

# Proliferative index and expression of CD38, Zap-70, and CD25 in different lymphoid compartments of chronic lymphocytic leukemia patients

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**Abstract:** Recent studies of chronic lymphocytic leukemia (CLL) show that malignant B cells proliferate at a rate similar to normal B lymphocytes. This is in apparent contradiction to the very low proliferation rate found in blood specimens from CLL patients. To address this problem, we studied the expression of Ki-67, CD38, CD25, and Zap-70 in different compartments of CLL patients. Using triple-color flow cytometry, we examined the expression of CD38, CD25, Zap-70, and Ki-67 antigens in the peripheral blood, bone marrow, spleen, and lymph nodes biopsies of patients with CLL, splenic marginal zone lymphoma (SMZL), and nonmalignant diseases. In parallel probes of lymph node/spleen biopsies and blood taken from one and the same patient, Ki-67 expression was 17 times higher. Among the whole cohort, we also found significantly higher Ki-67 expression in biopsies from lymph nodes and spleen ( $4.95\% \pm 0.55\%$ ), compared with bone marrow ( $1.88\% \pm 0.32\%$ ) and peripheral blood ( $0.45\% \pm 0.03\%$ ,  $P < 0.01$ ). In CLL patients, there are statistically significant correlations between the expression of CD38 and Ki-67 in bone marrow ( $P \leq 0.01$ ), Zap-70 and Ki-67 in blood ( $P \leq 0.01$ ), and Zap-70 and CD38 in blood ( $P \leq 0.01$ ). Patients with SMZL also showed a significant correlation between Ki-67 and CD38 expression ( $P \leq 0.01$ ) and between Ki-67 and Zap-70 expression ( $P \leq 0.01$ ). We show for the first time that proliferation of B lymphocytes in CLL patients is associated primarily with lymph nodes/spleen. Malignant cells in the blood represent only a subpopulation of nonproliferating and less-activated B cells in this disease.

**Keywords:** chronic lymphoid leukemia, CD38, Zap-70, Ki-67, bone marrow, lymph node

## Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by large clonal accumulation of B cells with the abnormal phenotype ( $CD5^+/CD23^+$ ) and low proliferation capacity.<sup>1</sup> As the disease is highly heterogeneous, efforts to identify subsets of patients have considered the mutational status of immunoglobulin heavy-chain variable regions ( $V_H$ ), Zap-70 expression,<sup>2</sup> cytogenetic abnormalities,<sup>3</sup> CD38 expression, p53 dysfunction,<sup>4</sup> as well as other approaches. Among cell-surface markers, both the expression and pathobiological role of CD38 in CLL have been the subject of numerous studies.<sup>5-7</sup> It has been established that high CD38 expression in B-CLL cells is associated with a worse clinical outcome.<sup>8-10</sup> Although CD38 is known to participate in signaling transduction in a number of different hematological cell types,<sup>11</sup> its biological role in CLL tumorigenesis remains unclear. Recent research suggests that a CD38-activated genetic program is relevant in proliferative responses and cell migration.<sup>12,13</sup>

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A number of biological markers have been recently described that address the prognosis of CLL disease and allow the identification of high-risk patients. However, the majority of these studies were limited by the use of peripheral blood samples. Another important negative prognostic marker in B-CLL is Zap-70, a 70-kDa zeta-chain protein normally essential for T-cell receptor signaling. Zap-70 expression was described in various B-cell malignancies and some subsets of normal B cells.<sup>14</sup> Flow cytometric evaluation of CD38 in combination with other negative markers such as Zap-70 is an important method for stratifying CLL patients in low-, intermediate-, and high-risk groups and is more straightforward than other technically complex mutational status assays. We analyzed a large cohort of CLL patients undergoing a routine diagnostic study and identified correlations on the primary level in the different lymphoid compartments. The objective of this study was to evaluate the possible correlations between activation markers CD38 and CD25, proliferation index, and Zap-70. We selected Ki-67 as a prominent marker for cell proliferation<sup>15</sup> and a negative prognostic factor in many types of cancer.<sup>16–22</sup> CD25, normally an activation marker on T cells, was reported to be upregulated on B cells from CLL patients.<sup>23–25</sup> Analysis of Ki-67 was of particular interest in relation to CD38, because data on the relationship between CD38 and Ki-67 expression in different B-cell lymphomas remain controversial.<sup>26–29</sup>

Despite the lack of an easily detectable proliferative compartment, recent studies of CLL patients who used oral administration of heavy water (<sup>2</sup>H<sub>2</sub>O) showed proliferation among B-CLL clonal cells to be substantially more rapid than realized previously with in vivo birth rate of 0.1%–1% of the total leukemic clone per day.<sup>30</sup> This apparently contradicts the low Ki-67 expression in CLL reported by several authors.<sup>27,28</sup> To evaluate the possibility that proliferation of B-CLL cells might be restricted to a particular compartment, we performed analysis of activation markers and Ki-67 expression in different biopsy specimens.

