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Restricted antigen recognition in B cell chronic lymphocytic leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) cells are considered to be derived from antigen-exposed B cells. To further explore the antigen-driven selection behind the leukemogenesis of CLL, we performed immunoglobulin (Ig) specificity screening of 7 CLL cell lines and 23 primary CLL clones from patient peripheral blood. We also included a recombinant monovalent monoclonal antibody (mAb) belonging to a subset of CLL cases with identical or semi-identical heavy chain complementarity determining region 3 (HCDR3) of the IGHV3-21 gene rearrangement. We found CLL mAb specificities against vimentin, filamin B, cofilin-1, proline-rich acidic protein 1, cardiolipin, oxidized low density lipoprotein and *Streptococcus pneumoniae* polysaccarides. These molecules are functionally associated with microbial infection and/or apoptotic cell removal. An antigen-driven selection would therefore imply that CLL B cell precursors are involved in the elimination and scavenging of pathogens and apoptotic cells, which could trigger the development of the disease.

The limited *in vitro* survival of CLL cells makes Epstein-Barr virus (EBV) immortalization of CLL cells a useful experimental model for studies on antibody-specificity screening. Considering the intricate procedure of EBV transformation of CLL cells and the many false cell lines used worldwide, we also wanted to characterize and evaluate the authentic origin of several previously established CLL cell lines and their normal lymphoblastoid counterparts. Three of the CLL cell lines tested were truly authentic (I83-E95, CLL-HG3 and CII), two had features of a biclonal Ig expression (232B4 and WaC3CD5+), one was only tentatively verified (PGA-1), whereas one cell line could not be verified (EHEB) due to lack of original patient cells for comparison. Two of the presumed normal lymphoblastoid cell lines tested were shown to be a neoplastic CLL clone. This study emphasizes the importance of

proper cell line authentication and we will continue to verify additional cell lines not yet proven authentic.

In conclusion, we provide evidence for natural Ab production by CLL cells and suggest that these cells might be derived from B cell precursors involved in the innate immunity and, thus, providing a first-line-defence against pathogens and in elimination of apoptotic cells.

Keywords: chronic lymphocytic leukemia, (auto)antigens, CLL cell lines

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List of papers

Paper I

Lanemo Myhrinder A*, Hellqvist E*, Sidorova E, Söderberg A, Baxendale H, Dahle C, Willander K, Tobin G, Bäckman E, Söderberg O, Rosenquist R, Hörkkö S, Rosén A. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. *Blood.* 111:3838-48; 2008

* Shared first authorship.

Paper II

Lanemo-Myhrinder A, Hellqvist E, Jansson M, Hultman P, Nilsson K, Jonasson J, Rosenquist R and Rosén A. Molecular authenticity of neoplastic and normal B cell line pairs established from chronic lymphocytic leukemia patients. Manuscript.

Abbreviations

Ab	antibody
Ag	antigen
AID	activation induced cytidine deaminase
ATF-2	activating transcription factor-2
BAFF	B cell activating factor
BCR	B cell receptor
Bp	base pair
C	constant
CDR	complementarity determining region
CLL	chronic lymphocytic leukemia
CLP	common lymphoid progenitor
CSR	class switch reaction
D	diversity
DNA-PK	DNA-dependent protein kinase
EBNA	EBV encoded nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
FISH	fluorescence *in situ* hybridization
Fo	follicular
FR	framework region
GC	germinal centre
HC	heavy chain
HSC	hematopoietic stem cell
IG	immunoglobulin
IGH	immunoglobulin heavy chain

IGK	immunoglobulin kappa chain
IGL	immunoglobulin lambda chain
J	joining
κ	kappa
λ	lambda
LC	light chain
LCL	lymphoblastoid cell line
LMP1	latent membrane protein-1
mAb	monoclonal antibody
MALDI	matrix assisted laser desorption ionization
MBL	monoclonal B cell lymphocytosis
MZ	marginal zone
N-nucleotide	non-template nucleotide
PCR	polymerase chain reaction
PRAP-1	proline-rich acidic protein-1
P-nucleotide	palindromic nucleotide
RAG	recombination activating gene
RSS	recombination signal sequences
SHM	somatic hypermutation
STR	short tandem repeat
TdT	terminal deoxynucleotidyl transferase
TOF	time-of-flight
V	variable
Zap70	zeta-chain (T cell receptor) associated protein kinase 70kDa

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Introduction

Chronic lymphocytic leukemia

B cell chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world, with approximately 500 new cases diagnosed annually in Sweden. It is more common in men than women and the risk of developing CLL is increased with age and the median age at diagnosis is 65 years. Most cases of CLL are sporadic, although familial CLL has been reported, with first degree relatives having higher risk of developing CLL [1,2]. The disease is characterised by proliferation and accumulation of CD5, CD19, $CD20^{low}$ and surface immunoglobulin $(Ig)^{low}$ expressing B cells, with a balanced homeostasis in patients with stable lymphocyte count and imbalanced homeostasis in patients with increasing lymphocyte count [3]. There is no unique genetic defect identified in CLL, although over 80% of the patients have cytogenetic lesions [4]. The most common are trisomy 12, del(11q), del(13q), del(17p), del(6q) and chromosomal translocations, with del(17p) having a worse prognosis often resistant to standard chemotherapy [4].

