Clonal heterogeneity in chronic lymphocytic leukemia cells: superior response to surface IgM cross-linking in CD38, ZAP-70-positive cells

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Patients with chronic lymphocytic leukemia whose cells express CD38 and ZAP-70 and utilize unmutated Ig VH region genes have a very poor prognosis. We studied whether cells expressing CD38 and ZAP-70 are more susceptible to stimulation through B-cell receptors than are cells that do not express CD38 and ZAP-70.

Design and Methods
CD38-positive and CD38-negative leukemic cells were separated from single cases and compared for their response to B-cell receptor cross-linking and ZAP-70 expression. Cohort studies were also carried out by measuring the apoptotic response to surface immunoglobulin M (IgM) cross-linking in 82 patients with chronic lymphocytic leukemia and the protein tyrosine phosphorylation induced by surface IgM in 21 patients.

Results
CD38-positive cells, isolated from cases of chronic lymphocytic leukemia classified as CD38-positive or CD38-negative, expressed more ZAP-70 than the corresponding CD38-negative cells, exhibited more robust protein tyrosine phosphorylation and had a greater tendency to apoptosis upon B-cell receptor cross-linking. In the cohort studies, surface IgM-induced protein tyrosine phosphorylation correlated significantly with CD38 and ZAP-70 expression and with the absence of Ig VH gene mutations. Apoptosis induced by surface IgM cross-linking correlated significantly only with the proportion of CD38-positive cells. Difficulties in finding more definitive correlations were probably related to imprecision in the *in vitro* test system and in the definition of cases as positive or negative.

Conclusions
Collectively, these data indicate that CD38-positive, ZAP-70-positive cells have a greater capacity for signaling through the B-cell receptor and suggest a function for B-cell receptor signaling in promoting chronic lymphocytic leukemia cell expansion, especially within the CD38-positive fraction of the leukemic clone.

Key words: chronic lymphocytic leukemia, CD38, ZAP-70, signal transduction, apoptosis

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Introduction

Chronic lymphocytic leukemia (CLL) has a heterogeneous clinical course and outcome.[1] Certain markers expressed by neoplastic cells can help to predict the prognosis of patients with this disease.[2,3] Patients whose cells utilize immunoglobulin heavy chain variable region (Ig VH) region genes with less than 2% somatic mutations (unmutated CLL), progress rapidly towards more advanced clinical stages, while patients whose cells express Ig V region genes with ≥2% mutations (mutated CLL) have a more indolent course.[4,5] CD38[4,6-8] and ZAP-70[9-12] are unfavorable prognostic markers in CLL. ZAP-70 is generally detected in a high percentage of cells from unmutated CLL and in a low proportion of cells from mutated CLL.[10-12] Albeit not absolutely, the expression or lack of expression of CD38 parallels the absence or the presence of Ig VH gene mutations, respectively.[4,8,13,14]

Despite these important clinical observations, the reasons why Ig VH gene mutation status and expression of ZAP-70 and CD38 influence or correlate with disease progression are still undefined. Cells from patients with CLL may express surface IgM with polyspecific (natural) antibody activity and bind to a variety of different antigens, including self-antigens.[15,16] This specificity, however, is detected rarely for surface IgM from mutated CLL cases, while it is common for surface IgM from unmutated cases.[17] These findings led to the hypothesis that the reaction between surface IgM of unmutated CLL cases and self-antigens induces cell activation and that continuous stimulation would favor expansion of unmutated CLL clones *in vivo*.[3] In contrast, cells from mutated cases would not be stimulated via their surface IgM, and their expansion would be more indolent. Tentatively, it could also be proposed that activation through surface IgM induces the expression of both ZAP-70 and CD38, inasmuch as both these structures are activation markers for normal B cells.[18-21] Moreover, they could be involved in the IgM-dependent signal transduction pathway,[18] although this issue is still controversial.[22,23]

The validation of the above model requires that cells from CLL cases with less favorable prognostic markers exhibit a viable surface IgM-dependent signal transducing pathway. In contrast, the same pathway does not need to function in cells from CLL cases with a more favorable prognosis, given its minor role in governing clonal expansion. A number of observations indicate that such differences exist between cells from cases with different prognoses. In our initial studies on CD38 expression, stimulation via surface IgM was more efficient in cells from CD38-positive cases and caused cell apoptosis *in vitro*.[24] Others found that signals delivered via surface IgM were more efficient in cells from unmutated, CD38-positive, ZAP-70-positive cases in various combinations.[25-27] Because of the importance of the issue, we have re-evaluated the relationships existing between expression of prognostic markers and capacity of surface IgM to signal the neoplastic cells. Two different approaches were used. With one, the CD38-positive and CD38-negative cells were purified from individual (mutated or unmutated) CLL cases and the two cell fractions tested separately *in vitro* for ZAP-70 expression and functional features. With the other, we looked for correlations between the expression of prognostic markers and response to surface IgM cross-linking in cohort studies using unfractionated CLL cells *in vitro*.

Design and Methods

Patients

Eighty-two patients with CLL from Genoa and Reggio Calabria were tested at diagnosis or, less often, at relapse. These patients, therefore, had not received therapy or had been free from therapy for at least 6 months. The diagnosis of CLL was based on standard criteria.[1] Flow-cytometric analyses documented co-expression of CD19, CD23 and CD5 and monotypic expression of the κ or λ chains. All patients gave informed consent to the study, which was approved by the respective Institutional Review Boards.

