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Commentary

Persistent B-Cell Stimulation or B-Cell Repertoire Anomalies? The Dilemma of the Origin of Chronic Lymphocytic Leukemia (CLL)

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1. Introduction

Chronic Lymphocytic Leukemia (CLL) is caused by the clonal expansion of CD5+ B lymphocytes in the circulation, peripheral lymphoid organs and bone marrow [1–4]. Although leukemic cells are clearly B-cells because of their expression of surface immunoglobulin (sIg) and B-cell markers such as CD19 and CD20, their phenotype, assessed by monoclonal antibody staining, does not classify them in any of the known B-cell subsets, posing difficulties for the identification of the cell of origin of CLL [5]. This is likely related to the constitutive activation of leukemic cells, which leads to the expression of several activation markers (of which CD5 represents a typical example) and confounds phenotypic identification. Despite these problems and the lack of knowledge of the molecular event(s) underlying the beginning of the leukemic process, several findings have contributed to elucidating the characteristic features of CLL cells and to propose theories on leukemogenesis.

The past definition of Chronic Lymphocytic Leukemia (CLL) was that of an accumulation of immune-incompetent lymphocytes that proliferate at a low pace [6]. The advent of DNA-labeling technologies with deuterated water in vivo led to a change in this view, since leukemic cells showed a substantial turn-over, with both cell proliferation and death [7]. Moreover, studies on surface Ig of leukemic lymphocytes indicated that they have a fully assembled B-cell Receptor (BcR) i.e., surface Ig capable of antigen recognition + adaptor molecules capable of activating the BcR-dependent pathway upon interaction with the appropriate ligand [8]. Therefore, CLL cells are not immune-incompetent in classic immunologic terms, i.e., uncapable of antigenic recognition via a functional BcR. The concept of the immune incompetence of leukemic cells was generated by the combined observation of the immunodeficiency status of most patients and by considerations on the potential incapacity of the leukemic cells to provide an adequate defense. Now, we know that leukemic cells, being monoclonal, would be unable to cover the antibody repertoire required for defense, even if capable of responding to external stimuli. As discussed below, patients' immunodeficiency must have different explanations.

Studies on the BcR-encoding genes have provided substantial information on the features and the mode of differentiation of CLL cells. In B-cells, the genes encoding for an antibody molecule, which also serves as BcR, are generated during ontogeny by a random recombination of one of the several copies of the germline IGHV genes with one IGHD gene and one IGHJ gene, that, together, form an IGHV-D-J rearrangement, which combines with a constant-region gene to encode for a complete heavy chain [9]. For light chains, recombination occurs among germline IGLV, IGLJ gene segments and constant-part genes. The process, which is different in different cells and enables a given B-cell to produce a single antibody molecule, invariably involves the addition of N sequences, which facilitate the joining of the various gene segments. The recombination of a variety of segments encoding the antibody variable region allows a very large number of antibody specificities

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to be attained. According to their monoclonal definition, leukemic cells from single patients share the same IGHV-D-J and IGLV-J rearrangements.

IGHV-D-J or IGLV-J rearrangements accumulate somatic mutations when mature B-cells are stimulated by specific antigens. This process, defined as Somatic Hypermutation, or SHM, occurs primarily although not necessarily in the Germinal Centers (GCs) of peripheral lymphoid tissues and is responsible for generating additional antibody specificities and for the affinity maturation of the antibodies produced [10,11]. CLL patients can be subdivided into two groups depending upon the presence (Mutated CLL, or M-CLL) or absence (Unmutated CLL, or U-CLL) of >2% of mutations in the IGHV-D-J rearrangement of the leukemic clone [12,13]. This indicates that the CLL clones from the two groups of patients follow different differentiation trajectories and that only those from M-CLL have possibly transversed the GCs. Notably, M-CLL has a less aggressive clinical course than U-CLL, a finding which is in line with the slower turn-over of leukemic cells determined by studies with deuterated water [7]. These findings also indicate that BcR features and functioning influence the rate of clonal expansion, a notion supported further by the observation that inhibitors of the BcR pathway are effective therapeutic agents in both M- and U-CLL [14].

