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## Antigen stimulation in the development of chronic lymphocytic leukemia\*

### Stymulacja antygenowa w patogenezie przewlekłej białaczki limfocytowej

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#### Summary

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world. The mechanism of the disease development still remains unrevealed. In recent years new unique molecular and clinical features of CLL have emerged leading to a unified hypothesis of CLL origin. Major progress in understanding CLL biology was made after identification of mutational status of immunoglobulin variable heavy chain (*IGHV*) genes, which also improved prediction of patients' clinical outcome. Preferential usage of *IGHV* genes has led to recognition of CLL-specific B cell receptors (BCRs), called stereotyped BCRs. Taken together, these data point to antigen stimulation of CLL progenitor cells. Studies on CLL antibody reactivity have shown affinity to molecular motifs on apoptotic cells and bacterial cell structures, supporting the current hypothesis of the CLL pathomechanism. In this paper we have summarized information available to date regarding current theory of cellular origin and pathology of CLL.

**Keywords:** CLL • chronic lymphocytic leukemia • pathogenesis • antigen • autoimmunity

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#### INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world and it represents about 25-30% of all leukemias. It affects mainly older people; the average age at the time of diagnosis is 65 years old [1,21,30,32,54]. In the course of CLL accumulation of B CD5<sup>+</sup> lymphocytes in peripheral blood, bone marrow, lymph nodes and spleen is observed [30,32,54].

It is known that CLL is a very heterogeneous disease. Some patients demonstrate signs of rapid progression and need immediate therapy, while many cases experience a more favorable course and never require treatment [1]. Clinical heterogeneity of CLL is a reflection of genetic and epigenetic abnormalities as well as the microenvironmental influence involved in its pathogenesis, but the mechanism of the disease still remains unrevealed [32,69].

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After the milestone discovery which was the mutational status of immunoglobulin variable heavy chain (*IGHV*) genes, immunoglobulin (Ig) genes became important prognostic markers allowing prediction of patient outcome. Comprehensive analysis of *IGHV* genes and definition of stereotyped B cell receptors (BCRs) have led to the most important theory of CLL pathogenesis known to date, namely the potential role of antigen (Ag) selection (fig. 1). Studies on CLL antibody (Ab) reactivity have provided hints about the nature of recognized by malignant cells Ags and what the cell of origin is [55,65].

### Cellular origin of CLL

Taking into account that CLL clones use mutated as well as unmutated *IGHV* genes, which divides patients into clinically distinct subgroups, a two-cell origin model can be considered. However, gene expression profiling (GEP) analysis shows a relatively small number of gene usage differences between unmutated CLL (U-CLL) and mutated CLL (M-CLL) in contrast to thousands of differences between healthy B lymphocytes [14]. The most distinctive phenotypic characteristic of CLL cells is expression of the CD5 molecule coupled with low levels of monoclonal surface immunoglobulins (sIg), usually class IgM or IgD; also the CD23 marker is present on leukemic cells. Although the precise origin of leukemic cells is still not clear, GEP and immunogenetic data suggest that CLL clones derive from competent B lymphocytes selected for clonal expansion and eventual transformation by multiple encounters and responses to antigens (fig. 1). Therefore the unified parsimonious hypothesis that CLL is a malignancy of the mantle zone (MZ) subpopulation has been adopted [7,26,27,55].

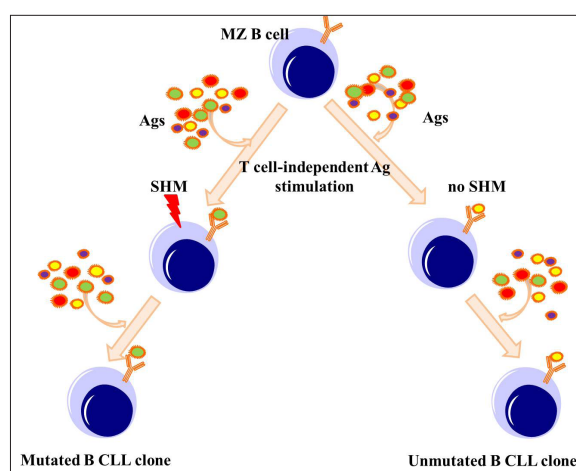
The features of MZ cells could explain the origin of CLL cells. MZ B cells are defined as  $IgM^{high}IgD^{low}$ , they can respond to bacterial polysaccharides in a T cell-independent manner, express either mutated or unmutated *IGV* genes, and the majority of human MZ B cells experience somatic hypermutations (SHM) [14,27,55]. The most significant feature of normal  $CD5^{+}$  B lymphocytes (B1) is an ability to produce polyreactive Ig that recognize variety of auto-antigens (autoAg) and cross-react with bacterial Ags. These natural auto-antibodies (autoAb) are encoded by the same group of *IGV* genes that are expressed in many cases of CLL. CLL cells and B1 cells also have in common other features, such as low expression of CD20 or myelomonocytic Ags – most frequently My4/CD14 [7,14,55].

