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*Original Article*

# Comparison of Bcl-2, CD38 and ZAP-70 Expression in Chronic Lymphocytic Leukemia

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**Abstract**: Chronic lymphocytic leukemia (CLL) was previously considered a uniform disease characterized by autonomous over-expression of bcl-2. Recently the pathogenic role of bcl-2 has been questioned and attention has turned to prognostic subtypes of CLL differing in CD38 and ZAP-70 expression. However, the relationship between bcl-2 and CD38 or ZAP-70 expression remains uncertain and was investigated using flow cytometric immunophenotyping of 50 CLL specimens. CLL cells were consistently bcl-2 positive but varied in expression level: mean fluorescence intensity (MFI) 45-152. Although there was no significant difference in bcl-2 expression between CD38 or ZAP-70 positive and negative specimens, an inverse correlation was identified between percentage of CD38 positive B-cells and bcl-2 MFI when all ($p<0.03$, $r^2=0.10$) and peripheral blood ($p<0.004$, $r^2=0.27$) samples were analyzed. While bcl-2 levels do not appear to be a major discriminator between indolent and more aggressive subtypes of CLL, CD38 and bcl-2 expression appear to be interrelated.
**Key Words**: bcl-2, CD38, chronic lymphocytic leukemia, ZAP-70

## Introduction

For many years chronic lymphocytic leukemia (CLL) was considered to result from the accumulation of resting, antigen naïve, B-cells [1]. Bcl-2 and its family members were proposed to play a key pathogenetic role by conferring resistance to apoptosis and thereby prolonging cell survival [2-4]. Since most CLL cases express high levels of bcl-2 protein, it was hypothesized that this reflected constitutive over-expression due to an intrinsic abnormality, akin to that seen in follicular lymphoma [1]. However, subsequent studies have revealed that bcl-2 expression varies between CLL specimens, can be altered by external cell stimuli, and appears to be under the control of a functioning regulatory pathway [2]. Therefore, it has been proposed that bcl-2 expression in CLL is not autonomous, might be maintained at least in part by ongoing microenvironmental stimuli, and may not play a major role in the pathogenesis of the disease [2, 5, 6]. In addition, although bcl-2 expression has been associated with a poor response to cytotoxic therapy for CLL, it does not appear to be a major determinant of clinical progression [1, 7-9].

More recently, two major prognostic subtypes of CLL have been recognized that are associated with differential expression of CD38 and ZAP-70. Further investigation into the biology of these newer prognostic markers has revealed that they may be associated with the ability of CLL cells to activate signaling pathways following stimulation of the B cell receptor (BCR) [10, 11]. These studies, as well as those investigating immunoglobulin heavy chain variable region usage, have revealed that not all CLL cells are naïve B-cells, and some display evidence of antigen exposure [9, 12-14]. Although, the precise mechanism for apparent differences in responsiveness to antigenic stimulation between the two major prognostic groups of CLL remains uncertain, and how these differences might affect

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prognosis is not known, it has been hypothesized that differential activation of the BCR in CLL cells might determine cell fate through stimulation of proliferation and survival pathways, including the bcl-2 associated anti-apoptotic pathway [9, 15-19]. Therefore, to further investigate the role of bcl-2 in CLL, the relationship of bcl-2 expression to that of CD38 and ZAP-70 was evaluated by flow cytometric immunophenotyping of 50 CLL specimens.

## Materials and Methods

The study was approved by the University of Pittsburgh Institutional Review Board. CD5 positive B-cell lymphoid neoplasms were identified among specimens analyzed in the clinical flow cytometry laboratory of the University of Pittsburgh Medical Center, Presbyterian Hospital. De-identified pathology reports and flow cytometry histograms were then reviewed to identify cases meeting the criteria for CLL as defined in the 2001 WHO classification of tumors of the hematopoietic and lymphoid tissues [20]. Fifty specimens were identified for further study: peripheral blood (30), bone marrow aspirate (15), lymph node biopsy (4), and pleural fluid (1).

