expressed during inflammation and (3) an overlapping group involved in both processes. Their expression can be induced by various stimuli, including growth factors and inflammatory cytokines. Besides these general aspects, chemokines are also associated to a variety of pathological processes. During tumourigenesis, they are known to play a crucial role informing and modifying the tumour stroma by inducing the infiltration of various hematopoietic cells (e.g. macrophages, natural killer (NK) cells, eosinophils, B and T lymphocytes) as well as fibroblasts and endothelial cells. They also contribute to the neovascularisation, the growth and the spreading of tumours [17].

2.1 Role of T-cells in the CLL microenvironment

The peripheral T-cell repertoire in CLL is significantly altered with a marked increase in oligoclonality in both CD4 and CD8 positive cells [28]. A multitude of *in-vitro* findings indicate that T lymphocytes are attractive candidates to play a role in the inhibition of the malignant B-cell apoptosis and to favour disease progression [29,30]. The weight of evidence points to a dialogue between malignant CLL B-cells and CD4^{pos} T-cells, based upon

bidirectional interactions that are regulated by adhesion molecules and chemokines and translate into the production of several cytokines by both cell types (reviewed by [26]). T-cell cytokines, including IL-4, IFN- γ , and IL-2 inhibit CLL B-cell apoptosis by upregulating Bcl-2 protein, reinforcing the concept that the ability of CLL cell to avoid apoptosis may be strongly influenced by external stimuli provided by the microenvironment [26].

Within pseudofollicular proliferation centers, proliferating leukemic lymphocytes are in contact with numerous CD3^{pos} T-cells, most of which are CD4^{pos}, and express CD40L, which can support the growth of CLL B-cells through CD40 ligation. CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily that is expressed by B-cells, dendritic cells and monocytes [31]. The stimulation of CD40 and interleukin 4 (IL-4) rescues CLL B-cells from apoptosis and induces their proliferation [32]. Moreover, CD40 crosslinking on CLL B-cells induces up-regulation of CD80 and CD54 and turns nonimmunogenic CLL cells into effective T-cell stimulators [33]. Later studies of Granziero *et al.* have shown that this proliferative CLL B-cells activated through CD40 also express survivin, a member of the family protein of inhibitor of apoptosis, (IAPs) [16]. This protein is the only IAP whose expression is induced in CLL B-cells by CD40L. The survivin positive cells have an extended survival, an increased proliferative rate and retain Bcl-2 positivity.

It is unclear why and how CD4^{pos} T-cells that gather in CLL pseudo-follicles are activated. Under normal circumstances, CD4^{pos} T-cells that cooperate with B lymphocytes in primary follicles recognize the antigenic peptide in the context of MHC-II class molecules (peptide MHCII binds to T cell receptor, TCR). This interaction results in the transient up-regulation of CD40L. However, T-cells in CLL patients exhibit defective immunological synapse formation which may account for the defects in T-cell helper function seen in earlier studies [30]. Whatever causes their activation, CD40L^{pos} T-cells are in close physical contact with CD40^{pos} CLL within proliferation centers [24]; hence the physiological stimulus provided by CD40L is available to malignant B-cells. Subsequent research has shown that these activated CD4^{pos} T-cells tend to assemble in pseudo-follicles attracted by the chemokines, CCL17 and CCL22 [22] and CCL3 and CCL4 [19,20] produced by proliferating CLL B-cells themselves, (Figure 1A).

In regard to CCL17 and CCL22, it is interesting that leukemic cells purified from LN and BM, but not from PB, constitutively express mRNA for both of them. The CD40-crosslinking of PB CLL cells induces the expression of these chemokines at RNA level [22]. Of them, CCL22 is released and is capable of attracting activated CD4^{pos} /CD40L^{pos} T-cells, while CCL17 is released only when IL-4 is added to the *in-vitro* system [16] (Figure 1A).

3. Role of stromal cells in the CLL microenvironment

T cells are not the only active responsible partners for leukemic B-cells. A number of adherent accessory cells present in different microenvironments are gaining increasing attention in the last years in the CLL progression. It has been convincingly demonstrated that a direct physical contact between BM stromal cells and leukaemic cells extends the survival of CLL B-cell [34]. Stromal cells are key regulators of normal B lymphopoiesis. However, even if they are known to provide binding sites and growth factors to developing B-cells, the precise nature of ligand-receptor interactions are not fully known. The interest has been initially focused upon

adhesion molecules. *In-vitro*, it has been shown that malignant CLL B-cells interact with BM stromal cells via $\beta 1$ and $\beta 2$ integrins [34]. This binding rescues CLL cells from apoptosis and extends their lifespan, suggesting a potential mechanism for the preferential *in-vivo* accumulation and survival of CLL cells within the BM.

