CD38 EXPRESSION LABELS AN ACTIVATED SUBSET
WITHIN CHRONIC LYMPHOCYTIC LEUKEMIA CLONES
ENRICHED IN PROLIFERATING B CELLS

by

Rajendra N. Damle\textsuperscript{1,2}, Sonal Temburni\textsuperscript{1}, Carlo Calissano\textsuperscript{1}, Sophia Yancopoulos\textsuperscript{1}, Taraneh Banapour\textsuperscript{1}, Cristina Sison\textsuperscript{3}, Steven L. Allen\textsuperscript{4,2}, Kanti R. Rai\textsuperscript{5,6}, and Nicholas Chiorazzi\textsuperscript{1,7}

\textsuperscript{1} Laboratory of Experimental Immunology, The Feinstein Institute for Medical Research, North Shore - LIJ Health System, Manhasset, NY 11030
\textsuperscript{2} Department of Medicine, New York University School of Medicine, New York, NY
\textsuperscript{3} Department of Biostatistics, The Feinstein Institute for Medical Research, North Shore - LIJ Health System, Manhasset, NY 11030
\textsuperscript{4} Monter Cancer Center, North Shore - LIJ Health System, Lake Success, NY 11042
\textsuperscript{5} Department of Medicine, Albert Einstein College of Medicine, Bronx, NY
\textsuperscript{6} Division of Hematology and Oncology, Long Island Jewish Medical Center, North Shore - LIJ Health System, Lake Success, NY 11040
\textsuperscript{7} Departments of Cell Biology and of Medicine, Albert Einstein College of Medicine, Bronx, NY

Running title: CD38 expression in CLL subclones
ABSTRACT

CLL cells are thought to have diminished cell cycling capacity, a view challenged by their phenotypic resemblance to activated human B lymphocytes. The present study addresses the cell cycling status of CLL cells, focusing on those leukemic cells expressing CD38, a molecule involved in signaling and activation that also serves as a prognostic marker in this disease. CD38+ and CD38− members of individual CLL clones were analyzed for co-expression of molecules associated with cellular activation (CD27, CD62L and CD69), cell cycle entry (Ki-67), signaling (ZAP-70), and protection from apoptosis (telomerase and Bcl-2).

Regardless of the size of the CD38+ fraction within a CLL clone, CD38+ subclones are markedly enriched for expression of Ki-67, ZAP-70, human telomerase reverse transcriptase, and telomerase activity. Although the percentage of cells (~2%) entering the cell cycle, as defined by Ki-67 expression, is small, the absolute number within a clone can be sizeable and is contained primarily within the CD38+ fraction. Despite these activation/proliferation differences, both CD38+ and CD38− fractions have similar telomere lengths suggesting that CD38 expression is dynamic and transient. These findings may help explain why high percentages of CD38+ cells within clones are associated with poor clinical outcome.
INTRODUCTION

Chronic lymphocytic leukemia (CLL) results from amplification and accumulation of clonal CD5+ B cells. Although initially thought to be homogenous in manifestations and mechanisms, it is now clear that CLL is quite heterogeneous. Subgroups can be defined by differences in IgVH gene mutations, CD38 and ZAP-70 expression, presence of chromosomal abnormalities, and p53 dysfunction, with cases expressing unmutated IgVH genes (“U-CLL”), elevated numbers of CD38+ or ZAP-70+ cells, deletions at 17p and 11q, or impaired p53 activity having worse clinical outcomes. Among cell surface markers, expression of CD38 and its relevance to the pathobiology of CLL has been the subject of intense study. It is now clear that this molecule binds CD3, enabling important cell-cell interactions that signal activation and survival pathways in normal and leukemic lymphocytes and antigen presenting cells.

Despite the heterogeneity of expression of various molecular and cellular markers in CLL, the disease appears relatively homogeneous by gene expression profiling. Only a small number of genes are differentially expressed between U-CLL and M-CLL, implying that all CLL clones likely derive from antigen-experienced/memory-like B cells. Similarly, different gene expression signatures distinguish CLL cases defined by CD38 and ZAP-70 expression.

A paradoxical feature of circulating CLL cells is the expression of multiple features of activated, antigen-experienced B cells by lymphocytes that are mostly arrested in the G0/G1 phase of the cell cycle. Although the majority of CLL cells from most cases express activation-related and certain cell cycle-related markers, surprisingly low percentages of Ki-67 expressing cells have been found in the blood of CLL patients compared to those observed in
other lymphoid malignancies. Furthermore a proliferative compartment exists in CLL, although this probably resides in the solid tissues. Of note, data derived using tissue microarrays suggest that most CLL cells exist in late G1 phase (cyclin E+) and a surprising number of cells exist in the S (cyclin A+) and G2/M phases (Cyclin B1+) of the cell cycle. These data are at variance with other studies mentioned above and may support a difference in cell cycle progression between circulating and tissue bound CLL cells.