Flow cytometric immunophenotyping was performed as previously described [21] using, at a minimum, the following combinations of antibody-fluorochrome combinations: kappa-fluorescein isothiocyanate (FITC)/lambda-phycoerythrin (PE)/CD20 peridinin chlorophyll (PerCP), FMC7-FITC/CD23-PE, CD2-FITC/CD8-PE/CD4-PerCP, and CD5-FITC/CD10-PE/CD19-phycoerythrin-cyanin 5 (PC5) (Becton Dickinson, San Jose, CA and Immunotech Beckman Coulter, Miami, FL). Data was acquired using Becton Dickinson FACSCalibur flow cytometers and analyzed using CellQuest software (Becton Dickinson, San Jose, CA).

Flow cytometric analysis of CD38 expression on CD19 positive cells was performed for routine clinical purposes on all but one case using the following three color combination: CD5-FITC (clone L17F12, Becton Dickinson, San Jose, CA), CD38-PE (clone HB7, Becton Dickinson, San Jose, CA), and CD19-PC5 (clone J4.119, Immunotech Beckman Coulter, Miami, FL). A plot of forward versus side scatter was used to identify cells falling in the "lymphoid region". The lymphoid cells were then displayed on a plot of CD38 versus CD19. CD38 was considered positive if greater than 20% of B-cells demonstrated staining. The proportion of B-cells that represented hematogones and/or plasma cells was determined through identification of cells co-expressing CD19 and CD10 or co-expressing CD19 and very bright intensity CD38. Since CD10 was not present in the same tube as the combined CD38 and CD5, and hematogones may demonstrate some staining for CD5, particular attention was paid to excluding bright intensity CD38 events from the determination of CD38 percent when CD10 positive hematogones exceeded 10% of CD19 positive B-cells.

Flow cytometric analysis for ZAP-70 was performed using a four color flow cytometric assay as follows. Briefly, cells were incubated with CD19-PE (clone 4G7, Becton Dickinson, San Jose, CA), CD3-PerCP protein (clone SK7, Becton Dickinson, San Jose, CA) and CD5 Allophycocyanin (clone L17F12, Becton Dickinson, San Jose, CA) on ice for 15 minutes. After washing with phosphate-buffered saline azide solution, cell pellets were resuspended, fixed, and permeabilized (Fix and Perm Kit, Caltag, Burlingame, CA). The permeabilized cells were then incubated with ZAP-70 fluorescein isothiocyanate (clone 1E7.2, eBiosciences, San Diego, CA) for 15 minutes at room temperature. Cells were washed and acquired on the flow cytometer. Cells falling in the "lymphoid region" on a plot of forward versus side scatter were displayed on dot plots of ZAP-70 versus CD3 and ZAP-70 versus CD19. Quadrant markers were set on the CD3/ZAP-70 dot plot so that virtually all of the T-cells were positive for ZAP-70. The proportion of ZAP-70 positive, CD19 positive B-cells was then determined by comparing with this T-cell derived threshold [22]. ZAP-70 was considered positive if greater than 20% of B-cells demonstrated staining above this threshold.

Flow cytometric analysis of bcl-2 expression was performed on all specimens using the three color method previously reported [23] using the following antibodies: bcl-2-FITC (clone 124, DAKO, Carpinteria, CA), CD5-PE (clone L17F12, Becton Dickinson, San Jose, CA), and CD19-PC5 (clone J4.119, Immunotech Beckman Coulter, Miami, FL). Following selection of events falling in the "lymphoid region" identified on forward-scatter versus side-scatter plots, mean fluorescence