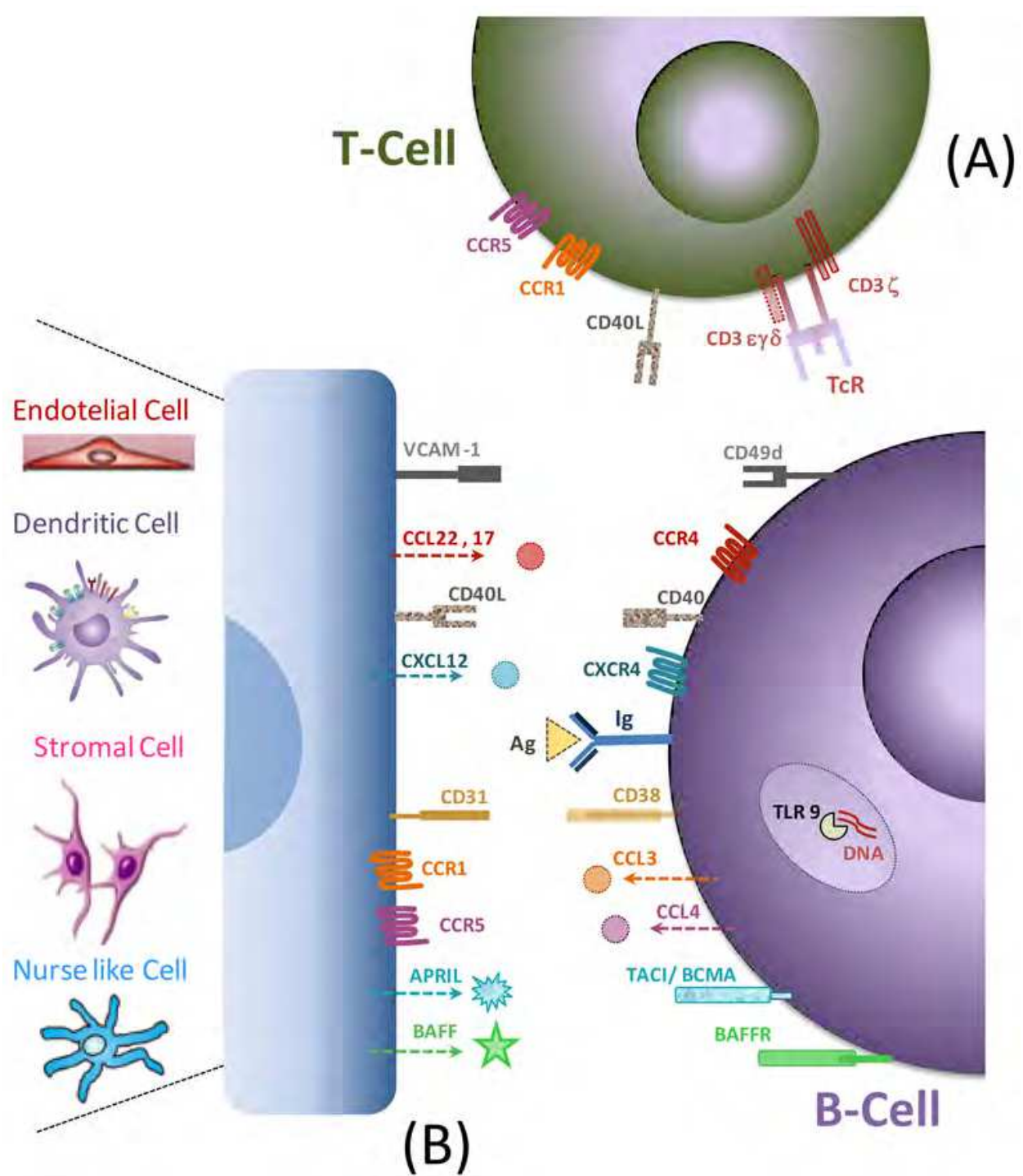


Fig. 1. The microenvironment stimuli on CLL B-cells . Main signaling interactions regulating the survival and the proliferation of leukemic clone. A) T-cells signals to CLL B-cells. B) Endothelial, dendritic and nurse like cells signals to CLL B-cells.