CLL is distinguished from other B lymphoproliferative disorders by using the scoring system described by Matutes et al., in which 4 or 5 of the $CD5^{+}$, $CD23^{+}$, $FMC7^{-}$, $CD22^{low}$ and surface Ig^{low} markers should be present at diagnosis [5]. CLL is an incurable and heterogeneous disease clinically divided into two subgroups based on the presence or absence of somatic mutations in the variable region of the immunoglobulin heavy-chain (IGHV) gene. Patients with mutated IGHV genes have an indolent variant of the disease, while unmutated IGHV genes are associated with a more aggressive clinical course, although exceptions

exist [6,7]. Therapy is given to symptomatic and advance-stage disease patients, with monoclonal antibody (mAb) therapy in combination with chemotherapy or haematopoietic stem cell transplantation being most common [8]. The heterogeneity in the clinical behaviour of the disease makes prognostic prediction of the clinical outcome in early disease very important. FISH analysis and the staging system Rai and Binet are used today [9,10]. The use of IGHV gene mutation analysis is widely accepted, but it is far from being used routinely due to the time consuming and expensive method. Other markers, such as Zap70 and CD38, which are correlated with unmutated CLL [7,11,12] and an inferior clinical progression have been suggested [12-14]. The correlation is not absolute, however, making these markers more suitable as independent prognostic markers.

Antigens in CLL

The origin of the CLL cell and the mechanisms behind the malignant transformation of normal B cells into CLL cells is unknown. The presence of mutations in the IGHV genes in about half of the CLL patients [15], as well as the gene expression profile [16] and surface membrane phenotype [17] identified in both unmutated and mutated CLL, points at an antigen-experienced history of the B CLL cell. A unique phenomenon for CLL is the biased usage of certain IGHV genes and the presence of subgroups of CLL with identical or stereotyped heavy chain complementarity determining region 3 (HCDR3) sequences [6,15,18-21]. Considering the low probability (10^{-12}) of two distinct B cells having identical B cell receptors (BCRs) by pure chance and the importance of HCDR3s in antigen binding, the identification of identical and stereotyped HCDR3 strongly implies an antigen-selection in CLL. One may therefore assume that the CLL progenitors are exposed to antigen previously or during their transformation into leukemic cells, thus the identification of the Ig

specificity would contribute to the mapping of the leukemic process. With greater knowledge of the origin and malignant transformation events underlying the disease, better therapies could be achieved and the prognosis would be improved. In order to understand and discuss the possible origin of the CLL cell, a general background of the IG genes and B cell development will be given in the following sections.

Structure and function of the Immunoglobulin molecule

The basic structure of Ig molecules comprises four polypeptide chains, two light (L) and two heavy (H) chains chemically bounded by disulfide bridges (Figure 1A) [22-24]. In humans, the L chains contain one N-terminal variable (V) and one C-terminal constant (C) domain, whereas the H chains are composed of one N-terminal variable and three (IgG, IgA, IgD) or four (IgM and IgE) C-terminal constant domains. There are two types of L chains, κ chains and λ chains. The constant part of the Ig molecule belongs to one of five isotypes, i.e. μ (IgM), γ (IgG), α (IgA), ε (IgE), δ (IgD). The two to three constant domains of the C-terminal portion of the two H chains constitute the Fc-fragment. In humans, the germ-line V domain of the IGH chain is coded by the V (variable), D (diversity) and J (joining) gene segments located on chromosome 14 (Figure 1B). The L chain V domain is encoded by V and J segments located on chromosome 2 for the κ chain and on chromosome 22 for the λ chain. The basic function of the variable region is the binding of antigenic epitopes via hypervariable subregions referred to as complementarity determining regions (CDR1, CDR2 and CDR3), which are flanked by less variable subregions called framework regions (FR1, FR2, FR3 and FR4). The CDR1 and CDR2 regions are coded by V regions, whereas CDR3 is coded by the V-(D)-J recombination junctions and contributes most to the diversity of the antigen-binding site. The principle functions of the

Fc-fragment are receptor-mediated phagocytosis, cytotoxicity and release of inflammatory mediators, receptor-mediated transport through mucosa and placenta and complement activation. The B cell receptor (BCR) is composed of membrane Ig and the co-receptors CD79a and CD79b, which transmit signals that drive the B cell response.