Almost 100% of circulating neoplastic cells are in the  $G_0$  phase of the cell cycle, but there is a characteristic asynchronism observed between the resting position of CLL cells and their activated phenotype. Resting malignant cells express molecules associated with B cell activation, such as CD23 and CD27. What is more, in leukemic cells there was found mRNA for *IL-1 $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-7*, *IL-8*, *IL-10*, *IL-13*, *IFN- $\gamma$* , *TNF*, *GM-CSF* and *TGF- $\beta$ 1*, but the role of cytokines in development of the disease is not clear. CLL

cells do not respond to any of the cytokines; they remain in a resting position [7].

However, a MZ origin of CLL cells faces difficulties. There are some differences in the surface phenotype between normal B and CLL cells. MZ B cells express surface IgM and IgD, but with phenotype of  $CD5^{-}CD23^{-}CD22^{+}$ , that differs from leukemic cells. This discrepancy could reflect up-regulation of CD5 and CD23 upon activation as CLL cells are constantly activated according to GEP and immunogenetic analysis. Also, expression of CD21, a receptor for the Epstein-Barr virus (EBV), cannot be explained by MZ origin of CLL. EBV immortalizes normal B cells regardless of CD5 expression. But it is almost impossible to immortalize CLL cells with EBV, even though EBV-receptor binding activity and EBV uptake of malignant cells are complete [7,14,27].

In contrast to current theory of cellular origin in CLL, recently published data have suggested that the disease initiating event may occur in hematopoietic stem cells. Apparently hematopoietic stem cells obtained from CLL patients and transplanted to xenograft models have a predisposition to generate clonal B cells with a CLL-like phenotype and certain *IGHV* genes usage [26,41].



**Fig. 1.** Schematic illustration of chronic lymphocytic leukemia development; MZ B cell – mantle zone B cell; Ags – antigens; SHM – somatic hypermutation; CLL – chronic lymphocytic leukemia. Mantle zone B cells independently of T cells can be exposed on autoAgs stimulation. Some of these B cells might undergo SHM and/or isotype class switching, others not, which leads to the development of either mutated or unmutated CLL clones expressing B cell receptors characteristic of particular autoAg.

### Immunoglobulin genes in CLL

The discovery that somatic mutations of the Ig variable region genes occur in a subset of CLL patients and that the absence or presence of these mutations significantly correlates with clinical outcome was a milestone in understanding the biology of CLL [37,62]. In general, patients with mutated *IGHV* genes follow a more indolent course compared to those with unmutated *IGHV*

genes, who have more progressive leukemia, adverse cytogenetic profiles, clonal evolution and resistance to therapy [44]. Based on these observations, it was speculated that the U-CLL cells derive from pregerminal center or naïve B cells, while M-CLL cells originate from postgerminal center or memory B cells. However, this theory, as mentioned above, is not supported by GEP analysis, which shows that CLL cells, independently of mutational status, and memory B cells have similar *IGHV* gene expression profiles. It is also known that malignant cells carry antigen experienced (CD27) and activated (CD23, CD25, CD69, CD71) phenotype of slg [33].

Since 1999, when Chiorazzi's, Hamblin's and Stevenson's independent groups confirmed that the *IG* gene repertoire in CLL cells is not random and that SHM among these genes do not occur uniformly, several studies have established that *IGHV* mutational status has prognostic value independent of the clinical stage. However, it is observed that relative frequencies of individual genes significantly vary among subsets. Performed analysis of *IGHV* rearrangement genes has revealed a striking association between particular gene usage and clinical/phenotypic features or outcome for selected subsets of CLL patients. And this points to antigen binding as a critical issue in determining clinical presentation [4,57].

A number of studies have confirmed biased usage of the *IGHV1-69* gene in CLL. Although over-usage of *IGHV1-69* is observed also in other B cell proliferative disorders, it is important that *IGHV1-69* is the most frequent gene over-represented in U-CLL patients [11,65]. Several alleles of this gene has been found. They can be grouped into two types based on different sequences that encode the second complementarity-determining region (CDR2), but only 1 type of allele appears to be generally used in CLL – 51p1 [40,65]. Moreover, *IGHV1-69* rearrangements are preferentially biased towards the usage of *IGHD3-3*, *IGHD2-2* as well as *IGHJ6* genes [37,44,65]. Another unusual molecular characteristic of *IGHV1-69* rearrangements is a longer than average CDR3 sequence, which in that case have 19 codons [40,65]. These features of *IGHV1-69* over-usage are clearly different from those seen in B cells of the normal elderly population [65]. It has been reported that apart from *IGHV1-69* also other genes from the *IGHV1* family are over-used among U-CLL patients, namely: *IGHV1-2*, *IGHV1-18*, *IGHV1-3*, *IGHV1-46*, *IGHV7-4-1*. The prognosis for patients with over-expression of the mentioned genes is poor [37].