The PB of CLL patients has been shown to contain cells that *in-vitro* can differentiate into adherent nurse-like cells, endowed with the capacity of protecting the attached leukaemic B-cells from spontaneous apoptosis [21]. Blood-derived nurse cells protect CLL B-cells from apoptosis by utilizing a mechanism dependent on SDF-1 (CXCL12), a CXC chemokine that is constitutively secreted by BM stromal cells and regulates B lymphopoiesis upon binding its receptor CXCR4 (CD184). CXCR4 is consistently over expressed by CLL B-cells [21]. In this sense, a recent work of Vaisitti et al., clearly shows that CD38 synergizes with the CXCR4 pathway supporting the working hypothesis that migration is a central step in disease progression and that expression of CD38 is correlated to this expression [35].

Using gene expression profiles comparing CD38^{pos}/CD49d^{pos} versus CD38^{neg}/CD49d^{neg} CLL B-cells, Zucchetto *et al.* [19] showed an over expression of the CCL3 and CCL4 chemokines in leukemic cells from the CD38^{pos}/CD49d^{pos} subset. CCL3 and CCL4 are up-regulated by CD38 engagement in CD38^{pos}/CD49d^{pos} CLL B-cells and also CCL3 was found to be expressed by CLL B-cells from bone marrow biopsies (BMB) of CD38^{pos}/CD49d^{pos} but not CD38^{neg}/CD49d^{neg} cases. High levels of CCR1 and, to a lesser extent, CCR5, the receptors for CCL3 and CCL4, were found in CLL-derived monocyte-macrophages. Consistently, CCL3 induced monocyte migration and CD68+ macrophage infiltration was particularly high in BMB from CD38^{pos}/CD49d^{pos} CLL B-cells. Conditioned media from CCL3-stimulated macrophages induced endothelial cells to express vascular cell adhesion molecule-1 (VCAM1), the CD49d ligand, likely through TNF- α over production. These effects were apparent in BMB from CD38^{pos}/CD49d^{pos} CLL, where lymphoid infiltrates were characterized by a prominent meshwork of VCAM-1+ stromal/endothelial cells. It appears that the CD31/CD38/ZAP-70 axis may represent a point of convergence of proliferative and migratory signals. CD38/CD31 interactions are followed by a marked upregulation of the semaphorin family member CD100, which in turn interacts with the plexin B1 ligand expressed by stromal cells and contributes to further sustain proliferation and survival of CLL B-cells [36].

Underlying the role of stromal cells in the CLL survival signals, a recent work of Zuchetto et al., show that T-cells do not emerge as relevant players in CCL3/CCL4-driven dynamics in CLL BM microenvironment. Rather, this work proposes that CCL3/CCL4 chemokines preferentially target monocytes/macrophages, which are recruited by this/these chemokine/s, in the context of microenvironmental sites of CCL3/CLL4-producing CLL [19]. CCL3 and CCL4 are small (8–10 kDa), structurally related chemokines that, under normal conditions, are secreted by mature hematopoietic cells. Biologically, CCL3 and CCL4 have overlapping effects and act as potent chemoattractants for monocyte, macrophages, dendritic, T, and natural killer cells [37]. Highlighting the importance of the expression of these chemokines in CLL progression, a recent work of Sivina *et al.*, proposes CCL3 chemokine as a novel prognostic marker in CLL, suggesting that its evaluation might become useful for risk-assessment in patients with CLL [38] (Figure 1B).

Leukemic CLL B-cells are not only exposed to signals delivered by accessory, non-malignant cells in the lymphoid tissues, but they are also capable of sensing pathogen associated molecular patterns through a variety of membrane or cytosolic receptors. Toll-like receptors (TLR) are probably the best characterized. TLR7 and TLR9, which recognize single stranded RNA and bacterial DNA respectively, are virtually always expressed (Figure 1). Other evidence which reinforces the importance of the microenvironment on the survival of B-

cells came from Decker *et al.* These authors have been shown that stimulation of CLL B-cells with an analog of bacterial DNA (CpG -ODN) induces the expression of cyclin D2 and cyclin D3 and reduces the expression of p27-*kip1* associated with cell cycling. Both cyclins were associated with *cdk4*, which is the catalytic partner of D-type cyclins in normal B cells. Moreover, immune complexes consisting of cyclin D2-*cdk4* or cyclin D3-*cdk4* were both functional and phosphorylated the RB protein *in-vitro* [39].