CLL cell isolation and culture

Peripheral blood mononuclear cells from CLL patients were isolated by Ficoll-Hypaque (Seromed, Biochrom KG, Berlin, Germany) density-gradient centrifugation. If CLL cells (CD5/CD19/CD23 triple positive cells) constituted less than 90% of the total cells, T cells, NK cells and monocytes were removed by treatment with CD3, CD56, CD16, and CD14 monoclonal antibodies (Becton Dickinson, San Diego, CA, USA) followed by magnetic bead separation (Goat Anti-Mouse IgG Dynabeads, Dynal Biotech ASA, Oslo, Norway).[19,28] CLL cells were cultured in RPMI 1640 medium (GIBCO, Invitrogen Life Technology, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, Seromed Biochrom), penicillin, streptomycin, and L-glutamine (Seromed Biochrom), at the concentration of 2×10^6 cells/mL at 37°C in an atmosphere containing 5% CO_2.

Immunofluorescence

Direct immunofluorescent staining was performed with CD19-FITC/PE, CD23-PE, CD38-PE, CD5-Cy-Chrome (Becton Dickinson & Co., Sunnyvale, CA, USA), IgM-PE (Caltag Laboratories, Burlingame, CA, USA), and κ-FITC and λ-PE (DakoCytomation, Glostrup, Denmark) monoclonal antibodies. The cells were analyzed using a FACSCalibur flow-cytometer (Becton Dickinson & Co). Kappa and lambda light chains were detected by triple staining with CD19 RPE-CY5. The proportion of CD38-positive leukemic cells was determined by triple staining for CD19-FITC, CD38-PE, and CD5-Cy-Chrome. The cut-off value used to distinguish between CD38-positive or CD38-negative cases of CLL was 30% positive cells.

Determination of ZAP-70 expression

Protein extracts were obtained from CLL cells isolated as above by exposure to lysis buffer (9 M urea, pH 7, 50 mM Tris). Protein concentrations were determined using the Bio-Rad protein assay (Bio-rad Laboratories, Hercules, CA, USA) and 10 μg of protein were used for western blotting. This was carried out using a mouse monoclonal antibody to ZAP-70 (clone 2F3.2 Upstate, Lake Placid, NY, USA) as described previously.[19] CLL samples were divided into three groups: ZAP-70 strong (40-100% positive cells), ZAP-70 weak (20-40% positive cells), and ZAP-70 negative (less than 20% positive cells). These percentages were determined by comparing the intensity of the ZAP-70 band observed in a given CLL case with that of a mixture of different concentrations of purified T cells with mononuclear cells depleted of T and NK cells as described previously.[19]

In order to detect CD38 and ZAP-70 expression simultaneously by flow-cytometry, CLL cells were first incubated with CD3, CD19, and CD38 monoclonal antibodies, fixed, permeabilized with fix and perm reagents (Caltag Laboratories) and exposed to a ZAP-70 monoclonal antibody (1.5 μg) (Upstate) or to a CD33 monoclonal antibody (negative control). Data are expressed as relative fluorescence intensity (RFI) i.e, mean fluorescence intensity of cells stained with the ZAP-70 monoclonal antibody/mean fluorescence intensity of control monoclonal antibody.

Fractionation of CLL cells according to CD38 expression

In these experiments, CLL cell suspensions were consistently depleted of T and NK cells and monocytes as described above. CD38-positive, -weak and -negative cells were separated from purified CLL cells using the MiniMACS system (Miltenyi Biotec Inc., USA).[19] Briefly, the cells were exposed to CD38-PE monoclonal antibody followed by anti PE-coated magnetic micro-beads. The cells were passed first through an LS column that retained high CD38-expressing cells. Cells not retained in the column were subsequently filtered through a LD column to separate low CD38-expressing cells from CD38-negative cells.

Apoptosis assays

Spontaneous apoptosis and apoptosis induced by goat anti-μ chain antibody (aμ-Ab, 10 μg/mL) (Southern Biotechnology Associates, Birmingham, AL, USA) or etoposide (VP-16, 50 μmol/L) (Vepesid; Bristol-Myers Squibb, Rome, Italy) were measured by annexin-V or propidium iodide staining as reported elsewhere.[29] The optimal concentrations of aμ-Ab or VP-16 to induce apoptosis were determined in preliminary titration experiments. Apoptosis induced by aμ-Ab was expressed as a percentage increase over spontaneous apoptosis: *i.e.* (% apoptosis induced by aμ-Ab - % spontaneous apoptosis) / % apoptosis induced by aμ-Ab x 100. Tests were also carried out in the presence of CD40L-transfected fibroblasts (clone 3T3 pIRES/CD40L) as reported elsewhere.[30]

Ig VH gene analyses

CLL Ig VH gene usage and mutations were determined using cDNA as reported previously.[31] A difference of 2% or more from the germ-line configuration represented the cut-off value to classify a given CLL case as *mutated*.[4,5,31]

Protein tyrosine phosphorylation detection and measurement

Cells (10^8 cells/mL) were exposed to aμ-Ab at 37°C for 1, 5 or 10 minutes, and lysed by adding an equal volume of 2% NP-40, 20 mM NaH_2PO_4, 2 mM EDTA, 2 mM PMSF, and 2 μM sodium pervanadate in saline. Nuclei-free cell extracts from 10^6 cells were fractionated by sodium dodecylsulfate electrophoresis on 10% polyacrylamide gels under reducing conditions, and then transferred electrophoretically onto nitrocellulose membranes (Hybond C Extra; Amersham, UK).[29] Tyrosine phosphorylated proteins were detected by incubating the membranes with 0.2 μg/mL biotin-conjugated anti-phosphotyrosine antibody (clone PY99, Santa Cruz Biotechnology) in 1% milk-TBST, followed by 0.3 μg/mL peroxidase-labeled streptavidin (DakoCytomation) in 1% milk-TBST.