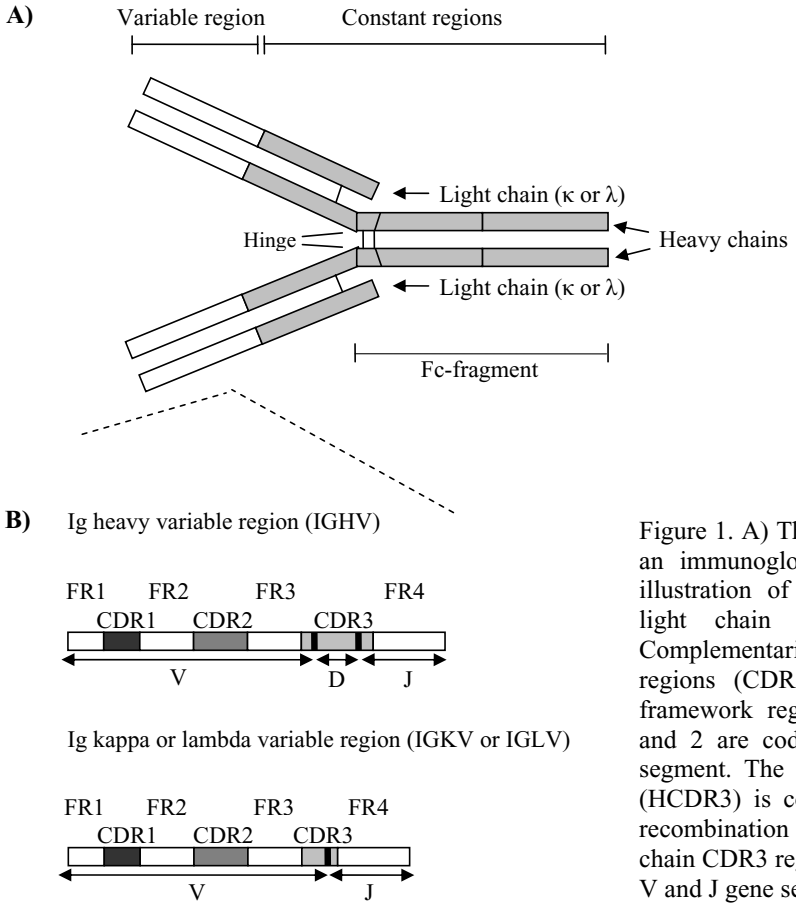

Figure 1. A) The basic structure of an immunoglobulin. B) Detailed illustration of the Ig heavy and light chain variable domains. Complementarity determining regions (CDRs) are flanked by framework regions (FRs). CDR1 and 2 are coded by the V gene segment. The heavy chain CDR3 (HCDR3) is coded by the V(D)J recombination junction. The light chain CDR3 region is coded by the V and J gene segments.

IG gene rearrangement and B lymphocyte development

Antigen independent B cell maturation in the bone marrow

B lymphocytes derive from pluripotent hematopoietic stem cells (HSC) in fetal liver and fetal/adult bone marrow [25]. The subsequent development of naïve B cells from common lymphoid progenitors (CLPs) is a multistep process characterized by the combination of V, (D), J and C genes into functional heavy and light chains, referred as IG gene rearrangement (Figure 2) [22-24]. There are 38-46 functional IGHV genes, 23 IGHD genes, 6 IGHJ genes and 9 IGHC genes that can be combined into a functional IG heavy chain. 34-38 functional IGKV, 6 IGKJ and one IGKC gene that can be rearranged into functional IG kappa light chains, and 29-33 IGLV, 4-5 IGLJ and 4-5 IGLC functional genes that can be rearranged into IG lambda light chains. The first B lineage cells are called progenitor B cells (pro-B cells). They rearrange the IGHD-HJ segments in the early stage, followed by IGHV to IGHD-HJ joining in the later stage and become precursor B cells (pre-B cells). The heavy chain is coexpressed with a surrogate light chain on the cell surface of pre-B cells and constitutes the pre-B cell receptor (pre-BCR). The pre-BCR stimulates proliferation of the pre-B cell and initiates the V-J rearrangement of the light chain in the small pre-B cell.

Antigen dependent B cell maturation in the peripheral compartment

Once a complete IgM molecule is expressed on the B cell surface, the cell is defined as an immature B cell. Immature B cells that survive selection for self tolerance in bone marrow and periphery undergo further differentiation and co-express IgD and IgM and become mature or naïve B cells, which circulate between blood and secondary lymphoid tissues [26,27]. If the naïve B cell encounters and becomes activated by a T cell-dependent foreign antigen it starts

to proliferate and differentiate into Ab producing plasma cells and memory B cells in the proliferative centra of secondary lymphoid organs [26,27]. This process is called germinal center (GC) reaction and is characterized by clonal expansion, somatic hypermutation (SHM) of IG V genes, class switch recombination (CSR) at the IGH locus and an additional selection process of the increased affinity maturation [26]. Somatic hypermutations are characterized by point mutations through base exchanges in all three CDRs of the IG V regions [28]. The number of these mutations is increased during secondary and tertiary Ab response thereby enhancing the chance to generate high affinity Abs. B cell clones with high affinity Ig molecules are preferentially expanding, giving rise to affinity maturation of Abs. CSR occurs at the intronic switch sites between the VH-D-JH unit and the IGHC gene segment and results in the expression of another isotype of the constant domain and, hence another effector function of the antibody [29,30]. The affinity induced deaminase (AID) is essentially involved in both CSR and SHM [28-30]. B cells encountering T cell-independent antigens outside germinal centers differentiate and proliferate into plasma cells without acquiring SHM or CSR [26].