Questions remain as to how many cells bearing evidence for cellular activation actually enter and complete the cell cycle. Since analyses of bulk populations limit the extent to which properties of members of cell populations can be understood, efforts are now focusing on fractionating CLL clones and defining differences in cellular components. In this regard, despite their monoclonal origin, highly purified CD38+ and CD38− subpopulations derived from the same CLL patient exhibit distinct gene expression signatures.

In an attempt to address this dilemma and to quantify the percentage of cells that enter the cell cycle, we have studied differences in expression of Ki-67 in relation to ZAP-70, Bcl-2, and surface membrane activation marker expression in CD38+ and CD38− subclones within a series of CLL clones from various patients differing in their overall CD38 status. We have also assessed differences in the replicative history and potential of CD38 subpopulations within individual CLL cases. These studies identify a close association between CD38 expression and increased Ki-67 and ZAP-70 positivity suggesting that CD38+ clonal members are more activated and could more frequently enter the cell cycle than their counterpart CD38− cells. Although CD38+ cells exhibited greater telomerase activity than companion CD38− cells, these two cell subsets did not differ in telomere length, implying that CD38 expression is a temporal feature of the cells’ activation state that can change over time.
MATERIALS AND METHODS

Patients and healthy donors

The Institutional Review Board of the North Shore - LIJ Health System approved these studies. Following informed consent and in accordance with the Declaration of Helsinki, venous blood was collected from 95 randomly chosen patients with CLL for whom Ig V gene DNA sequence data was available. Leukocyte-enriched fractions of blood donated by 20 normal volunteers matched for age with the CLL cases (≥60 years) were purchased from Long Island Blood Services, Melville, NY; these samples were negative for HIV and HBV antigens. PBMC were separated from heparinized venous blood and leukocyte-enriched fractions by density gradient centrifugation using Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) and cryopreserved for future use, using a programmable cell-freezing machine (Cryomed, Mt. Clemens, MI).

Analysis of surface membrane and intracellular antigens by flow cytometry

The following fluorochrome conjugated mAbs were used: anti-CD5-APC, anti-CD27-FITC, anti-Ki-67-FITC, anti-Bcl-2-FITC and anti-CD62L-FITC (BD Pharmingen, San Diego, CA); Simultest Leucogate, fluorochrome-conjugated isotype control mAbs and anti-CD19-perCP, anti-CD38-PE and anti-CD69-FITC (BD Biosciences, San Diego, CA) and anti-ZAP70-FITC (eBioscience, San Diego, CA). Unconjugated polyclonal anti-TERT antibody was purchased from Calbiochem, USA. Cryopreserved PBMC from 50 CLL cases were subjected to four-color immunofluorescence staining that included in each set mAbs to CD38, CD5 and CD19 along with, either anti-CD27, anti-CD62L or anti-CD69 mAbs. PBMC from the entire cohort of 95 CLL were studied for expression of intracellular antigens (Ki-67, ZAP-70 and Bcl-
2) by incubating with mAbs reactive with CD38, CD5 and CD19, permeabilizing and fixing with Cytofix/Cytoperm reagent (BD Biosciences, San Diego, CA), and then incubating with either antibodies to Ki-67, ZAP-70, Bcl-2 or isotype control mAbs for an additional 25 minutes at 4°C. Cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry systems, San Jose, CA).

For analyses of Ki-67 expression, mAb B56 (BD Pharmingen) was used; this mAb reacts with the same epitope as mAb MIB-1. To define optimum conditions for Ki-67 detection and, in particular, levels of Ki-67 expression in various stages of the cell cycle defined by DNA content, we examined tonsillar MNC that contain activated cells at different stages of maturation. Cells in S and G2/M were selectively enriched in Ki-67 expression compared to cells in the G0/G1 phase of the cell cycle (data not shown).

**Fluorescence activated cell sorting**

Cryopreserved PBMC from 20 CLL cases were incubated with mAbs specific for CD19, CD5 and CD38 and with fluorochrome-conjugated isotype control mAbs for 25 minutes at 4°C, and subjected to flow sorting on a FACS-Aria fluorescence activated cell sorter (Becton Dickinson Immunocytometry systems). CD19+CD5+CD38- and CD19+CD5+CD38+ subsets from each case were processed for telomere length and telomerase quantification.