The sequencing of IGHV-D-J rearrangements from large numbers of CLL clones has led to the clarification of the CLL BcR repertoire also in comparison with that of normal B-cells (reviewed in [15]). The repertoire of CLL clones differs from that of normal B-cells in the usage of IGHV-D-J segments and of their combinations [16].

Moreover, CLL clones from different patients have quasi-identical BcRs, defined as stereotyped BcRs [17–20]. Several hundreds of stereotypes have been identified and grouped into subsets, although the most frequently used are limited in number. Twenty-nine subsets identified as "major" represent 13.5% of the CLL repertoire, whereas subsets identified as "minor", constitute the 27.7% of the repertoire. Thus, at least 41% of the CLL repertoire is represented by stereotyped BcRs [21], while the proportion of CLL-like stereotypes is very low in the normal B-cell repertoire [22]. For the classification in a given subset, two different BcRs must have a Heavy-Chain Complementarity-Determining Region 3, or HCDR3, amino-acid identity of >50% and an amino-acid similarity of approximately 70%, a shared length of HCDR3, the utilization of the same IGHV gene (or of IGHV genes of the same clan) and the utilization of the same kappa or lambda light-chain type and of IGLV-J rearrangements with the same features of the CDR3 described for the H chain [21].

The features of the antibody repertoire impacts the BcR specificity of CLL cells. Many CLL clones produce poly-reactive BcRs [8,15]. Poly-reactivity means that a single antibody reacts to different antigens, including self-antigens like IgG and platelets, single- and double-stranded DNA, RNA, insulin, etc. When secreted, poly-reactive antibodies classify in the group of "natural antibodies" found in the sera of all mammalian species, which constitute the first line of defense against the incoming pathogens. CLL BcRs also react with self-antigens normally located inside the cells and exposed and oxidized during apoptosis. Normally, the antibodies against these antigens, including vimentin, tubulin, filamin B and non-muscle myosin IIA, serve to clear apoptotic cells, although when utilized as BcRs, they may cause cell stimulation, particularly in CLL, where apoptotic cells are abundant. CLL cell BcRs also react with antigens commonly recognized by antibodies present in Systemic Lupus Erythematosus (SLE) patients. Moreover, several BcRs can react with antigens of micro-organisms, such as those utilizing a combination of IGHV3-7 and IGKV2-24 genes, which recognize the β-(1,6)-glucan of the commensal yeast species. Exposure to this glucan causes CLL cell proliferation in vitro, indicating the importance of commensal micro-organisms for CLL cell growth [23]. BcRs reacting with antigens exposed by apoptotic cells cross-react with several microbial antigens. Finally, CLL BcRs have the ability of self-aggregating, since they recognize epitopes within the BcR expressed by the same leukemic clone. The self-aggregation of BcRs at the surface membrane of leukemic cells delivers activation signals. This phenomenon, referred to as "autonomous signaling", is detectable in virtually all CLL clones irrespective of their IGHV mutational status [24].

The stimuli delivered by the BcR, together with micro-environment signals, can account for the expression of activation markers by CLL cells, including CD27, which led to the classification of CLL (both M- and U-CLL) as a memory B-cell disorder, although this concept is now debated (see ref. [15] for details). Moreover, these stimuli may cause leukemic cell expansion in patients with full-blown CLL. Such expansion can facilitate the accumulation of cytogenetic lesions, such as del13q, del11q, del17p and trisomy 12, or mutations in *TP53*, *BIRC3*, *SFB31, NOTCH1* and in other genes [25]. These lesions seem late acquisition in the disease course, as indicated by their sub-clonal distribution, and are unlikely to be driver mutations for the initial phases of the disease.

