The role of antigen in the development of B-cell chronic lymphocytic leukemia
Hoogeboom, R.

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An in vitro culture system that selectively induces plasmacytoid differentiation and antibody secretion of B-cell chronic lymphocytic leukemia

Robbert Hoogeboom¹, Roy J.A. Reinten¹, Jan-Jaap Schot², Jeroen E.J. Guikema¹, Richard J. Bende¹, Carel J.M. van Noesel¹

¹ Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
² Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
Abstract

Antigen-specific B-cell receptor (BCR) signaling has been implicated in the pathogenesis of B-cell chronic lymphocytic leukemia (CLL). Identification of the cognate BCR ligands driving leukemogenesis in CLL is of pivotal importance for our understanding of this disease and may provide novel therapeutical modalities. However, identification of these ligands as yet requires recombinant in vitro production of CLL-derived immunoglobulins (Ig), which is a laborious and time-consuming method. Here, we present a novel and rapid method to produce soluble leukemia-derived Igs in vitro. In vitro stimulation of CLL cells with a combination of CD40 and TLR ligands induced plasmacytoid differentiation and IgM secretion. The produced IgM concentrations in the culture supernatants were sufficiently high to enable antigen specificity studies. We show that four out of seven IgMs derived of unmutated CLL (U-CLL) displayed self and/or poly-reactivity in tissue microarrays and in ELISAs, whereas none of four in vitro produced IgMs of mutated CLL (M-CLL) were polyreactive. In conclusion, this study provides a convenient and straightforward method to selectively produce CLL-derived IgM by in vitro cell culture, suitable for ligand identification.
In vitro production of CLL-derived immunoglobulins

Introduction

B-cell chronic lymphocytic leukemia (CLL), the most common leukemia in western adults\(^1\), is an incurable clonal expansion of CD5\(^+\)CD19\(^+\) B lymphocytes. Clinically, two types of CLL are being distinguished, based on the expression of either somatically mutated or unmutated immunoglobulin heavy chain variable region (\(IGHV\)) genes, which are associated with favorable and unfavorable prognoses, respectively\(^2\,^3\). CLL cells proliferate in so-called proliferation centers in lymph nodes. Here, CLL cells have gene expression profiles indicative of constitutive B-cell receptor (BCR) signaling\(^4\), an observation supported by the remarkable therapeutic effects of agents that interfere with the BCR signaling pathway, i.e. Bruton’s tyrosine kinase (Btk) and spleen tyrosine kinase (Syk) inhibitors\(^5\,^6\).

Several lines of evidence point towards a pivotal role for antigen-dependent BCR signaling in the development of CLL. The \(IGHV\) gene repertoire in CLL is biased to frequent usage of \(IGHV1-69\), \(IGHV3-7\) and \(IGHV4-34\)\(^7\) and over 30% of CLL can be grouped in subsets based on similarities of the amino acid sequences in the highly variable complementary determining region (CDR3)\(^8\,^9\,^10\,^11\,^12\,^13\). These stereotypic \(IGHV\) may harbor shared somatic mutations\(^13\) and are often paired with distinct immunoglobulin light chain variable region (\(IGLV\)) gene rearrangements\(^16\,^17\). Altogether, the observation that groups of CLL express highly homologous BCRs is generally considered as a strong indication that distinctive antigens are involved in their development.

