BCR ligation induced by IgM stimulation results in gene expression and functional changes only in IgV_H unmutated chronic lymphocytic leukemia (CLL) cells

Anna Guarini,[1] Sabina Chiaretti,[1] Simona Tavolaro,[1] Roberta Maggio,[1] Nadia Peragine,[1] Franca Citarella,[2] Maria Rosaria Ricciardi,[1] Simona Santangelo,[1] Marilisa Marinelli,[1] Maria Stefania De Propris,[1] Monica Messina,[1] Francesca Romana Mauro,[1] Ilaria Del Giudice,[1] and Robert Foà[1]

Divisions of [1]Hematology, and [2]Genetics, Department of Cellular Biotechnologies and Hematology, Sapienza University of Rome, Rome, Italy

Chronic lymphocytic leukemia (CLL) patients exhibit a variable clinical course. To investigate the association between clinicobiologic features and responsiveness of CLL cells to anti-IgM stimulation, we evaluated gene expression changes and modifications in cell-cycle distribution, proliferation, and apoptosis of IgV_H mutated (M) and unmutated (UM) samples upon BCR cross-linking. Unsupervised analysis highlighted a different response profile to BCR stimulation between UM and M samples. Supervised analysis identified several genes modulated exclusively in the UM cases upon BCR cross-linking. Functional gene groups, including signal transduction, transcription, cell-cycle regulation, and cytoskeleton organization, were up-regulated upon stimulation in UM cases. Cell-cycle and proliferation analyses confirmed that IgM cross-linking induced a significant progression into the G_1 phase and a moderate increase of proliferative activity exclusively in UM patients. Moreover, we observed only a small reduction in the percentage of $subG_{0/1}$ cells, without changes in apoptosis, in UM cases; contrariwise, a significant increase of apoptotic levels was observed in stimulated cells from M cases. These results document that a differential genotypic and functional response to BCR ligation between IgV_H M and UM cases is operational in CLL, indicating that response to antigenic stimulation plays a pivotal role in disease progression. (Blood. 2008;112:782-792)

Introduction

Chronic lymphocytic leukemia (CLL) is a disease characterized by an extremely heterogeneous clinical course; some patients may live for many years without requiring any treatment, whereas others, despite therapy, experience a rapid progression and in some cases an unfavorable fate.[1,2]

Several biologic parameters have allowed the risk stratification of CLL patients.[3] One of the most reliable parameters is represented by the presence or absence of a significant level of somatic mutations within the immunoglobulin (Ig) variable heavy (V_H) region genes. CLL patients with an unmutated IgV_H status (UM-CLL) show a worse prognosis, whereas patients with mutated status show better prognosis.[4,5] In line with this, gene expression analyses have shown a unique signature associated with mutated (M-CLL) and UM-CLL cells, and have highlighted an up-regulation of several genes linked to cell cycle and cell signaling in UM-CLL.[6,7] Other biologic indicators of a poor clinical behavior are represented by genomic aberrations,[8] surface expression of the CD38 antigen,[4,9] and the intracytoplasmic presence of the ZAP-70 protein.[10,11] CD38[12] and the tyrosine kinase ZAP-70 have been shown to increase B-cell receptor (BCR) signaling in UM-CLL,[13,14] and the coexpression of CD38 and ZAP-70 appears to correlate with a very strong activation of BCR signal transduction.[15,16] These markers of progressive disease seem to strengthen the hypothesis that BCR signaling plays an important role in the proliferation and maintenance of the malignant B cells.

The reasons for disease initiation and progression have so far not been fully elucidated. It has been suggested that antigen stimulation, along with interactions with accessory cells and cytokines, may represent the promoting factor that stimulates proliferation of the neoplastic cells, thus protecting them from apoptosis. These effects may differ in distinct patients and could lead to the disparity of clinical behavior in this disease.[17] Furthermore, there is evidence indicating that CLL may be sustained by an antigen-driven process based on a restriction of the repertoire, as well as by shared antigen-binding motifs used by neoplastic B cells.[17] In vitro cross-linking of BCR molecules with antibodies to IgM mimics the engagement of antigens with BCR and transmits signals to the cell nucleus.

In view of the different clinical and biologic behavior of M-CLL and UM-CLL IgV_H genes, in the present study we investigated the gene expression profile changes, as well as the functional modifications in the proliferation and apoptotic rate, and cell-cycle induction upon BCR ligation with immobilized IgM in these 2 distinct subgroups of CLL.

Methods

Patients

Twenty CLL patients, 10 females and 10 males, with a median age of 51.5 years (range, 30-84 years) were evaluated at the time of presentation

Submitted December 5, 2007; accepted April 16, 2008. Prepublished online as *Blood* First Edition paper, May 16, 2008; DOI 10.1182/blood-2007-12-127688.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. CLL patients' clinical characteristics

CLL patient label	Sex	Age at study, y	Binet stage	IgV_H status	IgV_H gene	ZAP-70, %	CD38, %	FISH analysis
CLL-1	F	59	B	Unmutated	3-33	90	3	Negative
CLL-2	M	59	A	Unmutated	1-69	65	76	del13q, del11q
CLL-3	M	45	A	Unmutated	3-09	74	0	NE
CLL-4	M	49	B	Unmutated	1-69	76	1	del11q
CLL-5	M	59	B	Unmutated	3-73	10	8	del13q, del14q, del6q
CLL-6	F	45	A	Mutated	4-34	0	1	del13q
CLL-7	F	41	B	Mutated	2-05	4	0	del13q
CLL-8	M	34	A	Mutated	3-72	0	0	del13q, del14q
CLL-9	F	39	B	Mutated	4-34	19	1	del11q
CLL-10	M	58	A	Mutated	4-34	0	3	Negative
CLL-11	F	58	B	Unmutated	3-11	81	21	del13q
CLL-12	M	60	B	Mutated	5-51	9	1	Negative
CLL-13	F	44	A	Unmutated	3-09	0	10	+12
CLL-14	F	57	A	Mutated	3-30	0	2	del13q
CLL-15	F	45	A	Mutated	4-34	3	0	Negative
CLL-16	F	56	B	Mutated	2-70	10	0	del14q
CLL-17	F	51	A	Mutated	3-21	1	0	del13q
CLL-18	M	84	A	Unmutated	4-39	77	0	NE
CLL-19	M	52	B	Unmutated	1-69	0	2	del11q, +12
CLL-20	M	30	B	Unmutated	4-34	32	78	del13q, del6q

NE indicates not evaluated.

before any treatment. This study was approved by the Institutional Review Board of "Sapienza" University of Rome. Informed consent to the blood collection and to the biologic analyses included in the present study was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of CLL was based on the presence in the peripheral blood of more than 5×10^9/L lymphocytes that expressed a conventional CLL morphology and immunophenotype (CD5/CD20$^+$, CD23$^+$, weak CD22$^+$, weak sIg$^+$, CD10$^-$). According to Binet staging system, 10 patients were in stage A and 10 in stage B. Patients' characteristics are summarized in Table 1. Routine analyses included the IgV_H gene mutational status,[18] ZAP-70[10,11] and CD38 evaluation by flow cytometry,[9] fluorescence in situ hybridization (FISH) analysis for the identification of cytogenetic aberrations involving 11q22-23, 13q14, 6q21, and 17p13 regions and trisomy 12, and p53 sequencing.[18]

CLL cell separation and stimulation

Blood samples were collected from CLL patients. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque density centrifugation (Nycomed Pharma, Oslo, Norway). Freshly isolated PBMCs were subsequently enriched in CD19$^+$ B cells (> 98%) by standard positive selection using a specific anti-CD19 antibody conjugated with magnetic beads (Miltenyi Biotech, Auburn, CA). Purified B cells were cultured in 96-well U-bottom plates (Corning, New York, NY) coated overnight at 4°C with 50 μg/mL anti–goat F(ab′)$_2$ IgG developed in rabbit (Sigma-Aldrich, St Louis, MO). B lymphocytes were plated at 5×10^5 cells/well in RPMI 1640 medium (Cambrex BioScience, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, South Logan, UT), 0.3 mg/mL L-glutamine, and 1% Pen-strepto (Euro-Clone, Pavia, Italy). BCR stimulation was performed by adding a goat F(ab′)$_2$ anti–human IgM (μ-chain specific; Sigma-Aldrich) at a final concentration of 10 μg/mL for 24 hours; in some selected experiments, stimulation was carried out for 6 hours and/or 48 hours.