The above considerations raise issues regarding the events facilitating the emergence of the CLL BcR repertoire. Does this repertoire result from a process of antigen stimulation/selection of mature B-cells? Or do anomalies in the early stages of B-cell maturation cause the formation of a special BcR repertoire, prone to be stimulated by self-antigens? The distinction is not trivial, since in the former case, transforming mutations will occur in mature B-cell clones induced to proliferate by antigenic stimulation, posing the issue of the mode of selection of the clones causing CLL. In the latter case, early transforming mutations can alter the subsequent B-cell differentiation process, leading to anomalous BcR repertoire formation. This could facilitate cell stimulation by certain antigens, including self-antigens, and the accumulation of further transforming mutations in the proliferating cells. In this article, we shall discuss the evidence in support of one or the other of the two options.

2. Origin of CLL from Mature B-Cells: The Role of Persistent Antigenic Stimulation

This hypothesis implies that persistent antigenic stimulation leads to the expansion/selection of certain B-cell clones, which will be subsequently the target of transforming genetic mutations. This clonal selection is likely a multistep phenomenon, with oligoclonal accumulation of partially transformed clones preceding the emergence of a dominant clone that defines the disease (Clinically Relevant Clone, or CRC) (see Figure 1A). The issue with this hypothesis is the following: how can persistent stimulation support cell proliferation and favor the accumulation of transforming mutations? There are several examples of malignant transformation occurring during a response to a pathogen in humans, the most known of which is gastric lymphoma arising during *H. pylori* infection [26]. The patients present an oligoclonal B-cell accumulation, preceding full-blown gastric lymphoma, which frequently disappears if the infection is eradicated with antibiotic therapy. Infection with *C. burneti*, *C. psittaci*, *B. burgdorferi* and *C. pylori* also relates to lymphoma insurgence following a similar pathogenetic mechanism [27]. The major difference with these conditions is that many antigens are responsible for cell stimulation in CLL as opposed to a single infectious agent. The possibility of a reaction against self-antigens initiating the phenomenon is in part supported by murine models focused on the so-called B-1 B-cells [28]. Under normal conditions, B-1 B-cells are constitutively activated, express surface CD5 and other activation markers, mount T-cell-independent responses leading to the predominant production of IgM, are self-replenishing, utilize a repertoire with abundant poly-reactive and quasi-identical antibodies and seed in the peritoneal cavity. Notably, murine B-1 B-cells cells utilizing poly-reactive BcRs are capable of autonomous signaling, which can in part explain their expansion [29]. B-1 B-cells are distinct from B-2 B-cells, which do not share the above features and mount T-cell-dependent responses, characterized by antibody affinity maturation and the production of Ig of different classes.