Another very frequent *IGHV* gene in CLL is *IGHV4-34*, usually over-expressed in M-CLL, although *IGHV4-34* is also used at high frequency among healthy individuals [29,33,44,65]. There are 2 different types of *IGHV4-34* rearrangements reported: with short CDR3 associated with *IGHJ4* gene usage and with a long CDR3 sequence coexisting with *IGHJ6* over-expression [65]. Several scientific groups have established that more than 1% of CLL patients over-using *IGHV4-34* with long CDR3 also over-express the *IGKV2-30* gene [29,33,44]. In this sub-

set CDR3 is positively charged and enriched in aromatic amino acids. What is more, VH and VK domains among this group display very characteristic patterns of SHM with frequent introduction of acidic residues, typically glutamic and aspartic residues [29]. Of note, all antibodies with *IGHV4-34* sequences within the *IGHV4-34/IGKV2-30* subset recognize N-acetyllactosamine (NAL) and are autoreactive in the germline state to the determinant of the I/I blood group antigen. Carrying intact NAL-binding motifs could possibly mean that these CLL cells were bound and stimulated for clonal expansion by NAL-containing epitopes of autoAgs and exoAgs [29,50]. Furthermore, patients belonging to this subset, in comparison with other cases, are significantly younger at the time of diagnosis and follow an indolent course of malignancy, rarely needing any treatment [29,33,44]. Among the *IGHV4-34/IGKV2-30* subset distinct GEP with generalized low expression of genes involved in proliferation and regulation of the cell cycle was observed, which might explain the indolent course of CLL [33]. It has been reported that about 6% of CLL Abs express IgG as a result of deletional isotype switch. Interestingly, this feature concerns CLL IgG with *IGHV4-34* and *IGHV4-39* gene over-usage [37,62].

*IGHV3* family genes are also very frequently over-expressed in CLL cells, together with *IGHV1-69* and *IGHV4-34*. Especially *IGHV3-21*, *IGHV3-23* and *IGHV3-7* are highly used and, what is more, have the highest mutational load in CLL [19]. The *IGHV3-21* subgroup is very interesting as it has been proven that these genes are an exception from the prognostic classification of *IGHV* mutational status and were proposed as an independent adverse prognostic factor [19,55,65]. *IGHV3-21* genes are usually expressed in M-CLL, although they can also be found in U-CLL. The whole subset of *IGHV3-21*, expressed by 11% of CLL patients, is associated with poor survival. Surprisingly, among M-CLL cases median survival is even shorter compared to U-CLL [11,19,29,33,37,40,44,55,65]. About 50% of patients with *IGHV3-21* rearrangements have a very homologous, short CDR3 region, in most cases composed of 9 amino acids with a conserved motif DANGMDV. Additional analysis of this subset has shown restricted usage of *IGLV3-21/IGLJ3* light chain genes with highly homologous CDR3 [19,50,55,65]. In contrast to the *IGHV3-21/IGLV3-21* subset there is a second subgroup of *IGHV3-21* genes with heterogeneous CDR3 rearrangements and light chain gene usage, characterized by variable CD38 expression and alternating clinical outcome [29]. M-CLL patients belonging to *IGHV3-21/IGLV3-21* clearly demonstrate more aggressive leukemia, shorter time to treatment and shorter time to progression compared to other M-CLL *IGHV3-21* [19,29,55,65]. Several studies have revealed the biological basis for the disease's aggressiveness. All current well-known negative prognostic markers, such as Rai stages III and IV, abnormalities in chromosomes 11 and 17, and expression of CD38 and ZAP-70, were more frequently found in patients with *IGHV3-21/IGLV3-21* rearrangements [37,44,55,70]. Furthermore, GEP analysis of CLL with *IGHV3-21* versus non-*IGHV3-21* showed

up-regulation of genes involved in transcription, cell cycle and protein kinase activity (*PPP5C*, *E2F4*, *SMARCD1*, *ARHGEF1*, *TYMS*, *HMGAI*, *EIF4G1*, *VIPR1*, *RND1*, *PPP1R15B*) in the *IGHV3-21* subset. Also in this subset down-regulation of genes involved in repression of transcription and negative control of the cell cycle (*SPEN*, *SAP18*, *TBL1X*, *MXI-1*, *CDKN1B*, *CDKN2D*, *SMAD2*, *RUNX1*, *TGFB2*, *REERG*, *ABI1*, *PMP22*) was demonstrated [19,24,65]. In summary, all these data indicate that the *IGHV3-21* subset represents an independent biological and possibly prognostic entity of CLL [24,65].