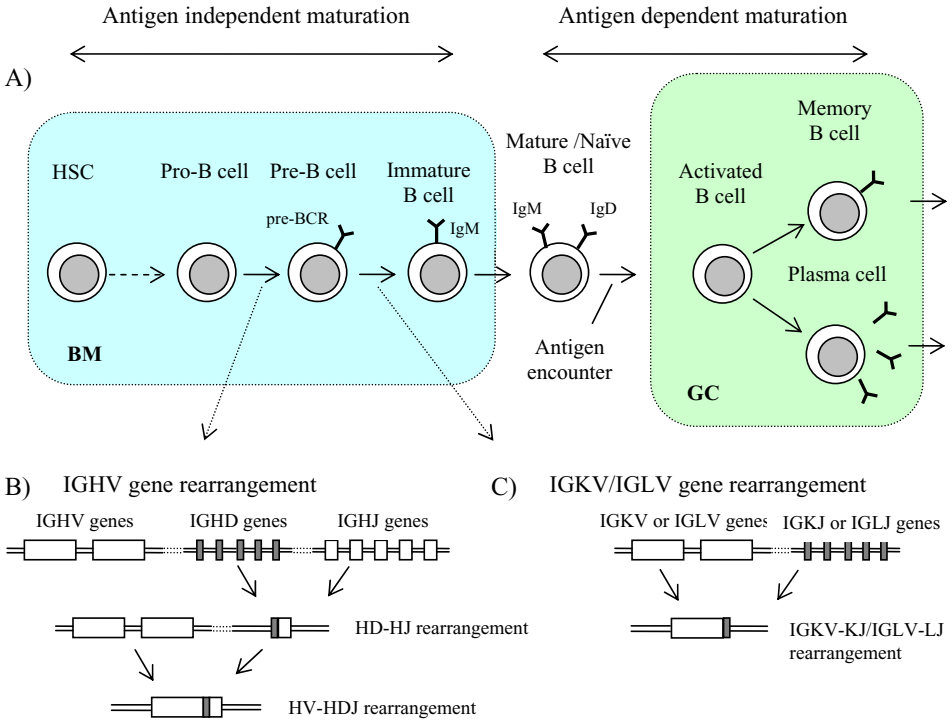

Figure 2. A) Antigen independent and dependent B cell development from pluripotent hematopoietic stem cells in bone marrow to terminal differentiated memory B cells and plasma cells in periphery. B) IG gene rearrangement of heavy chain in the pro-B cell. C) IG gene rearrangement of light chain in the pre-B cell. HCS=hematopoietic stem cell; BM=bone marrow; GC=germinal centre; IGHV=IG heavy variable chain; IGKV=IG kappa variable chain; IGLV=IG lambda variable chain.

Regulation of IG gene rearrangement and B cell development

The IG gene rearrangement is regulated by a recombinase complex that includes the recombination-activating genes 1 and 2 (RAG1 and RAG2), with heavy chains being rearranged before light chain and kappa light chain generally before lambda light chain [22,24,31,32]. RAG1 and RAG2 introduce double strand breaks into recombination signal sequences (RSSs) flanking each V, D and J gene segment and combine these in a 12/23 rule. The complete recombinase complex includes next to RAG1 and RAG2 the ubiquitously

present DNA-dependent protein kinase (DNA-PK), DNA-ligase IV, Ku70/Ku80 and terminal deoxynucleotidyl transferase (TdT), which participate in the joining process of the coding sequences by N- and P-nucleotide addition and DNA ligation. Once a successful rearrangement of a heavy or a light chain has occurred, no further rearrangement will take place, which ensures that only one functional heavy and light chain is expressed by B cells. This process is called allelic exclusion and is regulated by the activation and inactivation of RAG1 and RAG2, which is influenced by the pre-BCR expressed by pre-B cells. In the majority of cases, V, (D), J segments do not join in phase with the reading frame, resulting in a non-productive rearrangement. B cells that are self-reactive or have non-productive rearrangements passes several rounds of additional light chain rearrangement and IGHV-DJ rearrangement until a functional or non-self light and heavy chain has been produced, a process that is known as receptor editing and IGHV replacement. If no functional or non-self rearrangement takes place, the B cell will undergo apoptosis.

Lymphocyte development is positively and negatively regulated by various cytokines and transcription factors. The transcription factors Pax5, E2A and EBF are crucial for early B cell development [33], while the level of BCR signaling, Notch 2 and B cell activating factor (BAFF) is critical for development of the three functionally distinct mature B cell populations in a later stage; B-1 B cells, marginal zone (MZ) B cells and follicular (Fo) B cells [27,34]. Clonal expansion of one single neoplastic B cell causes leukemia/lymphoma development.

Generation of antibody diversity

The first stage of Ab diversity is generated by the many combinatorial events that can occur in the heavy and light chain loci. Although not all IGHV, IGKV

and IGLV genes are used with the same frequency and combine to stable Ig molecules, the number of productive heavy- and light chain combinations may account for approximately $2x10^6$ possibilities [22,35]. The joining process of the coding sequences is imprecise which further generates different productive combinations, especially in the CDR3 hypervariable region, where addition of N- and P-nucleotides through TdT activity increases the Ab diversity significantly. This junctional diversity as well as SHM further increases the Ab variability to 10^9-10^{12} possible combinations [22,35]. The probability of finding the same light and heavy chain rearrangement in two independent B cells by chance is, thus, 10^{-12}.