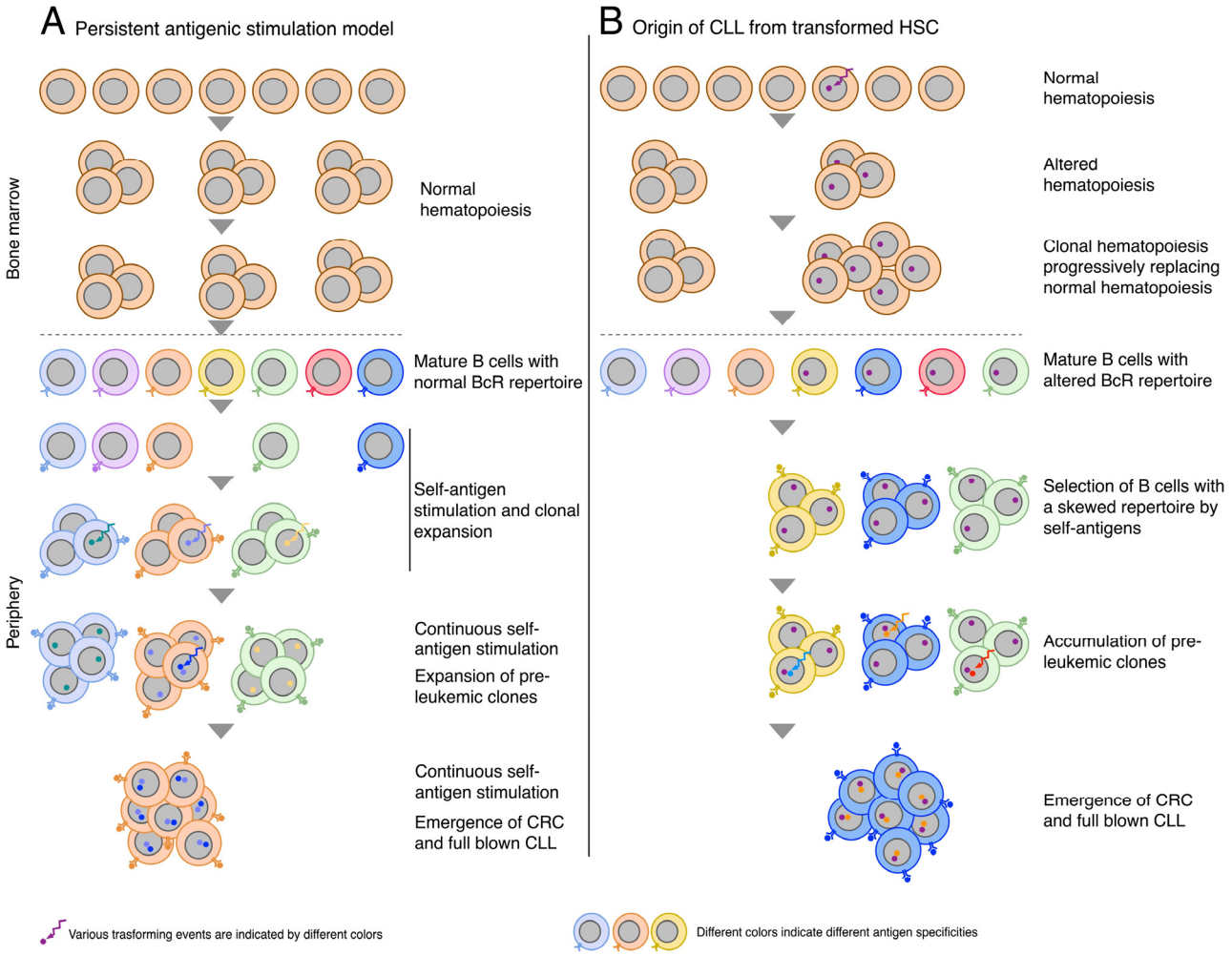

Figure 1. Two models to explain the origin of CLL. (**A**) *Persistent antigen stimulation model.* Because of excessive stimulation by self-antigens, possibly out of T-cell control, cells with poly-reactive/self-reactive BcRs, present in normal B-cells, expand, becoming the target of transforming events. The process occurs in a stepwise manner, leading to progressive and different accumulation of transforming mutations in various clones. The clone most fit for survival/expansion will eventually emerge as the Clinically Relevant Clone (CRC), leaving behind other partially transformed clones as fingerprints of the process. (**B**) *Model of origin from transformed HSCs.* The initial transforming lesion(s) occurs at the hematopoietic stem cell (HSC) level with two major consequences, clonal hematopoiesis (CH) and altered lymphopoiesis, which causes the accumulation of self-reactive clones that would be normally deleted. The encounter with self-antigens drives the expansion of self-reactive cells, which become concomitantly the target of additional transforming events. The competition between the various clones culminates in the emergence of the CRC as the best fit for survival/expansion.