Finally, not only signals delivered by stromal cells appear to be essential in the microenvironment crosstalk with the leukemic B lymphocyte. Cytokine array and enzyme-linked immunosorbent assay studies revealed increased expression of soluble CD14 by monocytes in the presence of CLL B-cells. This work shows that monocytes help in the survival of CLL B-cells by secreting soluble CD14, which induces nuclear factor κ β activation in these cells [40].

Overall, these data provide a link between microenvironmental factors and the proliferation/apoptosis dilemma of CLL B-cells. CLL is now revealing itself to be an environment-dependent hematological malignancy. This idea could be in agreement with a model of selective survival of certain clonal submembers, which would receive survival signals in these particular lymphoid sites.

3.1 Other microenvironment soluble factors involved in CLL progression

Several works in the last years, display the importance of soluble factor regulating the balance between stability and progression of this disease. It is known that CLL B-cells themselves can secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin (Ang) which are involved in the formation of new blood vessels. These newly formed vessels are characterized by increased permeability, and thus contribute to disease dissemination [12]. CLL B-cells can also express receptors for some of these pro-angiogenetic factors, including VEGF receptors VEGFR1 and 2 as well as the Ang-receptor. Signaling through these receptors significantly prolongs cell survival [41]. Additionally, it has been described that thioredoxin (Trx) is expressed in LN of CLL patients and that this expression can increase the CLL survival clone. In this work, the authors found that adding Trx at CLL B-cells increased in a dose-dependent fashion the release of TNF- α , which has been suggested to be an autocrine growth factor for these cells. Secretion of TNF- α maintained Bcl-2, and diminish the apoptosis in the CLL B-cells. [42].

4. Proliferative pool in CLL

It is well established that CLL is a heterogeneous disease: some patients experience a slowly progressive clinical course, but most will eventually enter an advanced phase requiring repeated treatment. Different groups have suggested that cytoskeletal organization, cellular adhesion and the migratory potential of the leukemic clone regulate tissue distribution of CLL cells, possibly influencing a patient's outcome [43,44]. This highlights the significance of topographical issues in disease progression and provides convincing evidence that CLL B-cells with enhanced motility are associated with aggressive disease. Independent confirmation of these results comes from data generated in patients, showing that a

significant proportion of the leukemic clone proliferates and that proliferation occurs predominantly in lymphoid organs.

Messmer and col. clearly demonstrate that a proliferative compartment exists in CLL, [11] although major part probably resides in the solid tissues [14]. Further, it is self-evident that the accumulated CLL B-cells in the PB are constantly nourished by an upstream proliferation cell compartment. It is reasonable to assume that the balance between the two compartments may be at the bases of the highly variable clinical course of CLL, which may behave as a stable and indolent monoclonal lymphocytosis, or as an aggressive disease.

At present, two proliferative subsets related to disease progression has been described in CLL. Chiorazzi's group proposed that the subset CD38 positive/Ki67 positive CLL B-cells could be a proliferative pool in this disease [14]. Additionally a recent work of Palacios *et al.*, also describe a proliferative subset in UM CLL patients characterized by the presence of active class switch recombination process and anomalous expression of the Activation-Induced cytidine Deaminase (AID) enzyme [15].

4.1 Proliferative CD38 positive CLL B-cells

Despite the large number of surface markers described in the CLL, the expression of CD38 and its association with the disease has been intensively studied. CD38 is accepted as a dependable marker of unfavorable prognosis and as an indicator of activation and possibly proliferation of CLL cells at the time of analysis. Leukemic clones with higher numbers of CD38 positive cells are more responsive to BCR signaling and are characterized by enhanced migration. *In-vitro* activation through CD38 drives CLL proliferation and chemotaxis, via activation of a signaling pathway that includes ZAP-70 and ERK1/2. *In-vivo* interaction of CD38 with CD31, its cognate receptor, have an important role in cell-cell interactions activating survival pathways in normal and leukemic lymphocytes [45].

An important work of Chiorazzi's group highlights the cell-cycling status of CLL cells, focusing on those leukemic cells expressing CD38 [14]. In order to going deeper in this area Pepper *et al.* extended these observations by comparing gene profile of CD38^{pos} and CD38^{neg} CLL B-cells of a single patients. The results showed that CD38^{pos} CLL cells possess a distinct gene expression profile compared with their CD38^{neg} sub-clones. CD38^{pos} CLL B-cells relatively overexpress vascular endothelial growth factor (VEGF), which is associated with increased expression of the anti-apoptotic protein Mcl-1 [13]. Detailed characterization of the proliferating CLL B-cell convincingly demonstrated a close association between CD38 expression and increased percentages of Ki-67 and ZAP-70 positive cells, suggesting that CD38^{pos} clonal members are more highly activated and prone to enter the cell cycle than their negative counterpart [13].