RNA extraction and oligonucleotide microarray

After 24 hours of incubation, unstimulated (US-CLL) and stimulated (S-CLL) cells were lysed and total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. In selected experiments, RNA was extracted also after 6 and 48 hours of stimulation.

To assess RNA quality, 2 μL RNA from each sample was analyzed by electrophoresis on agarose gel; for all samples the 260:280 ratio was more than 1.8, as required for microarray analysis.

HGU133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA) were used to determine gene expression profiles. The detailed protocol for sample preparation and microarray processing is available from Affymetrix.[19]

Statistical methods for microarray analysis

Oligonucleotide microarray analysis was performed with dChip software (http://www.dchip.org, Dana-Farber Cancer Institute, Boston, MA), which uses an invariant set normalization method. The array with median overall intensity was chosen as the baseline for normalization. Model-based expressions were computed for each array and probe set using the PM-MM model.[20]

Nonspecific filtering criteria for unsupervised clustering required the expression level to be higher than 100 in more than 30% of the samples and the ratio of the standard deviation (SD) to the mean expression across all samples to be between 0.5 and 1000. Hierarchic clustering was used as described by Eisen et al.[21]

To specifically identify genes differentially expressed between US-CLL and S-CLL samples in different subgroups of CLL, a *t* test was applied. Probe sets were required to have an average expression of 100 or more in at least one group, a fold change of 1.5 or more, and a *P* value of .05 or less. Furthermore, to strengthen the robustness of these results, the false discovery rate (FDR) was calculated over 5000 permutations. All experimental and microarray data can be found at Ematologia La Sapienza.[22]

Real-time quantitative polymerase chain reaction

Total RNA (1 μg) was retrotranscribed using the Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA). Real-time quantitative–polymerase chain reaction (Q-PCR) analysis was performed using an ABI PRISM 7700 sequence detection system and the SYBR green dye (Applied Biosystems, Foster City, CA) method, as previously described.[23] Real-time PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 minute; for 40 cycles. For each sample, C_T values for *GAPDH* were determined for normalization purposes, and delta C_T (ΔC_T) between *GAPDH* and target genes was calculated. Primers were designed using the Primer Express 1.0 software (PE Biosystems). The following primers were used: 5′ *GAPDH*, 5′-CCACCCATGGCAAATTCC-3′; 3′ *GAPDH*, 5′-GATGGGATTTCCATTGATGACA-3′; 5′ *SYK*, 5′-ATGGAAAAATCTCTCGGGAAGAA-3′; 3′ *SYK*, 5′-TGGCTCGGAT-CAGGAACTTT-3′; 5′ *ZAP-70*, 5′-GCACCCGAATGCATCAAC-3′; and 3′ *ZAP-70*, 5′-GACAAGGCCTCCCACATG-3′.

Cell-cycle analysis

Cell-cycle distribution changes were evaluated using the acridine orange (AO) technique, as previously described.[24] The percentage of cells in G_0, G_1, S, and G_2M and the mean RNA content of $G_{0/1}$ cells were determined by measuring simultaneously the DNA and RNA total cellular content. RNA content of $G_{0/1}$ cells was expressed as RNA-index (RNA-I $G_{0/1}$), determined as the ratio of the mean RNA content of $G_{0/1}$ cells of the samples multiplied by 10 and divided by the median RNA content of control lymphocytes. G_0 cells were defined as cells with an RNA content equal to or lower than that of control normal peripheral blood lymphocytes.

Flow cytometric analyses were carried out using a FACSCan flow cytometer (Becton Dickinson) operated at 488 nm, which detects green (F_{530}-DNA) and red ($F_{>620}$-RNA) fluorescence. Data acquisition and analysis (10 000-20 000 events) were performed with the CellQuest software (Becton Dickinson). Cell-cycle distribution was analyzed using the ModFit LT software (Verity Software House, Topsham, ME).

In vitro proliferation assay

For each patient, 5×10^5 $CD19^+$ B cells in 200 μL/well in 96-well microtiter plates were seeded in triplicate and cultured for 24 and 48 hours in the presence or absence of anti-IgM. After the indicated time of culture, cells were incubated with 0.037 MBq [^{3}H]thymidine/well (GE Healthcare, Little Chalfont, United Kingdom) for 18 hours, harvested, and counted using a beta-counter (Packard Bioscience, Groningen, The Netherlands). Results are expressed as the ratio of the mean counts per minute (cpm) of S-CLL versus US-CLL.

Apoptosis assays

Apoptosis was measured by 2 different methods: after 24 hours, cultured cells were double stained with FITC-conjugated annexin-V/propidium iodide (Immunotech Research, Vaudreuil-Dorion, QC). After staining, apoptotic cells were evaluated by flow cytometry and the data analyzed using the CellQuest software (Becton Dickinson). Furthermore, apoptosis was measured after 24 to 48 hours of culture upon stimulus by evaluating the sub-$G_{0/1}$ peak on DNA-frequency histograms using the AO technique described in "Cell-cycle analysis," which measures apoptotic cells based on the decreased stain ability of apoptotic elements in DNA green fluorescence (sub-$G_{0/1}$ peak on DNA frequency histograms) coupled with a higher RNA red fluorescence (which is common to chromatin condensation),[24,25] cell debris being excluded from the analysis on the basis of their forward light scatter properties.

Statistical analysis for proliferation studies and apoptosis

The 2-sided Student *t* test was used to evaluate the significance of differences between groups. Results are expressed as the means plus or minus SD.

Results

Microarray analysis reveals a different responsiveness profile between IgV_H UM and M CLL cases upon BCR cross-linking

To evaluate the effects of BCR stimulation on CLL cells, we performed gene expression profile studies on $CD19^+$ purified US-CLL and S-CLL cells isolated from 10 CLL patients (CLL-1-10) with different clinical and biologic features and stimulated the cells for 24 hours (Table 1). Our first analysis used an unsupervised approach: applying nonspecific filtering criteria to all samples, 673 probe sets, corresponding to 635 genes, were selected. As shown in Figure 1, unsupervised hierarchic clustering based on the expression of this set of genes identified 2 major clusters: the first included exclusively CLL patients with an UM configuration of the