The majority of CLL carrying unmutated \(IGHV\) (U-CLL) express low-affinity BCRs that are polyreactive, recognizing self- and exo-antigens, such as DNA, LPS, insulin, apoptotic cells, oxidized LDL, and the cytoskeletal antigens myosin and vimentin\(^18\,^19\,^20\,^21\). In contrast, BCRs of CLL carrying mutated \(IGHV\) (M-CLL) are usually not polyreactive, although for individual cases of M-CLL reactivity towards Hep2 cells, vimentin and Streptococcus pneumonia polysaccharide serotype 14 was identified\(^19\,^20\,^22\). Interestingly, for three stereotypic M-CLL subsets, a cognate antigen has been identified. Two subsets were found to have high affinity for the Fc-tail of the self-antigen IgG, so called rheumatoid factors\(^24\,^25\). In addition, we recently identified a novel \(IGHV3-7\)-expressing M-CLL subset that is highly specific for the fungal cell wall component beta-(1,6)-glucan\(^26\). Most importantly, stimulation with their respective cognate antigens, i.e. human IgG and beta-(1,6)-glucan, induced \textit{in vitro} proliferation in primary tumor cells of these M-CLL subsets\(^25\,^26\), indicating that stimulation by these antigens may also drive CLL expansion \textit{in vivo}.

To identify cognate antigens of the membrane-expressed immunoglobulins (Igs) of CLL, we have previously made use of a laborious and time-consuming method for recombinant production of CLL IgM as soluble antibodies in the myeloma cell.
line SP2/0\textsuperscript{25-28}. Other investigators generated recombinant antibodies in non-B-cell lineage cell lines, which has the disadvantage that this may lead to differently glycosylated Igs as compared to the Igs produced by B-lineage cell lines or primary CLL cells \textit{in vivo}\textsuperscript{19-21,23}. In addition, recombinant Igs are often produced as IgG or as incomplete F(ab) fragments, resulting in an avidity, affinity and possibly even a specificity that is different from the Igs expressed by the CLL cells\textsuperscript{29}. \textit{In vitro} production of Igs by cultured primary CLL cells would overcome most of these disadvantages. Previous attempts to produce soluble Igs by \textit{in vitro} stimulation of CLL cells have been hampered by rapid spontaneous apoptosis\textsuperscript{30} and/or yielded minute amounts of secreted Igs\textsuperscript{22,31,32}. Prolongation of \textit{in vitro} survival and enforced induction of plasmacytoid differentiation might resolve these problems.

Many groups have studied plasmacytoid differentiation of CLL cells. \textit{In vivo}, CLL cells appear to be blocked for terminal plasmacytoid differentiation\textsuperscript{33}. \textit{In vitro}, however, plasmacytoid differentiation of CLL cells can be induced by stimulation with toll-like receptor (TLR) ligands\textsuperscript{34}, activated T cells\textsuperscript{35,36}, T-cell-derived cytokines\textsuperscript{37,38}, and mitogens\textsuperscript{39,40}. \textit{In vitro} survival of CLL cells can be prolonged by supplying CD40-ligand (CD40L)\textsuperscript{41,42}, bone marrow stromal cells\textsuperscript{43} or nurse-like cells\textsuperscript{44}. However, no reports exist on how to exploit these methods to efficiently produce high concentrations of CLL-derived Igs.

In this study, we present a method to produce CLL-derived IgM by costimulation with CD40 and TLR ligands. Using this system, ample concentrations of secreted CLL-derived IgM were obtained from both unmutated and mutated CLL. The CLL Igs obtained were subsequently subjected to initial screens of ligand identification.

**Results**

**Combined stimulation of B-cell chronic lymphocytic leukemia cells by toll-like receptor and CD40 ligands induces plasmacytoid differentiation and immunoglobulin secretion**

Attempts to produce CLL-derived Igs by \textit{in vitro} culture of primary tumor cells are generally hampered by spontaneous apoptosis and low yields\textsuperscript{22,30-32}. To overcome spontaneous apoptosis, PBMCs of 20 CLL patients (10 with mutated and 10 with unmutated \textit{IGHV}) were cultured on a feeder layer of irradiated, CD40L-expressing 3T3 fibroblasts. After four weeks of culture, IgM was detected by ELISA in the cell culture supernatants of 16 of 20 patients at an average concentration of 0.4 μg/ml (Figure 1A). However, all but one CLL culture were below an arbitrary threshold of 2 μg/ml, i.e. a concentration amply sufficient to allow testing by most immunological techniques aimed at cognate antigen identification. We concluded that CD40
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stimulation alone does not yield sufficient amounts of CLL-derived IgGs.