Peroxidase activity was detected by chemiluminescence. Each tyrosine phosphorylated protein band was analyzed using the 1D Image Analysis Software version 3.5 (Kodak). Band data were generated by analyzing unsaturated autoradiographic images, creating regions of interest (ROI) based on the centroid and calculating pixel intensity values of the interior of the ROI. Data were then expressed as a ratio between a ROI and its reference value. In detail, each band (180 kD, 150 kD, 120 kD, 70 kD, 60 kD, 55 kD, 50 kD, 30kD), observed following aμ-Ab stimulation at the 1, 5 and 10 minute time points, was compared with the corresponding ROI of the unstimulated samples.

When correlations between prognostic markers and B-cell-receptor-induced protein tyrosine phosphorylation were studied, the total amount of phosphorylation was calculated by summing the phosphorylation of the single bands (1D Image Analysis Software version 3.5, Kodak)

Statistical analysis

All statistical calculations were performed using the statistical package SPSS for Windows, release 11.5, 2002 software (SPSS UK, Working, Surrey, UK). When ZAP-70, CD38, mutational status, and IgM mean fluorescence intensity were considered as binary variables, statistical comparisons were performed using two-way tables for the Fisher's exact test and multiway tables for Pearson's χ^2 test. The non-parametric Kruskal-Wallis and Mann-Whitney U-tests were employed to test the differences between two or more groups. Spearman's rank correlation coefficient (rho) was used to assess the strength of

the straight-line association between the different variables. A value of $p \leq 0.05$ was considered significant for all statistical calculations.

Results

Co-expression of CD38 and ZAP-70 by the same CLL cell subset

The prognostic markers of CLL cases studied are summarized in *Online Supplementary Table 1)*. In general, CLL cases characterized by high CD38 expression also had high levels of ZAP-70, whereas those with low to absent levels of CD38 also had low to absent ZAP-70 levels. Likewise, mutated CLL had low to absent CD38 and ZAP-70 levels, whereas the unmutated cases had high CD38 and ZAP-70 levels. When a 30% cut-off was used for CD38 and if ZAP-70-negative and -weak cases were pooled, a 73.1% concordance in expression of these markers was detected (*data not shown*). Collectively, these data suggested that, in most CLL clones, ZAP-70 and CD38 were co-expressed by the same cells. The cells from 23 CLL cases were, therefore, selected for further studies. Nine cases were CD38-positive (one was ZAP-70 negative, three ZAP-70 weak and five ZAP-70 strong) and 14 were CD38-negative (five were ZAP-70 negative, seven ZAP-70 weak and two ZAP-70 strong). The cells from these cases were incubated with monoclonal antibodies to CD38, ZAP-70, CD3 and CD19 and analyzed by flow-cytometry. As expected, CD3-positive T cells were also ZAP-70-positive (Figure 1A). CD19-positive B cells were either CD38-positive or CD38-negative with variation depending upon the different cases. The expression of CD38 correlated with that of ZAP-70; in particular, the mean fluorescent intensity for ZAP-70 was always higher in the CD38-positive cells than in the CD38-negative cells. The fluorescent intensity of ZAP-70 in the leukemic B cells from the 23 cases studied was also expressed as a ratio (RFI) over the control fluorescence observed with an anti-CD33 monoclonal antibody. The mean RFI values of the CD38-positive cells were significantly higher than those of the CD38-negative cells (Figure 1B) thus confirming that the cells with the highest ZAP-70 expression were among the CD38-positive cells.

Next, cells from eight cases (GE164, GE173, RC21, RC22 RC23, PZ1, RC18 and GE161) were fractionated according to CD38 expression. A technique making use of monoclonal antibody-coated beads was employed, since it is quicker than FACS sorting and the cells remain more viable for *in vitro* studies. Different CD38 levels were demonstrated by flow-cytometry in the three fractions recovered, *i.e.*, CD38-high (FR1), CD38-low (FR2) and CD38-negative (FR3) (Figure 1D). Cells from all fractions co-expressed CD5 and CD19 (Figure 1D), a finding which confirms that they were CLL cells and excluded contamination with T or NK cells. This is consistent with the fact that highly purified CLL cells were used. Analyses for ZAP-70 by western blotting demonstrated strong correlations between CD38 and ZAP-70 expression in the different fractions. Figure 1C shows the results of a typical test confirmed on all of the other CLL cases mentioned above.

Differences in protein tyrosine phosphorylation after surface IgM cross-linking in CLL cell fractions with different CD38 expression

Cell fractions expressing different levels of CD38 were prepared from CLL cells (RC21, RC22, GE173, RC4, and RC7) depleted of T, NK cells and monocytes. These fractions were tested for protein tyrosine phosphorylation following stimulation with aμ-Ab and for surface IgM expression by flow-cytometry. In each case, we took care that the number of purified CLL cells at the beginning of the fractionation procedure was high enough to yield at least 10×10^6cells/fraction to allow sufficient protein recovery for the western blot. As shown in Figure 2 A-D, which reports the results of a typical experiment out of five performed with different CLL cases, cells with the highest CD38 levels also exhibited the most efficient protein tyrosine phosphorylation activity. Differences in surface IgM expression were consistently observed, the CD38-positive cells being those with the highest surface IgM levels (Figure 2). The protein tyrosine phosphorylation observed in CD38-positive cells in the absence of aμ-Ab (or in the presence of an unrelated monoclonal antibody, not shown) did not differ from that of CD38-negative cells at any of the times tested. The few bands observed in the western blot could be attributed to the background caused by the biotinylated reagent employed to develop the gels (in Figure 2 A, B and D, lanes 1, 3 and 5). Binding of CD38 to the cell surface during separation did not, therefore, cause protein tyrosine phosphorylation *per se*, confirming that the CD38 monoclonal antibody used, unlike other CD38 monoclonal antibodies, did not deliver activation signals.[24]