## Materials and methods

### Patients and sample collection

Blood samples were collected from 202 consenting patients (88 females, 114 males) with CLL. This study included CLL patients seen at the Russian Hematology Scientific Centre between January 2006 and January 2009. Diagnosis of B-CLL was established according to standard morphologic and immunophenotypic criteria.<sup>31,32</sup> Subject ages ranged from 36 to 80 years (median = 60 years),

and the male-to-female ratio was (1.3:1). The majority of patients (n = 187) were untreated at the time of blood collection. Additional bone marrow aspirates (n = 44) and biopsies of lymph nodes (n = 32) and spleen (n = 9) were collected from consented CLL patients. There was no difference in Binet and Rai stages, nor were there significant differences in age or gender between patient groups from which blood samples or bone marrow samples or lymph node sample for biopsy were taken. Patient selection was based on a definitive diagnosis of CLL, and all patients had high- or intermediate-risk CLL. A control group consisting of 32 patients was identified with lymph node and spleen biopsies that were free of any malignant process. The biopsies were processed and stained with a three-color antibody panel in a fashion identical to that described below for CLL patient samples. A second group consisting of 14 patients with splenic marginal zone lymphoma (SMZL) and 7 patients with large B-cell lymphoma were selected from the clinical patients at our institution. Their diagnoses were also established according to standard morphologic and immunophenotypic criteria.<sup>31</sup>

### Immunophenotyping for CD38 and CD25 expression

Fresh samples were taken for surface marker and cytoplasmic marker analysis after obtaining informed consent. Mononuclear cells were obtained from peripheral blood and bone marrow by lysing erythrocytes in ammonium solution via density centrifugation and then kept in phosphate-buffered saline with 0.5% bovine serum albumin. Mononuclear cells from biopsy material were obtained using Medimachine (BD Biosciences, San Jose, CA, USA) and suspended in a Hanks solution. Surface staining was performed according to manufacturer's protocols using three-color staining. Monoclonal antibodies (direct conjugates) specific for CD19, CD5, CD23, and CD10 were used to define B-CLL phenotype. Antibodies against CD38 and CD25 were used in phycoerythrin (PE) conjugates. Approximately, 500,000 mononuclear cells were stained by adding 10–20 µL of conjugated antibodies, followed by incubation for 30 min at 4°C. Cells were stained with a mixture of fluorescently labeled antibodies: CD3-FITC/CD19-PE/CD45-PE-Cy5; CD5-FITC/CD38-PE/CD19-PE-Cy5; CD10-FITC/CD23-PE/CD19-PE-Cy5; CD20-FITC/CD25-PE/CD19-PE-Cy5.

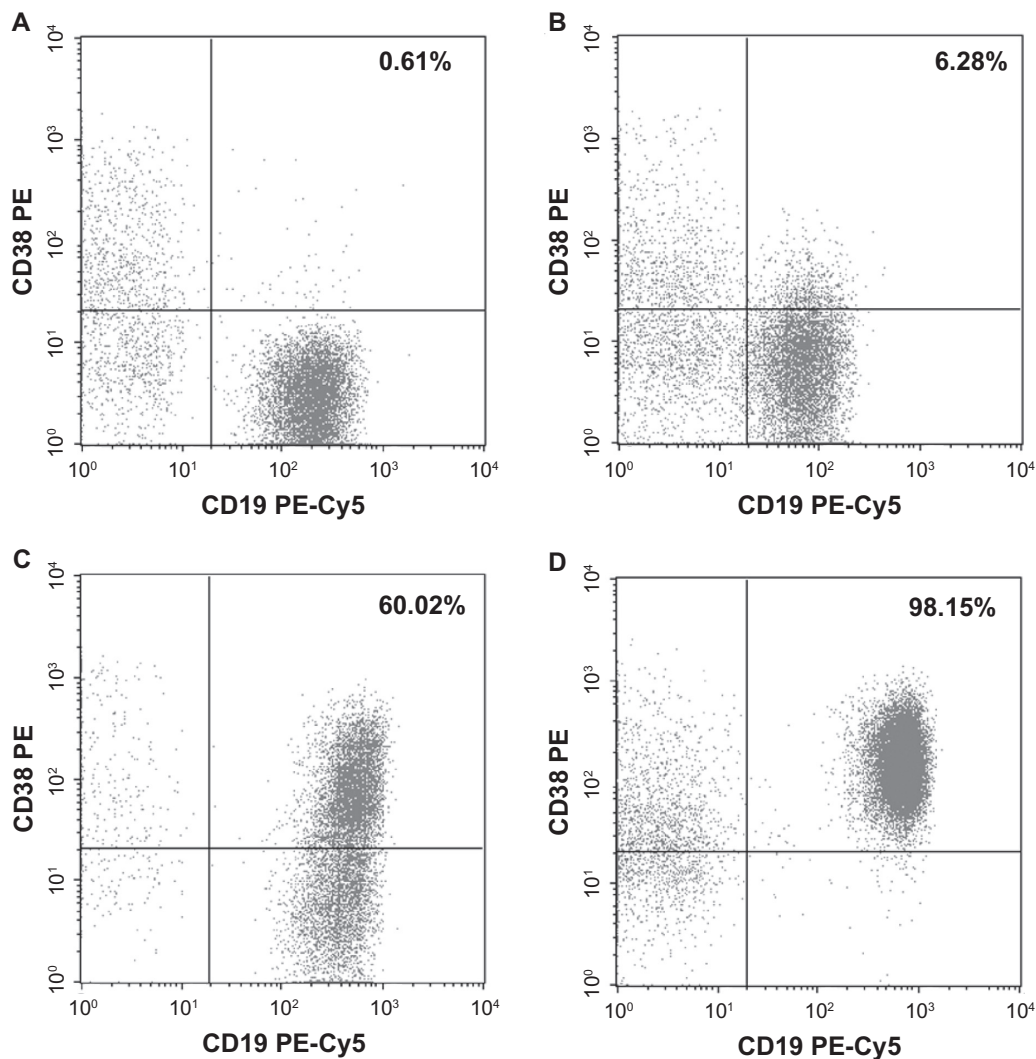
Between 30,000 and 50,000 cells were examined for each probe using the FACSCalibur flow cytometer (BD Biosciences). Gating and data analysis were performed

