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REVIEW ARTICLE

Novel Insights in Chronic Lymphocytic Leukemia: Are We Getting Closer to Understanding the Pathogenesis of the Disease?

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ABSTRACT

Chronic lymphocytic leukemia (CLL) has unique epidemiologic, biologic, and clinical features. The progressively emerging picture leads us to consider that the critical genes for malignant CLL cells are those regulated by a number of microRNAs revealed by refined cytogenetic and molecular studies, and that the key molecule is the B-cell receptor (BCR). The hypothesis that CLL cells might be selected by some sort of antigenic pressure is strengthened by numerous findings indicating that a BCR-mediated stimulation plays a relevant role in the natural history of the disease and that autoantigens, as well as molecular structures instrumental in eliminating and scavenging apoptotic cells and pathogenic bacteria, may be relevant in triggering and/or facilitating the evolution of CLL. An important question is whether the tiny monoclonal B-cell populations phenotypically similar to CLL (that occur in the peripheral blood of about 3.5% of healthy individuals and are termed monoclonal B lymphocytosis) might be a critical step in the development of CLL. All relevant events of CLL occur in tissues in which a number of cellular and molecular interactions shape a microenvironment conducive to the accumulation of malignant cells and favor the organization of proliferating cells in focal aggregates of variable size that form the pseudofollicular proliferation centers. Given the impact that understanding the pathogenesis of CLL might have on the development of new treatments, the purposes of this review are to discuss whether the novel insights in CLL are leading us closer to understanding the tenet of the disease; to define the emerging new, stimulating questions; and to unfold the major challenges that still need to be addressed.

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INTRODUCTION

The unique epidemiologic, biologic, and clinical features of chronic lymphocytic leukemia (CLL) have long tantalized both biologists and clinicians. The scientific community is now facing an unprecedented flurry of information that appear to dissect the distinctiveness of CLL and have generated an unprecedented enthusiasm about the possibility that we are coming close to understanding the tenet of the disease. Given the impact that such understanding might have onto the development of new treatments, it is appropriate to discuss how really close we presently are to unraveling the pathogenesis of CLL and to unfold the major challenges that still need to be addressed.

B-CELL RECEPTOR AND CLL

The hypothesis that CLL cells might be selected by some sort of antigenic pressure[1] already emerged from a number of studies showing that the immunoglobulin heavy chain variable region (*IGHV*) gene repertoire of CLL is highly restricted and biased as compared to normal adult B-cell repertoire.[2] The possibility that this difference may simply reflect the pattern of *IGHV* gene usage among the elderly, as most patients with CLL actually are, has not been formally ruled out. Still, novel experimental data, including the occurrence of somatic mutations of *IGHV* genes[3,4] as well as the phenotypic[5] and expression profiling signatures,[6] further strengthen the idea that a B-cell receptor (BCR)-mediated stimulation plays a relevant role in the natural history of the disease. Some CLL cases, mainly those expressing the *IGHV1-69* gene, have been consistently found to share closely homologous if not identical (stereotyped) complementarity determining region 3 (CDR3) sequences on IGH and light (L) chains.[7-15] CDR3 defines the specificity of BCR for the vast majority of antigens (Ag) and is unique for each B lymphocyte and its progeny, hence the probability that two individual B cells may express identical BCR by pure chance is extremely low (10^{-9} to 10^{-12}). Although this feature may again reflect a

limited heterogeneity of the IG repertoire in the elderly, it has to be appreciated that a remarkable BCR similarity is by no means a rare phenomenon in CLL. It has been recently shown that HCDR3 stereotypes can be detected in more than 25% of unrelated and geographically distant CLL cases,[15,16] further indicating that the recognition of a limited set of structurally similar epitopes may select at least some leukemic clones. As unmutated cases show stereotyped CDR3 more frequently than mutated ones (approximately 40% *v* around 10%)[15] antigenic exposure appears to be involved in the pathogenesis of all CLL cases, regardless the IGVH mutational status. It is remarkable that the clinical outcome of some stereotyped subsets of patients, such as those using *IGHV3-21* or *IGHV1-69* genes, correlates with the presence of a distinct CDR3 sequence (ie, with the capacity to recognize a specific Ag), rather than with the IGVH mutational status.[15]