To promote plasmacytoid differentiation, the TLR7 ligand R848 was added to the cultures in combination with CD40 stimulation. After four weeks of culture with CD40L and R848 stimulation, an increased IgM secretion was detected in nearly all cell cultures as compared to cultures without addition of R848. The supernatants of 10/20 cultures had reached IgM concentrations above the arbitrary threshold of 2 μg/ml with an average of 20.1 μg/ml (Figure 1B). Three cultures even contained IgM levels of more than 100 μg/ml. Ig-producing cultures were established of both the unmutated (6 of 10) and mutated (4 of 10) subgroups of CLL. Interestingly, two of the productive cultures, CLL102 and CLL616, were from CLL that expressed IGHV-rearrangements belonging to the stereotypic BCR subsets 29 (IGHV4-34/D6-19/JH3, M-CLL) and 5 (IGHV1-69/D3-10/JH6, U-CLL), respectively. A similar frequency (5/9) of Ig-producing cultures was obtained by addition of the TLR9 ligand CpG-DNA ODN2006. The concentrations reached up to 13 μg/ml with an average of 4.7 μg/ml (Figure 1C). Combined stimulation with R848 and CpG resulted in 8/12 productive cultures with an average of 9 μg/ml (Figure 1D). Addition of T-cell-derived

Figure 1: In vitro IgM production by primary CLL cells.
In vitro IgM production of CLL cells stimulated with CD40L (A), or CD40L combined with R848 (B), CpG (C), R848 and CpG (D) or IL2 and IL4 (E).
IL-2 and IL-4 to CD40L, induced IgM secretion in 8/12 CLL cultures with an average concentration of 40.8 μg/ml (Figure 1E).

After 8 days of culture with CD40 and TLR ligands, samples were drawn and the cells were mounted on glass slides by cytocentrifugation and stained with Giemsa’s solution. The majority of cells had obtained the typical morphological features of plasmacytoid differentiation, i.e. an enlarged cytoplasm with excentrically located nuclei and a conspicuous perinuclear hof indicative of an enlarged Golgi apparatus (Figure 2). Interestingly, culturing of CLL cells on irradiated CD40L-expressing fibroblasts alone was also sufficient to induce some degree of plasmacytoid differentiation, whereas no plasmacytoid cells were observed on slides mounted with fresh, uncultured CLL cells.

The secreted IgMs are derived from the B-cell chronic lymphocytic leukemia cells

To confirm that the secreted IgMs were derived from the CLL cells, Igκ and Igλ ELISAs were performed. Isotype restriction, defined as a four-fold excess of one isotype, was observed in 10/12 (80%) cultures generated by CD40 and TLR stimulation (Figure 3A-B). In these ten cultures, the secreted IgL isotype matched the IgL expressed by the leukemic clone as determined by PCR, cloning and sequencing (Figure 3A and Table 1). IgG concentrations were generally low (Figure 3A), except for cultures with CLL684, which also contained high levels of both Igκ and Igλ. In contrast, in only 4/8 (50%) supernatants generated by CD40 stimulation combined with IL-2 and IL-4, a clear isotype restriction was seen (Figure 3B), suggesting that a combination of CD40 and TLR stimulation is the most efficient among the methods studied. Overall, cultures of seven U-CLL and four M-CLL generated sIgMs of sufficient quality and concentration to enable cognate antigen identification (Table 1).