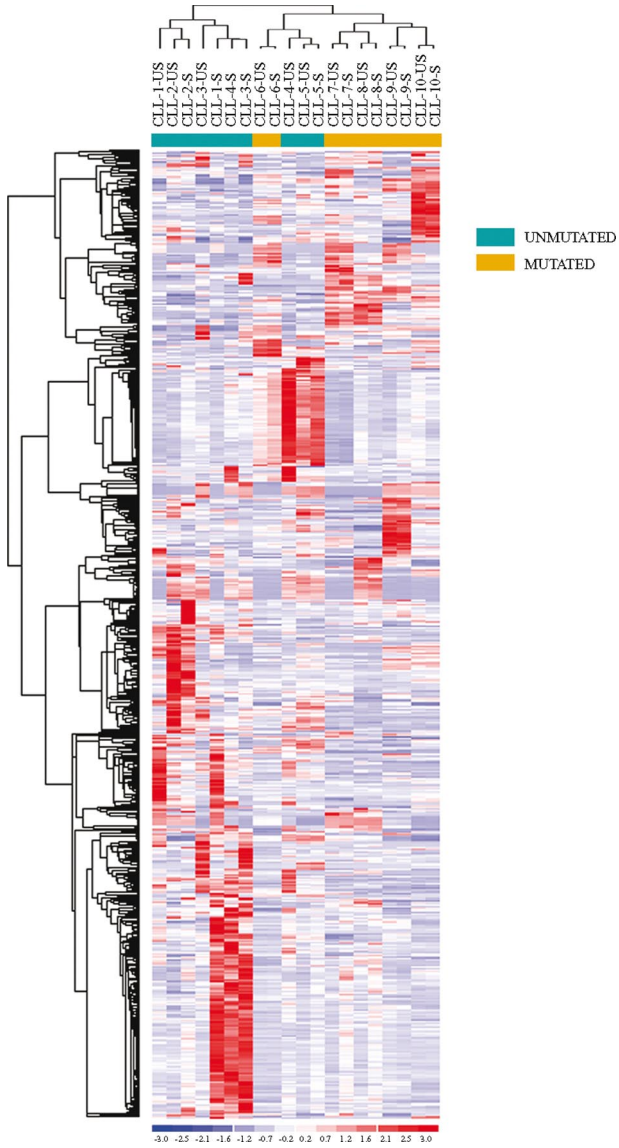

Figure 1. Unsupervised clustering of unstimulated (US) and stimulated (S) CLL samples. Unsupervised clustering of the unstimulated (US) and stimulated (S) CLL samples evaluated by gene expression profiling upon BCR cross-linking. Relative levels of gene expression are depicted with a color scale: red indicates highest levels of expression; blue, lowest levels of expression.

IgV_H genes, whereas the second cluster included all the IgV_H M cases and a single UM patient (CLL-5).

Second, to identify genes that were modulated upon BCR stimulation, we performed a *t* test between US-CLL and S-CLL samples. This analysis selected 71 differentially expressed genes: it is important to underline that this approach unequivocally showed that BCR stimulation induces relevant changes mostly within IgV_H UM patients (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We thus performed a supervised analysis to compare US-CLL and S-CLL samples within IgV_H UM and M cases. In the IgV_H UM cases, the *t* test identified 197 genes differentially expressed upon BCR cross-linking, the majority being highly expressed in the stimulated cells (Figure 2). A large set of these genes, reported in Table 2, codify for proteins involved in BCR signaling and/or in BCR activation; in particular, among the more represented functional groups, we found several genes involved in signal transduction *(TNFAIP3, DUSP2, DUSP4, DUSP10, NPM1, SYK, CXCR4,*

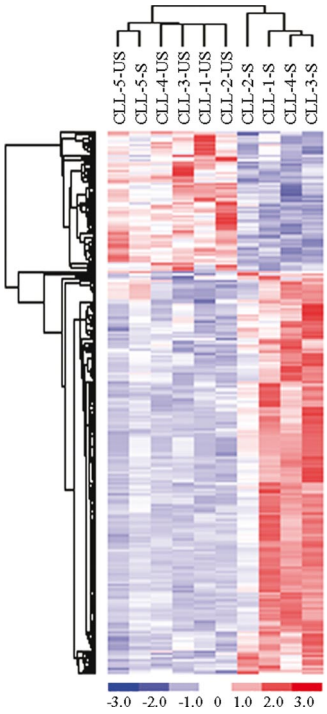

Figure 2. Comparison between unstimulated (US) and stimulated (S) samples in CLL unmutated (UM) patients. Identification of 197 genes differentially expressed between unstimulated (US) and stimulated (S) samples in CLL unmutated (UM) patients.

CCL3, CCL4, HOMER1), regulation of transcription *(EGR1, EGR2, EGR3, NME1, NME2, ZNF238, TOP1MT),* and metabolism *(LDHB, HSP90AB1, PSMCs, ATP2A3).* Moreover, different expression levels were identified also in a set of genes involved in cell-cycle regulation *(CCND2, CDK4, PTPN6, CHES1)* and cytoskeleton organization *(ACTB, ACTG1, K-ALPHA1, TUBB, BICD2).* Notably, after 6 hours of stimulation, we already observed an increase of a set of these genes (*EGR3, NR4A1, DUSP4, LRMP, CD39,* and an unknown sequence), suggesting that gene expression changes start to occur relatively early and increase over time (Figure S2). Contrariwise, when the same approach was applied to US-CLL and S-CLL cells within IgV_H M cases, no genes were selected in this analysis.

Overall, these results highlight that, whereas CLL cells from IgV_H UM patients are responsive to BCR cross-linking, this is not the case for CLL cells from IgV_H M patients, as these cells appear to be anergic to such stimulus.

Response to BCR stimulation is correlated to IgM expression

As previously mentioned, through a supervised approach it was possible to identify a set of genes modulated after BCR stimulation. Unsupervised clustering suggested that 3 samples (CLL-5, CLL-7, and CLL-8) showed an anomalous behavior, with 2 IgV_H M-CLL cases displaying gene modulation upon BCR stimulation and 1 IgV_H UM-CLL patient with very few changes occurring after stimulation, indicating that other factors may influence signaling through BCR. To explain this phenomenon, different clinicobiologic features were investigated, namely CD38 and ZAP-70 expression, as well as cytogenetic aberrations. The only parameter that was highly related to the response to BCR cross-linking was represented by the IgM levels (evaluated by microarray analysis). In fact, although IgM could be detected in all CLLs analyzed at both the RNA (Figure S3) and protein (data not shown) level, CLL cases that did not respond to BCR ligation showed lower IgM expression levels. Furthermore, IgM mRNA expression levels were not affected by BCR ligation.

Evaluation of SYK and ZAP-70 expression by Q-PCR analysis

Among the different tyrosine kinases involved in BCR activation, SYK represents an early signaling intermediate of this cascade in B cells.[26] ZAP-70, a tyrosine kinase of the Syk family, plays a role in increasing BCR signaling after cross-linking of IgM in CLL.[13] To investigate the relative expression changes of these kinases upon BCR engagement, *SYK* and *ZAP-70* expression levels in US-CLL and S-CLL cells were evaluated, after 24 hours of IgM stimulation, by Q-PCR. This analysis was performed on 10 CLL samples (5 IgV_H UM: CLL-1-5; 5 IgV_H M cases: CLL-6-9 and CLL-12). Q-PCR results are reported in Figure 3: *SYK* expression levels decreased in S-CLL samples compared with US-CLL samples. Moreover, the down-modulation of this gene was statistically significant ($P = .009$) exclusively in the CLL IgV_H UM cases. Contrariwise, no significant differences in *ZAP-70* expression levels were observed after stimulation in both IgV_H UM and M CLL cells. Overall, these findings indicate that, upon BCR engagement, *SYK*, but not *ZAP-70*, is differentially modulated between IgV_H UM and M CLL cases. Hence, these data further corroborate the microarray results that show a downmodulation of *SYK* and no changes in *ZAP-70* expression levels.

IgM cross-linking acts by inducing cell-cycle progression of primary CLL cells from IgV_H UM patients

To evaluate the effects of IgM cross-linking on cell-cycle distribution changes, cell-cycle analysis was performed on US-CLL and S-CLL cells after 24 and 48 hours upon stimulus. As shown in Figure 4A, IgM cross-linking induced a considerable proliferative activity on primary CLL cells from IgV_H UM patients (7 cases: CLL-1, CLL-2, CLL-11, CLL-13, CLL-18, CLL-19, and CLL-20), as shown by a statistically significant increase of cells in the G_1 phase from 1.06% ± 1.50% in US-CLL cells to 2.74% ± 2.50% in S-CLL cells ($P = .037$) after 48 hours of exposure to anti-IgM. Conversely, resistance to anti-IgM–mediated cell-cycle progression was observed in CLL cells from IgV_H M patients (5 cases: CLL-9, CLL-12, CLL-14, CLL-15, and CLL-17): from 0.28% ± 0.31% in US-CLL cells to 0.28% ± 0.26% in S-CLL cells (Figure 4B).