Different apoptotic capacity of CLL cells expressing different CD38 levels

Purified CLL cells fractionated as above were tested for apoptosis after surface IgM cross-linking. Cells from the CD38-low and especially those from the CD38-high cell fractions exhibited greater aμ-Ab-induced apoptosis than that of CD38-negative cells (Figure 3). The apoptotic capacity of the CD38-negative cells was not, however, impaired since they could be induced into apoptosis by exposure to VP-16. Figure 3 shows the data regarding cells cultured for 48 h and tested for apoptosis by propidium iodide staining, although similar results were obtained with cells cultured for 24 h and stained with annexin-V. The high values of spontaneous apoptosis seen in the fractions with high CD38 levels (FR1 in Figure 3) could indicate an intrinsic propensity to apoptosis or be conse-

quent to prior exposure to CD38 monoclonal antibody. To investigate this issue further, cells from cases GE164 and GE161 were exposed to CD38 monoclonal antibody under the same conditions used for cell separation. Separation was omitted and the cells were tested for apoptosis. The spontaneous apoptosis (48% *vs* 47.7% and 45.2% *vs* 36.1% for cases GE164 and GE161, respectively) and aμ-Ab-induced apoptosis (36.7% *vs* 33.5% and 2% *vs* 0.5% over the spontaneous apoptosis for cases GE164 and GE161, respectively) observed in the samples pre-treated with CD38 monoclonal antibody were not significantly different from those of untreated control cells. The relatively high levels of spontaneous apoptosis in CD38-positive sub-clones was, therefore, likely related to intrinsic features of the cells. The different cell fractions were also tested for aμ-Ab- induced apoptosis in the presence of CD40L-trasfected fibroblasts. In these conditions, the spontaneous apoptosis of FR1 cells fell from 40% to 11%. Nevertheless, a substantial induction of apoptosis by aμ-Ab was observed (Figure 3C).

Response of unfractionated CLL cells to aμ-Ab stimulation in vitro

Next, we investigated correlations between the capacity to respond to surface IgM cross-linking and expression of prognostic markers in unfractionated CLL cells from