**Table 1** CD38, ZAP70 and bcl-2 expression in CLL

| Patient | Specimen Type | % CD38+ B-cells | CD38 Result | % ZAP+ B-cells | ZAP Result | bcl-2 MFI B-cells | bcl-2 MFI non-B lymphoid cells | bcl-2 MFI Ratio | bcl-2 Result |
|---|---|---|---|---|---|---|---|---|---|
| 1 | BM | 93 | Pos | 94 | Pos | 142 | 56 | 2.51 | H |
| 2 | BM | 9 | Neg | 0.3 | Neg | 152 | 79 | 1.92 | H |
| 3 | BM | 100 | Pos | 27 | Pos | 116 | 64 | 1.81 | H |
| 4 | BM | 27 | Pos | 9 | Neg | 126 | 44 | 2.85 | H |
| 5 | BM | 46 | Pos | 21 | Pos | 128 | 66 | 1.94 | H |
| 6 | BM | 14 | Neg | 1 | Neg | 99 | 72 | 1.39 | L |
| 7 | BM | N/A | N/A | 27 | Pos | 104 | 45 | 2.33 | H |
| 8A[1] | BM | 54 | Pos | 5 | Neg | 106 | 45 | 2.37 | H |
| 9 | BM | 19 | Neg | 6 | Neg | 112 | 57 | 1.97 | H |
| 10 | BM | 6 | Neg | 3 | Neg | 78 | 60 | 1.30 | L |
| 11 | BM | 56 | Pos | 7 | Neg | 139 | 70 | 1.97 | H |
| 12 | BM | 68 | Pos | 3 | Neg | 60 | 36 | 1.67 | L |
| 13 | BM | 16 | Neg | 3 | Neg | 55 | 52 | 1.06 | L |
| 14 | BM | 71 | Pos | 1 | Neg | 52 | 52 | 1.00 | L |
| 15 | BM Bx | 68 | Pos | 82 | Pos | 83 | 47 | 1.76 | H |
| 16A[2] | LN | 98 | Pos | 98 | Pos | 82 | 41 | 2.00 | H |
| 17 | LN | 78 | Pos | 79 | Pos | 72 | 33 | 2.18 | H |
| 18 | LN | 4 | Neg | 70 | Pos | 134 | 91 | 1.46 | L |
| 19 | LN FNA | 13 | Neg | 21 | Pos | 80 | 57 | 1.40 | L |
| 20 | PB | 6 | Neg | 8 | Neg | 95 | 55 | 1.73 | H |
| 21 | PB | 34 | Pos | 0.5 | Neg | 121 | 77 | 1.56 | L |
| 22 | PB | 12 | Neg | 15 | Neg | 100 | 51 | 1.98 | H |
| 23 | PB | 95 | Pos | 13 | Neg | 59 | 50 | 1.18 | L |
| 24 | PB | 93 | Pos | 41 | Pos | 93 | 85 | 1.10 | L |
| 25 | PB | 2 | Neg | 6 | Neg | 137 | 71 | 1.93 | H |
| 26 | PB | 68 | Pos | 21 | Pos | 116 | 53 | 2.21 | H |
| 27 | PB | 67 | Pos | 4 | Neg | 75 | 53 | 1.43 | L |
| 28 | PB | 10 | Neg | 3 | Neg | 85 | 51 | 1.67 | L |
| 29 | PB | 68 | Pos | 1 | Neg | 70 | 56 | 1.26 | L |
| 30 | PB | 1 | Neg | 0.5 | Neg | 95 | 75 | 1.26 | L |
| 31 | PB | 1 | Neg | 1 | Neg | 106 | 55 | 1.93 | H |
| 32 | PB | 1 | Neg | 2 | Neg | 143 | 70 | 2.02 | H |
| 33 | PB | 0.3 | Neg | 0.6 | Neg | 137 | 62 | 2.19 | H |
| 34 | PB | 0.6 | Neg | 0.5 | Neg | 132 | 75 | 1.78 | H |
| 35 | PB | 10 | Neg | 0.2 | Neg | 86 | 63 | 1.37 | L |
| 36 | PB | 5 | Neg | 2 | Neg | 112 | 60 | 1.87 | H |
| 37 | PB | 64 | Pos | 11 | Neg | 69 | 53 | 1.31 | L |
| 38 | PB | 61 | Pos | 9 | Neg | 68 | 59 | 1.16 | L |
| 39 | PB | 18 | Neg | 7 | Neg | 117 | 69 | 1.70 | H |
| 40 | PB | 2 | Neg | 1 | Neg | 97 | 55 | 1.75 | H |
| 41 | PB | 7 | Neg | 2 | Neg | 68 | 49 | 1.39 | L |
| 42 | PB | 96 | Pos | 3 | Neg | 45 | 38 | 1.17 | L |
| 43 | PB | 7 | Neg | 2 | Neg | 78 | 45 | 1.72 | H |
| 44 | PB | 20 | Pos | 3 | Neg | 81 | 44 | 1.82 | H |
| 45 | PB | 1 | Neg | 1 | Neg | 96 | 39 | 2.47 | H |
| 16B[2] | PB | 57 | Pos | 2 | Neg | 113 | 74 | 1.53 | L |
| 46 | PB | 20 | Neg | 7 | Neg | 150 | 64 | 2.36 | H |
| 47 | PB | 30 | Pos | 28 | Pos | 74 | 48 | 1.54 | L |
| 8B[1] | PB | 23 | Pos | 1 | Neg | 124 | 47 | 2.61 | H |
| 48 | Pleural Fluid | 28 | Pos | 1 | Neg | 56 | 61 | 0.92 | L |