**Quantification of telomere length and telomerase activity.**

A Flow-FISH protocol detailed elsewhere was utilized to quantify mean telomere lengths in purified cell populations. Telomerase activity was assessed in the flow-sorted CD19+CD5+CD38- and CD19+CD5+CD38+ subsets using the TRAPeze telomerase detection kit.
(Chemicon International, Temecula, CA) as described\textsuperscript{37}; this approach employs the telomere repeat amplification protocol (TRAP; ref.\textsuperscript{38}).

\textbf{Statistical analyses}

Expression of Ki-67 and other markers was compared between healthy donors and CLL cases using the Mann-Whitney test. Comparison across CD38 groupings (negative, low, intermediate, high) with respect to Ki-67 and ZAP-70 expression was carried out using the Kruskal-Wallis test. Upon finding significant differences across groups, a Bonferroni-like adjusted pair wise comparison was made to determine which groups differed from one another. Differences between percentages of cells expressing the same marker within CD38-based subsets were evaluated using the Wilcoxon Signed rank test. The Spearman’s rank correlation coefficient was calculated to determine the strength of association between various parameters.
RESULTS

Expression of Ki-67 by CD5$^+$ normal and leukemic B cells

We analyzed Ki-67 expression among PBMC from a cohort of 95 randomly selected CLL cases and 20 age-matched healthy donors to identify cells that had traversed the G0/G1 phase (Figure 1A). On average, the CLL clones contained 1.77% Ki-67-expressing cells (range 0.1 - 13.8%), which was a significantly higher percentage than that of CD5$^+$ B cells from normal subjects (average = 0.23%; range 0.0 - 0.98%; $P < 0.001$, Kruskal-Wallis test).

Expression of Ki-67 in CD38$^{\text{high}}$ and CD38$^{\text{low}}$ CLL clones

Because CD38 is upregulated with activation and maturation of normal human B lymphocytes$^{39}$ and its expression is intimately linked to cellular activation$^{17}$ and disease course$^{2}$ in CLL, we determined the relationship between expression of Ki-67 and CD38 within a leukemic clone. The 95 CLL cases were divided into subgroups based on the percentage of CD5$^+$CD19$^+$ cells expressing CD38 (Figure 1B) by selectively gating and analyzing FACS data obtained after incubation with mAb reactive with CD19, CD5, CD38, and Ki-67. Fifty eight percent (55/95) of the clones contained $<30\%$ CD38-expressing leukemic cells (CD38$^{\text{low}}$) and 42% (40/95) of the clones contained $\geq30\%$ CD38-expressing cells (CD38$^{\text{high}}$). As shown in Figure 1C, the average number of Ki-67$^+$ cells was significantly greater in CD38$^{\text{high}}$ cases (mean: 2.66%; range: 0.11-13.80%) than in CD38$^{\text{low}}$ cases (mean: 1.06%; range 0.0-5.63%; $P < 0.001$, Kruskal-Wallis test). Of note, the average number of Ki-67$^+$ cells was also significantly greater in U-CLL cases (mean: 2.05%; range: 0.0 – 13.8%) than in M-CLL cases (mean: 1.23%; range 0.0 – 3.98%; $P < 0.001$, Kruskal-Wallis test) (data not shown).
Expression of Ki-67 by CD38+ and CD38- cells within CLL clones

Recent studies suggest that CD38+ and CD38- members of CLL clones differ in gene expression and certain cellular functions, e.g., signaling through the BCR. Therefore Ki-67 expression by CD38+ and CD38- cells of the same 95 CLL clones was analyzed. To accomplish this, two regions corresponding to both ends of the CD38 expression spectrum were demarcated by FACS (Figure 2A): region R3 that contains the majority of the CD19+CD5+CD38+ fraction and region R4 that contains the majority of the CD19+CD5+CD38- fraction.

The percentage of cells expressing Ki-67 in CD19+CD5+CD38+ and CD19+CD5+CD38- fractions within CD38low and CD38high cases was tabulated (Figure 2B). Significantly higher percentages of CD38+ cells expressed Ki-67 than CD38- cells (mean Ki-67 expression in CD38+: 4.79%, range: 0.1-26%; mean Ki-67 expression in CD38-: 0.87%, range: 0-4.4%; P < 0.001; Signed Rank test), regardless of the type of case analyzed (i.e., CD38low and CD38high).

Surprisingly, even in CD38low cases, a sizeable fraction of CD38+CD5+ cells expressed Ki-67 (mean 5.57 ± 0.6 %; n = 55), reflecting entry into the cell cycle (arrow, Figure 2B, left side).