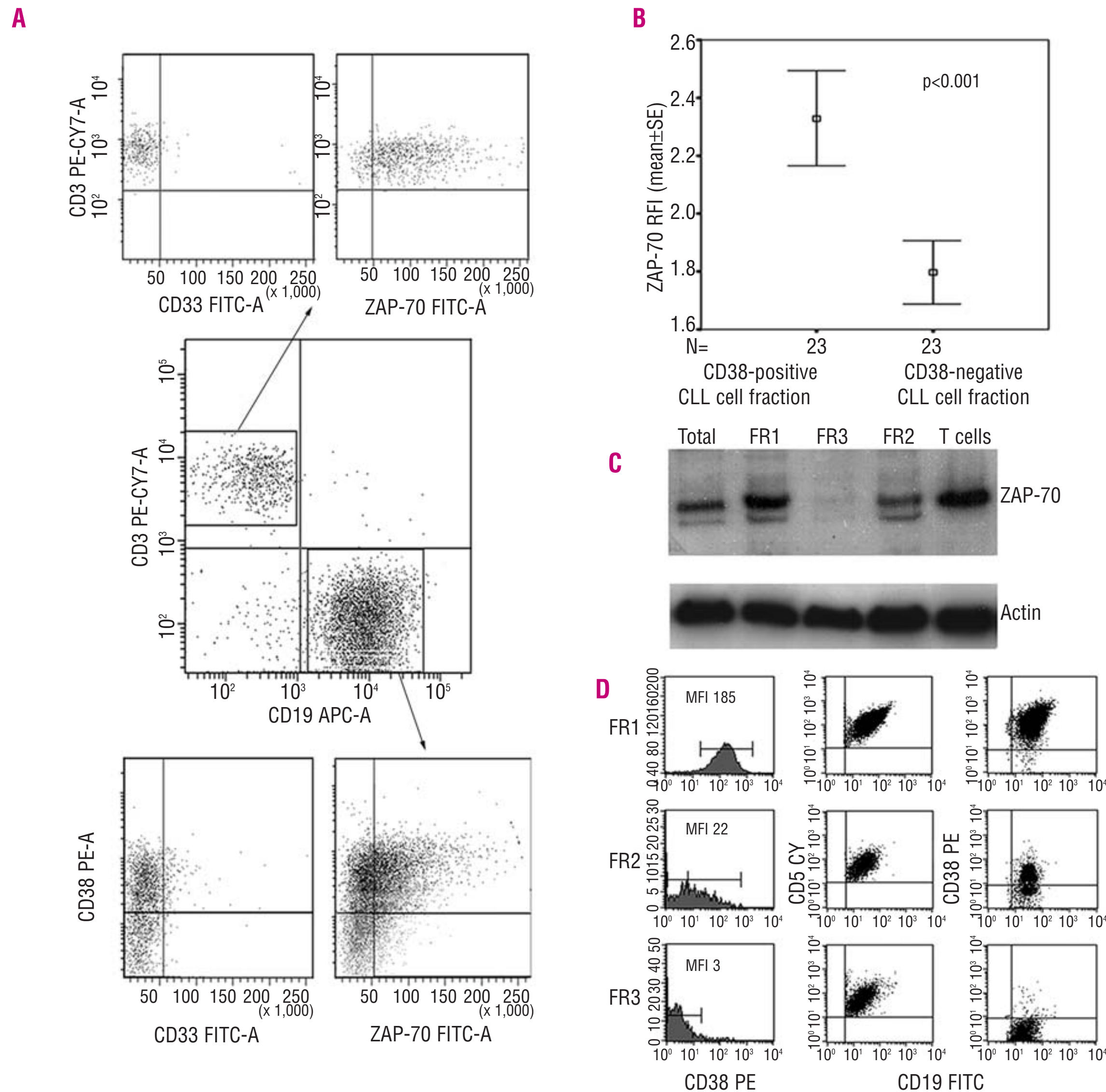

Figure 1. Co-expression of CD38 and ZAP-70 by cells from single CLL cases. (A) Cells from a representative CLL case (RC21) were stained for CD3, CD19, CD38 and ZAP-70. T cells (CD3-positive) and B cells (CD19-positive) were gated and analyzed for ZAP-70 expression. (B) CLL cells from 23 cases were stained as in A. CD19-positive cells were gated and ZAP-70 expression was determined in the CD38-positive and CD38-negative cells. The values are expressed as RFI. Statistical analyses were performed using the Mann-Whitney non-parametric test. (C and D) CLL cells from case RC18 were separated into three fractions [FR1 (CD38-high), FR2 (CD38-intermediate), FR3 (CD38-negative)] and analyzed for ZAP-70 expression by western blotting (C) or for CD38, CD5 and CD19 by flow-cytometry (D). For the comparison of ZAP-70 expression by western blotting 10μg of protein were loaded in each of the lanes indicated.

82 patients. CLL cases could be subdivided into three groups; in the first group, [group A, 40 cases (48.7%), *Online Supplementary Table 1*], aμ-Ab treatment induced apoptosis which was at least 20% above the spontaneous values. In the second group [group B, 33 cases (40.2%), *Online Supplementary Table 1*], the apoptosis induced by the aμ-Ab was less than or equal to the 20% threshold set for inclusion in group A. This cut-off was selected because the distribution of values of aμ-Ab-induced apoptosis of the pooled CLL cases exhibited a significant gap between 20% and 27% apoptosis. In the third group [group C, 9 cases (11%), *Online Supplementary Table 1*], aμ-Ab treatment caused an inhibition of spontaneous apoptosis (*i.e.*, the apoptosis observed was at least 10% below that of untreated controls). This cut-off value was selected arbitrarily based on the consideration that variations in spontaneous apoptosis in different samples from the same CLL patients rarely exceed 5%. The results shown in *Online Supplementary Table 1* were obtained with cells cultured for 48 h and stained with propidium iodide; comparable data were observed with cells exposed to aμ-Ab for 24 h and stained with annexin-V (*not shown*). These experiments were repeated in 12 cases, following an interval of 1 to 2 years. The response to aμ-Ab treatment in the repeat experiments was comparable to that observed in the first tests. Correlations between aμ-Ab-