EBV immortalization of CLL cells

Proliferating immortalized lymphoblastoid cell lines (LCLs) are readily established by *in vitro* infection of B lymphocytes with Epstein-Barr virus (EBV) [36]. CLL cells, however, are considered resistant to EBV transformation due to inability of inducing expression of the viral plasma membrane protein, latent membrane protein 1 (LMP1), in the EBV infected CLL cell, even though the viral Epstein Barr nuclear antigen (EBNA) proteins are expressed. EBNA2 is the main regulator of the LMP1 expression and it binds to the LMP1 promotor in a complex with other proteins [37]. The absence of LMP1 is explained by the inability of EBV to induce expression of c-Jun and activating transcription factor-2 (ATF-2), two proteins known to operate in the complex with EBNA2 [38]. The lack of c-Jun and ATF-2 in *in vitro* cultured CLL cells, thus, prevent EBNA2 to bind to the promoter and induce the expression of LMP1, resulting in prevented establishment of immortalized CLL cell lines. A few CLL cell lines have, however, been successfully established [39-43]. Wendel-Hansen et al. presented a strategy in which EBV infection was followed by exposure to a

cytokine mix known to induce proliferation. This combination induced expression of both EBNA and LMP1 and several CLL cell lines were successfully established [43].

Aims

The aim of the study was to investigate the antigen-driven selection in the leukemogenesis of chronic lymphocytic leukemia. More specifically:

Paper I – To characterize in detail the antigen specificity of CLL cells using EBV transformed CLL cell lines and *ex vivo* CLL cultures.
Paper II – To investigate the genotype and phenotype of 12 CLL derived lymphoid cell lines, of which several were used in paper I, and explore their authentic neoplastic origin.

Material and Methods

Material

Twelve cell lines derived from seven CLL patients were previously established in our laboratory or collected from others [39-43]. These were I83-E95 (CLL), I83-LCL (LCL), CII (CLL), CI (LCL), WaC3CD5^{+} (CLL), Wa-osel (LCL), 232B4 (CLL), 232A4 (LCL), PGA1 (CLL), PG/B95-8 (LCL), CLL-HG3 (CLL) and EHEB (CLL). The CLL cell lines where analyzed for their Ig specificity in paper I, whereas all cell lines where characterized and analyzed for their authentic neoplastic origin in paper II. A recombinant monovalent IGHV3-21 mAb was also included in paper I, as well as 23 primary CLL clones from peripheral blood mononuclear cells (PBMCs) of CLL patients attending the Hematology Clinic of Linköping University Hospital.

Methods

A general overview of the principles and procedures of the methods used in this thesis will be given in the following section. Details are available in paper I and II.

Fluorescence in situ hybridization (FISH) analysis

FISH was used in paper II to detect cytogenetic abnormalities of the cell lines.
Methodological principles and procedures: Absence or presence of specific DNA sequences are detected and localized in order to identify chromosomal abnormalities [44]. Interphase or metaphase chromosome preparations are

attached to microscope slides. Repetitive DNA sequences are blocked by incubating chromosomes with short DNA segments. Fluorescence conjugated probes are hybridized to the chromosomes and the results are visualized and quantified by using a microscope.

Immunoglobulin gene analysis

IG gene analysis was used for characterization and verification of authenticity of the cell lines in paper II.

Methodological principles and procedures: The IGHV (and IGKV/IGLV) region of DNA or RNA/cDNA is sequenced to provide clinical information related to prognosis in CLL, e.g. mutational status [22]. Extracted DNA or RNA is amplified by PCR and the IG clonality is examined by separating the PCR products by gel electrophoresis. The products are sequenced using PCR and a DNA sequencer and the resulting IGHV sequence is submitted for database search. The IMGT®, the international ImMunoGeneTics information system®, (http://imgt.cines.fr) [45] is the international reference in immunogenetics and immunoinformatics.

High resolution short tandem repeat (STR) analysis

STR analysis was used in paper II in order to determine whether the CLL clone and its normal counterpart originated from the very same patient.

Methodological principles and procedures: Short tandem repeats (STRs) is a class of DNA polymorphisms with repetitive units of 2 to 10 nucleotides adjacent to one another [46]. By analysing several STR loci and counting the number of specific STR repetitive units in a given locus a unique genetic profile of an individual is provided. PCR is used to amplify specific polymorphic regions of extracted nuclear DNA. The PCR fragments are analysed for the

number of STR sequences by gel electrophoresis or capillary electrophoresis. The size of the fragment reflects the number of repeats in the STR loci.

Flow cytometry

Flow cytometry was used for phenotype characterization of the cell lines in paper II and for analysis of Ig specificity of selected CLL mAbs towards apoptotic Jurkat cells as well as vimentin expressing macrophages in paper I.