However, further studies of the same laboratory failed to establish a strong correlation between the percentage of CD38^{pos} proliferating cells in CLL clones and survival and disease progression [46]. The fact that CD38 is expressed in a high percentage of tumoral cells in UM patients indicate that CD38^{pos} leukemic cells constitute a heterogeneous population including a small fraction of cells with an increased proliferative potential. Results from Messmer *et al.* show that leukemic CLL proliferating rates range from 0.08% to 1.7% [11] suggesting that not all CD38 positive cells, are proliferating.

The scenario outlined by these data indicates that the CD38^{pos} cells subpopulation involve a discrete and small subset of cells, also CD38 positive, that have recently exited a solid tissue, and have received freshly proliferation signals.

4.2 Proliferative AID positive CLL B-cells

Recent evidences from our group outline the importance of another cellular subset, characterized by an anomalous expression of the mutagenic molecule AID in a proliferative leukemic clone [15]. This protein is a B cell-restricted enzyme, induced principally through the contact of T and B-cells via CD40-CD40L interactions, despite that recent works also show that the innate immune response via TLR receptor is able to trigger their expression [47]. The physiological expression of this enzyme is responsible for somatic hypermutation (HMS) and class switch recombination (CSR) process in B lymphocytes [48]. However, the mutational activity of AID identifies this enzyme as the first genome mutator in humans with oncogenic potential [49]. Supporting this view, different works report that constitutive AID expression is associated with a loss in the target specificity and with lymphoproliferative disorders [49,50].

In the CLL disease we have reported that AID is anomalous expressed in the PB of some patients with UM VH genes, active CSR and clinical poor outcome [51]. Despite expression of a functional AID as assessed by an active CSR and mutations induced in the preswitch region, CLL B-cells in these patients did not succeed to achieve the SHM process [52]. Although clonal CSR has been described in CLL B-cells long ago [53,54] and different works have shown that this process occurs principally in patients with UM disease [52,55], the origin and the biologic implications of this subpopulation in the physiopathology of CLL remain elusive.

Because AID expression in CLL is associated with ongoing CSR in patients with UM disease, we investigated the relation of AID expression, CSR process, and microenvironment activation in the PB of CLL patients with different clinical profiles. Our results show that high expression of AID is almost exclusively restricted to the subpopulation of tumoral B-cells having an active CSR process (IgG^{pos} CLL B-cells). This subset expresses high levels of proliferation and antiapoptotic molecules such as Ki-67, *c-Myc*, and Bcl-2. In addition, this particular subset of leukemic cells display high levels of CD49d and CCL3/CCL4 chemokines, as well as a decreased expression of cell cycle inhibitor p27^{kip1} compared with their quiescent counterpart IgM B-cells. Finally, the presence of this subpopulation in patients with UM CLL is closely related to an aggressive course of the disease [15]. Additionally to this, over-expression of anti-apoptotic and proliferative molecules as well as expression of molecules implicated in the microenvironment interactions has also been established. Thus, this tumoral CLL subset appears to be a hallmark of a recent contact with an activated microenvironment exclusively found in UM CLL patients with a poor clinical outcome [15].

It is difficult to determine the precise role of these highly proliferating activated tumoral B-cells. Since the presence of this subset is clearly associated to poor prognosis, it might have an adjuvant role in the maintenance of the CLL proliferative pool. However, given their increased proliferative potential they should normally outnumber the IgM^{pos} cells and this is not the case. Thus, we could assume that these cells should undergo apoptosis