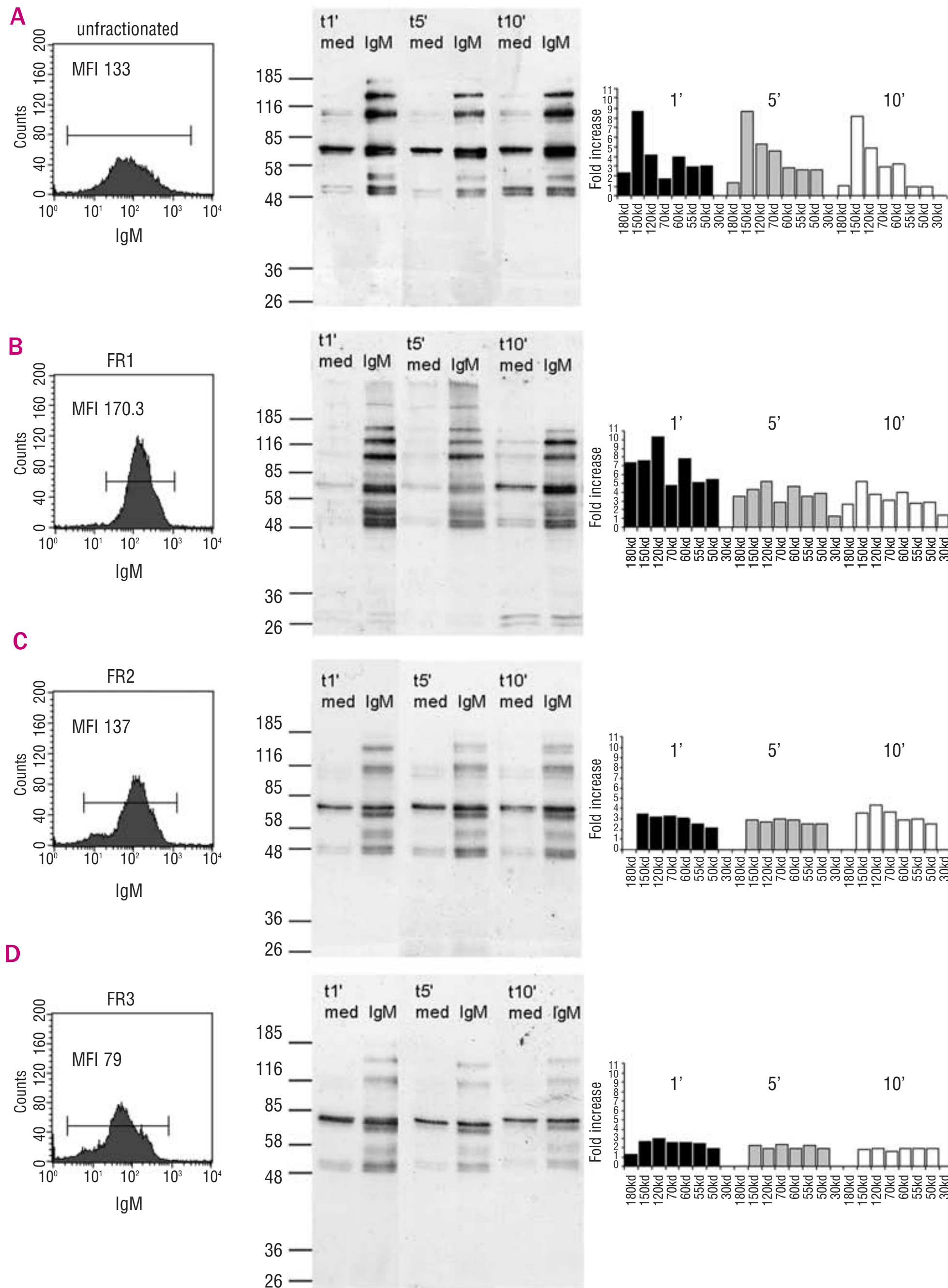

Figure 2. Protein-tyrosine phosphorylation in CLL cells fractionated according to CD38 expression. (A-D) Tyrosine phosphorylation at 1, 5 and 10 minutes following the indicated stimuli was analyzed by western blotting. Cells from case RC21 were separated into the indicated fractions as described in Figure 1. (A) Unfractionated cells. (B) CD38-high cells (FR1). (C) CD38-intermediate cells (FR2). (D) CD38-negative cells (FR3). The pixel intensity of the indicated tyrosine phosphorylated protein bands was measured, and plotted as the fold increase observed following aμ-Ab stimulation compared with that of the non-stimulated cells. The figure also shows surface IgM expression detected by flow-cytometry for each cell fraction. This experiment is representative of five tests carried out in different cases.

induced apoptosis and prognostic markers were investigated in groups A and B, but not in group C, which comprised only nine cases, with a heterogeneous distribution of markers (*Online Supplementary Table 1*). When cases from groups A and B were studied together (*Online Supplementary Figure 1 A,C,E*), a greater tendency to undergo apoptosis in response to aμ-Ab was observed in ZAP-70-positive, unmutated and CD38-positive CLL cases. These differences were statistically significant only for CD38 (p=0.035). There were more CD38-positive cases in group A (23/40; 57.5%) than in group B (9/33; 27.2%) (*Online Supplementary Figure 1B*). This difference was significant (p=0.017). Although there were more unmutated and ZAP-70 positive cases in group A than in group B, these differences were not statistically significant (*Online Supplementary Figure 1 D, F*).

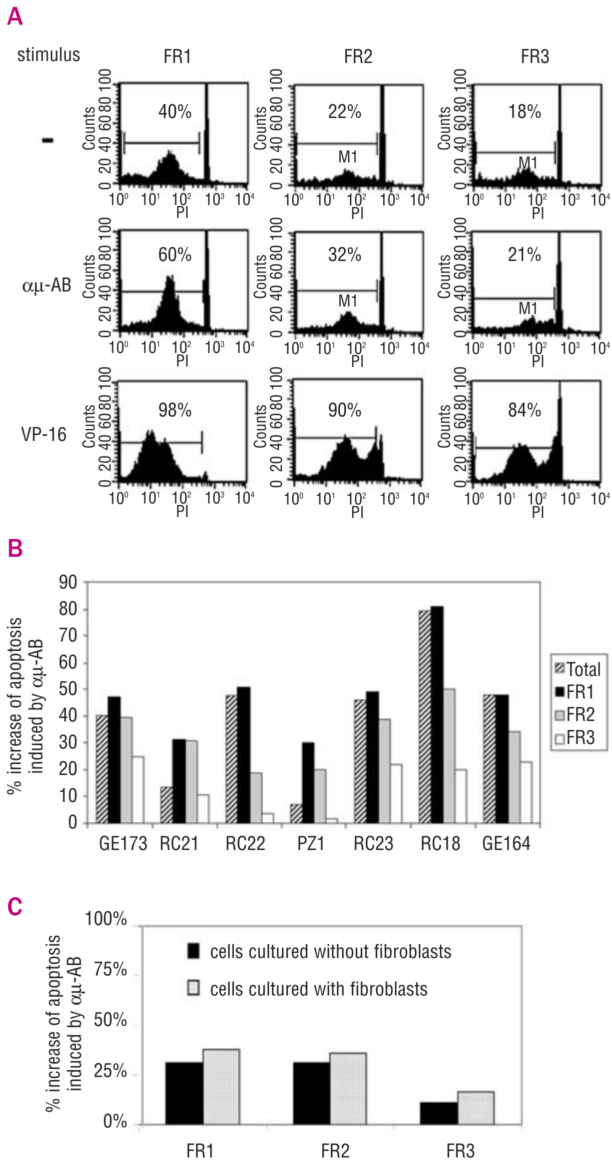

Figure 3. Differences in apoptotic capacity of CLL cells separated according to CD38 expression. CLL cells from case RC21 were fractionated into CD38-high (FR1), CD38-intermediate (FR2), and CD38-negative cells (FR3). (A) The three fractions were exposed to medium, aμ-Ab or VP16 for 48 h *in vitro*. Apoptosis was determined by propidium iodide staining. (B) Summary of percentage increase of apoptosis seen in all cell fractions from the cases tested following exposure to aμ-Ab. (C) Cell fractions obtained from case RC21 were exposed to aμ-Ab in the presence or absence of CD40L-transfected fibroblasts. Values are expressed as percentage increase of apoptosis following aμ-Ab stimulation.