BM, bone marrow aspirate; PB, peripheral blood; BM Bx, bone marrow biopsy; LN, lymph node; FNA, fine needle aspiration; Pos, positive; Neg, negative; H, high; L, low. [1]Bone marrow and peripheral blood samples are from same patient. [2]Lymph node and peripheral blood samples are from same patient.

intensity for bcl-2 was recorded for B-cells and CD19 negative non-B lymphoid cells i.e. T- and NK- cells. The ratio of mean fluorescence intensity of CLL cells compared to non-CLL lymphoid cells was calculated (MFI-R).

For statistical analyses, Pearson's correlation and t-tests were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

## Results

Fifty specimens that met the diagnostic criteria for CLL were identified (30 peripheral blood, 15 bone marrows, 4 lymph nodes, and 1

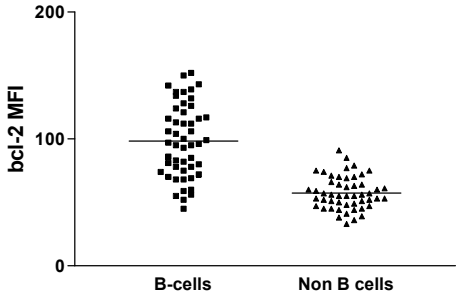


**Figure 1** Comparison between bcl-2 expression in B-cells and non-B lymphoid cells. B-cells demonstrate significantly higher bcl-2 MFI than non B-lymphoid cells ($p<0.0001$).

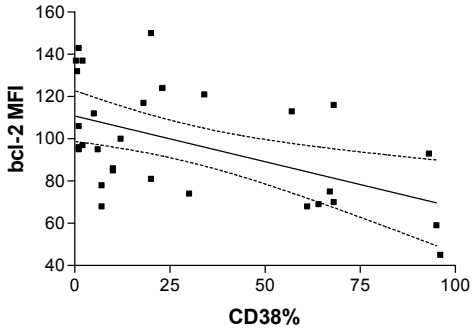


**Figure 2** Comparison between bcl-2 MFI and the percentage of CD38 positive B-cells in peripheral blood samples, demonstrating an inverse correlation ($p<0.004$, $r^2=0.27$).

pleural fluid) from forty-eight patients (34 males, 14 females, age range 35-86 years, mean age 66 years). The specimens included pre-treatment samples, obtained either at the time of diagnosis or subsequently, and post-treatment samples. CD19 positive/CD5 positive CLL-cells represented greater than 87% of all B-cells in the peripheral blood specimens. CD19 positive/CD5 positive CLL cells represented 98-100% of all B-cells in lymph node specimens, and 88-100% of all B-cells in bone marrow specimens. In two bone marrow specimens (from patients 5 and 9), hematogones represented greater than 10% of all B-cells.

CD38 was positive in 25/49 cases (51%) and ZAP-70 was positive in 12/50 cases (24%). Analysis of CD38 and ZAP-70 positivity among all CLL cases demonstrated a statistically significant correlation ($p<0.04$). Analysis using a lower cutoff for ZAP-70 positivity (greater than 8%) showed an even stronger correlation with CD38 expression ($p<0.01$). CD38 and ZAP-70 results were concordant in 31/49 cases (63%). Discordant results included 16 (32%) CD38 positive/ZAP-70 negative cases and 2 (4%) CD38 negative/ZAP-70 positive cases (**Table 1**).

CLL cells were bcl-2 positive in all specimens. Absolute and relative bcl-2 levels varied among the B-cells (MFI 45-152; MFI-R 0.92-2.85) and this variation was greater than that seen in non B-lymphoid cells (**Table 1**). In addition, bcl-2 MFI of B-cells was significantly greater than that of non B-lymphoid cells ($p<0.0001$) (**Figure 1**). There was no significant difference in bcl-2 expression by B-cell and non B-lymphoid cells between specimen types. A positive correlation between B-cell and non B-lymphoid cell bcl-2 MFI was observed for all specimens ($p<0.0001$, $r^2 = 0.3$), peripheral blood samples only ($p = 0.002$, $r^2 = 0.3$), and non-peripheral blood specimens ($p = 0.01$, $r^2 = 0.3$).