### BCR stereotypy

The BCR is a multimeric complex formed by sIg non-covalently associated with heterodimer Ig $\alpha$ /Ig $\beta$  responsible for signal transduction (CD79a/CD79b) [7,21,61]. The BCR is the key molecule that allows mature B lymphocytes to bind to specific Ag. A number of functional *IG* genes together with the mechanisms causing Ig diversification are responsible for huge variations of BCR structure and uniqueness of the Ig expressed by B lymphocytes [21,61].

A skewed repertoire of *IGHV* genes has been described in different types of B cell lymphoproliferative disorders, but CLL is a model leukemia for understanding oncogenesis by *IG* gene analysis. SHM patterns of *IGHV* are a characteristic process for BCRs undergoing Ag selection. Therefore it is suggested that antigen is involved in the development of leukemia by stimulating proliferation of B cells expressing BCR with Ig encoded by specific genes [4,44,57].

In the past decade several studies analyzing sequences of *IG* genes in CLL cells have confirmed that unrelated and geographically distinct patients might display similar *IGHV/IGHD/IGHJ* rearrangements with highly homologous, if not identical, CDR3. To describe the striking phenomenon that distinct Ig could be repeated in different subsets of CLL patients, in 2004 Chiorazzi's group applied the term 'stereotyped BCRs' [4,31,33,44,57]. The first criteria proposed by Chiorazzi's group were as follows: (i) usage of the same *IGHV/IGHD/IGHJ* germline genes, (ii) usage of the same *IGHD* gene reading frame and (iii) VH CDR3 amino acid identity  $\geq 60\%$  in line with established bioinformatics concepts for evaluation of sequence conservation in protein sequences, such as amino acid substitution matrices [4,44]. Most studies on stereotyped BCRs are focused on *IGHV* genes, particularly HCDR3, based on the fact that the more similar primary HCDR3 sequences of independent Igs are, the more similar are their folding and specificities. Further research was performed using different techniques in order to establish new subsets of patients with stereotyped BCRs [4,57].

In 2007 Stamatopoulos et al. [59] published an analysis of 916 CLL cases of Mediterranean origin with total analysis of 927 *IG* genes. They found that 20% of CLL patients carrying stereotyped BCRs can be grouped in

one of 48 stereotyped subsets. The clustering algorithm that was used in the study has been modified, essential criteria being  $\geq 60\%$  HCDR3 similarity and length, regardless of *IGHV* gene usage. It was observed that *IGHV* genes might be phylogenetically linked and carry related germline sequences, and therefore they can produce overall homologous VH domains when recombined with identical *IGHD* and *IGHJ* genes. For example, subset #1 is characterized by usage of the *IGHD6-19* and *IGHJ4* genes in association with different *IGHV* genes (*IGHV1-2*, *IGHV1-3*, *IGHV1-18*, *IGHV1-8*, *IGHV5- $\alpha$* , *IGHV7-4-1*) [4,29,44,55,59]. Based on the clustering approach, this study revealed that BCR stereotypy exists among unmutated as well as mutated cases with the frequency exceeding 25%. What is more, many different subsets with distinct BCR stereotypy were identified and the relative size of each subset might differ significantly. In the quoted paper the authors also described the phenomenon whereby subsets with stereotyped VH CDR3 share unique molecular and clinical features that can be characterized by restricted *IG* light chain genes usage and CDR3 properties. This once again raises the possibility that a particular antigen-binding site can be crucial in determining clinical features and prognosis for subsets of CLL patients [4,29,44,59]. Thus identification of shared amino acid patterns leading to BCR stereotypy could be useful in investigating the nature of selecting antigens and furthermore might impact allocation of patients into different groups with diverse biological and clinical characteristics. Due to progressively rising Ig sequence data, Stamatopoulos's group have developed a bioinformatic method based on sequence pattern discovery that allows for the identification of VH CDR3 similarities in disregard of *IGHV/IGHD/IGHJ* gene usage. This approach enabled recognition of more distant relationships between sequences forming the basis of subset discovery, on higher levels characterized by more widely shared sequence patterns and therefore greater size. Interestingly, high level clusters were found to be characterized by striking *IGHV* repertoire restrictions with only six *IGHV* genes, namely *IGHV1-69*, *IGHV1-3*, *IGHV1-2*, *IGHV3-21*, *IGHV4-34* and *IGHV4-39*, accounting for  $>80\%$  of CLL patients [4,44].