using CellQuest software (BD Biosciences) and FlowJo software (TreeStar Inc, Ashland, OR, USA). The level of CD38 expression was determined by selective gating as a percentage of CD5<sup>+</sup>CD19<sup>+</sup> cells expressing CD38 (Figure 1). The same method of sample preparation and three-color extracellular staining were used throughout the study period. The level of CD25 expression was determined in the same way using CD20<sup>+</sup> instead of CD19<sup>+</sup>.

### Intracellular flow cytometric analysis of Ki-67 (MIB-1) protein expression

CLL peripheral blood lymphocytes, bone marrow, and lymph nodes were analyzed by triple-color immunofluorescent staining using antibodies against Ki-67 (Clone MIB-1) and antibodies

against CD79a and CD3 (Ki-67-FITC/CD79a-PE/CD3-PE-Cy5). Briefly, purified lymphocytes were fixed in 1% freshly prepared paraformaldehyde (Sigma, St. Louis, MO, USA) for 5 min at room temperature. After fixation, the cells were permeabilized using a commercially available kit (BD Biosciences) for 10 min at room temperature. After staining with Ki-67 (MIB-1)-FITC for 25 min at 4°C, cells were washed again and analyzed on a FACSCalibur flow cytometer (BD Biosciences). At least 30,000 events were acquired from each sample. Negative isotype-matched controls (BD Biosciences) were used to exclude nonspecific events. Expression of Ki-67 was determined as a percentage of CD79a-positive cells. Only cases with a negligible percentage of normal B lymphocytes were included in analysis of the cytoplasmic marker.



**Figure 1** Representative flow cytometry profiles of CD38 expression in patients with B-CLL. CLL cells were analyzed for surface CD38 expression after incubation with directly conjugated anti-CD19-PE-Cy5, anti-CD38-PE, and anti-CD5-FITC antibodies. **A)** and **B)** Samples from two patients negative for CD38 expression (<30%). **C)** Sample of patient positive for CD38 expression (CD38<sup>intermediate</sup>, see text for details). **D)** Sample of patient positive for CD38 expression (CD38<sup>high</sup>, see text for details). Numbers are % of CD38-positive B cells.

## Analysis for Zap-70

Flow cytometric analysis of intracellular Zap-70 expression was performed on whole blood samples using the method described by Crespo and coauthors.<sup>33</sup> Briefly, cells were fixed and permeabilized using the BD Cytotfix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. Antibodies against Zap-70 (Clone 1E7.2) were used in PE conjugate. After staining with Zap-70 antibody, cells were washed again and stained with monoclonal antibody conjugates against CD79a and CD3 (BD Biosciences).

To quantify Zap-70 expression, we used the following method of analysis: the identification of the Zap-70<sup>+</sup> cell population was driven by the external isotype-matched control. Analysis of Zap-70 expression in T cells (positive control) was performed by calculating the ratio between the mean fluorescence intensity (MFI) of cells stained with anti-Zap-70 monoclonal antibodies and the MFI of the corresponding isotype-matched control. Biparametric dot plot graphs were obtained for 1) CD3 and Zap-70 or 2) CD79a and Zap-70. Cutoff for Zap-70 expression on B-CLL cells was set at 95% Zap-70 positivity of T cells (CD79a-FITC/Zap-70-PE/CD3-PE-Cy5).

## Statistical analysis

The nonparametric Spearman's rank correlation coefficient was applied to evaluate the possible correlation between the continuous variables Zap-70, CD38, CD25, and Ki-67 from blood, lymph nodes, and bone marrow samples from the CLL patient group and the control group with reactive

lymph nodes. All computations were carried out using the SSCP statistical program version 17.0 and GraphPad version 5.0 (La Jolla, CA, USA). A value of  $P \leq 0.05$  was considered significant for all statistical calculations.