An inverse correlation was identified between the percentage of CD38 positive B-cells and bcl-2 MFI when all samples ($p < 0.03$, $r^2 = 0.10$) and peripheral blood samples only ($p<0.004$, $r^2=0.27$) were analyzed. No statistically significant correlation was identified between bcl-2 MFI and CD38 percent when non-peripheral blood specimens were analyzed. An inverse correlation was identified between the percentage of CD38 positive B-cells and bcl-2 MFI-R for the peripheral blood samples only ($p < 0.002$, $r^2 = 0.30$) (**Figure2**). No statistically significant correlation was identified between bcl-2 MFI-R and CD38 percent when all specimens or non-peripheral blood specimens were analyzed. No significant correlation was demonstrated between the percentage of ZAP-70 positive cells and the CLL B-cell bcl-2 MFI or MFI-R in either the whole group or peripheral blood samples only. In addition, no statistically significant difference was demonstrated in B-cell bcl-2 MFI between CD38 positive vs CD38 negative cases (MFI 92±29 vs 106±27) and ZAP-70 positive and ZAP-70 negative cases (MFI 102±25 vs 97±30), even when a lower ZAP-70 cut-off of 8% was utilized.

Further evaluation of the frequency distribution of bcl-2 MFI-R demonstrated a break in the data separating two groups of samples that differed in bcl-2 status: bcl-2

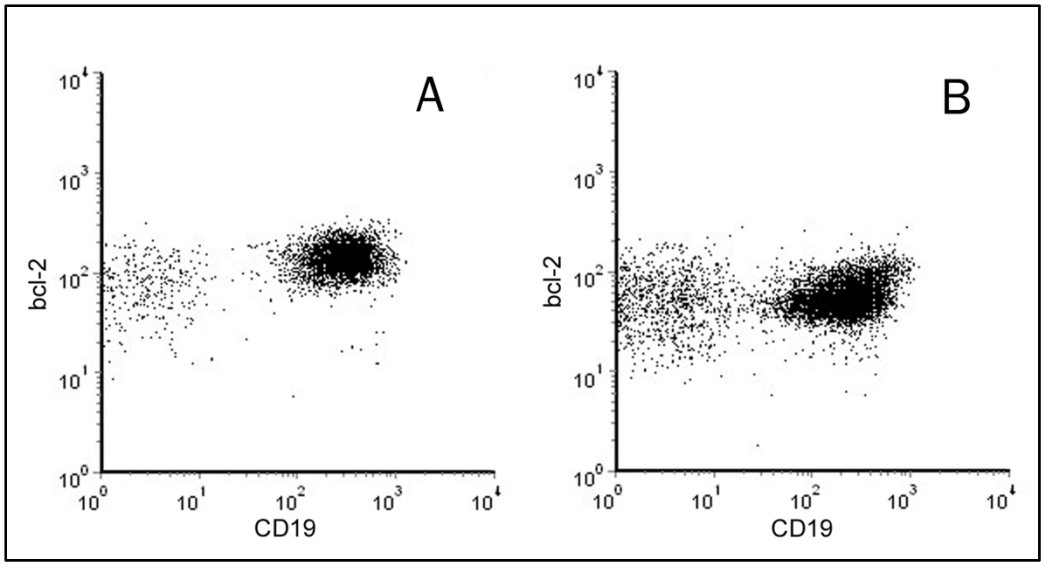


**Figure 3** Flow cytometric dot-plots illustrating bcl-2 expression of 19 positive B-cells and CD19 negative non B-lymphoid cells. (**A**) Sample with high bcl-2 expression in CLL cells (MFI-R > 1.7). (**B**) Sample with low bcl-2 expression in CLL cells (MFI-R < 1.7).

MFI-R greater than 1.7 (bcl-2 MFI-R high) (**Figure 3A**) and bcl-2 MFI-R less than 1.7 (bcl-2 MFI-R low) (**Figure 3B**). This cut-off point was confirmed using an imperfect gold standard analysis [24]. 28/50 cases (56%) were considered bcl-2 MFI-R high (MFI-R greater than 1.7). Although there was no statistically significant association between bcl-2 status (high versus low) and CD38 positivity or ZAP-70 positivity, only 43% of the bcl-2 high cases were CD38 positive compared to 59% in the bcl-2 low group.