Figure 2: In vitro plasmacytoid differentiation of CLL cells stimulated with CD40 and TLR7 ligands. Cytological specimen of unstimulated CLL cells or CLL cells stimulated with CD40L and R848. Note the enlarged cytoplasm, excentrically located nuclei and conspicuous perinuclear hof in the cells stimulated with CD40L and R848. Giemsa staining, 400x magnification.
Application of in vitro produced leukemia-derived IgM for antigen identification

To address the antigenic specificity of IgMs obtained by in vitro culturing of CLL cells, tissue microarrays (TMA) containing 21 different tissues from healthy human donors were stained. Two of seven (29%) U-CLL-IgMs showed staining of cells in nearly all tissues, confirming the notion that U-CLL frequently express polyreactive BCRs (Figure 4)\textsuperscript{19,20,22,28}. Eight CLL-IgMs, including those of four M-CLL, did not stain any of the 21 tissues in the TMA. CLL700, a U-CLL, specifically stained vacuoles in exocrine cells of the pancreas (data not shown). For comparison, 4/11 (36%) recombinantly produced U-CLL-IgMs showed polyreactive staining in TMA, whereas only one of twelve (8%) recombinant M-CLL-IgMs showed staining of multiple tissues (Table 2).

Next, self-reactivity of the in vitro produced CLL-IgMs was analyzed in ELISA. CLL616 and CLL233, that were both polyreactive in tissue arrays, showed anti-nuclear activity (ANA) and also bound antigens in a cytoplasmic lysate of the larynx epidermoid carcinoma cell line Hep-2 (Figure 5A-B). The recombinant IgMs of CLL46, CLL54 and CLL55, which were polyreactive in TMAs, also showed reactivity in both the ANA and the Hep2 ELISA. One U-CLL-IgM (CLL785) and one M-CLL-IgM (CLL697) reacted with the cell-lysate of Hep-2 cells, but did not show ANA reactivity (Figure 5A-B), suggesting that these IgMs also bind self-antigen(s) but are not polyreactive. Seven of the in vitro produced IgMs did not react with any of the tissues nor lysates tested, strongly suggesting that these CLL are not polyreactive and bind to, yet unknown, specific antigens.

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**Figure 3: Soluble IgM in culture supernatants originates from CLL cells.**

(A) Presence of IgM, Igκ, Igλ and IgG in cell culture supernatants of CLL cells stimulated with CD40L and R848. Plotted are OD450 values without subtraction of background. (B) Summary of the yields of in vitro produced IgM under the three conditions indicated.
Table 1: IGHV- IGKV- and IGLV-sequences of in vitro produced CLL IgMs

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<th>CLL</th>
<th>Mut</th>
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<td>IGLV3-21/JL3</td>
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<td>IGLV3-21/JL2</td>
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<td>C CSYAGSSTHV F</td>
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<td>IGKV2-24/JK2</td>
<td>C MGATQCPPT F</td>
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<td>IGLV3-10/JL2</td>
<td>C SYDPGSGQRF V</td>
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<td>IGLV4-60/JL3</td>
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<td>IGHV1-69/D5-12/JH4</td>
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<td>IGLV1-51/JL1</td>
<td>C QTWDRSLEYYV F</td>
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</table>

M: IGHV-mutated, U: IGHV-unmutated, RF: rheumatoid factor\(^5\); Subsets defined by Stamatopoulos et al\(^{12}\); nd: not determined
Discussion

In this study, we developed a novel and rapid method to induce plasmacytoid differentiation of CLL cells in vitro in order to obtain secreted CLL-derived IgMs. The main advantage of this method is that it avoids a laborious recombinant approach that may yield Igs with an altered glycosylation, valency, affinity and possibly even an altered specificity as compared to native Igs. We observed that stimulation of CD40 alone was sufficient to induce plasmacytoid differentiation in a small proportion of cells, but did not result in sufficiently high concentrations of secreted IgM. By combining CD40 with TLR stimulation, plasmacytoid differentiation was induced in virtually all cells and high concentrations of soluble CLL-IgMs were obtained in about half of the CLL cultures. Testing of the produced antibodies in IgL ELISAs demonstrated that the produced IgL chains matched those expressed by the CLL cells, indicating that the applied culture condition selectively induces plasmacytoid differentiation and Ig production of CLL cells. In vitro Ig-production was successful using CLL cells from both mutated and unmutated CLL patients (including two CLL that express stereotypic BCRs), suggesting that this method is applicable to a broad range of CLL.