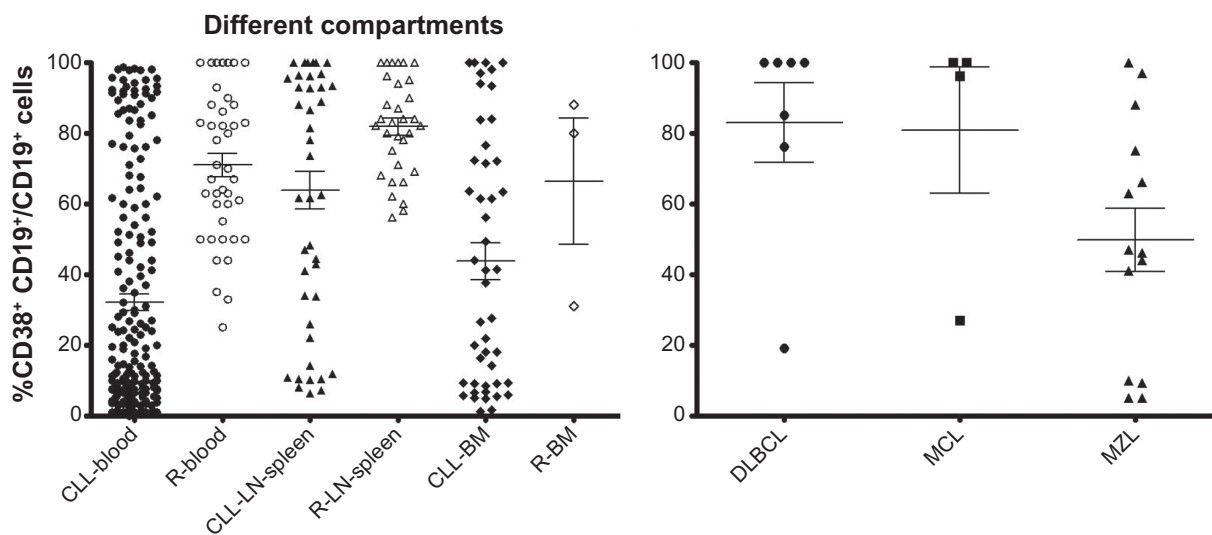
## Results

### CD38 expression

The level of CD38 expression in CLL in our cohort of patients varied from almost negligible to 100% (Figure 1). A leukemic cell population was considered positive for CD38 when it was  $\geq 30\%$  in accordance with previous reports.<sup>10,34,35</sup>

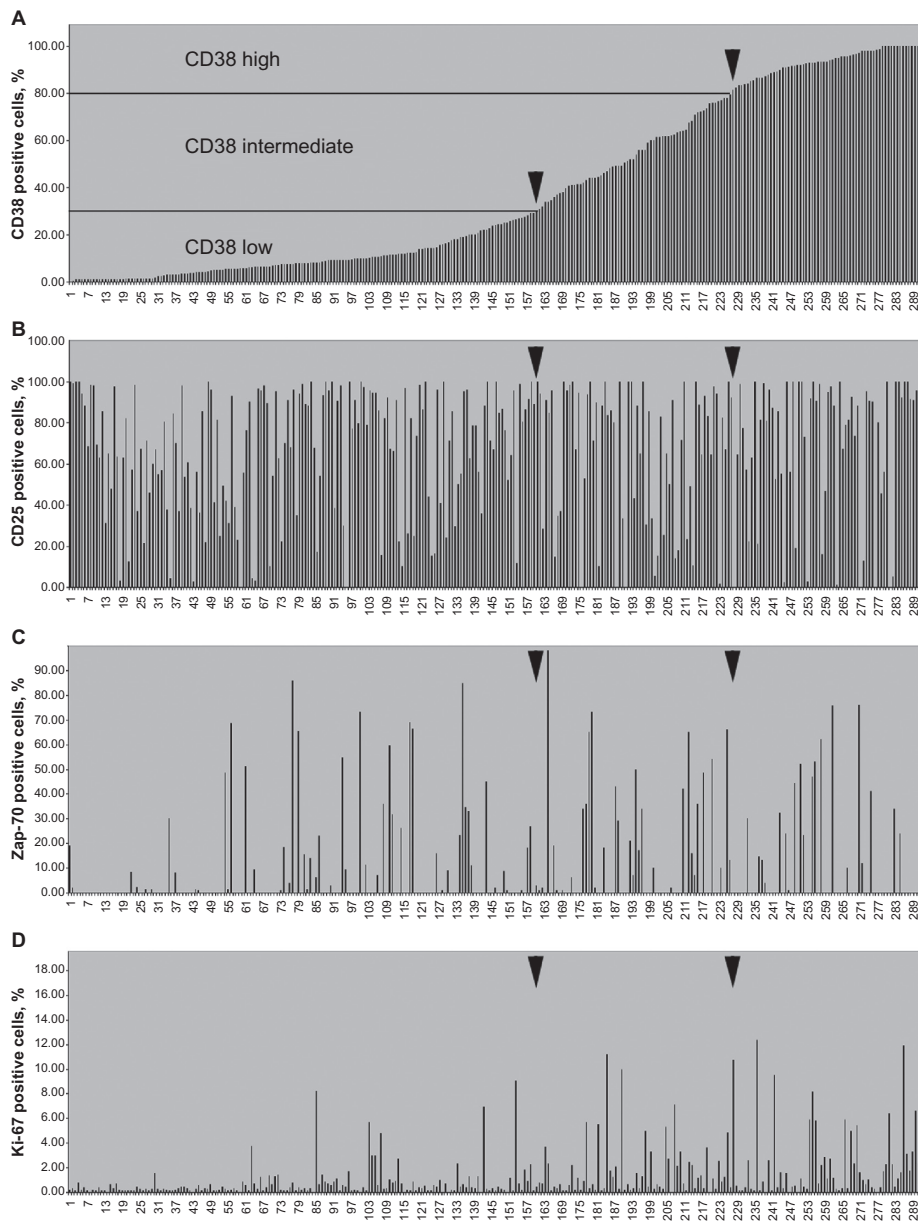
We found significantly higher CD38 expression in biopsies from lymph nodes and spleen compared with bone marrow biopsies and peripheral blood (mean percentage,  $63.7\% \pm 5.3\%$  vs  $43.7\% \pm 5.2\%$  and  $32.0\% \pm 2.3\%$  respectively;  $P < 0.01$ ) (Figure 2). No significant difference was found between lymph node biopsies ( $60.7\% \pm 11.5\%$ ;  $n = 36$ ) and spleen biopsies ( $64.4\% \pm 6.1\%$ ;  $n = 9$ ). Thus, we considered them one group for further analysis. Consequently, the percentage of CD38-positive patients identified by probing lymph nodes/spleen, bone marrow, and blood was 68.4%, 53.3%, and 38.6%, respectively;  $P < 0.01$ .