Leukemic cells from unmutated cases tend to produce polyreactive antibodies (Abs) similar to natural autoantibodies,[8,17] while most mutated CLL tend to produce oligomonoreactive Abs.[17] In numerous cases, the specificity of the Abs produced by CLL cells is autoreactive, including antirheumatoid factor, anti-DNA, anticardiolipin Abs,[7,8,11,15,17] or targets molecular motifs exposed on the surface blebs of apoptotic cells or microbial epitopes expressed on the coat of common bacteria.[11,15,17] These data indicate that autoAgs as well as molecular structures instrumental in eliminating and scavenging apoptotic cells and pathogenic bacteria may be relevant in triggering and/or facilitating the evolution of CLL. Such a relationship may not be a surprise considering that chronic Ag stimulation is involved in the onset of some chronic B-cell malignancies.[18] Both microbial and self Ag are implicated, examples being gastric mucosa associated lymphoid tissue lymphomas in the context of chronic *Helicobacter* Pilori infection[19] and non-Hodgkin's lymphomas (NHL) developing within salivary glands in Sjogren's syndrome[20] or within thyroid tissue in Hashimoto's thyroiditis.[21] A recent large-scale systematic assessment has revealed that, besides the development of rare NHL entities in affected tissues, specific autoimmune disorders are associated with NHL risk.[22] While recurrent CDR3 motifs in the IG sequences have not been detected in several lymphoproliferative disorders, including follicular lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma, and multiple myeloma, few cases of mucosa associated lymphoid tissue lymphomas were found to exhibit significant homology to rheumatoid factor (RF)–related CDR3.[15,23]

Most circulating CLL cells are in the G0/early G1 phase of the cell cycle.[24] At the same time they are phenotypically hyperactivated,[5] as if just stimulated and ready to undergo cell division. Thus, an important question is whether CLL cells have the capacity to transduce the signal they receive through BCR stimulation. CLL cells differ significantly in their in vitro capacity to signal through the BCR: some cases (most unmutated) carry more competent BCRs and others (usually mutated) appear to be unresponsive.[25-27] This may depend on the nature of the Ag as well as on the affinity of the receptor and it is not unreasonable to postulate that in the former cases an ongoing antigenic stimulation might promote CLL survival and possibly also growth via surface immunoglobulin–mediated signals, while in the latter cases a continuous binding of the Ag might lead to receptor desensitization and to an anergic state.[28] None of the BCR abnormalities described, including impaired glycosylation and folding of the μ and CD79a chains[29] and alternative splicing of *CD79b* gene,[30] appear to be the ultimate explanation for the molecular basis of this difference. It has recently been shown[31] that in a subset of patients with CLL that leukemic cells have a molecular profile, including a constitutive activation of MAP kinase and NFAT transactivation in the absence of AKT activation, that recapitulates the signaling pattern of anergic murine B cells suggesting that this profile might represent the molecular signature of anergic human B lymphocytes as well.

SPECIFIC GENE ABNORMALITIES ARE GRADUALLY COMING TO LIGHT

Refined cytogenetic studies are documenting the clinical importance of genetic subtyping CLL.[32] Still it is not yet clear which chromosomal abnormalities are primary and which are secondary events. The long sought CLL-specific gene alterations are gradually coming to light thanks to mouse models[33] and to the discovery that microRNA (*miRNA*) genes frequently reside in hot spots for chromosomal abnormalities in CLL cells[34] (as they do in other cancer cell types).[35]

The emerging view is that the main genetic alterations of CLL entail the deregulation of specific miRNAs that lead to transcriptional/post-transcriptional abnormalities. An example is provided by miRNA genes *miR-15a* and *miR-16-1* that are located at 13q14.3 (the site of the most frequent deletion in CLL) and are frequently deleted and/or downregulated in patients with CLL.[36] Both microRNAs appear to negatively regulate Bcl2 at post-transcriptional level and this repression appears to be enough to induce apoptosis.[37] The role of *miR-15a* and *miR-16-1* is also supported by a recent study of a mouse model of human CLL, the New Zealand black mice with autoimmune and B lymphoproliferative disease.[38]

Another emerging gene has been revealed by transgenic mouse models where the deregulated expression of the T-cell leukemia/lymphoma 1 (*Tcl1*) oncogene in B cells[33] causes the development of a disorder resembling aggressive (unmutated) human CLL with *IGVH* and *IGVL* genes displaying minimal levels of somatic mutations that react with autoAg or with microbial epitopes.[39] It is of interest that, in human CLL, high levels of Tcl1 expression correlate with unmutated IGVH status and with ZAP70 positivity.[40] Even more important the expression of Tcl1 appears to be regulated at least in part by miR-29 and miR-181, two microRNAs differentially expressed in CLL.[41]

The conclusion is that distinct miRNA signatures may map different subsets of patients classified according to disease progression implying that specific miRNA (and the genes they control) may be at the basis of the different variants of CLL.