once leaving the pseudo-follicles. A recent work suggesting a link between AID expression and B-cell apoptosis in GC favour this view [56]. In these conditions, the IgG^{pos} subset could reflect the existence of an active microenvironment leading to permanent stimulation of the IgM^{pos} pool, which would be turn on the CSR machinery maintaining this IgG^{pos} subset in the PB. Alternatively, an adjuvant role in the maintenance of the CLL IgM proliferative pool by this subset could be considered. Recently, evidence indicates that outside the GC, there is a fraction of AID^{pos} B-cells subset of interfollicular large B-lymphocyte and in the thymic medullae of tonsils [57]. Interestingly, these AID positive B-cells ongoing CSR form prominent cytoplasmic extensions, lending them to a “dendritic cell-like” appearance [57]. In this respect, unpublished results from our laboratory indicate that *in-vitro* stimulation with CD40L/IL-4 not only induces B-cells to proliferate, but also activates lymphocytes to adopt a morphological aspect of “pseudo-dendritic” cells expressing B-cell markers. If the stimulation through CD40L or other stimulation molecules are able to induce these “pseudo-dendritic” cells to become efficient antigen presenting cells remains elusive yet. Whatever the case, the hypothesis that in the UM subgroup stimulation of BCR takes place by an unknown auto-antigen [27,58] and that this is responsible for consecutives stimulations sustaining survival/expansion signals in the tumoral clone, results an interesting issue highlighted by these results.

In this context, we hypothesize that the survival signals of this AID^{pos} CLL B-cells subset could be constitutively triggered by the recognition of an autoantigen present in LN and/or BM (figure 2). In order to explain, why an active AID^{pos} tumor clone is unable to carry out the SHM process, we propose that an unidentified cofactor of AID is absent in the AID^{pos}, UM CLL subset. The correct expression of both, AID and its cofactor, enables the leukemic clone to achieve the SHM process. Once mutated, the clone loses its ability to recognize the autoantigen and, consequently it loses the possibility to receive pro-survival and proliferative signals (figure 2 panel A). In contrast, the expression of AID in the absence of its cofactor prevents BCR mutations, allowing a persistent interaction of the leukemic cells with the autoantigen (figure 2 panel B). The positive signaling through the BCR together with pro-survival and proliferative factors from the microenvironment leads to the accumulation of CLL B-cells and the progression of the disease. The high proliferation rate, the over-expression of AID and other factors, could favor DNA translocations and oncogenic mutations finally associated with progressive and refractory disease (figure 2 panel B).

Inhibition of apoptosis may occur *in-vivo* in pseudo-follicles observed in the lymph nodes, and in the cell clusters described in the bone marrow. These pseudo-follicles include proliferating B-cells in close contact with increased numbers of CD4 T-cells expressing CD40L, which is necessary for AID expression. These activated CD4 T-cells could be recruited by tumor B-cells through the expression of T cell-attracting chemokines such as CCL17 and CCL22 [22] and/or CCL3 and CCL4 [20]. Besides this, the CD38 and CD49d proteins appear to be important additional players interacting with nurse-like cells, stromal, and endothelial cells to complete the activation pathway within the proliferative centers [19]. Overall, these observations favor the view that certain cellular subsets in CLL could receive survival signals in the specific microenvironments, increasing their proliferative potential and consequently associated with a more aggressive disease.