The results of the joint Mediterranean-Scandinavian analysis of 1939 CLL patients were published in 2008. The authors employed the same criteria to assign patient sequences to subsets and reported an even higher proportion of patients belonging to different subsets. Almost 30% of sequences among the analyzed cohort were ascribed to one of 110 stereotyped subsets. The described study was focused on SHM patterns and based on the findings it has been concluded that among mutated subsets several of them showed distinctive mutations, which probably occurred due to the antigen response. These 'CLL-biased' and 'subset-biased' mutation patterns confirm the theory of antigen stimulation in the development of the *IG* gene repertoire in CLL and moreover show that beside CDR3 also other regions of V genes are involved in the antigen reaction (fig. 1).

Interestingly, a large proportion of stereotyped patients carried a recurrent somatically introduced deletion of a serine codon from VH CDR2. Comparison amongst a cohort of heterogeneous CLL *IGHV3-21* rearrangements with *IGHV3-21* sequences from non-malignant cells showed that the VH CDR2 serine codon deletion is subset-biased [4,50,55].

Subsequent studies on stereotypy were based on an algorithm developed by Stamatopoulos et al. [59] in 2007. In 2010 Darzentas et al. [20] analyzed HCDR3 sequences of 2800 patients with a slightly modified algorithm: amino acid identity between sequences was 50%, but with at least 70% similarity in terms of hydrophathy, volume properties and chemical characteristics [20,55]. Also in a recently published paper Stamatopoulos et al. [2] updated their clustering algorithm to test the Ig repertoire of 7424 CLL cases. Changes were addressed mainly to HCDR3 length and the exact location of shared patterns within the HCDR3 sequence. In this algorithm variant roles of CDR1 and CDR2 sequences in recognition and binding of Ags were taken into account [2,44].

Nevertheless, all aforementioned studies have confirmed that CLL patients can be divided into two separate categories, either with stereotyped or with heterogeneous *IG* genes in a ratio of 1:2. Furthermore, all of the performed analyses provided strong evidence for the existence of subsets with stereotyped BCRs encoded by different, although phylogenetically related, *IGHV* genes. It is worth mentioning that BCR stereotypy in CLL is fundamentally different from that established in other B cell malignancies. Defined sequence patterns among each subset are encoded by the germline sequences of the D-region and J-region of *IGHD-IGHJ* genes or by N-diversity regions of *IGHV-IGHD* and/or *IGHD-IGHJ* genes [4,9,13,44,57,61].

### Antigen stimulation

The above-described BCR stereotypy might suggest the role of superantigen in development of the disease (fig. 1). In addition to asymmetric usage of *IGHV* genes, conserved sequences in HCDR3 point to selection of conventional antigen [62]. Monoclonal antibodies found in the circulation of malignant patients were defined as similar to natural IgM Abs with low affinity to microbes, lacking in SHM and with autoreactivity/polyreactivity. Enzyme immunoassay techniques provided further information. Auto- and polyreactivity are conversely associated with the mutational load of *IG* genes in BCRs [15,37,44,51,55]. Interestingly, it was observed that monoreactivity of several M-CLL mAbs can be converted to polyreactivity by reversal of the Ab amino acidic structure to that of the germline gene, suggesting that most CLL B cells derive from normal B lymphocytes with poly- or autoreactive BCRs [35,37]. Chiorazzi's and Rosenquist's group have demonstrated that CLL mAbs could react with molecular motifs on apoptotic cells and bacteria [10,12,15,51,55].

Studies on CLL mAbs have revealed reactivity against oxidation-specific epitopes (table 1). These neoepitopes are formed during oxidation of lipids, proteins and DNA, which occurs in response to microbial infection or chronic inflammation for example. Oxidized low density lipoprotein (oxLDL) arises from conjugation of metabolites of lipid peroxidation and can be found on apoptotic cells and microbial surfaces. To date oxLDL has been reported to play a role in rheumatoid arthritis, Alzheimer's disease, atherosclerosis and hemochromatosis [16,47,55]. Recently this MDA-modified (malondialdehyde-modified) oxLDL epitope was found to be recognized by stereotyped CLL mAbs from subset #1 and subset #32 as well as non-stereotyped CLL mAbs *IGHV3-21UM*, *IGHV1-2UM* and *IGHV3-30M* [51,55]. Moreover Catera's group reported that stereotyped subset #1, #6, #8 and #9 CLL mAbs bind to oxidation epitope MDA, and these reactivities correspond with apoptotic cell binding [10,55]. These facts emphasize that most CLL mAbs have the capacity of binding different epitopes, all of which are generated in the process of oxidation [10,16,51,55].