Methodological principles and procedures: Fluorochrome conjugated Abs are used for analysis of single cells expressing one or several specific proteins, which allows simultaneous multiparametric analysis for counting, examining and sorting cells suspended in a fluid [47] . The combination of scattered and fluorescence light provide information about cell size (forward scatter), cell granularity (side scatter) and protein expression (fluorescence signals). Cells are immunostained in solution, washed and analysed by a flow cytometer.

Immunostaining

Immunostaining was used for CLL mAb specificity screening of paraffin-embedded human tissue samples and single cells, as well as for detection of vimentin expression on cell surface of CLL cells in paper I.

Methodological principles and procedures: Abs are used for detection of specific proteins in cell or tissue sections using fluorescence or non-fluorescence staining techniques [47]. Cells/tissues are fixed and blocked for reduction of unspecific binding. Abs are applied to the sections and the results are visualized by using microscopy or autoradiography. Antigen-retrieval may be used to increase antigen detection by breaking some of the cross-linkings created during fixation, allowing hidden antigens to be exposed.

Western blot analysis

Western blot analysis was used for further mAb-specificity screening of cell extract or protein macroarrays and for reconfirmation of identified CLL specific antigens in paper I.

Methodological principles and procedures: Abs are used for detection of specific proteins extracted from cell or tissue samples [47]. Proteins are separated by size using gel electrophoreses, blotted onto synthetic membranes and blocked for reduction of unspecific binding. Peroxidase conjugated Abs are commonly used for protein detection and are visualized by chemiluminescent reactions.

Enzyme-Linked ImmunoSorbent Assays (ELISA)

ELISA was used for mAb-specificity screening and antigen-specificity reconfirmation in paper I and for quantification and isotype determination of secreted Ig in paper I and II.

Methodological principles and procedures: ELISA allow for the detection of single antigens or Abs in a sample [47,48]. Abs/antigens attached to the bottom of a well are incubated with the antigen/Ab to be measured. Enzyme-linked Abs are used together with a substrate that causes a signal or colour change that is quantified by a spectrophotometer.

Affinity purification of proteins

Affinity purification was used to isolate one of the CLL specific antigens identified in paper I.

Methodological principles and procedures: Affinity purification utilizes specific binding interactions between molecules to isolate a target from a mixture of proteins [49]. The protein sample is incubated with the immobilized ligand

allowing for capture of the target molecule. Unbound samples are washed away from the solid support so that only proteins having specific binding affinity to the ligand are isolated. The target molecule is eluted from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs. A fraction of pure protein is retrieved.

Mass spectrometry

Mass spectrometry (MS) was used to identify the CLL mAb-specific antigens in paper I.

Methodological principles and procedures: Mass spectrometry provides structural characterization, such as peptide masses, peptide sequences and post-translational modifications of proteins [50]. The information is used to identify the protein by database searching. The most common techniques used to volatize and ionize the proteins and peptides are matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), which utilize laser and high voltage for ionization, respectively. The ionized proteins/peptides are then sorted in a mass analyzer. The time-of-flight (TOF) mass analyser measures the mass-to-charge (m/z) ratio by determining the time required for ions to reach the detector. The quadrupole mass filter commonly used with ESI also determines the m/z ratio of a protein or peptide but also allows for further fragmentation to yield information of the peptide sequence. Extracted proteins are separated by 1D or 2D gel electrophoresis and the protein of interest is excised from the gel. The protein is in-gel digested using proteases and the resulting peptides are eluted and concentrated. The peptides are analysed by MS and the protein is identified by matching a list of experimental peptide masses with theoretically calculated peptide masses in a database.

Luminex

Luminex was used for CLL mAb-specificity screening of *S. pneumoniae* capsular polysaccharides in paper I.

Methodological principles and procedures: The Luminex system is a flow cytometry based tool that permits the simultaneous measurement of many analytes from a single sample and is developed from traditional ELISA [48,51]. The technology uses colour-coded microspheres coated with different kinds of biomolecules that binds the analytes of interest. The internal dye of the microsphere and the reporter dye are detected by lasers in the Luminex analyser. The reporter molecule quantifies the biomolecular interaction that has occurred at the microsphere surface. Microspheres coated with capture molecules are incubated with the analytes of interest and the interactions are detected by fluorescent conjugated Abs by passing the samples through a Luminex analyser.