Murine B-1 B-cells (and not B-2 B-cells), when transferred from adult to neonatal mice, expand in the host, giving origin to oligoclonal B-cell populations which express CD5, utilize the same Ig allotypes as the donor cells and are believed to represent a pre-leukemic condition [30–32]. The interpretation of the data is that B-1 B-cells from adult mice, if placed in neonatal mice, are persistently stimulated by self-antigens and give raise to pre-leukemic clones in the absence of micro-environmental control. The transition from the pre-leukemic stage to leukemia is probably prevented by the short experimental time. Support to this notion is provided by observations on mice transgenic for the *T cell leukemia 1* or *tcl1* gene [33]. These mice, obtained following the observation of elevated TCL1 proteins in CLL cells, carry a *tcl1* transgene under the control of an IGHV promotor and an Eμ enhancer, and their B-cells have elevated TCL1 levels. In late periods of their life, these mice develop

a CD5+ B-cell lymphoproliferative disorder resembling CLL, a finding that underlines the requirement for a long latency period to allow for the selection of fully transformed clones. The cells of these lymphoproliferations utilize BcRs resembling those of U-CLL, which can deliver autonomous signaling [24].

The transfer of CD5+ B-cells from adult *tcl1* mice to neonatal normal recipients causes accelerated leukemia onset compared with that observed with CD5+ B-cells from normal mice. The process is accelerated even further when the donor mice are double-transgenic for *tcl1* and for an autoreactive BcR (e.g., to phosphatidylcholine or non-muscle myosin IIA) or carry a deletion of the *pten* gene, which negatively regulates the BcR [34,35]. Moreover, the drug-induced or genetic inactivation of BcR signaling results in delayed leukemia onset [34]. Notably, cytogenetic lesions reminiscent of CLL del13q- are observed in some of these murine leukemias [32]. Collectively, the data indicate the need for a cooperation between self-antigen stimulation and the upregulation of a promoting factor, like *tcl1*, facilitating cell proliferation. Genetic lesions, like the artificial *tcl1* upregulation of transgenic mice, which promotes cell proliferation, have not been so far detected in CLL. The demonstration of similar lesions, which may involve regulatory sequences, would make the model of persistent B-cell stimulation more plausible. These lesions may be somatically acquired or genetically transmitted. The latter option would provide an explanation for the occurrence of CLL in families, although studies on the molecular nature of genetic lesions have so far failed to provide mechanistic answers [36,37]. Moreover, a malfunctioning of the micro-environmental control on B-cell expansion can be postulated to go along with the similarities with the pre-leukemic stage induced by the injection of adult CD5+ B-cells in neonatal mice. Since CLL is a disease of the elderly, such altered control is possible, particularly at the T-cell level, given the reported failures of these cells with advancing age [38].

3. Concerns Regarding the Persistent Antigenic Stimulation Model

In the elaboration of the above models, the differences between human and murine CD5+ B-cells have been underestimated. Murine B-1 B-cells are a rather homogeneous population with BcR repertoire features resembling CLL already before any leukemogenic event. In contrast, these repertoire similarities have not been detected in any of the known human B-cell subpopulations. This is particularly true when CLL-like stereotypes are considered, which are in much lower proportions in all normal B-cell subpopulations, have a different distribution of their subsets and do not present the typical kappa/lambda chain restriction [22,39,40]. In addition, CD5 expression coincides with B-cell activation in humans, documented by the presence of additional activation markers on CD5+ B-cells. This notion is in line with the observation that CD5+ B-cells taken ex vivo have different phenotypic features, distinctive of various B-cell subsets, and that the B-cells of any subset can be activated in vitro to express CD5 [5]. Furthermore, CD5+ B-cells purified from normal individuals have a distribution of CLL-like stereotypes similar to that of naive B-cells that are the major component of CD5+ B-cells [22]. Thus, the lymphoproliferative disorders arising from B-1 B-cells in mice appear to originate from cells already sharing many features with their leukemic counterparts. The accumulation of B-1 B-cells is abundantly represented only in certain murine strains, raising the possibility that infectious agents (viral infections longitudinally transmitted?) or germ-line genetic lesions, like those of familial CLL, contribute to the proliferation of these cells and possibly to a pre-leukemic transformation. CD5+ B-cells with the features of murine B-1 B-cells are not observed in humans and thus cannot be the cells of origin of CLL.