Also microbial epitopes were found to be targeted by CLL sIgs [9,44,55]. In 2003 Binder et al. [5] revealed molecular mimicry between *Streptococcus pneumoniae* capsular polysaccharides and oxLDL (table 1). This phenomenon appears to be a crucial link between auto- and alloreactivity of mAbs [5,33,44,55]. What is more, CLL sIgs reactivity against certain Gram-positive and Gram-negative bacteria was shown, namely: *Streptococcus pyogenes*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterobacter cloacae* (table 1). Particularly high binding properties were noticed in *IGHV1-69UM* mAbs [5,44,55]. Lately, viral infections were suggested to drive subgroups of CLL patients. It was observed that persistent infections with EBV and CMV (cytomegalovirus) are associated with subset #4 *IGHV4-34* (table 1). Also *IGHV1-69* and *IGHV3-21* displayed capacity of binding to CMV pUL32 protein (table 1). Likewise HCV (hepatitis C virus) has been implicitly correlated with CLL (table 1) [33,44,45,55,60]. In a recent paper Hoogeboom et al. [38] identified a subset of patients expressing mutated *IGHV3-7* and semi-identical *IGKV2-24* genes, defined as subset V3-7Sh, recognizing  $\beta$ -(1,6)-glucan (table 1).  $\beta$ -(1,6)-glucan is a polysaccharide found in the cell wall of certain yeast, fungi, mushrooms and bacteria. It is known that  $\beta$ -(1,6)-glucan can be recognized by healthy Abs and activate the immune system. According to these data, 0.3% of all CLL cases belong to the mutated V3-7Sh subset and respond to the natural antigenic determinant shared by an extensive group of pathogens, e.g. commensal yeast species of *Candida*, *Trichosporon*, *Malassezia* and *Saccharomyces*, filamentous fungus *Aspergillus*, and also spores and conidia of *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Fonsecaea pedrosoi*, and *Rhizopus oryzae* [38].

A novel fact from the research on antigen affinity in CLL is that malignant mAbs with particular features can react with cytoskeletal proteins. With the exception of *IGHV4-54M*, which exhibits a nuclear binding pattern as observed on Jurkat cells, IgG1 mAbs react

with autoAgs of cytoplasmic and perinuclear origin [25,28,39,43,44,48,49,58]. Interestingly, all of these proteins have a functional connection with microbial infections and/or apoptotic cell eradication [55]. Nonmuscle myosin heavy chain IIA (MYHIIA) is an important protein in eukaryotic cells that plays a role in several cellular processes, including cell motion. Chu et al. [17,18] published two separate papers in *Blood* on the subject of MYHIIA. They established that MYHIIA is targeted by stereotyped mAbs from subset #6 with *IGHV1-69/IGHD3-16/IGHJ3* rearrangements (table 1). This 225 kDa protein is cleaved by caspases and rearranges in the cytoplasm, transferring to the surface of the cell during apoptosis, then recognition of MYHIIA by CLL mAbs becomes possible. Such MYHIIA-exposed apoptotic cells were defined as MEACs. Interestingly, MEAC binding was observed among U-CLL patients and high reactivity correlated with poor survival. Data obtained by Chu et al. [17,18] indicate that MEAC reactivity might determine CLL clone behavior [39,44,55,58].

Vimentin, a type III cytoskeletal protein, was found to function on the external side of the plasma membrane and to be secreted after bacterial infection. This protein not only maintains the architecture of cells, but also participates in transcellular migration, cell adhesion, cellular signaling and wound healing. Vimentin in a stress response can be exhibited on apoptotic primary T cells, neutrophils, platelets and oxLDL-binding macrophages [28,49,51,55]. Myhrinder et al. [51] found that subset #32 mAbs with *IGHV3-30.3UM* as well as mAbs with mutated *IGHV3-30* gene rearrangements react with vimentin (table 1) [51].

Filamin was the first discovered non-muscle actin binding protein. There are three isoforms of this protein: filamin A, B and C [25]. Isoforms A and B are cross linking proteins participating in over forty molecular interactions, both being exposed on the surface of apoptotic cells. Unmutated *HG3/IGHV1-2* mAbs were established to bind filamin B as well as oxLDL Ags and apoptotic Jurkat cells (table 1) [25,51,55].