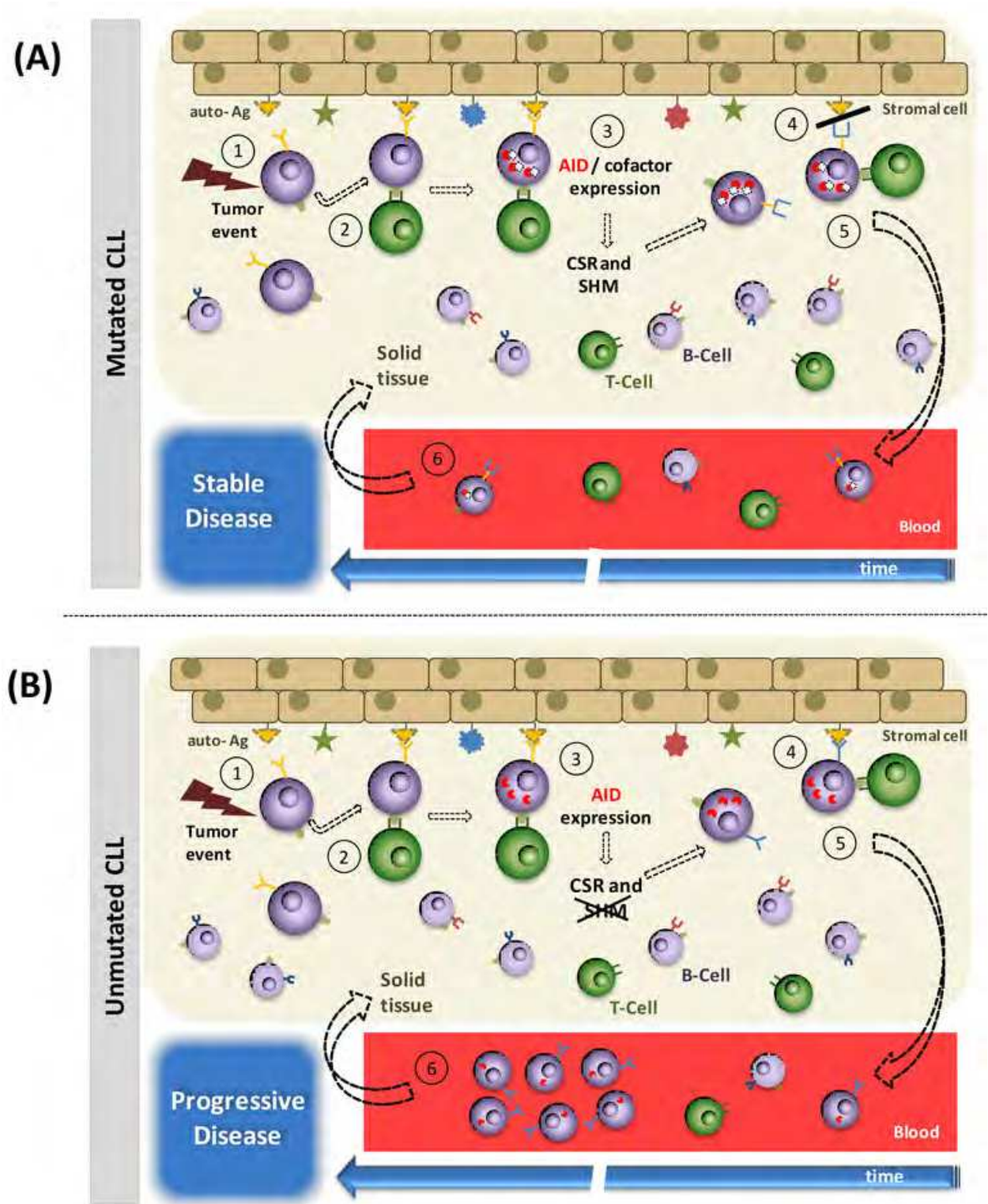


Fig. 2. Anomalous AID expression in UM CLL patients: potential role of autoantigen in the progression diseases.

The survival signals of the proliferative AID^{pos} CLL B-cells subset could be constitutively triggered by the recognition of an autoantigen (auto-Ag) present in LN and/or BM.

In the mutated cases we propose that after an unknown tumor event (1), the tumoral B-cell could recognize an auto-Ag through BCR and receive collaboration from other cells such as T follicular helper cells or antigen-presenting cell (APC) (2). At this level proliferation centers could be initiated and after this activation the tumor clone might trigger AID expression and its unknown partners in order to achieve SHM and CSR (3). Once mutated the VDJ regions of BCR, the leukemic clone loses its ability to recognize the auto-Ag (4) and, consequently also loses the possibility to receive survival and proliferative signals.

In UM patients, panel B, tumor event occurs in a B-cell (1), BCR of this leukemic cell recognizes the auto-Ag and is induced to proliferate with the help of another T-cells or APC. (2) The leukemic clone expresses AID and their partners, but not the specific cofactor necessary to achieve a correct SHM process (3). Constitutive AID expression in this scenario only is able to trigger CSR, but it cannot mutate the VDJ region of BCR (4). This persistent activation of the leukemic clone leads to the existence of this proliferative subset IgG^{pos}/AID^{pos}. The increasing number of these leukemic, switched cells in the proliferative centers leads to the leukemic cells extravasation to peripheral blood (5). These circulating cells might home to solid tissues eventually and thus, they would receive proliferation/survival signals again (6). Cycles of these last two events overtime, produce an increase in the number of proliferating AID^{pos} CLL B-cells (detectable in peripheral blood), which is considered as a hallmark of a proliferative and progressive leukemia.

5. Inflammation role in an activated CLL microenvironment

The relationship between antigen stimulation/inflammation and the natural history of CLL is not surprising considering that inflammation is involved in the initiation and progression of several chronic lymphoid malignancies of B-cell type [59].

Chronic inflammation and CLL are inter-related in many aspects. The malfunctioning of the immune system helps the first few cancer cells to establish into a full-fledged CLL. In comparison to normal B-cells, leukemic cells are rescued from apoptosis by bone-marrow stromal cells, signifying the selectivity of microenvironment for malignant cells. Compelling evidences show us that CLL progression is originated in an inflammatory microenvironment in which many cells (T-cells, stromal cells, monocytes, macrophage and dendritic cells) are all able to delivered survival signals supporting the tumoral clone. These microenvironmental responses are often brought about by the interplay of different chemokines, cytokines, transcriptional factors or post-translational modifications [9].