Stimulation with the TLR7 ligand R848 was more effective in inducing Ig secretion than stimulation with the TLR9 ligand CpG DNA (ODN2006), which may be due to a reported higher expression of TLR7 by CLL cells. Stimulation with TLR ligands alone was not tested in this study, as it was reported that TLR7 and TLR9 stimulation decrease survival of CLL cells in vitro. Combined stimulation with CD40, IL-2 and IL-4 resulted in higher production of CLL IgMs than the combinations of CD40 and TLR-
Table 2: IGHV and IGLV sequences of recombinant IgM of CLL

<table>
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<tr>
<th>Mut</th>
<th>CLL</th>
<th>Subset</th>
<th>IGHV-rearrangement</th>
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<td>IGHV4-34/D55-5/JH6</td>
<td>C ARWYPIDPMIRRYYYGMDV W</td>
<td>IGKV2-30/JK1</td>
<td>C MQGTHWPF F V +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>26V</td>
<td>IGHV4-34/D2-2/JH3</td>
<td>C ARGVPDCSSASCASLYFSDS W</td>
<td>IGKV1-6/JK2</td>
<td>C LDYNPPYT F V F</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>43</td>
<td>IGHV3-21/JH6</td>
<td>C ARQDNAMDV W</td>
<td>IGKV3-21/LJ3</td>
<td>C QQWDSLAVF V F</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>53</td>
<td>IGHV3-21/D2-15/JH6</td>
<td>C ARQANHMDV W</td>
<td>IGKV1-3-LJ3</td>
<td>C QQWDSLAVF V F</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>60</td>
<td>IGHV3-21/D1-26/JH6</td>
<td>C ARDANGMDV W</td>
<td>IGKV1-40/LJ2</td>
<td>C QQWSLAVF V F</td>
<td>–</td>
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<tr>
<td>M</td>
<td>64</td>
<td>IGHV4-34/D515-5/JH6</td>
<td>C VRYGDTAVKRRYYYGMDV W</td>
<td>IGKV1-30/JK4</td>
<td>C MQGTHWPF F V</td>
<td>–</td>
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</tr>
<tr>
<td>M</td>
<td>65</td>
<td>IGHV4-34/D4-11/JH6</td>
<td>C ARCGTPDRYRRPPPYYYGMDV W</td>
<td>IGKV2-30/JK2</td>
<td>C MQGTHWPF F V</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>81</td>
<td>RF</td>
<td>IGHV3-7/D3-22/JH3</td>
<td>C ARQDYDASATYNFNADFI W</td>
<td>IGKV3-15/JK1</td>
<td>C QHYMNWPWT F</td>
<td>–</td>
</tr>
<tr>
<td>M</td>
<td>82</td>
<td>V3-7sh</td>
<td>IGHV3-7/JH1</td>
<td>C GEINS G W</td>
<td>IGKV2-24/JK2</td>
<td>C MQATQIC F V</td>
<td>–</td>
</tr>
<tr>
<td>M</td>
<td>85</td>
<td>IGHV3-7/D3-10/JH4</td>
<td>C ARDTQARFTYNDSF W</td>
<td>IGKV1-44/LJ1</td>
<td>C AAWDDTSGHF V F</td>
<td>–</td>
<td></td>
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<tr>
<td>M</td>
<td>88</td>
<td>V3-7sh</td>
<td>IGHV3-7/D2-21/JH5</td>
<td>C VESAS W</td>
<td>IGKV2-24/JK4</td>
<td>C VQAHLF F V</td>
<td>–</td>
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<tr>
<td>M</td>
<td>89</td>
<td>V3-7sh</td>
<td>IGHV3-7/D6-25/JH1</td>
<td>C AEISTD W</td>
<td>IGKV2-24/JK4</td>
<td>C MQATQIC F V</td>
<td>–</td>
</tr>
</tbody>
</table>