DOES CLL HAVE A PRELEUKEMIC PHASE? THE ISSUE OF MONOCLONAL B LYMPHOCYTOSIS

The CD5+, CD23+, CD20low, sIgMlow cell phenotype is the hallmark of CLL. Quite recently, with the use of modern multiparameter flow-cytometric analyses, tiny monoclonal B-cell populations phenotypically very similar to CLL have been demonstrated in the peripheral blood (PB) of about 3.5% of healthy individuals[42,43] and are now named monoclonal B lymphocytosis (MBL).[44] MBLs are more frequent in male, increase in frequency with age,[42,43] and have a significantly higher incidence in the relatives of patients with CLL.[45] On these bases, it appears logical to hypothesize that MBL might be a precursor state for CLL, somehow reminiscent of the relationship between monoclonal gammapathy of

undetermined significance and multiple myeloma[46] or of the high frequency of IGH/BCL2 translocations in the PB of normal individuals, who do not have evidence of follicular lymphoma.[47]

Still, the incidence of MBL is at least 100 times greater than the incidence of CLL and one cannot take for granted that the appearance of monoclonality per se is a sign of genuine neoplastic transformation, as the progressive restriction of the immune repertoire is an aspect of the normal immune-senescence process.[48] It has also been shown that the clonal expansions of T lymphocytes detected in the elderly[49-51] may be associated with chronic and latent (eg, Epstein-Barr virus, cytomegalovirus) viral infections[52,53] and give rise to T-cell leukemias, though very rarely, suggesting that the age-related appearance of monoclonality may be an epiphenomenon of a long-time exposure to chronic antigenic stimulation.[54] In this context it is still unknown whether MBL, especially those detected in CLL relatives, are Ag-specific or polyreactive as in CLL.

It is not unreasonable to suggest that MBL might be a critical step in the development of CLL, possibly increasing the number of cells where the nondispensable transforming events occur. Still such a possibility raises the question whether MBL represent the expansion of the normal counterpart of leukemic B lymphocytes and a potential target for subsequent transforming hits or are already genuine (albeit incompletely transformed) neoplastic elements, waiting for the proper (maybe antigenic) stimulus for an actual expansion into a fully fledged leukemic phase. Again this issue is reminiscent of the relationships between monoclonal gammapathy of undetermined significance and overt myeloma and needs further investigation.

WHERE IT ALL HAPPENS: THE TISSUE MICROENVIRONMENTS

All relevant events of CLL occur in tissues where three main B-cell populations—small lymphocytes, pro-lymphocytes and paraimmunoblatsts—give rise to two major compartments (Fig 1).[55] Small lymphocytes form the accumulation compartment that is also poured in the PB and is the most evident and most studied clonal component. Presumably accumulating small lymphocytes are the offspring of an upstream proliferation compartment essentially represented by pro-lymphocytes and paraimmunoblatsts that cluster to form the pseudo-follicular proliferation centers (PC). PC are focal aggregates of variable size scattered in lymph nodes and to a lesser extent in the bone marrow (BM).[55-58] PC are the hallmark of CLL as they are not detected in any other B-cell malignancy, while it is of interest that they are also observed in inflamed tissues of patients with systemic autimmune/inflammatory disorders, such as rheumatoid arthritis[59] and multiple sclerosis,[60] reinforcing the interest for AutoAg stimulation in CLL. The number and the extent of PC are heterogeneous in different patients.[61] Although a direct association between PC prominence and prognosis has not been formally documented, a correlation between large numbers of prominent PC and a morphological diagnosis of atypical CLL has been reported,[62] and atypical CLL has been associated with poor prognosis. It has also been suggested that the number and the size of PC correlate with lymphocyte doubling time.[63] All these data taken into account the prevailing opinion is that PC are the source of much of the cellular generation in CLL.