Correlation between aμ-Ab-induced tyrosine protein phosphorylation and expression of prognostic markers

We analyzed possible correlations existing between protein tyrosine phosphorylation, which followed cross-linking of surface IgM, and expression of prognostic markers in 21 randomly selected patients (identified by an asterisk in *Online Supplementary Table 1*). In these cases, the extent of protein tyrosine phosphorylation correlated directly with the expression of CD38 (p=0.015) and ZAP-70 (p=0.032) and inversely with the presence of Ig VH gene mutations (p=0.03) (Figure 4 A, B, C). There was one outlier case with unusually high protein tyrosine phosphorylation. However, when this case was excluded, there was still a significant correlation between CD38 (p=0.049) or ZAP-70 expression (p=0.05) and Ig VH gene status (p=0.04). These correlations were statistically significant. The extent of protein tyrosine phosphorylation following surface IgM cross-linking did not relate to the classification of single cases in apoptosis group A or B (Figure 4 D).

Discussion

This study shows that CD38-positive leukemic cells expressed ZAP-70, exhibited a more efficient B-cell receptor-dependent signal transduction pathway, and were more apoptosis prone than the CD38-negative B cells from the same leukemic clone, which were also negative for ZAP-70. Some technical issues deserve comment. For example, stimulation through surface CD38 during separation could induce ZAP-70 expression giving the false impression of co-expression of the two markers. However, the demonstration of co-expression of the two markers by flow-cytometry in unfractionated cells and the consideration that ZAP-70 may not be induced on cells exposed to CD38 monoclonal antibody in the cold make this hypothesis very unlikely. The possibility that exposure to CD38 monoclonal antibody caused a facilitated stimulation by aμ-Ab also appears unlikely since cell exposure to CD38 monoclonal antibody did not induce protein tyrosine phosphorylation, and exposure of unseparated cells to anti-CD38 monoclonal antibody did not change the magnitude of the cells' apoptotic response to aμ-Ab stimulation. Both observations are consistent with the inability of the CD38 monoclonal antibody used here to stimulate B cells.[18,29]

Purified CD38-positive subclones exhibited the same

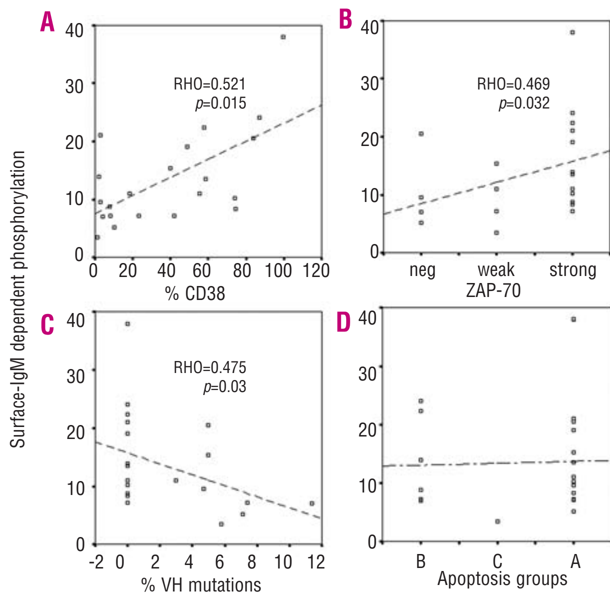

Figure 4. Protein tyrosine phosphorylation following surface IgM cross-linking: correlation with the expression of prognostic markers by CLL cells. (A, B, C) Correlation between protein tyrosine phosphorylation and expression of the indicated prognostic markers. The analysis was carried out on 21 cases indicated by an asterisk in Table 1. Protein tyrosine phosphorylation was detected by western blotting and measured by the 1D Image analysis software version 3.5 according to criteria explained in the Design and Methods section. (D) Correlation between protein tyrosine phosphorylation and apoptotic capacity according to the classification of single cases into group A, group B or group C.

features irrespective of whether they were isolated from mutated or unmutated CLL cases. This suggests that CD38-positive, ZAP-70 positive cells can be generated in all CLL clones, and that differences between CLL cases with a good or bad prognosis would lie in the number of cells exhibiting CD38 and ZAP-70 and the corresponding functional features. Since CD38-positive, ZAP-70-positive cells are those more prone to be stimulated through surface Ig, the present observations support the aforementioned model that antigenic stimulation may be instrumental in promoting clonal expansion *in vivo*[2,3] and do not contradict the notion that continuous antigenic stimulation may induce expression of ZAP-70 and/or CD38.[19-21] Finally, the data are in agreement with the observations that CD38-positve and CD38-negative cells from the same leukemic clones differ in their gene expression signatures.[32]

A few questions remain unanswered. For example, given the fact that the levels of cells expressing CD38 and/or ZAP-70 remain fairly constant with time, how can this homeostasis be maintained? If the expansion of cells capable of responding to surface IgM cross-linking is favored, one would expect that all CLL, either mutated or unmutated, would transform into CD38-positive, ZAP-70 positive cases in a relatively short time. Apoptosis of excessively stimulated cells would contribute to reinstate the balance, by eliminating a number of CD38-positive, ZAP-70 positive cells and favoring the permanence of non-activated cells. This possibility is supported by recent *in vivo* analyses of CLL cell birth and death kinetics.[28] Alternative, and not mutually exclusive, explanations could involve *tolerance* to stimuli of excessively activated cells[2] or accumulation of lesions of the IgM-dependent signal transduction pathway in these cells, particularly at the CD79 level.[33,34] Finally a slow, but steady transformation of CD38-negative, ZAP-70 negative cells into CD38-positive, ZAP-70 positive cells could occur *in vivo*.