M: IGHV-mutated, U: IGHV-unmutated, RF: rheumatoid factor; V3-7sh subset defined by Hoogeboom et al.; Subsets defined by Stamatopoulos et al.;
In vitro production of CLL-derived immunoglobulins

In addition, the frequency of productive cultures was higher with IL-2 and IL-4. However, IgL isotype ELISAs demonstrated the presence of non-CLL derived IgM, indicative of Ig secretion by contaminating non-CLL B-cells. These findings suggest that TLR ligands more selectively target CLL cells in this system. Purification of CLL cells prior to culture with IL-2 and IL-4 may overcome this disadvantage. Not all CLL responded to stimulation with CD40 and TLR ligands. CLL that do not secrete Igs in response to the conditions applied in this study may be susceptible to stimulation with CD40 in combination with other factors to induce plasmacytoid differentiation e.g. stimulation with mitogens\textsuperscript{39,40} or with different combinations of cytokines e.g. IL-2 and IL-15\textsuperscript{47}.

Previously, several studies have reported secreted CLL Igs that were obtained by in vitro culture\textsuperscript{22,31,32}. However, in none of the culture systems, Ig concentrations higher than 0.2 μg/ml were reached, which is generally too low for purification and specificity testing. Using our method, concentrations of over 100 μg/ml were reached with an average of 20 μg/ml, conveniently higher than our arbitrary threshold of 2 μg/ml. 2 μg/ml is comparable to the concentrations which are generally recommended for commercial antibodies (e.g. 2 μg/ml for flow cytometry, 1 μg/ml for immunohistochemistry and 0.2 μg/ml for ELISA). In a recent paper, Hwang and colleagues\textsuperscript{48} describe a method to increase the efficiency of Epstein-Barr virus (EBV) transformation to produce CLL-derived Igs. Using this method, on average 3.3 μg/ml of IgM was produced.

Our panel of newly generated CLL IgMs contained three U-CLL that were self-reactive in TMAs. The U-CLL IgMs of CLL233 and CLL616 stained cells in nearly all tissues,
indicating that these CLL are polyreactive. Four CLL-IgMs bound Hep-2 lysates in ELISA, suggesting that these CLL may also recognize cytoplasmic antigens. Two U-CLL and three M-CLL did not show any reactivity in our assays. Possibly these cases are monospecific for a pathogen-derived antigen, as we recently demonstrated for an \textit{IGHV3-7}-expressing subset of M-CLL\textsuperscript{26}. Overall, U-CLL were more frequently self-reactive as compared to M-CLL, which is in line with previous reports on CLL BCR specificities\textsuperscript{19,20,22,28}.

In conclusion, we have developed a rapid and convenient method for the production of CLL-derived IgMs. Combined stimulation with CD40L and R848 was the most effective condition studied. The concentration and purity of the majority of the produced IgMs was sufficient to investigate their antigenic reactivity. Poly- and self-reactive binding patterns were identified of 4/7 U-CLL IgMs and 1/4 M-CLL IgMs, respectively. Interestingly, several studies report on a potential correlation between CLL BCR specificity and disease aggressiveness\textsuperscript{49,50}. Convenient methods to produce CLL-derived IgM \textit{in vitro} may facilitate studies on CLL BCR specificity and subsequently may enable specificity-based assessment of prognosis. Moreover, subsets of CLL may specifically recognize drugable target antigens. Accelerated production of CLL-derived IgM may allow application of specificity-based therapeutic strategies for CLL.

Acknowledgements
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Materials and Methods

Patients and \textit{IGHV} sequences
Twenty CLL were randomly selected from the patient database at the Academic Medical Center (AMC) in Amsterdam, The Netherlands. \textit{IGHV} were sequenced according to the Biomed protocol\textsuperscript{51}. BCR sequences were analyzed using V-Quest, available online at www.imgt.org\textsuperscript{52}. Table 1 contains the \textit{IGHV}- and \textit{IGLV}-sequences of the CLL used in this study. This research was conducted in accordance with the ethical standards in our institutional medical ethical committee on human experimentation, as well as in agreement with the Helsinki Declaration of 1975, as revised in 1983. Accordingly, all patients signed informed consent.