Results and Discussion

The antigen-driven selection theory behind the evolution of CLL is a generally accepted hypothesis, but it has mainly been based on genetic and phenotypic data [6,15-21]. We used mAbs derived from CLL clones, either from peripheral blood of CLL patients or CLL cell lines (paper I) and showed that CLL mAbs were specific for vimentin, filamin B, cofilin-1, proline-rich acidic protein-1 (PRAP-1), cardiolipin, oxidized low density lipoprotein (oxLDL) and *Streptococcus pneumoniae* polysaccharides. These molecules or motifs are functionally associated with microbial infections and/or apoptotic cell removal [52-64]. An antigen-driven selection would therefore imply that precursor CLL B cells are involved in the elimination and scavenging of pathogens and apoptotic cells and that antigens may trigger the development of the disease. The CLL mAbs are, thus, reminiscent of natural Abs, which are described as low affinity polyreactive and autoreactive Abs lacking SHM. Natural Abs are involved in the scavenging/elimination of apoptotic cells and autoantigens as well as providing a first-line-defense against bacterial and viral infections [65,66]. Other reports on natural Ab production in CLL have been published [67-70] and Catera et al. recently confirmed our findings by identifying CLL mAb specificity against apoptotic cells and oxidized neoantigens, reminiscent of epitopes exposed on pathogens [71]. The restricted antigen recognition in mutated and unmutated CLL clones as well as the similar gene expression profile of the two CLL subsets, suggests a unique cellular origin of the CLL precursor cell. The self-renewing and long-lived $CD5^+$ B1-a B cell population described in mouse models is the main producer of natural Abs [66]. The CLL cells, at least the unmutated CLL cases, which lack SHM and produce polyreactive IgM Abs, have been proposed to arise from a human B-1a

equivalent due to the analogous CD5 expression. However, such a population has just tentatively been described in humans [72] and gene expression profile contradicts a human peripheral $CD5^+$ B cell population origin of CLL cells [11,16]. Another potential CLL progenitor cell is the MZ B cell. Similar to CLL cells, they are often autoreactive, express a BCR repertoire against oxLDL and produce IgM Abs against T cell independent type 2 antigens such as bacterial polysaccarides [27,34,73,74]. Unlike B1 cells, MZ cells express both unmutated and mutated IGHV genes [73,74], as do CLL cells. The $CD5^-$ phenotype displayed by MZ B cells [74], however, contradicts such cellular origin of CLL cells. On the other hand, CD5 has been described as a B cell activation marker [75] and the CD5 expression by CLL cells might therefore not necessarily be a prerequisite of the CLL progenitor. Clonal expansion of B cells phenotypically reminiscent of CLL cells, called CLL-phenotype monoclonal B-cell lymphocytosis (MBL), has been identified in 5.1% of healthy individuals of 62 to 80 years of age [76]. A biased usage of IGHV3-07, IGHV3-23 and IGHV4-34, as also seen in mutated CLL cases, and an overrepresentation of a mutated IGHV repertoire (88%) was also demonstrated in MBL. There is a higher incidence of MBL in relatives to CLL patients as compared to the general population [77,78], which would suggest that MBL is a possible CLL progenitor population. However, not all MBL with CLL-phenotype does progress into CLL and it is unknown whether MBL cells have encountered antigens and/or if they are polyreactive as CLL cells. Thus, further investigation is needed. The CLL gene expression profile and phenotype might be a result of the neoplastic transformation mechanisms of CLL and not a feature of any normal B cell subpopulation, which further complicates the mapping of the clonal origin of the CLL cell.

Circulating CLL cells are in the G_0/early G_1 phase of the cell cycle [79] and their survival and clonal expansion depends on BCR signals and co-signals from

accessory cells in the proliferative centra (pseudofollicles) [80,81]. However, the CLL cells differ in their responsiveness to antigen-BCR ligation, with unresponsive cells being more prominent in IGHV mutated CLL cases compared to unmutated CLL [82-85], despite the activated phenotype displayed by CLL cells [17]. This could reflect the possibility of the mutated CLL cells to reside in an anergic state, which in fact has been demonstrated in a proportion of CLL cases were constant phosphorylation of proteins involved in BCR signaling were correlated with unresponsiveness, although no correlation to mutational status was shown [86]. Moreover, Mockridge et al. showed that unresponsive cells had low sIgM level, and that the sIgM expression as well as the sIgM-Ca^{2+} signal capacity were restored when these CLL cells were cultured *in vitro* [85]. These facts point at the presence of an anergy-inducing BCR specific antigen operating *in vivo*. Alternatively, these cells may have become incapable of responding to antigens due to changed BCR structure caused by SHM of the BCR. The presence of an unresponsive CLL subset may reflect the importance of other cell surface markers for promoting cell survival and proliferating signals. CD5 has been shown to provide survival in some CLL cases [87,88] and IgD remains signal competent in both unmutated and mutated CLL cases, despite of an anergized status [85]. Receptors for cytokines and chemokines [81,89-91] as well as direct contact with accessory and stromal cells [91-93] can also supply survival signals. These scenarios could explain the heterogeneous prognostic outcome in CLL, where the Zap70, CD38 expressing and responsive unmutated CLL cells proliferate upon antigen-stimulation, causing a more aggressive progression compared with the unresponding mutated CLL cells.