The difference in CD38 expression between lymph nodes and blood was confirmed by analysis of parallel specimens (Table 1). In 18 out of 20 cases, the percentage of CD38-positive cells in the lymph nodes was higher than in the blood, and in 13 cases, it was at least 1.5 times higher. The overall difference in CD38 expression between the two groups of specimens was highly significant ( $P < 0.001$ ).

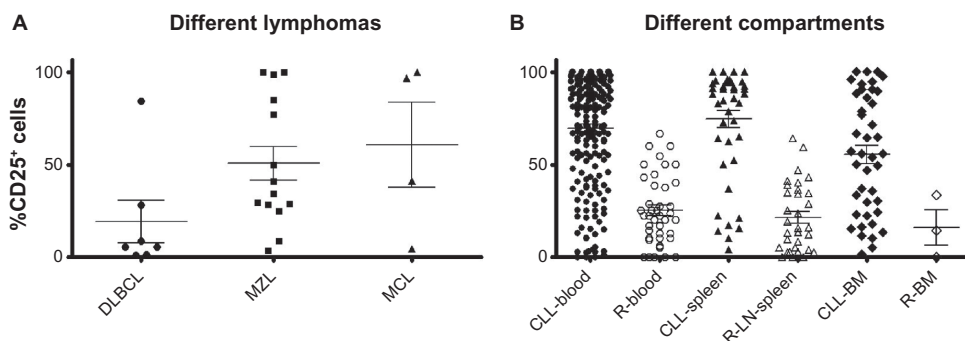


**Figure 2** Percentage of CD38<sup>+</sup> cells by lymphoid compartments in patients with B-CLL. Scattergram of CD38<sup>+</sup> cells in peripheral blood, spleen/lymph nodes, and bone marrow biopsies from CLL patients (the leftmost, center, and rightmost columns of each panel; filled circles, tetrahedrons, and triangles, respectively). Results are presented as the percentage of CD38<sup>+</sup> and CD19<sup>+</sup>, and the means are marked by solid lines.

**Abbreviations:** CLL, chronic lymphocytic leukemia; LN, lymph node; BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; MZL, marginal zone lymphoma.



**Figure 3** Percentage of different antigens (CD25, Ki-67, and Zap-70) stratified according to CD38 expression. Expression levels of CD25, Zap-70, and Ki-67 from B cells of CLL patients. CLL patients were divided into three groups according to the level of CD38 expression on their B cells: low (<30%), intermediate (30%–80%), and high (>80%).



**Figure 4** Percentage of CD25<sup>+</sup> B cells in patients with B-CLL and other lymphomas. Scattergrams of CD25<sup>+</sup> B cells in: **A**) Different B-cell lymphomas and **B**) different compartments in CLL patients and patients with reactive conditions. Results are presented as the percentage of CD25<sup>+</sup> B cells, and the means are marked by solid lines.  
**Abbreviations:** CLL, chronic lymphocytic leukemia; LN, lymph node; BM, bone marrow; DLBCL, diffuse large B-cell lymphoma; MZL, marginal zone lymphoma.

Patients in the SMZL group had slightly higher levels of CD38 expression on B cells ( $49.7\% \pm 8.9\%$ ;  $n = 14$ ) than CLL patients ( $38.6\% \pm 2.1\%$ ), and patients in the large B-cell lymphoma group had significantly higher levels of CD38 expression ( $82.0\% \pm 11.2\%$ ;  $n = 7$ ). The level of CD38 expression on B cells in the reactive specimens was  $75.3\% \pm 2.2\%$  ( $n = 76$ ). Hence, the level of CD38 expression in B-CLL lymph nodes/spleens was still lower than in reactive lymph nodes/spleens ( $63.7\% \pm 5.3\%$  vs  $81.8\% \pm 2.4\%$ ;  $P < 0.01$ ).

The cohort of CLL patients was further stratified according to the level of CD38 expression. Patients were divided into three groups according to the percentage of CD38-positive B-CLL cells (Figure 3): CD38<sup>low</sup> (<30% of positive cells) (Figure 1A and 1B), CD38<sup>intermediate</sup> (between 30% and 80% of positive cells) (Figure 1C), and CD38<sup>high</sup> (>80% of positive cells) (Figure 1D). From the whole cohort ( $n = 202$ ), 128 patients had <30% of CD38-expressing leukemic cells (CD38<sup>low</sup>), 41 patients were in the CD38<sup>intermediate</sup> group, and 33 patients were in the CD38<sup>high</sup> group. To further evaluate the relevance of CD38<sup>high</sup> and CD38<sup>low</sup>, we compared CD38 expression with other markers.