Cofilin-1 is another cytoskeletal protein that possibly plays a role in CLL pathogenesis. Cofilin-1 is a member of the actin binding and depolarizing family and it has been identified in a wide range of eukaryotic organisms. In an unphosphorylated form this protein has the ability to bind both globular and filamentous actin, promoting their dynamics for motility, development, polarity as well as cytokinesis [66,68]. In final stages of apoptosis cofilin-1 is exposed in apoptotic blebs. When oxidation of the protein is inhibited, oxidant-induced apoptosis is stopped [43,48,51]. Cofilin-1 was discovered to be targeted by three leukemic mAbs: unmutated Igs belonging to subset #5 *CII/IGHV1-69*, mutated *232B4/IGHV3-48* and subset #2 mAbs *rIGHV3-21M* (table 1) [51,55].

Despite strong evidence supporting the role of Ags stimulation in CLL ontogeny, it is still not clear when the

Ags reaction occurred in malignancy development and whether it was an incident taking place only once at a certain point of neoplastic transformation or it is an ongoing process. Sutton et al. [64] conducted an analysis of intraclonal diversification (ID) within *IG* genes through ongoing mutational activity. Results from ID study in other B-cell disorders point to ongoing SHM, proving that Ags constantly interact with neoplastic cells. The CLL ID data showed a little evidence for ongoing Ags stimulation in IgG and IgM B cells with the exception of subset #4, where all cells showed high levels of ID. These findings were confirmed by ID analysis of light chain genes in subset #4 cells. Therefore it seems likely that at least in particular subsets ongoing stimulation by Ags takes place after malignant transformation [46,52,55,64].

### Autoimmune events in CLL

Patients with CLL might develop autoimmune disorders, primary directed against hematopoietic cells. Autoimmune complications probably appearing as a result of an impaired immune system frequently are severe and life-threatening for CLL patients and require medical intervention even when CLL is still quiescent and does not need specific therapy [8,31,34,42,53,67].

Autoimmune hemolytic anemia (AIHA) is strongly associated with CLL, as are immune thrombocytopenia (ITP) and rarely pure red cell aplasia (PRCA). The occurrence of immune cytopenias has been reported in many recent studies to range up to 25%. The most common disease is AIHA, while ITP and PRCA are more rare [22,36].

There are a few clinical and biological characteristics of CLL that have been associated with an increased risk of autoimmune cytopenia. It is known that advanced stage of CLL and age of patients correlate with more frequent occurrence of AIHA [3,36]. AIHA and ITP have also been associated with poor prognostic factors, such as unmutated *IGHV* gene, high ZAP-70 expression and increased serum  $\beta$ 2-microglobulin level [36].

In the case of AIHA and ITP autoimmunity can be triggered by bystander non-malignant B cells or CLL cells. The etiology of anti-RBC Abs in CLL is not fully described yet, but several mechanisms have been proposed. Non-neoplastic B cells via T-cell mediated manner produce polyclonal high-affinity IgG that targets RhD, RhCcEe and B3 epitopes in AIHA and GpIIIb/IIIa in case of ITP. Then opsonized cells are destroyed via antibody-dependent cellular cytotoxicity. It is unknown which normal cells are involved in this process, but significant increases of CD4<sup>+</sup> T cells and NK cells in peripheral blood have been observed [22,63]. It has also been suggested that autoreactive T helper cells occurrence might be induced by autoAgs directly presented by CLL cells. In that case tumor necrosis factors, such as B-cell activator factor (BAFF) and proliferation-inducing ligand (APRIL), probably play an important role [22,36,63].

**Table 1.** Antigen reactivities reported in CLL

Antigen	Stereotyped BCRs	Non-stereotyped BCRs	References
<b>oxLDL</b>	<i>IGHV1-18UM</i>	subset#1	10, 51
	<i>IGHV1-2UM</i>	subset#1	
	<i>IGHV4-34M</i>	subset#4	
	<i>IGHV1-69UM</i>	subset#6	
	<i>IGHV4-39UM</i>	subset#8	
	<i>IGHV3-21UM</i>	subset#9	
	<i>IGHV1-2UM</i>	subset#28	
<b><i>S. pneumoniae</i>, <i>S. pyogenes</i>, <i>E. faecium</i>, <i>E. faecalis</i>, <i>E. cloacae</i></b>	<i>IGHV3-30.3UM</i>	subset#32	5, 51
<b>CMV</b>	<i>IGHV4-34M</i>	subset#4	44, 45, 60
<b>EBV</b>	<i>IGHV4-34M</i>	subset#4	44, 45
<b>HCV</b>	<i>IGHV4-59M</i>	subset#13	44
<b>β-(1,6)-glucan</b>	<i>IGHV3-7M</i>	V3-7Sh*	38
<b>MYHIIA</b>	<i>IGHV1-2UM</i>	subset#1	17, 18, 44
	<i>IGHV1-3UM</i>	subset#1	
	<i>IGHV1-18M</i>	subset#1	
	<i>IGHV1-69UM</i>	subset#6	
	<i>IGHV4-34M</i>	subset#4	
	<i>IGHV4-39UM</i>	subset#8	
	<i>IGHV1-69UM</i>	subset#9	
	<i>IGHV3-21UM</i>	subset#9	
<b>Vimentin</b>	<i>IGHV1-2UM</i>	subset#1	44, 51, 56
	<i>IGHV1-3UM</i>	subset#1	
	<i>IGHV1-5-7UM</i>	subset#1	
	<i>IGHV4-39UM</i>	subset#8	
	<i>IGHV3-30.3UM</i>	subset#32	
<b>Filamin B</b>	<i>IGHV1-5-7UM</i>	subset#1	51, 56
<b>Cofilin-1</b>	<i>IGHV3-21M</i>	subset#2	51
	<i>IGHV1-69UM</i>	subset#5	