Two patients had flow cytometric analysis performed on more than one type of specimens on two separate occasions (cases 8A/B and 16 A/B). Cases 8A and 8B (bone marrow and peripheral blood respectively) were both CD38 positive and bcl-2 MFI ratio high. Cases 16A and 16B (lymph node and peripheral blood respectively) were both CD38 positive but had divergent bcl-2 MFI ratios (**Table 1**).

## Discussion

Bcl-2 mediated inhibition of apoptosis has fallen from favor as a key factor in the pathogenesis of CLL because a uniform mechanism for autonomous bcl-2 over-expression has not been identified and bcl-2 expression appears to remain responsive to exogenous stimuli [1, 2]. For example, bcl-2 levels have been shown to decrease in response to treatment with chlorambucil, theophylline and nucleoside analogues, following phorbol-ester induced proliferation, and in association with spontaneous apoptosis in culture [5, 25, 26]. Additionally, spontaneous apoptosis of CLL cells is accompanied by a cascade of events that suggests the presence of an intact, functional, bcl-2 anti-apoptotic regulatory pathway [2]. Therefore, it has been proposed that bcl-2 expression, and prolonged cell survival, in CLL may at least in part represent a response to microenvironmental stimuli that include interactions with non-leukemic cells, lymphokines, and antigens [2, 5, 6]. Differences in CLL cell responsiveness to external stimuli also appear to be associated with expression of some of the newer prognostic markers. IgVH mutational status and CD38 expression are both related to the ability of CLL cells to signal in response to BCR cross-linking through ligation of membrane IgM [15, 16]. ZAP-70 has been shown to enhance IgM signaling through the BCR [17]. IgVH mutational status is associated with differential expression of several genes known to be modulated during BCR signaling [11]. Furthermore, it has been proposed that BCR signaling might affect prognosis through differential stimulation of cell survival or

proliferation pathways [9, 17, 19].

Our study investigated whether the level of bcl-2 expression might account for some of the differences between the major prognostic subtypes of CLL. Although an inverse correlation was demonstrated between bcl-2 MFI and CD38 percentage, no significant difference was identified in bcl-2 expression between CLL specimens differing in CD38 or ZAP-70 status (positive versus negative). These results suggest that bcl-2 is not a major determinant of the differences between the indolent and more aggressive subtypes of CLL. However, the proportion of ZAP-70 positive cases is lower than that reported for some previous studies [22] and the proportion of discordant CD38 positive, ZAP-70 negative cases is higher [17, 27, 28]. Therefore, the possibility that bcl-2 and ZAP-70 expression are correlated in some circumstances cannot be entirely excluded. Demonstration of an inverse correlation between the level of bcl-2 expression and the proportion of CD38 positive cells in CLL is of interest because both appear to belong to cellular pathways that are functional and responsive [2, 19]. Therefore, it is tempting to speculate that variable stimulation and responsiveness of CLL cells might result in some cases with many CD38 positive cells, down-regulated bcl-2 expression, and perhaps a predilection for apoptosis, and other cases with fewer CD38 positive cells, higher levels of bcl-2 expression, and perhaps a relative resistance to apoptosis.

CD38 is a transmembrane glycoprotein that is normally expressed at several stages of B-cell maturation: precursor B-cells, follicle center cells, and plasma cells. Normal resting peripheral blood B-cells express CD38 at a low level, but expression can be induced by cell activation [10, 14]. In normal B-cells, CD38 related signaling can lead to apoptosis or proliferation depending on the stage of cell maturation, nature of the stimulus, density of CD38 antigen expression relative to an apparent threshold, and the presence of cofactors. In precursor B-cells, where bcl-2 expression is typically low, CD38 signaling leads to apoptosis [29]. In contrast, although high CD38 expression in cultured follicle center cells is associated with spontaneous apoptosis, this can be abrogated by increased bcl-2 expression following CD38 ligation[29].