PC have an interesting microenvironment as pro-lymphocytes and paraimmunoblasts are interspersed with and surrounded by numerous CD3+ T cells,[55] many being CD4+ CD40L+ elements in

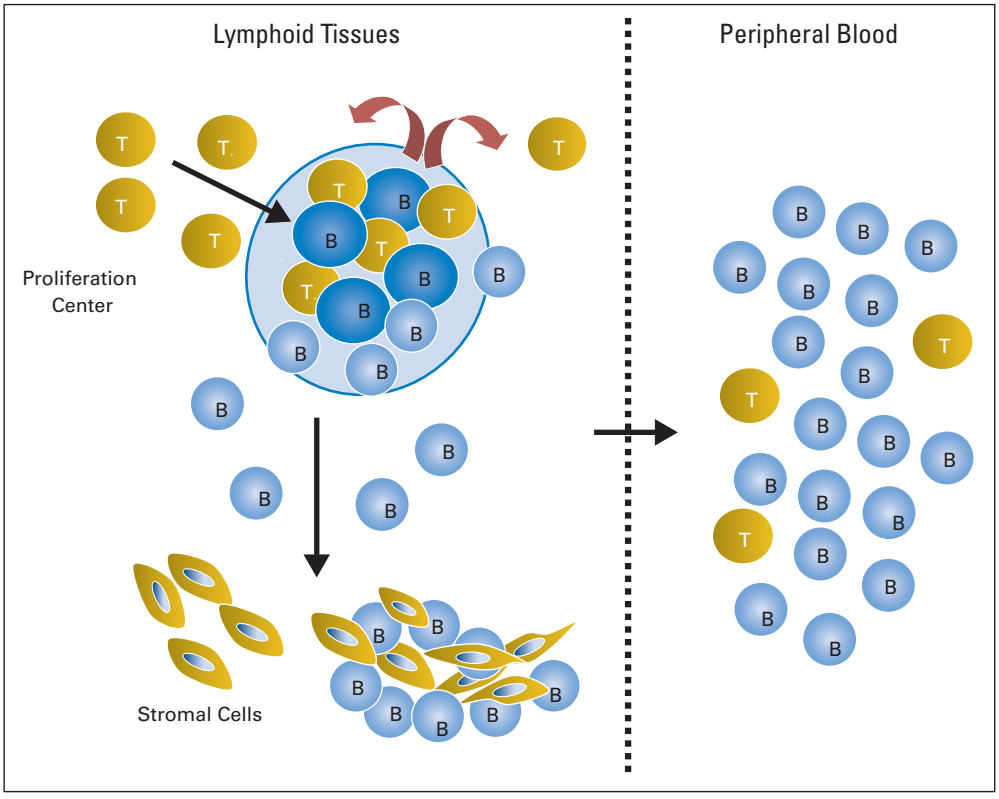

Fig 1. The tissue-oriented model. All relevant events of chronic lymphocytic leukemia (CLL) occur in tissues where leukemic cells can be exposed to (auto)Ag stimulation, are selected for clonal expansion and utilize microenvironment interactions to avoid apoptosis and acquire better growing conditions. In the tissues two major compartments exist. The proliferation compartment is essentially represented by large cells that cluster to form the pseudofollicular proliferation centers. In the proliferation centers the growth of leukemic cells is favored by an advantageous T-cell help. Small lymphocytes that interact with stromal cells to acquire extended survival are the offspring of the proliferation compartment. These small lymphocytes relentlessly accumulate and may flow in the peripheral blood through undefined mechanisms. The circulation and recirculation pattern of peripheral blood leukemic lymphocytes are unknown.

close contact with the proliferating malignant B cells.[64] A delicate network of follicular dendritic cells has also been observed in some PC,[56,57] while stromal cells and a number of accessory cells are found especially admixed with small lymphocytes.[65,66] Several findings suggest the possibility that T cells provide a short-term support which influences malignant B-cell proliferation, while stromal cells and accessory cells including the nurse-like cells provide a long-term support that favors the extended survival and accumulation of leukemic cells.[67-69]

All these data indicate the importance of tissue microenvironments. Still our understanding of the role of T-cells and stromal cells is just in its infancy. Despite CLL being a prototype B-cell malignancy, the overall pool of T cells is significantly increased in size and circulating T cells may frequently be oligoclonal.[70,71] It is unclear whether T-cell oligoclonality reflects the aging of the immune system[72] or is Ag driven. Individual expanded T-cell subsets may have specific tissue and architectural distributions, one example being the concentration of CD4+ T cells within PC. However, most but not all CD3+ T cells found within PC are CD4+ and by no means all CD4+ T cells express CD40L. This observation brings in the issue that functionally different T-cell subsets may be present in the tissues and that their functional role in their specific location is incompletely explored. One example is provided by regulatory T cells[73] that are increased in the PB of patients with CLL with advanced stage and unfavourable prognosis.[74] Another example is represented by the Vδ1 subset of γδ T lymphocytes that have been found increased in the PB of patients with nonprogressing CLL, whose cells were killed in vitro only when the Vδ1 ligands were expressed or upregulated on the surface of leukemic cells.[75] The tissue distribution of both regulatory T cells and Vδ1 T lymphocytes needs to be addressed.