Comparison of Ki-67 expression in CD38+ vs. CD38- cells of normal CD5+ B cells and CLL cells

Many normal human B cells express varying levels of CD38, regardless of CD5 co-expression. Therefore, we analyzed expression of Ki-67 by CD38+ and CD38- fractions of normal circulating CD5+ B cells and of CLL cells (Figure 2C) and then compared them with each other (Figure 2D). Ki-67+ cells are more often found in the CD38+ cells of both CLL and normal B lymphocytes (Figure 2C; Normal subjects: mean 0.23%; CLL: mean 4.79%; P <0.001, Mann Whitney test), and there are many more Ki-67+ cells in both CD38- as well as CD38+
fractions of CLL cells (Figure 2C) compared with normal CD5⁺ B cells (Ki-67% in CD38⁻: 0.0%; Ki-67% in CD38⁺: mean 0.23%; P <0.001, Mann Whitney test). Figure 2D (right side) depicts the same data obtained for the normal CD5⁺ B cells (Figure 2D, left side) but represented on a magnified scale to indicate that significant differences in Ki-67 expression exist even between CD38⁺ and CD38⁻ subsets of normal B cells. Among both CLL clones and normal B-cell populations, CD38 expression marks a CD5⁺ population containing more cells that have entered the cell cycle. This is the case even when the CD38⁺ cells are from CLL clones that have only a small CD38⁺ fraction (e.g., as low as 0.3%; Figure 2B).

**Expression of Ki-67 in CLL cells differing in density of cell surface CD38**

To determine whether Ki-67 expression is related to the density of CD38 expressed on the surfaces of different CD19⁺CD5⁺ CLL cells, we further divided FACS region R3 from Figure 2A into three arbitrary “slices” based on the intensity of CD38 expression, R4 = CD38low, R5 = CD38int, and R6 = CD38high (see Figure 3A for three representative CLL cases). Although all 95 CLL cases were analyzed for CD38 expression, data on CD38 intensity slices were tabulated only in cases in which >50 cells were detected in either R4, R5, or R6; therefore these analyses include data from 60 of the original 95 cases. The mean fluorescence intensity (MFI) values for CD38 expression in R6 were >3.5 fold greater than that of cells in R5 and 10 fold greater than that of R4. The MFI values for R5 were >3.5 fold greater than that of cells in R4.

The percentages of Ki-67-expressing cells in regions R3, R4, R5 and R6 for each of the 60 cases analyzed were significantly different from each other (Figure 3B and 3C; P <0.001, Kruskal Wallis test), indicating clearly a direct relationship between Ki-67 positivity and density of CD38 expression on CLL cells. This relationship existed in every case analyzed (Figure 3D).
Similarly, CD38 density defined by MFI was higher in the Ki-67\(^+\) subset of the clone compared to the Ki-67\(^-\) subset in each case (Figure 3E; \(P < 0.01\), Mann Whitney test).

**Expression of activation-related cell surface markers and Bcl-2 in CD38\(^+\) and CD38\(^-\) subsets of CLL clones**

In addition to CD38, CLL cells express other activation- and maturation-associated markers\(^{27,41,42}\), e.g., CD69 that is upregulated rapidly with cellular activation that retains cells in the vicinity of the inductive stimulus\(^{43,44}\), CD62L that is involved in cell adhesion and eventually lost from the cell surface after cell activation\(^{45}\), and CD27 that is reflective of a memory and an activated phenotype\(^{46,47}\). These markers were also analyzed in CD38\(^+\) and CD38\(^-\) fractions of individual CLL clones (Figure 4). The percentages of cells within cases expressing these markers were significantly different (CD27 – CD38\(^+\): mean 75.1%; CD38\(^-\): mean 58.8% in; \(P < 0.001\); CD62L – CD38\(^+\): mean 21.4%; CD38\(^-\): mean 13.5%; \(P < 0.001\); and CD69 – CD38\(^+\): mean 23.9%; CD38\(^-\): mean 20.8%; \(P < 0.01\); Signed Rank test), although the trend of differences between cells in the CD38\(^+\) and CD38\(^-\) subsets were not as consistent as found for Ki-67 expression.

**Expression of ZAP-70 in CD38\(^+\) and CD38\(^-\) subsets of CLL clones**

ZAP-70 expression in normal human B lymphocytes is linked to cellular activation\(^{48-50}\) and has an important cell-signaling role in CLL\(^{51-54}\). We therefore compared expression of ZAP-70 in CLL cells of the same 95 cases and CD19\(^+\)CD5\(^+\) cells from healthy elderly subjects. Significantly higher percentages of CLL cells expressed ZAP-70 compared with B lymphocytes from control subjects (Figure 5A).
Furthermore, none of the cells in the CD38⁻ subset from normal subjects expressed ZAP-70 (0/20), even though the percentage of ZAP-70-expressing cells in the CD38⁺ subset ranged from 0.3-11.2% (Figure 5B). In contrast, 98% (93/95) of CLL cases had a higher percentage of CD38⁺ cells expressing ZAP-70 than CD38⁻ cells within the same clones (Figure 5C). In individual cases, ZAP-70 expression exhibited strong positive correlation with both CD38 expression and Ki-67 expression (Table 1; \( P < 0.001 \), Spearman correlation). These findings were true regardless of percentage of the clonal members expressing CD38, further supporting the concept that expression of CD38 and ZAP-70 co-evolves.