### CD25 expression

Unlike CD38, expression of CD25 on B cells was significantly increased in CLL patients compared with other groups (CLL:  $68.4\% \pm 1.8\%$ ; patients with reactive conditions:  $23.4\% \pm 2.2\%$ ; SMZL:  $50.7\% \pm 9.2\%$ ; and diffuse large B-cell lymphoma (DLBCL):  $22.3\% \pm 13.0\%$ ) (Figure 4). This finding is in accord with previous reports.<sup>24</sup> The highest level of CD25 expression in CLL was found in lymph nodes and spleens, and the lowest level was in the bone marrow (Table 2). However, the difference between different compartments was insignificant ( $P > 0.2$ ).

### Zap-70 expression

Using T cells as an external positive control, we defined the percentage of B cells positive for Zap-70 in different CLL compartments (Figure 5). Expression of Zap-70 in different lymphomas and different compartments in CLL is summarized

**Table 1** Expression of different antigens (%) on CLL cells in peripheral blood and spleens/lymph nodes from the same patients ( $n = 20$ )

	CD38	Ki-67
Blood	$29.38 \pm 7.03$	$0.92 \pm 0.30$
Lymph nodes and spleens	$43.84 \pm 7.87$	$6.80 \pm 1.68$
Ratio <sup>1</sup>	$2.76 \pm 0.56$	$17.32 \pm 4.73$

**Notes:** Mean  $\pm$  SEM. <sup>1</sup>Ratios were calculated as means for all pairs of specimens.

**Table 2** Expression of different antigens (%) on CLL cells in peripheral blood, bone marrow, and spleens/lymph nodes

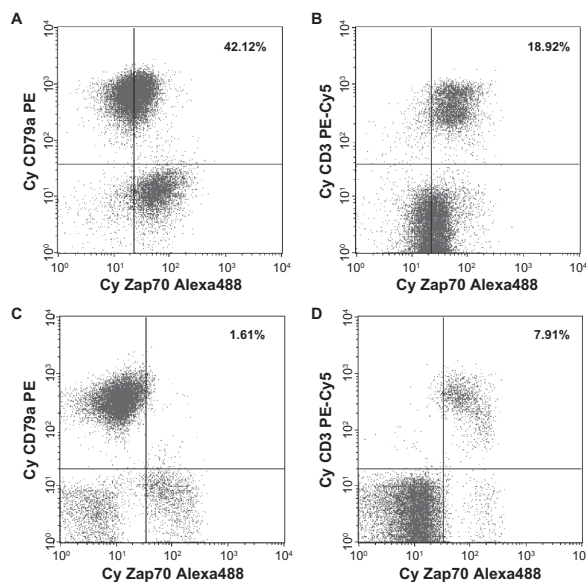
	CD38	Ki-67	CD25
Blood ( $n = 201$ )	$31.98 \pm 2.34$	$0.45 \pm 0.03$	$69.46 \pm 2.13$
Bone marrow ( $n = 42$ )	$44.22 \pm 5.30$	$1.88 \pm 0.32$	$55.40 \pm 4.93$
Lymph nodes and spleens ( $n = 42$ )	$63.71 \pm 5.32$	$4.95 \pm 0.55$	$75.01 \pm 4.63$
Reactive lymph nodes and spleens ( $n = 32$ )	$81.78 \pm 2.41$	$4.76 \pm 0.81$	$21.25 \pm 3.24$

**Note:** Mean  $\pm$  SEM.

in Figure 5. Percentages of Zap-70-positive B cells in the peripheral blood of CLL patients ranged from 0% to 85.4% (median = 13.4). The level of Zap-70 expression was significantly higher in the B lymphocytes of lymph nodes and spleens of B-CLL patients (range: 6%–98%; median = 36). Additionally, we assessed Zap-70 expression in samples from patients with SMLZ and large B-cell lymphoma. In SMZL samples, the percentage of Zap-70-positive B cells ranged from 0% to 28% (median = 0.5) and in DLBCL samples from 0% to 98% (median = 31). Control samples of peripheral blood from patients (B lymphocytes) without malignancies were negative for Zap-70 staining.

### Proliferative index

The proliferative properties of cells from peripheral blood and solid lymphoid tissues of patients with CLL were estimated



**Figure 5** Representative flow cytometry profiles of Zap-70 expression in patients with B-CLL. Peripheral blood cells from B-CLL patients were analyzed by flow cytometry after staining with isotype control or anti-Zap-70 antibodies. **A)** Dot plots of a Zap-70 negative case. **B)** Dot plots of a Zap-70-positive case. **C)** Forward and side scatter of whole blood. **D)** CD79a and Zap-70-Alexa 488 staining gated on R1 lymphocyte scatter gate.

by determining the expression of the proliferative antigen Ki-67. Some authors have previously reported a very low level of Ki-67 in circulating CLL cells.<sup>36</sup> Although our results confirm this, we found a rather high percentage of Ki-67-positive B cells in lymph nodes and spleens from CLL patients ( $4.94\% \pm 0.55\%$ ;  $n = 45$ ) (Figure 6) that was close to Ki-67 levels on B cells in reactive lymph nodes and spleen specimens ( $4.76 \pm 0.81$ ;  $n = 32$ ). In CLL bone marrow specimens, the percentage was only  $1.78 \pm 0.32$  ( $n = 44$ ;  $P < 0.01$ ), and in the peripheral blood, it was 10 times lower, only  $0.45\% \pm 0.03\%$  ( $n = 201$ ;  $P < 0.001$ ) (Table 1).