In our tests, B-cell receptor cross-linking more often caused apoptosis than survival of CLL cells *in vitro*. Why then should an apoptotic signal cause clonal progression rather than clonal abortion? Stimulation with aμ-Ab was carried out on purified B cells *in vitro*. Leukemic cells, activated by surface IgM cross-linking, may be driven into apoptosis *in vitro* because of the absence of anti-apoptotic signals, whereas the fate of the same cells *in vivo* would be determined by micro-environmental support.[35-38] This would be part of the homeostatic mechanisms alluded to above. The stimulation received by cells *in vivo* may, therefore, not be mimicked artificially *in vitro* and the specific functional outcome of aμ-Ab stimulation *in vitro* may be less important than the fact that a functional consequence can occur, with the stimulation viewed as a read-out system to measure the cells' susceptibility to signals delivered by surface IgM.

The major outcome of CLL cell stimulation by aμ-Ab has been variously reported to be apoptosis, cell survival or even proliferation. In other studies, both apoptosis and survival were observed, as in our study, albeit in different proportions. These differences could be related to different methods of stimulation used *in vitro*.[27,29,39-41] Although we were unable to see remarkable variations in responses when changing stimulation conditions [i.e., antibody concentrations, insoluble vs soluble reagents, intact IgG antibody vs. F(ab')2 fragments], these variables are difficult to control and may contribute to observed differences in results. Purification of the CLL cell suspensions and/or presence of accessory cells may also influence the outcome of stimulation via surface IgM; this represents an additional variable that is difficult to control when different studies are compared. Finally, the mode of B-cell receptor stimulation may influence the final outcome. We have recently observed that CLL cell stimulation through surface IgD induces cell survival more frequently than apoptosis (*unpublished findings*) leading to the conclusion that regulation of clonal size through B-cell receptor stimulation may be even more complex than so far believed.

The phenotypic heterogeneity of CLL clones demonstrated by the fractionation studies offers an explanation for the weaker correlations between marker expression and functional properties in the cohort studies. Clonal heterogeneity may cause imprecision in the definition of a CLL case as positive or negative for a given marker, and additional imprecision may be generated by different sensitivities of the tests and by the cut-off points selected. In

connection with this, it is of note that CD38 and ZAP-70 are generally measured as a percentage of positive cells in a given clone and that these values (and also the classification of a case), may change if mean fluorescent intensity values or RFI are considered, because of the heterogeneity of marker expression within the clone. These considerations may in part explain some discrepancies when the overlap between ZAP-70 and CD38 expression is measured within a CLL clone using one of the other approaches (see Figure 1). Functional tests, such as apoptosis, have an even higher degree of imprecision because of additional variability caused by cell preparation methods and cell viability. Finally, these biological tests measure phenomena resulting from complex chains of events, each of which can produce errors because of excessive amplification or decrease of a given signal. These considerations may help to explain why, at a cohort level, there was a stronger correlation between expression of prognostic markers and activation of the IgM-dependent signal transduction pathway measured by protein tyrosine phosphorylation than with apoptosis induced by stimulation via surface IgM.

The imprecision generated by technical errors may represent a valid, albeit partial, explanation for the discrepancies between marker expression and function. In fact, 21/78 (26.9%) CLL cases were discrepant for CD38 and ZAP-70 expression; this value correlates well with that reported by others.[9,11,42,43] Two of these cases with discrepant marker expression deserve comments; case RC1 had 3% CD38-positive cells and strong ZAP-70 expression, while case RC11 had 87% CD38-positive cells and was ZAP-70 negative. The cells from both cases had a viable surface IgM-dependent signal transduction pathway and were induced into apoptosis by surface IgM cross-linking. In relation to the model discussed above, the cell stimulation received *in vivo* may be different in the two cases, thereby causing the expression of either CD38 or ZAP-70. These observations also raise questions regarding the role of CD38 and ZAP-70 in the B-cell receptor-dependent signal transduction pathway, an issue already considered by others.[22,23] Nevertheless, the present studies support the notion that CD38 and ZAP-70 may be efficient indicators of a viable B-cell receptor-dependent signal transduction pathway in CLL cells and the data provide new clues to explain why correlations exist between these markers and an adverse prognosis.

Authorship and Disclosures

GC is the author taking primary responsibility for the paper, designed and performed most of the experiments, analyzed most of the data and drafted the manuscript. MC contributed to CLL Ig VH gene sequencing analysis, and revised the article critically. SM isolated CLL cells, performed culture experiments and drafted the article. MF performed protein tyrosine phosphorylation experiments and revised the article critically. MS and VC, EV and MG contributed to recruitment of CLL patients and revised the article critically. SZ contributed to acquisition of data, and revised the article critically. NC contributed to the analysis and interpretation of data and revised the article critically for important intellectual content. FM contributed to recruitment of CLL patients, statistical analysis of the data and revising the article critically. MF conceived the project, provided a critique of the experimental designs and assisted in data interpretation as well as undertaking the final revision of the manuscript. All authors approved the final version of the paper to be published. The order of the authors was arranged accordingly. The authors reported no potential conflicts of interest.

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