Induction of proliferation after IgM cross-linking

To corroborate the fact that by gene expression profiling a set of cell cycle–related genes is up-regulated in IgV_H UM cases, the effects on proliferation upon IgM cross-linking were measured by thymidine uptake. Primary cells from 7 IgV_H UM cases (CLL-1, CLL-2, CLL-11, CLL-13, CLL-18, CLL-19, and CLL-20) displayed a proliferative response after 24 and 48 hours of stimulation, with an increase of 2.7- and 1.9-fold change, respectively. At variance, no difference in thymidine uptake was observed in S-CLL and US-CLL cells from 4 IgV_H M patients (CLL-9, CLL-12, CLL-14, and CLL-15).

IgM cross-linking effects on apoptosis modulation

To explore if the proliferative effects of IgM cross-linking could be associated with a cytoprotective activity, apoptosis levels were also analyzed at 24 and 48 hours in IgV_H UM and M CLL cells using the annexin-V and AO techniques. In 5 IgV_H UM cases (CLL-1,

Table 2. Genes differentially expressed between unstimulated (US) and stimulated (S) samples in CLL unmutated (UM) cases

Probe set ID	Gene symbol	*P*	Fold change	Gene function	Expression in UM cases upon IgM stimulus
201030_x_at	*LDHB*	.001	1.57	Tricarboxylic acid cycle metabolism	High
213757_at	Unknown	.004	1.80	Unknown	High
213214_x_at	*ACTG1*	.004	2.22	Cell motility	High
201694_s_at	*EGR1*	.006	9.42	Regulation of transcription, DNA-dependent	High
202314_at	*CYP51A1*	.006	2.13	Electron transport	High
235122_at	Unknown	.009	2.31	Unknown	High
209218_at	*SQLE*	.010	1.93	Electron transport	High
200790_at	*ODC1*	.010	2.40	Polyamine biosynthesis	High
217869_at	*HSD17B12*	.011	1.68	Steroid biosynthesis	High
204440_at	*CD83*	.011	4.77	Immune response	High
211058_x_at	*K-ALPHA-1*	.011	1.55	Microtubule-based movement	High
209795_at	*CD69*	.013	2.06	Defense response	High
201064_s_at	*PABPC4*	.014	1.59	RNA processing	High
219971_at	*IL21R*	.014	2.67	Natural killer–cell activation	High
211724_x_at	*FLJ20323*	.014	1.76	Unknown	High
226397_s_at	*TBC1D7*	.016	2.58	Unknown	High
221750_at	*HMGCS1*	.016	1.68	Lipid metabolism	High
242260_at	*MATR3*	.016	2.68	Unknown	High
205249_at	*EGR2*	.017	15.50	Regulation of transcription, DNA-dependent	High
222062_at	*IL27RA*	.018	1.82	Immune response	High
201892_s_at	*IMPDH2*	.018	1.93	Metabolism	High
224707_at	*C5orf32*	.018	1.52	Unknown	High
212803_at	*NAB2*	.019	1.96	Regulation of transcription, DNA-dependent	High
206206_at	*CD180*	.019	1.80	Immune response	High
200801_x_at	*ACTB*	.019	1.57	Cell motility	High
208696_at	*CCT5*	.019	1.68	Protein folding	High
208057_s_at	*GLI2*	.020	1.79	Regulation of transcription, DNA-dependent	High
208680_at	*PRDX1*	.020	2.00	Cell proliferation	High
204794_at	*DUSP2*	.022	8.42	Protein amino acid dephosphorylation	High
223751_x_at	*TLR10*	.022	1.94	Immune response	High
201516_at	*SRM*	.022	1.95	Spermidine biosynthesis	High
201268_at	*NME2*	.022	1.77	Regulation of transcription, DNA-dependent	High
208693_s_at	*GARS*	.023	2.58	Protein biosynthesis	High
212811_x_at	*SLC1A4*	.023	2.10	Transport	High
209959_at	*NR4A3*	.023	2.06	Regulation of transcription, DNA-dependent	High
201587_s_at	*IRAK1*	.024	1.60	Signal transduction	High
202340_x_at	*NR4A1*	.024	1.69	Regulation of transcription, DNA-dependent	High
237753_at	Unknown	.025	2.96	Unknown	High
209836_x_at	*BOLA2*	.026	1.81	Unknown	High
200953_s_at	*CCND2*	.026	2.72	Regulation of progression through cell cycle	High
229659_s_at	*PIGR*	.026	1.99	Protein secretion	High
201859_at	*PRG1*	.027	1.99	Unknown	High
224406_s_at	*FCRL5*	.027	1.76	Unknown	High
226459_at	*PIK3AP1*	.027	1.65	Unknown	High
202391_at	*BASP1*	.027	2.62	Unknown	High
204621_s_at	*NR4A2*	.027	9.65	Regulation of transcription, DNA-dependent	High
200068_s_at	*CANX*	.028	1.61	Angiogenesis	High
213793_s_at	*HOMER1*	.028	6.07	Phospholipase C activating pathway	High
201577_at	*NME1*	.028	3.22	Cell cycle	High
201079_at	*SYNGR2*	.028	2.04	Unknown	High
227291_s_at	*BOLA3*	.029	1.80	Unknown	High
201013_s_at	*PAICS*	.029	2.38	Purine nucleotide biosynthesis	High
212038_s_at	*VDAC1*	.029	2.21	Transport	High
1568618_a_at	*GALNT1*	.030	1.98	Protein amino acid glycosylation	High
213638_at	*PHACTR1*	.030	3.93	Unknown	High
217848_s_at	*PPA1*	.030	2.83	Metabolism	High
223361_at	*C6orf115*	.030	3.83	Unknown	High
213925_at	*C1orf95*	.031	2.77	Unknown	High
224677_x_at	*C11orf31*	.031	1.74	Cell redox homeostasis	High
225913_at	*KIAA2002*	.031	1.50	Protein amino acid phosphorylation	High
207668_x_at	*PDIA6*	.032	1.75	Protein folding; cell redox homeostasis	High
227353_at	Unknown	.032	1.67	Unknown	High
226167_at	*SYT7*	.032	1.76	Transport	High
215346_at	*CD40*	.032	1.55	Regulation of B-cell proliferation	High
206115_at	*EGR3*	.032	15.41	Regulation of transcription, DNA-dependent	High
200064_at	*HSP90AB1*	.033	1.79	Protein folding	High

Genes are rank-ordered according to their *P* value.