Likewise, the role of chemokines and chemokine receptors in shaping a conducive microenvironment as well as the lineage affiliations of the still incompletely defined accessory cells collectively defined stromal cells has to be delineated and the molecular mechanisms through which they favor the extended survival and accumulation of leukemic cells has to be more precisely defined.

(AUTO)AG STIMULATION AND THE UNANSWERED QUESTIONS

The concept of autoAg stimulation as a causal event has to be reconciled with the fact that in each given patient with CLL all leukemic cells carry the same monoclonal BCR, hence the same potential reactivity. Still only a tiny proportion (the one present in the PC) appear to be able to enter the cell cycle. The implication is that Ag stimulation might lead to B-cell (hyper)activation as a consequence of the Ag binding to BCR but has not the capacity to induce an actual proliferation. Hyperactivated CLL cells are in a paradoxical situation as in kinetic terms they are mostly in the G0/early G1 phase. Ag stimulation might be able to continuously tickle individual cells without having the capacity to promote their further entrance into the cell cycle thereby leading cells at the decisional crossroad between apoptosis and proliferation. Accordingly Ag stimulation would be a preparing but not the ultimate triggering event to enter the cell cycle and differentiation.

A potential abnormality may involve the signal transduction system and especially the connections that link BCR stimulation, cell activation, and the cytoskeleton modification[76,77] that the cell has to acquire in order to proliferate and move. It is possible that cytoskeleton and signal transduction abnormalities may also contribute to give CLL cells a special fragility which would explain why circulating elements may appear in PB films as cellular debris known as smudge cells or Gumprecht's shadows.[78]

Further, even if most experimental efforts have been concentrated on the proliferation and apoptosis it cannot be overlooked that an abnormality in cell differentiation may explain a major difference between autoAg-driven CLL clones and the polyclonal autoreactive B cells observed in organ-specific and systemic autoimmune disorders. The latter produce and secrete sizable amounts of autoAbs while CLL cells do not. Hence, a critical aspect of CLL clonal expansion is the lack of differentiation to cells capable of producing significant amounts of AutoAbs that might somehow buffer the autoAg. The implication is that the triggering Ag keeps on stimulating the clone which grows in an attempt to provide new cells capable of neutralizing the autoAg but fails because the cells do not produce enough autoAbs to neutralize the autoAg. There is no feedback from the system that is then geared to perpetuate itself. Where the autoAgs are located and why the proliferation occurs only in areas that take the form of PC is unknown.

TISSUE-ORIENTED MODEL

We may try to put together the existing data in a model that aims at pinpointing the crucial elements so-far emerged and that deserve further investigation. One possibility (perhaps the simplest one starts from the notion that autoreactive CD5+ B cells are found in the marginal zone of peripheral lymphoid tissues.[79] (Auto)Ag stimulation might select one target cell from this pool and promote its expansion which may end prematurely, or become recognized as MBL or directly progress to overt CLL; the possibility that some MBL may progress to fully fledged CLL awaits further investigation. It is conceivable that the fate of the selected target cell is under the control of genes, such as *Tcl1*, and/or is influenced by specific alterations of the miRNA pattern. Still the extent of these influences has to be defined.

The tissues are the actual sites where leukemic cells can be exposed to (auto)Ag stimulation and selected for clonal expansion. It is plausible to believe that clonal cells are able to utilize microenvironment interactions to avoid apoptosis and acquire better growing conditions. In the tissues they can receive an advantageous T-cell help essentially in the PC and interact with stromal cells to progressively accumulate before flowing in the PB (Fig 1). Numerous questions concerning the central role of microenvironment and PC in the disease replicative history are unanswered. We have to understand which mechanisms lead to the patchy and scattered development of PC in lymph nodes and BM. As it is naïve to consider that PC are directly promoted by Ag stimulation, an alternative possibility is that different microenvironmental features may more or less fortuitously converge to allow PC organization. Over time further genomic aberrations develop, likely prevalent in cases with high birth rate and/or prominent PC, that may stop the need for Ag tickling or mimic the consequences of Ag stimulation.