When expanded to cell cultures *in vitro*, the lack of supportive signals received from the *in vivo* microenvironment will drive the CLL cells to rapidly undergo spontaneous apoptosis. This makes establishment of EBV immortalized CLL cell lines a useful tool in experimental models for studies on molecular

mechanisms during the leukemogenesis. Considering the intricate process of EBV transformation of CLL cells and the reports on a very high incidence (15-30%) of false cell lines (cross-contaminated) used worldwide in bio-medical laboratories [94,95], it is of great importance to verify the neoplastic and true origin of CLL cell lines. We performed a careful characterization of the authentic origin of several previously established CLL cell lines (paper II). Two of the cell lines, I83-E95 and CLL-HG3, were truly authentic as shown by identical IGHV rearrangement with patient *in vivo* CLL clone. The CII cell line had sustained trisomy 12 and CD5 expression, as reported in the original description of the CLL patient. The cell line was also shown to belong to subset 5 with stereotype HCDR3 and considering that this is a unique phenomenon in CLL, this CLL cell line could also be regarded as truly verified. The PGA1 cell line and the PG patient, from whom the cell line was established, were only sparsely described in primary reference and none of the markers in the Matutes scoring system used for diagnosis of CLL were described. The PGA1 cell line had maintained the 47,XY,+12 karyotype originally described but had lost the ring chromosome 15. Thus, our data only partially verify an authentic origin of the PGA1 cell line. The dissimilar CLL definition used in the 1980s, when the PG patient was diagnosed with CLL, and the absence of CLL characteristic markers shown by us, allow us to question the CLL diagnosis of the PG patient. The 232B4 cell line had different IGHV rearrangement compared to the patient *in vivo* CLL clone, which could indicate a false (contaminated) cell line. However, it had the same STR pattern as the 232A4 cell line established from the same patient, and the 325bp long IGHV fragment identified in the patient sample could possibly present the 325 bp long IGHV segment of the 232B4 cell line. If that is the case, the dual IGHV rearrangement could imply a biclonal CLL, which has previously been described in several CLL cases [96-100]. This has, however, not yet been proven and further analysis is intended to verify a neoplastic and true origin of the 232B4 cell line. A biclonal CLL was also

suggested when analyzing the WaC3CD5$^+$ cell line. We showed that the Wa-osel cell line, which was assumed to be the normal LCL counterpart to WaC3CD5$^+$, actually was the malignant clone since this cell line had identical IGHV rearrangement to patient *in vivo* CLL clone. Still, the WaC3CD5$^+$ cell line originated from the patient peripheral blood as shown by STR analysis, and it showed presence of CD5 and del(13q), which were some of the markers used to diagnose the patient with CLL. We also showed that WaC3CD5+ belonged to subset 32. This subset constitutes only a few CLL cases and has different IGHV usage and is therefore only considered as a potential subset and, hence, not a proof of a CLL origin. WaC3CD5$^+$ was in the original study isolated from patient's peripheral blood by anti-CD5 labelled magnetic beads and could therefore represent a minor CLL population of the CLL patient or a normal CD5$^+$ B cell population. It is also tempting to speculate of an MBL origin of the WaC3CD5$^+$ cell line, and that this population developed into the CLL clone from which the Wa-osel cell line was established. This has, however, not been proven and requires further extensive studies. We could not verify a neoplastic origin of the EHEB cell line due to loss of CD5 expression, lack of definitive markers and/or access to patient blood cells. The lack of CLL markers and the identical STR-pattern with corresponding CLL cell line, indicated a normal B cell origin of the 232A4 and PG/B95-8 LCL sister cell lines. The monoclonal B cell selection caused by EBV-transformation procedures and the appearance of chromosomal abnormalities occurring in long-term cell cultures may, however, raise the question whether to use these LCL cell lines as normal counterparts to the CLL cell lines in experimental models.

Conclusions

Paper I

In conclusion, the Ig specificities and the restricted antigen recognition identified by us and others, support that the $CD5^+$ B CLL cells produce natural Abs involved in the elimination of apoptotic cells and pathogenic bacteria. Repeated infections, such as pulmonary infections which actually has been linked to CLL [101], may trigger the onset of the disease. These infections could induce immunological cross-reactivity against self-antigens and/or presentation of apoptotic cell material and neoepitopes in solid tissues by stromal or other cells. The inability of the CLL cells to eliminate the antigen-trigger could cause continuous antigen-drive of the CLL clone, leading to proliferation and accumulation of the leukemic cells, where a more rapid proliferation reflects the more aggressive outcome seen in some CLL patients.

Paper II

Three of the CLL cell lines tested were truly of authentic neoplastic origin (I83-E95, CLL-HG3 and CII), two had features of a biclonal CLL origin (232B4 and WaC3CD5+), one was partly verified (PGA-1), whereas one cell line could not be verified (EHEB). Two of the presumed normal LCLs tested showed characteristics with the neoplastic CLL clone. This emphasizes the importance of cell line authentication and we intend to perform further research to verify a neoplastic and true origin of the cell lines that not yet has been proven truly identified.

Future perspectives

We and others have shown strong molecular evidence for CLL being derived from antigen-experienced B-cells. However, it is not yet proven whether the antigen-exposure affects the leukemic transformation and the survival of these cells or is an early selection procedure. CLL precursors are probably continuously activated by (auto) antigens through their BCR. This causes enhanced risk of genetic lesions in the BCR-mediated signal transduction pathway, which may contribute to the leukemic process. It would be of great interest to study these phenomena by *in vitro* stimulation of CLL cells by the antigens that we have identified in this study.

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