Table 2. Genes differentially expressed between unstimulated (US) and stimulated (S) samples in CLL unmutated cases (continued)

Probe set ID	Gene symbol	*P*	Fold change	Gene function	Expression in UM cases upon IgM stimulus
217850_at	*GNL3*	.033	1.54	Regulation of progression through cell cycle	High
211714_x_at	*TUBB*	.033	1.70	Cell motility	High
201585_s_at	*SFPQ*	.034	1.56	Regulation of transcription, DNA-dependent	High
201000_at	*AARS*	.034	2.22	Protein biosynthesis	High
204674_at	*LRMP*	.034	2.51	Vesicle targeting	High
236099_at	Unknown	.034	7.75	Unknown	High
212691_at	*NUP188*	.034	1.54	Unknown	High
203880_at	*COX17*	.035	1.52	Protein folding	High
202081_at	*IER2*	.035	1.80	Unknown	High
200625_s_at	*CAP1*	.035	1.70	Actin cytoskeleton organization and biogenesis	High
209441_at	*RHOBTB2*	.036	1.50	Signal transduction	High
212129_at	*NIPA2*	.036	1.56	Unknown	High
224468_s_at	*C19orf48*	.036	1.65	Unknown	High
200822_x_at	*TPI1*	.036	1.52	Metabolism	High
226034_at	Unknown	.036	11.09	Unknown	High
222494_at	*CHES1*	.037	1.51	Cell cycle	High
206760_s_at	*FCER2*	.037	1.69	Unknown	High
202110_at	*COX7B*	.037	1.59	Electron transport	High
205114_s_at	*CCL3*	.037	38.34	Calcium ion homeostasis	High
200629_at	*WARS*	.037	6.41	Protein biosynthesis	High
204014_at	*DUSP4*	.037	20.11	Protein amino acid dephosphorylation	High
208758_at	*ATIC*	.038	1.60	Purine nucleotide biosynthesis	High
221923_s_at	*NPM1*	.038	1.51	Intracellular protein transport	High
202643_s_at	*TNFAIP3*	.038	2.00	Antiapoptosis	High
216323_x_at	*H2-ALPHA*	.038	1.79	Microtubule-based movement	High
223054_at	*DNAJB11*	.038	1.66	Protein folding	High
228320_x_at	*CCDC64*	.038	1.81	Unknown	High
224903_at	*CIRH1A*	.038	1.56	Transport	High
221563_at	*DUSP10*	.038	3.70	Protein amino acid dephosphorylation	High
243931_at	*CD58*	.038	1.95	Cell-cell adhesion	High
209473_at	Unknown	.038	1.90	Unknown	High
209104_s_at	*NOLA2*	.039	1.73	rRNA processing	High
226264_at	*SUSD1*	.039	4.75	Unknown	High
201114_x_at	*PSMA7*	.039	1.54	Microtubule-based movement	High
201263_at	*TARS*	.039	1.95	Protein biosynthesis	High
220966_x_at	*ARPC5L*	.039	1.69	Regulation of actin filament polymerization	High
210162_s_at	*NFATC1*	.040	1.67	G1/S transition of mitotic cell cycle	High
201195_s_at	*SLC7A5*	.041	4.25	Amino acid metabolism	High
205599_at	*TRAF1*	.041	1.60	Signal transduction	High
1559067_a_at	Unknown	.041	4.61	Unknown	High
217809_at	*BZW2*	.042	1.65	Regulation of translational initiation	High
209383_at	*DDIT3*	.042	1.96	Regulation of progression through cell cycle	High
214430_at	*GLA*	.042	1.79	Metabolism	High
226633_at	*RAB8B*	.042	1.60	Regulation of transcription, DNA-dependent	High
201622_at	*SND1*	.042	1.58	Regulation of transcription, DNA-dependent	High
208885_at	*LCP1*	.043	2.38	Actin filament bundle formation	High
224654_at	*DDX21*	.043	2.11	Regulation of transcription, DNA-dependent	High
205269_at	*LCP2*	.043	1.79	Immune response	High
212295_s_at	*SLC7A1*	.043	3.51	Amino acid metabolism	High
225676_s_at	*WDSOF1*	.044	1.79	Unknown	High
244261_at	*IL28RA*	.044	1.52	Negative regulation of cell proliferation	High
214096_s_at	*SHMT2*	.044	1.98	Glycine metabolism	High
228238_at	*GAS5*	.044	1.61	Unknown	High
201198_s_at	*PSMD1*	.044	1.58	Regulation of progression through cell cycle	High
202246_s_at	*CDK4*	.045	1.62	Regulation of progression through cell cycle	High
223058_at	*FAM107B*	.045	1.59	Unknown	High
218239_s_at	*GTPBP4*	.045	1.50	Ribosome biogenesis	High
212671_s_at	*HLA-DQA1*	.045	1.70	Immune response	High
200807_s_at	*HSPD1*	.045	1.71	Protein folding	High
202149_at	*NEDD9*	.045	2.11	Regulation of progression through cell cycle	High
217739_s_at	*PBEF1*	.046	2.42	Signal transduction	High
201422_at	*IFI30*	.046	1.84	Immune response	High
229437_at	*BIC*	.046	2.23	Unknown	High
202397_at	*NUTF2*	.046	1.59	Protein transport	High
201923_at	*PRDX4*	.047	1.92	I-kappaB phosphorylation	High
204032_at	*BCAR3*	.047	1.68	Regulation of progression through cell cycle	High

Genes are rank-ordered according to their *P* value.

Table 2. Genes differentially expressed between unstimulated (US) and stimulated (S) samples to CLL unmutated cases (continued)

Probe set ID	Gene symbol	*P*	Fold change	Gene function	Expression in UM cases upon IgM stimulus
202421_at	*IGSF3*	.047	3.31	Unknown	High
201761_at	*MTHFD2*	.048	4.17	Metabolism	High
201947_s_at	*CCT2*	.048	1.52	Regulation of progression through cell cycle	High
200634_at	*PFN1*	.049	1.56	Cytoskeleton organization and biogenesis	High
215967_s_at	*LY9*	.049	1.85	Cell adhesion	High
223207_x_at	*PHPT1*	.049	1.64	Dephosphorylation	High
204103_at	*CCL4*	.049	17.24	Cell motility	High
213734_at	Unknown	.049	1.84	Unknown	High
201762_s_at	*PSME2*	.049	1.86	Immune response	High
204744_s_at	*IARS*	.050	2.23	Protein biosynthesis	High
206687_s_at	*PTPN6*	.050	2.02	Protein amino acid dephosphorylation	High
220987_s_at	*C11orf17*	.001	1.53	Protein amino acid phosphorylation	Low
222150_s_at	*LOC54103*	.002	1.57	Unknown	Low
226101_at	*PRKCE*	.004	1.57	Protein amino acid phosphorylation	Low
235170_at	*ZNF92*	.004	1.74	Regulation of transcription, DNA-dependent	Low
228762_at	*LFNG*	.004	1.96	Development	Low
212774_at	*ZNF238*	.005	1.52	Regulation of transcription, DNA-dependent	Low
225768_at	*NR1D2*	.005	1.70	Regulation of transcription, DNA-dependent	Low
220341_s_at	*LOC51149*	.005	1.66	Unknown	Low
33304_at	*ISG20*	.006	1.64	DNA and RNA catabolism	Low
225656_at	*EFHC1*	.006	1.72	Calcium ion homeostasis	Low
229070_at	*C6orf105*	.007	1.61	Unknown	Low
213154_s_at	*BICD2*	.007	1.73	Protein biosynthesis	Low
234725_s_at	*SEMA4B*	.007	1.63	Development	Low
208206_s_at	*RASGRP2*	.008	1.60	Regulation of cell growth	Low
226068_at	*SYK*	.010	1.62	B-cell receptor signaling pathway	Low
228793_at	*JMJD1C*	.014	1.53	Regulation of transcription, DNA-dependent	Low
225623_at	*KIAA1737*	.014	1.56	Unknown	Low
206170_at	*ADRB2*	.014	1.58	Signal transduction	Low
227020_at	*YPEL2*	.016	1.63	Unknown	Low
201853_s_at	*CDC25B*	.017	1.85	Regulation of progression through cell cycle	Low
228377_at	*KLHL14*	.017	1.55	Unknown	Low
219073_s_at	*OSBPL10*	.018	1.61	Lipid transport	Low
226106_at	*RNF141*	.019	1.61	Regulation of transcription, DNA-dependent	Low
1562089_at	*GLYATL1*	.019	2.09	Unknown	Low
209829_at	*C6orf32*	.019	1.61	Unknown	Low
214366_s_at	*ALOX5*	.020	1.64	Electron transport	Low
222557_at	*STMN3*	.021	1.78	Signal transduction	Low
238604_at	Unknown	.022	1.51	Unknown	Low
205510_s_at	*FLJ10038*	.022	1.55	Unknown	Low
230224_at	*LOC644353*	.023	1.77	Unknown	Low
211919_s_at	*CXCR4*	.023	1.50	Signal transduction	Low
228549_at	*PLEKHG4*	.023	1.78	Metabolism	Low
219505_at	*CECR1*	.026	1.57	Development	Low
225802_at	*TOP1MT*	.026	1.62	DNA topologic change	Low
238376_at	*DEXI*	.027	1.60	Unknown	Low
235432_at	*NPHP3*	.029	1.51	Lipid metabolism	Low
208914_at	*GGA2*	.030	1.55	Intracellular protein transport	Low
208438_s_at	*FGR*	.030	1.53	Protein amino acid phosphorylation	Low
238429_at	*TMEM71*	.030	1.81	Unknown	Low
219574_at	*MARCH1*	.030	1.60	Unknown	Low
207522_s_at	*ATP2A3*	.037	1.53	Calcium ion transport	Low
213839_at	*KIAA0500*	.037	1.85	Unknown	Low
239292_at	Unknown	.038	1.82	Unknown	Low
230997_at	*TTC21A*	.038	1.84	Unknown	Low
229383_at	Unknown	.040	1.55	Unknown	Low
1569703_a_at	*CORO1C*	.043	1.93	Actin cytoskeleton organization and biogenesis	Low
225051_at	*EPB41*	.044	1.56	Actin cytoskeleton organization and biogenesis	Low
225360_at	*TRABD*	.044	1.59	Unknown	Low
235385_at	Unknown	.047	1.80	Unknown	Low
228465_at	Unknown	.047	1.51	Unknown	Low
239533_at	*GPR155*	.047	1.63	Signal transduction	Low
207571_x_at	*C1orf38*	.049	1.54	Cell adhesion	Low
219452_at	*DPEP2*	.050	1.88	Proteolysis	Low