**Telomerase activity and telomere length of flow-sorted CLL cells**

Telomerase activity is enhanced on activation of normal⁵⁵,⁵⁶ and leukemic B cells³⁷,⁵⁷. Therefore, telomerase activity was quantified in B cells from CLL clones using the TRAP assay. Figure 6A indicates a modest direct relationship between telomerase activity and percentage of cells in CLL clones expressing CD38 (\( r = 0.35, P < 0.01 \)). The analyses were refined by purifying CD5⁺CD38⁺ and CD5⁻CD38⁻ B cells from 20 CLL cases using FACS and the same gating approach illustrated in Figure 2A and quantifying telomerase activity in these two subsets. In every instance, flow-sorted CD38⁺ CLL cells possessed higher telomerase activity than CD38⁻ cells from the same clone (Figure 6B).

Telomerase activity requires functional, human telomerase reverse transcriptase (hTERT) protein⁵⁸. To determine if differences in percentage of hTERT⁺ CLL cells in a bulk population are at the root of the observed differences in enzymatic activity, we analyzed the percentage of CLL cells expressing hTERT by flow cytometry. The product of the percentages of cells expressing hTERT and the intensity of its expression was calculated and termed “estimated
telomerase activity”. TPG activity obtained from the TRAP assays showed a positive correlation with these arbitrary units in CD38+ cells but not in CD38− cells (Figure 6D). In addition, there was a positive correlation between percent Ki-67-expressing cells with telomerase activity ($r = 0.515$, $P = 0.02$) and ZAP-70 expression ($r = 0.26$, $P < 0.01$).

Notably, despite the differences in hTERT protein and telomerase activity between the CD5+CD38+ and CD5+CD38− subsets, mean telomere lengths of these fractions were comparable within each clone (CD38+: range 1.66 – 5.98, mean 3.34 kb; CD38−: range 1.59 – 6.25, mean 3.36 kb; $P = 0.39$; Figure 6C). This finding suggests that CD38+ and CD38− subclones are linked and represent a continuum, distinguished by transient and activation-related expression of CD38.
DISCUSSION

The majority of circulating CLL cells exhibits features of pre-activated cells. Their surface membranes are decorated with molecules found on stimulated B lymphocytes, with specific antigens displayed differing between U-CLL and M-CLL. Based on telomere length, the cells have replicated multiple times and clearly more than B cells from healthy subjects matched for age. Finally, in vivo labeling of CLL cells reveals birth of a limited but significant number of new leukemic cells.

Based on these findings, one would expect that CLL cells are cycling. Surprisingly, few cells in the blood have progressed beyond the G0/G1 phase of the cell cycle, although more cells in solid tissues may have done so. Although our experiments were carried out on B cells from the peripheral blood since adequate numbers of solid tissue samples were not available, based on the studies mentioned, the size of the proliferative component in tissues is likely greater than we have estimated for the blood.

Because clonal members can be heterogeneous in expression of genes and markers related to cellular activation and adhesion, in particular CD38, this intraclonal heterogeneity may translate into differences that determine which cells enter the cell cycle and may help to explain contradictory results. Early hints of such differences came from studies of the pattern of CD38 expression by members of CLL clones indicating that the presence of a distinct CD38+ population within a leukemic clone, regardless of its apparent size, identified patients who would have more aggressive disease.
Consequently, we examined how differences in expression of markers of activation by CLL cells correlate with entrance into the cell cycle; in particular we utilized expression of Ki-67, a nuclear protein that is upregulated in the G1, S, G2 and M phases of the cell cycle but is absent from resting cells (G0 phase; ref. 65). Significantly higher percentages of CD5+B cells from CLL cases, than CD5+B cells from controls, expressed Ki-67 (Figure 1A). When the CLL cases were divided into two groups based on a cut-off of ≥ or < 30% CD38-expressing cells within a leukemic clone, CD38_{high} cases exhibited remarkably more Ki-67^{+} cells (Figure 1C). Similarly, U-CLL clones contain more Ki-67^{+} cells than M-CLL clones (not shown).