The murine models provide explanations for the origin of U-CLL, given the similarities between murine leukemia and U-CLL. The complexity of the process of Somatic Hypermutation (SHM) and the more indolent nature of the IGHV-mutated leukemic cells may prevent the formation of full-blown M-CLL-like disease in mice. Alternatively, M-CLL could follow a different pathogenetic pathway not occurring in the murine models. That M-CLL cells follow different trajectories of origin is suggested by several observations, the

most important being the finding that stereotypes that have SHM in CLL cells also have SHM in normal cells and that the same occurs for stereotypes without SHM, indicating that BcR rearrangement can determine the subsequent differentiation pathway leading to SHM or not [22].

The persistent antigenic stimulation model tenets that leukemogenesis is a stepwise process, favoring initial oligoclonal B-cell accumulation followed by the emergence of a CRC. This stimulation/selection process should leave behind fingerprints, like persistent oligoclonal B-cell accumulation. This notion could be supported by the frequent findings of additional B-cell clones, besides the Clinically Relevant Clone (CRC), in the circulation in patients with CLL or Monoclonal B-cell Lymphocytosis (MBL), universally considered a pre-leukemic condition [41–45]. However, more studies seem necessary to investigate the structural relationships between the CRC and the additional clones that could support an ongoing process of stimulation/selection.

4. Origin of CLL Involving HSCs

A hypothesis differing from the above implies that the initial steps of CLL origin occur in the hematopoietic stem cells (HSCs) (see Figure 1B). This is in line with CLL being a disease of the elderly and with the finding of HSCs increasingly accumulating mutations with advancing age [46]. The model tenets that transformed HSCs have unbalanced differentiation toward the B-cell lineage, although they can still differentiate along different lineages, generating clonal hematopoiesis (CH). CH is initially limited and accompanied by normal hematopoiesis but may become predominant with advancing disease. In this respect, the origin of CLL would not differ from that of other myeloproliferative disorders, such as polycythemia or thrombocythemia, which have CH with predominant differentiation toward a single cell lineage [46]. The molecular nature of the early transforming events of HSCs is not clarified, and the model remains speculative; however, these events could involve mutations of exonic or regulatory sequences involved in the regulation of hematopoiesis, which are difficult to detect. These mutations can be somatically acquired, or genetically transmitted, a condition characterizing familial CLL.

Because of their descendance from transformed HSCs, cells responsible for B-cell lymphopoiesis could be altered, causing anomalies in the deletion process of self-reactive cells, which expand owing to continuous self-antigen stimulation. This causes accumulation of multiple B-cell clones, several of which are self-reactive and are further selected for survival/expansion. The outcome includes the appearance of a predominant CRC (characterizing the disorder as a B-cell leukemia) and of additional B-cell clones at different transformation levels.

The model which shifts the attention from mature B-cells to HSCs in the origin of CLL is not entirely new. The initial steps of the origin of follicular lymphoma (FL) have been traced in B-cells in early maturation stages, where IGHV-D-J rearrangement could facilitate the translocation of the *bcl-2* oncogene to the IG locus (a disease-defining lesion). B-cells with translocated *bcl-2* nevertheless mature further to the GC stage until the accumulation of further transforming mutations causes the onset of full-blown FL [47,48]. An analogous origin was hypothesized for those Burkitt Lymphomas (BLs) in which the joining of the translocated *c-myc*, characterizing the disease, involves the IGHV gene segments, indicating that the translocation occurred in the early maturation stages, concomitantly with IGHV-D-J rearrangement [49]. Yet, the cells of full-blown BL have the features of mature B-cells and are phenotypically like centro-blasts.