CD38 expression in CLL is variable and has received attention as a prognostic indicator. CD38 positive CLL has been associated with more advanced stage disease, shorter time to first treatment, poor response to CT, and shorter overall survival [30-33]. However, the distinction between CD38 positive and CD38 negative CLL is not always straightforward because within a single specimen CLL cells may display heterogeneous expression of CD38 or include two discrete populations with disparate CD38 expression i.e. biphasic CD38 expression, and expression can also vary over time, with treatment, and between different specimen types [30, 32-38]. Therefore, it has been suggested that CD38 expression in CLL is more than just a prognostic marker, and akin to expression by normal peripheral blood B-cells, may reflect the level of cell activation. *In vitro*, CD38 expression by CLL cells can be induced by cytokine stimulation, although the ability of CLL cells to upregulate CD38 expression in response to stimulation with IFN alpha differs between CD38 positive, negative, or bi-phasic cases [36, 37]. *In vivo*, CD38 expression in CLL appears to correlate with other phenotypic markers of activation, and even within a single specimen, CD38 positive and negative CLL cells have been noted to differ in their degree of activation [36-38]. Thus, the correlation identified in the current study between bcl-2 expression and CD38 percent, rather than CD38 status, might reflect biologic differences in CLL cell responsiveness at a cellular level. CD38 and bcl-2 expression were evaluated in the current study using separate flow cytometric tubes and different surface and cytoplasmic staining procedures. Therefore, direct comparison of cellular expression was not possible. Although one previous study evaluating CD38 positive and CD38 negative CLL cells within the same specimen did not identify an association with bcl-2 expression, the results were expressed as an average of bcl-2 expression across all specimens and therefore did not take into account variation in bcl-2 expression between individual specimens or cells [38]. More numerous studies have evaluated CLL specimens for an association between apoptosis and bcl-2 expression or CD38 expression. An inverse relationship has been demonstrated between bcl-2 expression and the propensity of CLL cells to undergo spontaneous apoptosis *in vitro*, including more recent studies showing an association between micro-RNA miR-15a/miR-16-1 induced down-regulation of bcl-2 expression in

CLL and apoptosis [2, 39-42]. High expression of both of these micro-RNAs in CLL has been associated with adverse prognostic factors including high levels of ZAP-70 expression and unmutated IgVH [43]. Although several studies have evaluated CD38 expression and apoptosis, they have yielded conflicting results that may relate to differences in study design, including whether apoptosis is spontaneous or induced [16, 19, 44-46]. In addition, these studies have only compared CLL cases that differ in CD38 status (positive versus negative) rather than CD38 percent and therefore have not addressed potential differences at a cellular level.

Of interest, our study also demonstrated a correlation between the level of bcl-2 expression in CLL-cells and non-CLL lymphoid cells. Although analytical variation between specimens cannot be entirely excluded, it is possible that T-cell and CLL B-cell expression of bcl-2 could be affected by common factors. To further address the possibility of analytical variability between cases in the current study, the bcl-2 MFI-R was calculated as previously reported by Menendez, using non-CLL lymphoid cells as an internal control [40]. Utilizing the MFI-R, an inverse correlation was demonstrated with percent CD38 positive B-cells for peripheral blood samples only. Although it is unclear why this association was lost when other specimen types were included in the analysis, this may reflect microenvironmental differences in T-cell, as well as CLL B-cell, populations. For example, PB and LN specimens obtained from a single study patient (16A and B) demonstrated divergent MFI-R results that may reflect such differences. It is well recognized that non-tumoral T-cells are altered in B-cell CLL and it has been proposed that these T-cells may provide an important stimulus to CLL cell survival and growth, possibly through cytokine feedback circuits [6]. In addition, CLL involving lymphoid tissues and bone marrow specimens often has proliferation centers that contain subsets of CLL-cells and associated T-cells with distinctive phenotypic features [6, 47]. Therefore, the MFI-R is possibly an over simplification and it will be of interest to further investigate the relative expression of bcl-2 and CD38 in subsets of CLL-cells and T-cells in the PB and different tissue compartments.

In summary, the current study demonstrated an inverse relationship between the percent of CD38 positive cells and the level of bcl-2 expression. Although bcl-2 does not appear to be a major determinant of the differences between the main prognostic subtypes of CLL, these results suggest that BCR signaling and anti-apoptotic pathways may be interrelated and that bcl-2 mediated inhibition of apoptosis might be more important in cases of CLL with fewer CD38 positive cells. However, given the dynamic, and transient, nature of CD38 expression it will be of interest to more directly investigate the relationship of CD38 and bcl-2 expression at a cellular level, and correlate the pattern of staining with other markers of cell activation, proliferation, and apoptosis.

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