We further analyzed Ki-67 expression within CD38^{+} and CD38^{-} subclones of each individual CLL case. Whether obtained from a CLL case or control subject, proportionally more Ki-67^{+} cells were contained in the CD38^{+} than the CD38^{-} fraction (Figure 2C and D). Even CD38^{-} cells in CD38_{low} cases showed significant numbers of Ki-67-expressing cells (Figure 2B), suggesting that CD38 expression labels cells in an activated state that have crossed the G_{0}/earlyG_{1} boundary of the cell cycle. Finding that the percentages of Ki-67^{+} cells within the clone increased with the cell surface density of CD38 (Figure 3 C and D) suggests that a cell’s level of CD38 expression reflects its extent of activation as well as its proliferative capacity. These findings are consistent with the enhanced transcription of other activation markers (e.g., CD18, CD49d, CD20, and subunit 5 of the anaphase-promoting complex/cyclosome) in leukemic B cells from CD38_{high} CLL patients^{66}.

In addition, we quantified expression of CD69, CD62L and CD27 in CD38^{+} and CD38^{-} subsets of the clones from a cohort of 50 CLL cases. Significant differences were observed in percentages of cells expressing these markers within the subsets, although these paired values did not follow a consistent trend of lower in CD38^{-} and higher in CD38^{+} in individual cases (Figure...
4A, B and C). It was somewhat surprising to find increased numbers of CD62L+ cells in the CD38+ fraction, since CD62L expression wanes after cellular activation. However because CD62L and CD69 are involved in retaining lymphocytes at the site of stimulation43,44,45, the levels of these two molecules on circulating CD38+ cells might be less than those in the solid tissues and therefore might indicate that CD38+ cells expressing these markers are recent emigrants from such sites. We are currently testing this possibility using in vivo cellular labeling67.

Anti-apoptotic proteins such as Bcl-2, Bax, Bak, BAD, and Mcl-1 that might contribute to prolonged survival in vivo are upregulated in CLL cells68,69. However the fact that CD38+ and CD38− cells did not differ with respect to expression of Bcl-2 suggests that this protein does not confer a selective survival advantage to CD38-expressing cells, especially in light of the finding that CD38− cells die faster than CD38+ cells in vitro35.

Since ZAP-70 expression is influenced by activation state and is also one of a few genes differentially expressed between the IgVH gene mutation patient subgroups, we examined its association with CD38 expression. Our observation on elevated percentages of ZAP-70+ cells in the CD38+ subset of the clone compared with those in CD38− CLL cells (Figure 5B and C) within a majority of cases corroborates earlier findings70, consistent with the suggestion that CD38+ cells might have a better ability to transduce BCR-mediated signals40 with the help of simultaneous ZAP-70 expression51,52. Although ZAP-70 was not detected in CD38− cells from healthy controls, it was found to a low extent in the CD38+ cells from the same normal individuals, as reported earlier by others48-50. It is interesting that in a minority of CLL cases there were more ZAP-70+ cells in the CD38− fraction of the clone, suggesting that ZAP-70 expression may be retained in a subpopulation of CD38+ cells that have lost CD38 expression.
In this regard, the finding that highly purified CD38+ and CD38- subfractions of CLL clones showed no differences in mean telomere length (Figure 6C) suggests that the replicative histories of these two fractions are not different and supports the notion that these fractions may represent a continuum, distinguished by transient, activation-related expression of CD38.

Thus, regardless of the percentage of CD38+ cells in a patient’s CLL clone, CD38 expression is linked to cell activation and labels a proliferative component defined by Ki-67 expression. On average, 1.77% of the leukemic cells in the cases studied expressed Ki-67 and the majority of these cells were within the CD38+ fraction (4.8% of CD38+ CLL cells co-expressed Ki-67 vs. 0.88% of CD38- CLL cells). We need to emphasize, however, that the circulating CD38+Ki-67+ component is small in relation to the percentage of cells within a clone (~2%). Nevertheless, it may represent a sizeable number of cells in toto. Since the size of a CLL clone in vivo ranges from 10^{12} to 10^{14} cells, this percentage then represents ~10^{10} - 10^{12} cells. However, ongoing cell death appears to balance out this continuing proliferation in most patients, thereby maintaining absolute cell numbers and explaining the often slow changes in lymphocyte counts in vivo. Since primarily CD38+ CLL cells co-expressed Ki-67, one would anticipate that this fraction would be enriched in proliferating cells; indeed our preliminary data suggest that the CD38 marks the proliferative compartment in CLL, based on incorporation of $^{2}$H label.