Culture conditions
Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Paque (GE Healthcare, Uppsala, Sweden) according to manufacturer’s instructions. 4 x 10\textsuperscript{5} cells
per well were cultured in 400 μl for 28 days in the presence of 0.5 x 10^5 γ-irradiated CD40L-expressing 3T3 fibroblasts and stimulated with 1 μg/ml of the TLR7 ligand R848 (Invivogen, San Diego, CA, USA), 1.5 μg/ml of the TLR9 ligand CpG ODN 2006 (Invivogen, San Diego, CA, USA) or 100 ng/ml of IL-2 and IL-4 (Prospect, Ness-Ziona, Israel).

Recombinant immunoglobulin production
RNA was isolated from PBMCs using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, cDNA was synthesized using Pd(N)6 random primers. IGHV and IGLV genes were amplified using IGHV-, IGKV-, IGLV-, IGHJ-, IGKJ- and IGLJ-specific primers. Recombinant, soluble IgM (slgM) of 23 CLL patients were produced using plgH(μ) and plgL(κ) expression vectors as described previously. In brief, the rearranged IGHV and IGLV genes of each of these leukemias were cloned into the plgH(μ) and plgL(κ) vectors, respectively. For production of recombinant antibodies, 10 μg plgH(μ) and 10 μg plgL(κ) was linearized with PvuI and co-transfected into SP2/0 myeloma cells by electroporation. Subsequently, transfected cells were selected in medium containing 400 μg geneticin per ml.

ELISA
Supernatants were screened for secreted slgM, using mouse anti-human IgM (MH15), Igκ (MH19), Igλ (MH29) and IgG (MH16) antibodies (all from Sanquin, Amsterdam, The Netherlands), by ELISAs as described. Using this ELISA, Ig concentrations as low as 0.01 μg/ml can be detected. For testing reactivity to Hep2 cells, Hep2 cells were lysed for 1 hour at 4°C in lysis buffer containing 1% triton X-100, 5 mM EDTA, 20 mM Tris-HCl and 150 mM NaCl in the presence of a protease inhibitor cocktail (Roche, Mannheim, Germany). Subsequently, the protein concentration was determined by bicinchoninic acid assay and 15 μg/ml was coated in Costar EIA microtiter plates (Corning Inc, Corning, NY, USA) overnight at 4°C. Antigen reactivity for specific antigens was assessed by coating antigens at 4 μg/ml. After blocking with 1% BSA for 30 minutes at 37°C, wells were incubated with serial dilutions of CLL cell culture supernatants for 1 hour at room temperature, followed by incubation with HRP-conjugated mouse anti-human IgM (Clone MH15, Sanquin, Amsterdam, The Netherlands) and developed as described previously. Absorbance at 450 nm is plotted without subtraction of background. Anti-nuclear activity (ANA) was determined by the QUANTA-LITE® ELISA kit (Inova, San Diego, CA, USA). Reactivity was detected as described for other ELISAs above.
**Immunohistochemistry**

1.5 x 10^5 of cultured cells were mounted on a glass slide by cytocentrifugation for 4 minutes at 500 rpm, fixed with acetone and stained with Giemsa’s solution. Paraffin-embedded tissues from healthy donors were obtained from the AMC and assembled in tissue microarrays (TMA) using a manually operated TMA device (Beecher Instruments, Silver Spring, MD). After antigen retrieval (Citrate buffer, 20 min at 120°C), slides were stained overnight with CLL cell culture supernatants at 1 μg/ml in PBS. Mouse anti-human IgM (Clone MH15, Sanquin, Amsterdam, The Netherlands) was used as secondary antibody. Subsequently staining was visualized using Powervision⁺ (ImmunoVision Technologies, Duiven, The Netherlands).

**References**

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