Genes are rank-ordered according to their *P* value.

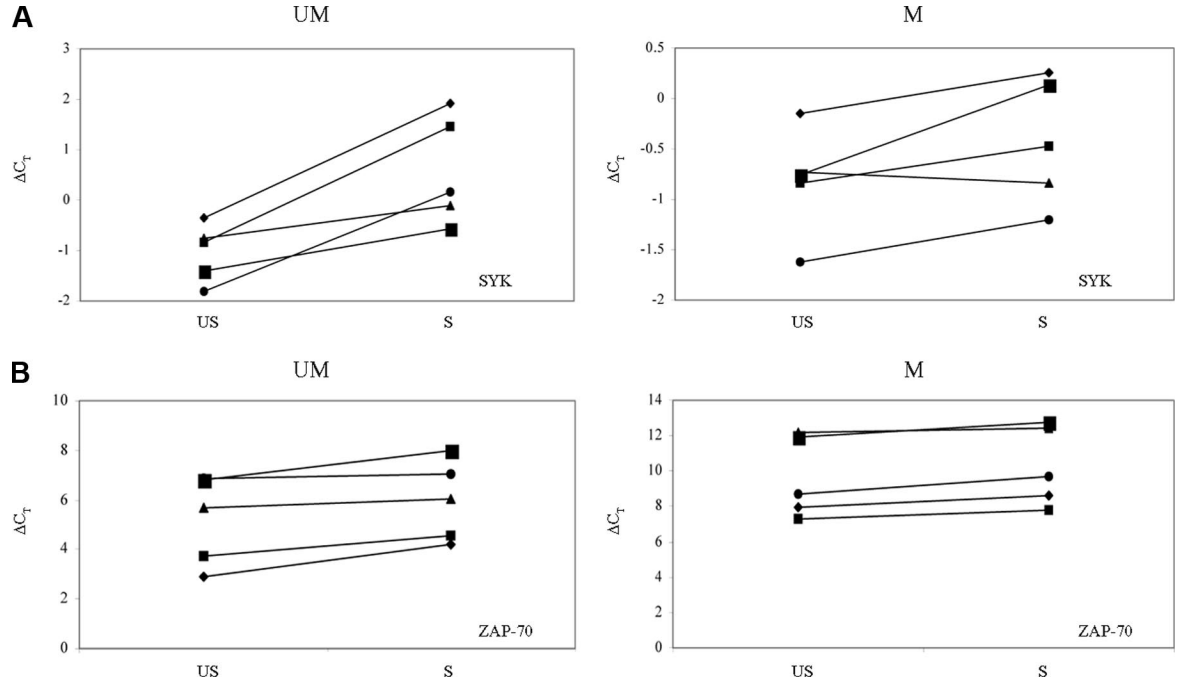

Figure 3. SYK and ZAP-70 changes upon stimulus. SYK (A) and ZAP-70 (B) expression upon 24 hours of BCR stimulation between unstimulated (US) and stimulated (S) samples in CLL unmutated (UM) and mutated (M) cases. Gene expression values are expressed by ΔC_T values: low ΔC_T values correspond to high gene expression levels.

CLL-2, CLL-4, CLL-5, and CLL-8), IgM cross-linking induced a decrease in apoptosis. In fact, at 24 hours upon BCR engagement, the level of apoptosis in S-CLL cells was 55.4% plus or minus 20.8%, whereas the value observed in US-CLL samples was 68.7% plus or minus 15.0%. At variance, 4 IgV_H M cases (CLL-6, CLL-7, CLL-10, and CLL-16) became sensitive to IgM-triggered apoptosis at 24 hours, with a mean percentage of annexin-V–binding cells of 50.3% plus or minus 17.9% as opposed to the basal rate of spontaneous apoptosis of 17.7% plus or minus 9.3%. These findings were also confirmed when apoptosis levels were evaluated using the AO technique. In fact, within the 7 IgV_H UM cases (CLL-1, CLL-2, CLL-11, CLL-13, CLL-18, CLL-19, and CLL-20) IgM cross-linking induced a decrease in the percentage of $subG_{0/1}$ cells in 4 of 7 patients (from 41.12% ± 16.9% in US-CLL cells to 33.71% ± 11.7% in S-CLL cells; $P = .06$; data not shown). Contrariwise, a significant increase of apoptosis, from 33.42% plus or minus 21.1% in US-CLL cells to 44.72% plus or minus 27.3% in S-CLL cells ($P = .04$), was observed at 48 hours in 4 of 5 CLL cells from IgV_H M patients (CLL-9, CLL-12, CLL-14, and CLL-15). A cytoprotective effect of IgM cross-linking was observed in the last IgV_H M sample (CLL-17: from 35.5% in US-CLL cells to 23.2% in S-CLL cells).

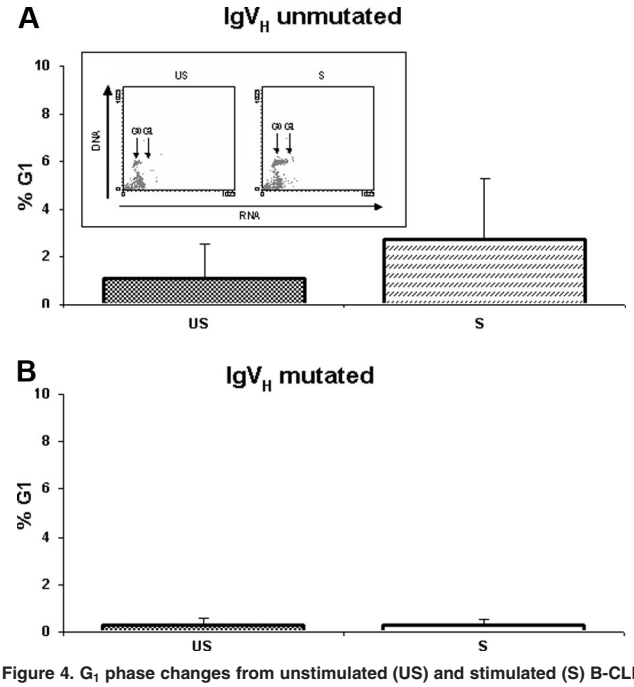

Figure 4. G_1 phase changes from unstimulated (US) and stimulated (S) B-CLL cells. G_1 phase changes (mean percentages ± SD) from unstimulated (US) and stimulated (S) B-CLL cells were evaluated using the AO technique after 48 hours of culture in (A) IgV_H unmutated and (B) IgV_H mutated samples. Representative experiment obtained from an IgV_H unmutated patient is shown in the box.