Collectively our findings may help to explain why the presence of high percentages of CD38+ leukemic cells within a CLL clone is associated with aggressive disease and poor clinical outcome, since presumably it is from this fraction that new, more dangerous chromosomal abnormalities evolve. Moreover, combining quantification of Ki-67+ cells with CD38+ cells may provide even greater prognostic effectiveness.
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Authors’ Contributions: Rajendra N. Damle: Designed and performed research, analyzed data, and wrote paper
Sonal Temburni, Taraneh Banapour: Performed research
Carlo Calissano and Sophia Yancopoulos: Wrote paper
Cristina Sison: Analyzed data
Steven L. Allen, Kanti R. Rai: Analyzed data and wrote paper
Nicholas Chiorazzi; Designed research, analyzed data, and wrote paper

The authors declare that they have no competing financial interests.
REFERENCES


53. Richardson SJ, Matthews C, Catherwood MA, et al. ZAP-70 expression is associated with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukemia (B-CLL). Blood. 2006;107:3584-3592.


56. Igarashi H, Sakaguchi N. Telomerase activity is induced in human peripheral B lymphocytes by the stimulation to antigen receptor. Blood. 1997;89:1299-1307.


TABLE 1

Correlation of ZAP-70 expression with CD38+ and Ki-67+ cells in individual CLL clones

<table>
<thead>
<tr>
<th>CD38 expression</th>
<th>Ki-67 expression</th>
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<tr>
<td>r = 0.244</td>
<td>r = 0.274</td>
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<td>P = 0.017</td>
<td>P = 0.007</td>
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Percentages of cells co-expressing ZAP-70 with either CD38 or Ki-67 in individual cases were subjected to Spearman correlation test.
FIGURE LEGENDS

Figure 1: **Ki-67 expression in CD5+ B cells from healthy elderly subjects and CLL patients**

PBMC from 20 healthy elderly subjects and 95 CLL cases were analyzed for expression of CD5, CD38, CD19 and Ki-67. **A.** Percentage of CD5+CD19+ cells from normal controls and CLL cases expressing Ki-67 (P < 0.001, Mann Whitney test). **B.** Percent CD38+ B cells in CLL clones designated CD38<sub>low</sub> (55/95) and CD38<sub>high</sub> (40/95) based on a 30% cut-off. **C.** Significant differences in Ki-67 expression between CD38<sub>low</sub> and CD38<sub>high</sub> CLL cases (P <0.001, Mann Whitney test).

Figure 2: **Ki-67 expression by CD38+ and CD38- B cells within individual CLL cases**

PBMC from 95 CLL cases and 20 elderly healthy donors were stained as in Figure 1. **A.** Dot plots of CD5 and CD38 expression by B cells from 3 CLL cases and one healthy donor. **B.** **Left:** Lines connect data points for percent Ki-67+ cells in CD38<sup>- </sup> and CD38<sup>+</sup> subsets within each CLL case in the CD38<sub>low</sub> subgroup. Note: detection of Ki-67<sup>+</sup> cells even in the CD38<sup>- </sup>subset of CD38<sub>low</sub> cases (arrow); **Right:** Lines connect data points for percent Ki-67<sup>+</sup> cells in CD38<sup>- </sup> and CD38<sup>+</sup> subsets within each CLL clone in CD38<sub>high</sub> subgroup. **C.** Lines connect data points for percent Ki-67<sup>+</sup> cells in CD38<sup>- </sup> and CD38<sup>+</sup> subsets of all CLL cases. **D.** **Left:** Lines connect data points for percent Ki-67<sup>+</sup> cells in CD38<sup>- </sup> and CD38<sup>+</sup> subsets within each healthy subject. **Right:** Same data from left half drawn to a smaller scale.

Figure 3: **Ki-67 expression in relation to density of CD38**

PBMC from CLL cases were stained as described for Figure 1 and contour plots depicting expression of CD5 and CD38 in CD19<sup>+</sup> cells of CLL cases were generated. **A.** CD38<sup>+</sup>
fraction was divided into 3 regions - R4, R5 and R6 - referred to as CD38\textsuperscript{low}, CD38\textsuperscript{int} and CD38\textsuperscript{high}, respectively. Percentages of Ki-67 expressing cells were scored only if at least 50 cells fell within marked regions. B. Percentages of Ki-67-expressing cells in CD38\textsuperscript{low}, CD38\textsuperscript{int} and CD38\textsuperscript{high} subsets from 60/95 CLL cases. C. Average ± S.E. of values from B. D. Lines connect data points depicting percent Ki-67\textsuperscript{+} cells in each CD38 subset for each of 60 CLL cases. E. In every case studied, CD5\textsuperscript{+}Ki-67\textsuperscript{+} cells showed higher density of CD38 compared to CD5\textsuperscript{+}Ki-67\textsuperscript{-} cells from the same case.