However, the model for the origin of CLL proposed here differs from those of FL and BL, since in these lymphomas, the initial transforming event is placed concomitantly with IGHV-D-J rearrangement in early B-cell progenitors, whereas the cell giving origin CLL is indicated as an HSC. Although supported only in part by experimental evidence, the involvement of HSCs could help provide a mechanistic justification for clinical conditions of CLL not otherwise explained, such as non-immune-mediated cytopenias and/or bone marrow failures, which may be consequent to CH [44]. In addition, the anomalous

maturation of B-cells, derived from transformed HSCs, could prevent self-reacting B-cell deletion, facilitating autoimmunity (caused by clones different from the CRC), and/or induce a restricted antibody repertoire in the B-cells different from the CRC facilitating hypogammaglobulinemia. Both conditions frequently characterize CLL.

5. Supporting Evidence and Concerns Regarding the Origin from HSCs

The first indications of HSC involvement were provided by Kikushige and colleagues [50] in 2011, reporting an expansion of pre-B-cells in the bone marrow of CLL patients. Furthermore, they described that purified HSCs (CD34+ cells) gave rise to the oligoclonal accumulation of B-cells if engrafted in immune-deficient mice. Notably, the original CRC of the patient was not detected among the engrafted cells, possibly because of the lack of support of activated T cells, which are mandatory for CRC engraftment but were not injected into mice [51]. The pre-leukemic cells developing in mice had multiple IGHV-D-J rearrangements, the expansion of which was thought to be related to stimulation/selection by the mouse micro-environment, different from that of humans. These experiments were performed at a time when a complete repertoire analysis to compare clones developing in mice with those of CLL could not be carried out by NGS. Moreover, possible genetic lesions shared by the different MBL-like clonal expansions of mice and the cells from the patients could not be investigated by single-cell analyses to provide more stringent evidence for clonal correlations.

Additional evidence is based on observations that cells of different lineages purified from patients with CLL share the same genetic lesions as the CRCs. This provides a demonstration for CH occurring in CLL, although it is difficult to exclude contamination of non-lymphoid cells by leukemic cells in these experiments. These issues, which were duly pointed out by the authors of the studies, who also carried out the controls feasible at the time, could probably be now overcome by using single-cell technologies that allow for the concomitant detection of genetic lesions, IGHV-D-J rearrangements and cell lineage markers [52,53].

Finally, the finding of more than a single B-cell clone in CLL and MBL can be taken as evidence for leukemogenesis starting in early differentiation stages of B-cells, possibly prior to or concomitantly with the IGHV-D-J rearrangement [41,42,44,45].

6. Concluding Remarks

In the past, CLL was almost universally believed to begin in mature B-cells with the collaboration of signals from the BcR. The discussion was focused on which human B-cell subset was the human counterpart of murine CD5+ B-cells (B-1 B-cells), the likely target of the transformation. This model has raised criticism related to the rather optimistic parallels between the B-1 B-cells in mice and the CD5+ B-cells in humans and to the lack of a convincing explanation for the origin of the CLL repertoire. These difficulties have led to the search for other models, focused on the transformation beginning in the HSCs. These imply both deranged B-cell maturation favoring the appearance of an anomalous B-cell repertoire and CH and can explain the pathophysiology of several pathological phenomena of CLL. Although supported by experimental observations, the final evidence for these models awaits the contribution of studies with newer technologies, possibly at the single-cell level. In addition, the definition of the problem awaits elucidation of the early molecular lesions causing CLL, which have not been so far detected despite extensive DNA-sequencing studies.

7. Origin of the Article

The idea of this commentary and of the two models of CLL origin stemmed from discussions on the function of BcR in CLL. MF wrote a draft reporting the ideas of the group. The text was discussed again by all the authors and changed when needed. All authors have read and agree with the published version of the manuscript.

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