Discussion

The heterogeneous clinical course of CLL represents a fascinating biologic problem, quite unique to this disease within the spectrum of hematologic malignancies. Many speculations have been put forward in an attempt to explain disease progression versus disease stability. In the present study, we sought to explore the possibility, frequently hypothesized,[17,27] that antigen stimulation, or response to antigenic stimulation, may play a pivotal role in the expansion of the leukemic clone.

Gene profiling has proven very useful in clarifying different important aspects of CLL pathogenesis and prognosis. By using this technique it has, in fact, been shown that CLL is a unique disease,[6,7] that ZAP-70 represents an important prognostic marker,[6,10,11] and that a gene signature is associated with CD38 expression[28] and with disease progression[29]; from a functional point of view, it has also been shown that CLL cells from IgV_H unmutated cases display a profile that resembles that of activated B cells.[7] Therefore, we first used this technique to identify molecular events that may play a role in the progression of the disease and

subsequently confirmed the results obtained by performing functional in vitro experiments. We used an experimental model, based on the stimulation of the BCR of CLL cells with an IgM immobilized antibody, that can mimic the in vivo conditions of progression physiopathology.[30] Remarkably, our study shows that although IgM stimulus induces significant gene expression changes in the UM-CLL subgroup, no changes are observed in the M-CLL subgroup. The strongest gene expression changes occurred, as expected, in genes involved in signal transduction—in particular, in several DUSP members, namely *DUSP2*, *DUSP4*, and *DUSP10*, which are important regulators of the MAPK pathway,[31] as well as *CCL3* and *CCL4*, important regulators of calcium homeostasis and motility.[32,33] Similarly, we observed a consistent up-regulation of several members of the EGR family (*EGR1*, *EGR2*, and *EGR3*), NR4A family (*NR4A1*, *NR4A2*, and *NR4A3*), and *NME1* and *NME2*, involved in transcription regulation,[34-37] pinpointing that these processes are strongly affected by IgM stimulation.

In line with previously published data,[6] gene expression profiling also detected an upmodulation of genes involved in cell-cycle regulation coupled with cell-cycle progression. In fact, although for many years CLL has been considered a malignancy of mature lymphocytes with an extended survival but a low proliferative potential, it has been recently documented in vivo, using a nonradioactive method to measure CLL cell kinetics, that CLL cells are nonquiescent,[38] indicating that CLL is an overall dynamic disease.

It has also been demonstrated that immunostimulatory DNA oligonucleotides induce cell-cycle progression, proliferation, and enhanced survival in patients with progressive disease and a UM IgV_H profile.[39] On the contrary, the same stimulation induced a cell-cycle arrest and leukemic cell apoptosis in the majority of cases with stable disease and M IgV_H. Furthermore, Longo et al[40] performed an analysis of cell-cycle regulatory protein expression after stimulation and showed that cyclin D3, which plays a role in the G_1 phase, increased in all stimulated CLL cells; contrariwise, cyclin A, which is expressed in the S phase of the cell cycle, is induced only in CLL cells that proliferate after stimulation; the AKT and ERK kinases appear to play a key role in these events.[39,40]

In line with these recent findings, our results indicate that IgM stimulation induces the modulation of several genes involved in signal pathways controlling survival and proliferation in UM-CLL. In particular, we observed (1) the increased expression of *NFATC1* that participates in the ERK and JNK MAPK pathways[41]; (2) the downmodulation of *CDC25B* involved in the p38 MAPK pathway[42]; and (3) the upmodulation of a set of genes involved in cell proliferation,[43] the most important being *CCND2* and *CDK4*. These results are also in agreement with Muzio et al[44] who recently reported that a subset of CLL patients display an activation of ERK1/2 and an increased transactivation of NF-AT, upon Ig ligation.

Cell-cycle analysis upon IgM stimulation confirmed the progression of cell cycle exclusively in UM-CLL cells; as a matter of fact, in agreement with Deglesne et al,[30] it was possible to highlight an increase in the percentage of cells in the G_1 phase, although the rate of cells in the S phase was, at this time point, unmodified. These results were further corroborated by the proliferation assay that showed a proliferative response only in UM-CLL cases upon BCR cross-linking.

It has been shown that the response to IgM stimulus is proportional to the levels of IgM exposed on the cell surface.[45] Accordingly, we could document that the transcriptional levels of IgM are higher in UM-CLL cells and appear to be the parameter that better correlates with responsiveness to BCR stimulation. This finding supports the hypothesis that the IgM levels play a more important role than CD38 and ZAP-70 expression in the stimulus response. In our study, the CD38 antigen was expressed on the surface of the leukemic cells only in 3 patients; although the number of $CD38^+$ patients is small, we propose that CD38 expression may contribute primarily to the secondary events that follow stimulation. Similarly, in the past it was thought that ZAP-70 could play a major role in BCR signal transduction. It has now been unequivocally demonstrated that BCR signaling predominantly uses the tyrosine kinase SYK and that ZAP-70 contributes only to enhance the reaction and to prolong the persistence of the stimulus.[46] Consistent with these findings, in our study the levels of *ZAP-70* expression did not change upon stimulation in UM-CLL and M-CLL cells. Contrariwise, a significant difference in *SYK* expression levels was observed at 24 hours upon stimulus exclusively in UM-CLL cells, confirming published data[47] on the predominant role of the SYK molecule in the process of BCR signal transduction. Finally, we found un up-regulation of *SHP-1* (alias *PTPN6*),[48] a gene that interacts with ZAP-70.

The role of apoptosis in CLL progression is still debated and contradictory data have been obtained on IgM S-CLL and US-CLL cells. In the past, an enhanced apoptosis in CLL cells before and after IgM stimulus, partly dependent on CD38 expression, has been reported.[49,50] More recently, our group has demonstrated that leukemic cells taken from patients with stable disease are more susceptible to enter apoptosis than leukemic cells obtained from progressive patients[24]; other authors have shown that CLL cells show defects in apoptosis induction and have correlated this phenomenon with the high activity of Lyn, a tyrosine kinase involved in signal transduction, that is constitutively phosphorylated in leukemic cells.[51]

Gene expression analysis showed that, upon stimulation, a very small set of genes involved in the apoptotic pathway is up-regulated only in UM-CLL cells, suggesting that apoptosis may represent a minor phenomenon upon IgM ligation. Interestingly, the genes hereby identified exert an antiapoptotic function. In fact, the increase of genes correlated to the TNF and NFKB signaling pathways, namely *CD40*, a member of the TNF receptor, *TRAF*, a TNF receptor-associated factor, and *TNFAIP3*, a gene involved in the negative regulation of NFKB pathway, suggests that these genes interact in mediating apoptosis inhibition.[52-54] To confirm these results, we analyzed the apoptotic rate of CLL cells after IgM stimulation; whereas in M-CLL cases the number of apoptotic cells significantly increased after 48 hours upon ligation, in UM-CLL cells at 24 and 48 hours after IgM stimulation there was a trend toward a reduction in the apoptotic rate. These results indicate that IgM stimulation contributes to the survival of UM-CLL cells, whereas in M-CLL cases no antiapoptotic signal is induced and therefore cells die by speeding up their apoptotic program.