\*defined by Hoogeboom et al. [38]; BCR – B cell receptor; *IGHV* – immunoglobulin variable heavy chain; oxLDL – oxidized low density lipoprotein; *S. pneumoniae* – *Streptococcus pneumoniae*; *S. pyogenes* – *Streptococcus pyogenes*; *E. faecium* – *Enterococcus faecium*; *E. faecalis* – *Enterococcus faecalis*; *E. cloacae* – *Enterococcus cloacae*; CMV – cytomegalovirus; EBV – Epstein-Barr virus; HCV – hepatitis C virus; MYHIIA – nonmuscle myosin heavy chain IIA.

In the case of PRCA in CLL the mechanism is not well defined, but it appears to be humorally mediated [22].

Non-hematological autoimmune events in CLL are rare. To date in numerous reports cases of rheumatoid arthritis, systemic lupus erythematosus, ulcerative colitis, pernicious anemia, Sjogren’s syndrome, Grave’s disease, nephritic syndrome, pemphigus, glomerulosclerosis and paraneoplastic neurological syndrome have been described [34,53].

The process underlying development of autoimmune disorders in CLL is complex. It requires interactions

between neoplastic cells, pathologically functioning T cells, the microenvironment and the immune system. Occurrence of autoimmune disorders in CLL is not fully understood yet, but autoreactivity against formed elements of blood appears to be related to the ability of malignant cells to process and present Ags derived from blood cells [3,36].

#### Alternative approach to CLL pathomechanism

Recently Duhren-von Minden et al. [23] presented a novel theory for CLL development. According to their data, CLL is driven by antigen-independent, cell-autono-

mous signaling. Apparently HCDR3 interacts with intrinsic motifs, such as a sequence in the second framework region (FR2), within the same or nearby BCRs, consequently inducing cell-autonomous signaling independent of extrinsic epitopes. Moreover, HCDR3 sequences derived from leukemic BCRs are able to transfer autonomous signaling capacity to other BCRs. Irrespectively of *IGHV* mutational status and BCR stereotypy, in all CLL sIgs autonomous signaling was observed [13,23]. In *Cell Research* Chiorazzi and Efremov commented on this newly discovered signaling as Ag-dependent. According to the authors, the described interaction of HCDR3 with epitopes can be considered as an example of autoreactivity, where selection of B cells with HCDR3 sequences recognizing FR2 autoepitopes takes place [13]. Therefore the newly discovered interaction of HCDR3 sequences with the FR2 region may be a confirmation of the current antigen-driven CLL development hypothesis.

## CONCLUSIONS

Aforementioned evidence indicates the importance of Ag stimulation in the leukemogenesis of CLL. Analysis

of BCRs has provided considerable information about malignant cells and the possible mechanism underlying the biology of the disease. Available data show that most likely CLL cells after BCR-mediated stimulation, irrespectively of *IGHV* mutational status, express activated membrane markers and secrete various cytokines similarly to MZ Ag-experienced B cells, therefore promoting expansion of respective CLL clones. The BCR stereotypy and studies on Ag reactivity suggest that CLL cells originate from B cells involved in rather innate functions, such as MZ B cells, as expressed Abs bind to autoAgs exposed on apoptotic cells or conserved microbial epitopes. The thesis on Ag stimulation via BCR is also supported by skewed usage of *IGHV* genes found in neoplastic BCRs. Prognostic value of *IGHV* mutational status and existence of subsets of patients expressing stereotyped BCRs enforce this theory [7,31,44,55].

Although new facts in regard of CLL biology and cell origin are continuously emerging, the primary cause of leukemia still needs to be investigated for better understanding of CLL etiology.

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