**Figure 4: Expression of cell activation- and apoptosis-related molecules in CD38\textsuperscript{+} and CD38\textsuperscript{-} cell subsets within CLL cases**

PBMC from 50 CLL cases were analyzed for expression of CD27, CD62L, or CD69 by CD5\textsuperscript{+}, CD19\textsuperscript{+}, and CD38\textsuperscript{+} cells. A, B, and C show significant differences in expression of CD27, CD62L, and CD69, respectively, within CD38\textsuperscript{+} and CD38\textsuperscript{-} subsets of the clone (CD27 and CD62L\textsuperscript{-} P <0.001, CD69 - P <0.01). D. Means ± S.E. for percent of Bcl-2\textsuperscript{+} cells (left) and means ± S.E. for MFI of Bcl-2 expression (right).

**Figure 5: ZAP-70 expression in CD38\textsuperscript{+} and CD38\textsuperscript{-} cells**

PBMC from 20 elderly healthy subjects and 95 CLL cases were incubated with mAbs to CD5, CD38, CD19, ZAP-70 or appropriate isotype control mAbs. CD19\textsuperscript{+}CD5\textsuperscript{+} cells exhibiting a ZAP-70 staining in excess of the isotype control mAbs were considered ZAP-70\textsuperscript{+}. A. Significant differences in percentage of CD5\textsuperscript{+}CD19\textsuperscript{+} cells expressing ZAP-70 (P < 0.001, Mann Whitney test). These data were further analyzed to determine differences in expression of ZAP-70 between the CD38\textsuperscript{-} and CD38\textsuperscript{+} populations in healthy controls (B) and in CLL cases (C).
Significant differences exist between ZAP-70 expression within CD38⁻ and CD38⁺ subsets from healthy donors and CLL cases ($P < 0.001$, Kruskal-Wallis test).

**Figure 6: Telomerase activity and telomere length of flow-sorted populations**

**A.** Telomerase activity was assayed in B cells from 60 CLL cases using TRAP assay and percentage of CD38⁺ CLL cells plotted versus TPG (total product generated) units in the same case. CD19⁺CD5⁺ cells from 20 CLL cases were sorted into CD38⁺ and CD38⁻ subpopulations and processed for quantification of telomere length and telomerase activity. **B.** Lines connect data points that indicate telomerase activity in the cell subsets of each individual case. **C.** Lines connect data points for mean telomere lengths of flow-sorted CD38⁺ and CD38⁻ CLL cells. **D.** Significant positive correlation ($r = 0.465$, $P < 0.045$) exists between estimated telomerase activity and observed telomerase activity (TPG units) in CD38⁺ cells from 20 CLL cases shown in **B** and **C**; this correlation does not exist in the CD38⁻ cells.
Figure 1

A

Percent Ki-67-expressing CD5+B cells

Healthy controls
(n=20)

B-CLL cases
(n=95)

B

Percent CD38-expressing CLL cells

CD38\textsuperscript{low} cases

CD38\textsuperscript{high} cases

CLL cases (n=99)

C

Percent Ki-67-expressing CLL cells

CD38\textsuperscript{low} CLL cases
(n=55)

CD38\textsuperscript{high} CLL cases
(n=40)
Figure 2

A

CD38 expression
CD5 expression

CLL 910
CLL 899
CLL 896
Healthy control

B

Percent Ki-67 expressing CLL cells

CD38<sup>neg</sup> CD38<sup>pos</sup>

C

Percent Ki-67-expressing CD<sup>+</sup> B cells

CD38<sup>neg</sup> CD38<sup>pos</sup>

D

Percent Ki-67-expressing CD<sup>+</sup> B cells

CD38<sup>neg</sup> CD38<sup>pos</sup>
Figure 4

A. CD27 expression

B. CD62L expression

C. CD69 expression

D. % Bcl-2+ MFI: Bcl-2

Percent positive CLL cells

CD38\textsuperscript{neg}  CD38\textsuperscript{pos}

CD38\textsuperscript{neg}  CD38\textsuperscript{pos}

CD38\textsuperscript{neg}  CD38\textsuperscript{pos}

CD38\textsuperscript{neg}  CD38\textsuperscript{pos}
Healthy controls (n=20)

B-CLL cases (n=95)

Percent ZAP-70-expressing CD5+ B cells

Figure 5

A

B

C

Percent ZAP-70-expressing CD5+ B cells

Percent ZAP-70-expressing CLL cells
Figure 6

A

B

C

D
CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells

Rajendra N Damle, Sonal Temburni, Carlo Calissano, Sophia Yancopoulos, Taraneh Banapour, Cristina Sison, Steven L. Allen, Kanti R. Rai and Nicholas Chiorazzi

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