Since the difference in Ki-67 expression might be related to variation in patient characteristics (clinical stage, etc), we made direct comparisons of the levels of Ki-67 from parallel peripheral blood and lymph node/spleen biopsies obtained from 20 patients. The level of proliferation in the lymph nodes/spleens was significantly higher compared to the blood (Table 2;  $P < 0.005$ ), and this difference was even greater than in independent specimens (Table 1).

Our study indicated that the low level of Ki-67 in peripheral blood may represent a subpopulation of quiescent malignant B cells, while their real proliferation occurs in the lymph nodes and spleen. This view is supported by previous reports of the recovery time for B-CLL cells obtained by nonradioactive labeling.<sup>30</sup>

### Correlation analysis of CD38, CD25, Zap-70, and Ki-67 expression in different compartments

We analyzed possible correlations among CD38, Zap-70, Ki-67, CD20, and CD25 expression in peripheral blood, bone

marrow, and spleens with lymph nodes. A correlation was found among CD38, Zap-70, and Ki-67 in B-CLL (Table 3). However, the correlation between Zap-70 and Ki-67 appeared to be insignificant. In the lymph node and spleen, a positive correlation was found only in the CD38<sup>intermediate</sup> group.

In patients with SMZL, expression of CD38 correlated with Ki-67 ( $r = 0.557$ ;  $P < 0.01$ ) but not with Zap-70 (Table 4).

Levels of CD25 and CD38 expression demonstrated a positive correlation only in lymph nodes and spleens of patients with CLL ( $r = 0.321$ ;  $P \leq 0.05$ ), but there was no significant correlation in blood or bone marrow ( $P > 0.2$ ).

Creating three groups based on CD38 expression levels allowed further evaluation of the relationship among the expression of Ki-67, CD25, and Zap-70. In the overall cohort of patients, Ki-67 was highest in the CD38<sup>high</sup> group. In blood specimens, Ki-67 was highest in the CD3<sup>intermediate</sup> group and lowest in the CD38<sup>low</sup> group. Zap-70 in the whole cohort as well as in blood specimens was only minimal in the CD38<sup>low</sup> group, slightly higher in CD38<sup>intermediate</sup>, and maximal in the CD38<sup>high</sup> group. Average expression of CD25 was nearly the same in all three groups (Table 2).

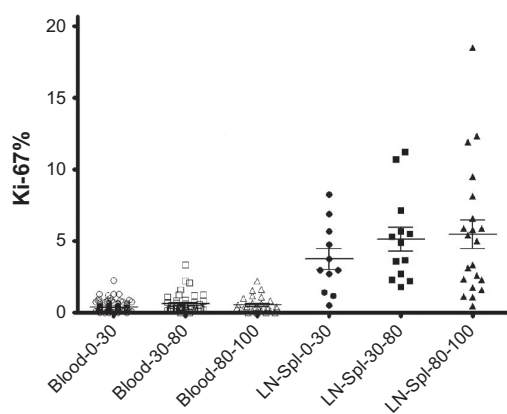
Our study indicates that in CLL, the levels of expression of CD38 and Ki-67 are significantly higher in lymph nodes/spleens and bone marrow compared to the peripheral blood.

## Discussion

Most cells circulating in the blood of B-CLL patients are in the G0 phase of the cell cycle, although some of them do express activation markers (CD38 and CD25).

CD38 is a multifunctional surface molecule that is expressed on activated B cells and is involved in signaling transduction and adhesion.<sup>5</sup> There is a general consensus that CD38 expression in blood cells is a negative prognostic marker for patients with CLL.<sup>8,9,34,37</sup> CD38 expression in blood cells is considered positively correlated with the majority of negative prognostic markers for CLL, including Zap-70,<sup>38,39</sup> elevated soluble CD23, and cytogenetic abnormalities.<sup>35,40,41</sup> However, the literature contains no data on comparison of expression of CD38 and proliferative markers in different tissue compartments in CLL.

We compared the levels of CD38 and proliferation markers in different lymphoid compartments of B-CLL patients and control groups of patients, including those with SMZL, large B-cell lymphoma, as well as patients without malignancies. When the percentages of CD38<sup>+</sup> cells were plotted for the entire patient population (data not shown),



**Figure 6** Comparison of Ki-67 versus CD38 in different compartments. Scattergrams of Ki-67<sup>+</sup> B cells in peripheral blood and spleen/lymph node biopsy specimens from CLL patients with CD38 low, intermediate, and high levels. Results are presented as the percentage of Ki-67<sup>+</sup> B cells, and the means are marked by solid lines. **Abbreviations:** LN, lymph node; Spl, spleen.