We have also to clarify the fate of proliferating cells in the PC, how many of them die in situ and how many transit into the accumulation compartment. Data are being gathered to indicate that the clone is more dynamic than anticipated in terms of proliferation and apoptosis. Investigations on telomere length and telomerase activity in PB

cells[80,81] as well as in vivo kinetic studies[82] indicate that all CLL cases (especially the unmutated) have an extensive replicative history.

Finally, it is critical to establish which mechanisms promote the flow of resting cells from the tissue accumulation compartment into the PB (Fig 1). This is an important clinical question as in small lymphocytic lymphoma the malignancy is essentially compartmentalized in the tissues with large nodes and spleen but few circulating malignant cells. Conversely, peripheral lymphoid organs may be just minimally enlarged while a variable proportion of malignant cells, in some cases astonishingly large, is detected in the PB.

PERTURBATION OF RESIDUAL NORMAL IMMUNE CELLS

In this overall context it cannot be overlooked the uniquely profound and still unexplained perturbation of residual normal immune cells that leads to the progressive development of hypogammaglobulinaemia and to the occurrence of pathogenic autoAbs, which are polyclonal, hence produced by normal residual B cells. Surprisingly they target virtually only Ag expressed by hematopoietic-derived cells,[83,84] especially red cells and platelets, frequently causing autoimmune hemolityc anemia and/or thrombocytopenia while systemic autoimmune disorders are exceptional.[85,86]

Functionally different subsets of T cells (regulatory T cells and γδ T lymphocytes need to be added to the list) may be operating in the tissues and entail physical and biochemical interactions with leukemic B cells. We have to take into account several observations showing that CLL themselves are capable to induce a profound immunosuppression by perturbing specific T-cell subsets.[87-89] One clear example is the observation that contact with leukemic cells causes specific significant changes in both CD4 and CD8 T cells which lead to a profound impairment of their function.[90] It has also been suggested[28] (but not yet proved) that CLL B cells might behave as regulatory B cells[91] resulting in suppression of antibody responses. Were CLL cells able to behave as immunoregulatory B cells, the disease-associated immunosuppression and the accompanying abnormalities of T-cell functional activity would find a common explanation.

All these findings taken into account it is not unreasonable to postulate that abnormalities of specific T-cell subsets may explain the progressive development of hypogammaglobulinemia and the generation of polyclonal pathogenic autoAbs. The reason why these autoAbs are mainly causing autoimmune blood disorders remains a mystery.

NEW, STIMULATING CHALLENGES

The emerging picture leads to new, stimulating challenges. As the key molecule for malignant CLL cells is the BCR the definition of the nature of the Ag becomes a crucial aspect. It is even more important to assess to what extent Ag stimulation is relevant and in which phase of the disease (early development *v* progression), where it occurs and through which mechanisms. It is also essential to prove or disprove whether a (still unknown) genetic abnormality may mimic the effects of Ag stimulation in CLL cells. To this end, the elucidation of the pattern of miRNA signature in MBL and the proper characterization of the genes regulated by the miRNA involved in CLL is critical. The role of MBL as CLL precursor state has to be proved. Were this the case the central question would become to define which genes are switched on (off) in the cases of MBL that plan to become overt CLL.

As the microenvironment is an attractive target for innovative treatment strategies it has to be considered that BM and secondary lymphoid organs have entirely different microenvironments, each finely tuned to serve the specific organ function through the activity of different cell types and the expression of different genes. This heterogeneity dictates the necessity to establish proper culture systems to reproduce in vitro the in vivo situation of different microenvironments. The microenvironment-specific culture systems may become useful tools for testing new drugs. In any case, their effects onto CLL cell survival and growth need to be validated in mouse models. Another microenvironment-related challenge is the definition of the rules that govern the recirculation pattern of peripheral blood leukemic lymphocytes.

We do not know whether CLL is a malignancy driven by elusive, rare leukemic stem cells perhaps lurking in specific tissue areas or whether it is a mature, Ag-stimulated clonally expanded B-cell population that recirculates between blood and the tissues, where it is exposed to growth signals and (auto)Ag. This issue also brings in the ultimate challenge, to understand the relationship between (auto)Ag-triggered events of cell activation and malignant transformation. Answering this question will not only lead to a biology-based treatment of CLL, but may also allow a more profound (re)definition of the relationships between the immune system and tumor development.

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