The inflammatory chemokines are expressed in inflamed tissues and signal for recruitment of neutrophils. On the other hand, homeostatic chemokines produced constitutively in distinct tissue microenvironments to sustain traffic of mature lymphocytes in lymphoid and nonlymphoid tissues [17]. Despite the protective function it has on the CLL B-cells through apoptosis inhibition this factor also allows the spontaneous migration of malignant cells towards BM stromal cells, suggesting that CLL B-cells may utilize this mechanism to infiltrate the BM [21]. SDF-1 and other chemokines such as CCL3 and CCL4 secreted proteins, appear to form a pro-survival circuitry by regulating leukocyte trafficking, extravagating into sites of tissue inflammation and maintaining extended lymphocyte survival [19].

Cytokines are signaling key mediators of inflammation or an immune response, involved in accelerating inflammation and also are present in high levels in CLL patients. They are classified as pro-inflammatory (IL1, IL6, IL15, IL17, IL23 and TNF- α [61,62]), or anti-inflammatory (IL4, IL10, IL13, transforming growth factor(TGF β) and TNF- α depending on their function in tumorigenesis [60]). Another work, recently performed by Schulz *et al.* [61] touch upon the issue of inflammatory cytokines and signaling pathways associated with CLL survival. Consistent with this possibility inflammatory cytokines genes are upregulated in this work. Among these genes chemokine (C-C motif) ligand 2 (CCL2) was shown to be induced in monocytes by the presence of CLL cells *in-vitro*.

In addition to chemokines and cytokines, the key mediators of inflammation-induced cancer include activation of transcription factors. There are a wide range of transcriptional factors that bind to the promoter region of target genes and activate transcription of these oncogenes. Aberrant expression of the transcription factors like MYC, STAT and NF- κ B are associated to inflammatory immune response but also in carcinogenesis and poor prognosis in CLL [62].

The fact that inflammatory receptors such as Toll-like receptors (TLR) can be engaged concomitantly with the BCR, it becomes reasonable to presume that TLR may also play a role in BCR co-stimulation of CLL cells. Indeed, bacterial lipopeptides protect CLL cells from spontaneous apoptosis mediated by TLR signaling [63]. On the other hand, post-translational modifications may affect the activity and longevity of the proteins anti- and pro-apoptotic proteins in an inflammatory microenvironment. Bcl-2 protein undergoes phosphorylation at sites Thr56, Thr69, Ser70, Thr74 and Ser87 in response to different stimuli [64]. Taken together, the extracellular signals from cytokines and chemokines, the contribution of transcriptional factors and post-translational modifications on anti-apoptotic proteins ultimately form a complex network to deliver microenvironmental support to the malignant cells [9].

6. Conclusion

Important progress resulting in high levels of clinical and even molecular remissions has been recently achieved in CLL treatment. However, CLL remains an incurable disease. Compelling evidence suggests that crosstalk with accessory cells in specialized tissue microenvironments, such as the BM and secondary lymphoid organs, favours disease progression by promoting malignant B-cell growth and drug resistance. We are starting to understand which genes, molecules and accessory cells are involved in CLL B-cell/microenvironment interactions and what roles they play. Nevertheless, we need a more proper knowledge about the signals received and/or transmitted by CLL B-lymphocyte, interacting with T lymphocytes, and/or with stromal, endothelial, dendritic and nurse-like cells in the particular CLL microenvironment. Therefore, understanding the crosstalk between malignant B-cells and their milieu could give us new keys in the cellular and molecular biology of CLL that can finally lead to novel strategies in the treatment of this disease.

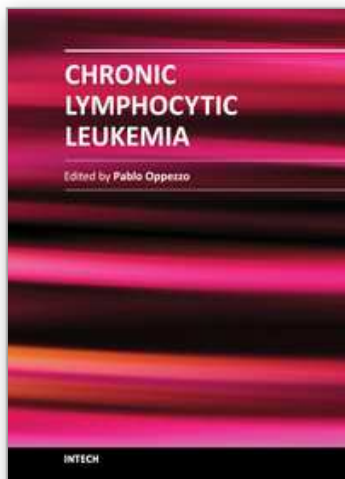
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B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancytopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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