**Table 3** Expression of different antigens (%) stratified by CD38 peripheral B-CLL blood, bone marrow, and spleen and lymph nodes

	CD38 <sub>low</sub> -PBL	CD38 <sub>intermed</sub> -PBL	CD38 <sub>high</sub> -PBL	Bone marrow CLL	Spleen, lymph nodes CLL
Zap-70	18.4 ± 2.9 (n = 68)	29.6 ± 4.6 (n = 31)	32.1 ± 5.0 (n = 21)	29.6 ± 12 (n = 6)	41.1 ± 9.7 (n = 9)
Ki-67	0.36 ± 0.34 (n = 127)	0.66 ± 0.1 (n = 42)	0.53 ± 0.09 (n = 30)	2.48 ± 0.77 (n = 42)	4.95 ± 0.55 (n = 42)
CD25	70.0 ± 2.5 (n = 127)	69.7 ± 5.2 (n = 42)	67.3 ± 6.1 (n = 30)	55.4 ± 4.9 (n = 42)	75.0 ± 4.6 (n = 42)

Note: Mean ± SEM.

the patients could be divided into three groups, putting thresholds at 30% and 80% of CD38-positive cells. Patients with 30% or more B cells were considered positive, 30%–80% intermediate positive, 80% high positive, and those with <30% were considered negative.

The expression of CD38 is higher in solid lymphoid organs compared with peripheral blood.<sup>42,43</sup> We found the expression of CD38 to be different in solid lymphoid organs than in blood, and highest in lymph nodes and spleen biopsies of B-CLL patients. In parallel specimens (lymph node/blood), in 6 out of 20 cases, the difference in the expression of CD38 was large enough to change the grade (from negative to positive and from intermediate to positive high).

Together with CD38, Zap-70 was recently recognized as an independent negative prognostic factor for CLL.<sup>14</sup> Zap-70, normally expressed in T cells and natural killer cells,<sup>44</sup> has been reported to be present in human-activated B cells<sup>33</sup> and also to be expressed by clonal CD19<sup>+</sup>CD5<sup>+</sup> B-CLL cells. Recently, it has been shown that Zap-70 phosphorylates after CD38 ligation is a limiting factor for this signaling pathway.<sup>45</sup> In accordance with previous studies reporting a correlation between CD38 and Zap-70 in peripheral blood, we confirmed a correlation between CD38 and Zap-70 expression in blood and bone marrow, but not in lymph nodes or spleens.

Previous reports have shown that CD25 expression is increased in CLL lymphocytes.<sup>23,24</sup> Our study revealed a significant correlation between CD25 and CD38 expression

only in spleens and lymph nodes of CLL patients and no correlation between CD25 and CD38 in blood. In addition, no correlation was found between CD25 and Ki-67 or Zap-70 among our patients.

Although several studies report that CLL cells from most patients express certain activation-related<sup>28,46</sup> and cell cycle-related markers,<sup>26,47–49</sup> data on Ki-67 expression is highly controversial.<sup>26,27</sup> Our data showing a 10-fold higher expression of Ki-67 (the mean percentage of expression) in solid tissues (lymph nodes and spleen) of CLL patients are in accordance with the kinetics study of the Messmer Group<sup>30</sup> and the contention of early CLL researchers<sup>50,51</sup> that a proliferating component in CLL resides in the solid tissues. Along with histological findings on the presence of proliferation centers in lymph nodes and in the bone marrow,<sup>52</sup> our data support the hypothesis that the proliferating leukemic cells in CLL always reside in solid lymphoid organs, that is, lymph nodes and spleens. Although our findings show a significant correlation between CD38 and Ki-67 expression in bone marrow, the relationship between CD38 and Ki-67 in lymph nodes/spleens was more complicated. Although the mean percentage of CD38-positive and especially Ki-67-positive populations in this compartment were the highest, we found no significant correlation between them.

In conclusion, our findings give further evidence that activation level of CLL cells does not represent proliferation potential and proliferation of CLL is restricted to the lymph nodes.

**Table 4** Spearman's rank order correlations of CD38, Ki-67, CD25, and Zap-70 expression in different lymphoid compartments of CLL patients

Correlation pairs	Lymph nodes and spleen, CLL	Bone marrow, CLL	Blood, CLL	Lymph nodes and spleen, nonmalignant	Marginal zone lymphoma
CD38/Zap-70	0.051 (n = 9)	0.886 (n = 6)*	0.359 (n = 106)**		−0.044 (n = 14)
CD38/Ki-67	0.027 (n = 42)	0.528 (n = 42)**	0.246 (n = 201)**	−0.295 (n = 32)	0.557** (n = 14)
Zap-70/Ki-67	0.100 (n = 9)	0.771 (n = 6)	0.471 (n = 106)**		0.458** (n = 14)
CD25/CD38	0.321 (n = 42)*	−0.087 (n = 42)	0.070 (n = 200)	0.216 (n = 32)	−0.072 (n = 14)
CD25/Ki-67	−0.271 (n = 42)	−0.206 (n = 41)	0 (n = 200)	−0.324 (n = 32)	−0.103 (n = 14)
CD25/Zap-70	−0.136 (n = 9)	−0.100 (n = 5)	0.084 (n = 105)		−0.362* (n = 14)

Notes: \*Significant at  $P = 0.05$ ; \*\*Significant at  $P = 0.01$ .

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## Disclosure

The authors report no conflicts of interest in this work.

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