Our results differ from those reported in a recent paper,[55] where the authors evaluated the gene expression profile of B cells from healthy donors and CLL patients at several, but short, time points after IgM stimulation. Their data indicate that an increased apoptosis can be observed in CLL cells compared with normal B cells; furthermore, the authors showed, rather unexpectedly, an up-regulation of proapoptotic genes in samples from patients with aggressive disease. Among the reasons that may contribute to the divergent results, it must be noted that the

patients' clinicobiologic features differ profoundly between the 2 studies. More importantly, in our experiments, more prolonged IgM stimulation time points have been evaluated, and these experimental conditions, presumably, allowed the documentation of a positive correlation between the leukemic cell behavior and the neoplastic expansion; it must be underlined that at 6 hours upon stimulus, we already observed an increase in the expression levels of a small set of genes, and these levels were then reconfirmed at 24 hours. Finally, 2 technical issues may have contributed to the different results obtained: first, in our experimental model an immobilized IgM was used, whereas Vallat et al[55] used a soluble IgM; second, from a statistical point of view, the presence of a third group of samples (ie, normal B lymphocytes) may have interfered with data normalization and, therefore, with gene expression profiling data analysis.

Overall, our data support the hypothesis that the stimulation of a self- or non–self-antigen is a contributing factor toward maintaining the disease.

In conclusion, the results of this study challenge the belief that CLL is a disease characterized by a defective apoptosis and accumulation of malignant cells. Indeed, we show that, unlike in M-CLL cases, UM-CLL cells respond to signals delivered by surface IgM by up-regulating several genes associated with cell cycle and allowing cell growth and expansion, thus ultimately contributing to the unfavorable clinical course of CLL patients with an UM IgV_H profile.

Acknowledgments

This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC; Milan, Italy), Ministero dell'Istruzione, Università e Ricerca (MIUR, Rome, Italy), Programi di Ricera di Interesse Nazionale (PRIN; Rome, Italy), Fondo per gli Investimenti della Ricera di Base (FIRB; Rome, Italy), and Fondazione Internazionale di Ricerca in Medicina Sperimentale (FIRMS, Torino, Italy).

Authorship

Contribution: A.G. designed research, analyzed the data, and wrote the paper; S.T. and S.C. performed gene expression profile and molecular experiments, and contributed to the preparation of the paper; R.M., N.P., S.S., M.M., M.S.D.P., M.R.R., and M.M. performed functional cell experiments; F.C. analyzed the data and contributed to the preparation of the paper; F.R.M. and I.D.G. analyzed patients; R.F. reviewed the design of the study, analyzed and discussed the results, and critically revised the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Robin Foà, Division of Hematology, Via Benevento 6, 00161 Rome, Italy; e-mail: rfoa@bce.uniroma1.it.

References

1. Calligaris-Cappio F, Hamblin TJ. B-cell chronic lymphocytic leukemia: a bird of a different feather. J Clin Oncol. 1999;17:399-408.
2. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med. 1995;333:1052-1057.
3. Gentile M, Mauro FR, Guarini A, Foa R. New developments in the diagnosis, prognosis and treatment of chronic lymphocytic leukemia. Curr Opin Oncol. 2005;17:597-604.
4. Damle RN, Wasil T, Fais F, et al. Ig V gene status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999;94:1840-1847.
5. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig VH genes associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848-1854.
6. Rosenwald A, Alizadeh AA, Widhopf G, et al. Relation of gene expression phenotype to immunoglobulin mutation genotype in B cell chronic lymphocytic leukemia. J Exp Med. 2001;194:1639-1647.
7. Klein U, Tu Y, Stolovitzky GA, et al. Gene expression profiling of B cell chronic lymphocytic leukaemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001;194:1625-1638.
8. Döhner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000;343:1910-1916.
9. Gentile M, Mauro FR, Calabrese E, et al. The prognostic value of CD38 expression in chronic lymphocytic leukaemia patients studied prospectively at diagnosis: a single institute experience. Br J Haematol. 2005;130:549-557.
10. Crespo M, Bosch F, Villamor N, et al. ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia. N Engl J Med. 2003;348:1764-1775.
11. Rassenti LZ, Huynh L, Toy TL, et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med. 2004;351:893-901.
12. Deaglio S, Capobianchi A, Bergui L, et al. CD38 is a signaling molecule in B-cell chronic lymphocytic leukemia cells. Blood. 2003;102:2146-2155.
13. Chen L, Widhopf G, Huynh L, et al. Expression of ZAP-70 is associated with increased B-cell receptor signalling in chronic lymphocytic leukemia. Blood. 2002;100:4609-4614.
14. Chen L, Apgar J, Huynh L, et al. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. Blood. 2005;105:2036-2041.
15. Damle RN, Temburni S, Calissano C, et al. CD38 expression labels an activated subset within chronic lymphocytic leukemia clones enriched in proliferating B cells. Blood. 2007;110:3352-3359.
16. Deaglio S, Vaisitti T, Aydin S, et al. CD38 and ZAP-70 are functionally linked and mark CLL cells with high migratory potential. Blood. 2007;110:4012-4021.
17. Chiorazzi N, Rai RR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005;352:804-815.
18. Guarini A, Gaidano G, Mauro FR, et al. Chronic lymphocytic leukemia patients with highly stable and indolent disease show distinctive phenotypic and genotypic features. Blood. 2003;102:1035-1041.
19. Affymetrix. GeneChip Expression Analysis Technical Manual. http://www.affymetrix.com/support/technical/manual/expression_manual.affx. Accessed June 19, 2008.
20. Li C, Wong WH. Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc Natl Acad Sci U S A. 2001;98:31-36.
21. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A. 1998;95:14863-14868.
22. Ematologia La Sapienza. http://www.ematologialasapienza.org/default.asp?Chiave = &Chkart = 375&Label = 0210.0030.0020.0070.0010&LV = 0210.0030.0020.0070. Accessed June 16, 2008.
23. Husson H, Carideo EG, Neuberg D, et al. Gene expression profiling of follicular lymphoma and normal germinal center B cells using cDNA arrays. Blood. 2002;99:282-289.
24. Ricciardi MR, Petrucci MT, Gregorj C, et al. Reduced susceptibility to apoptosis correlates with kinetic quiescence in disease progression of chronic lymphocytic leukaemia. Br J Haematol. 2001;113:391-399.
25. Darzynkiewicz Z, Bruno S, Del Bino G, et al. Features of apoptotic cells measured by flow cytometry. Cytometry. 1992;13:795-808.
26. Law CL, Sidorenko SP, Chandran KA, et al. Molecular cloning of human Syk: a B cell protein-tyrosine kinase associated with the surface immunoglobulin M-B cell receptor complex. J Biol Chem. 1994;269:12310-12319.
27. Messmer BT, Albesiano E, Efremov DG, et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J Exp Med. 2004;200:519-525.
28. Durig J, Nückel H, Hüttmann A, et al. Expression of ribosomal and translation-associated genes is correlated with a favorable clinical course in chronic lymphocytic leukemia. Blood. 2003;101:2748-2755.
29. Fernandez V, Jares P, Salaverria I, et al. Gene expression profile and genomic changes in disease progression of early-stage chronic lymphocytic leukemia. Haematologica. 2008;93:132-136.
30. Deglesne PA, Chevallier N, Letestu R, et al. Survival response to B-cell receptor ligation restricted to progressive chronic lymphocytic leukemia cells irrespective of ZAP-70 expresssion. Cancer Res. 2006;66:7158-7166.
31. Teng CH, Huang WN, Meng TC. Several dual specificity phosphatases coordinate to control the magnitude and duration of JNK activation in signaling response to oxidative stress. J Biol Chem. 2007;282:28395-28407.
32. Richardson RM, Pridgen BC, Haribabu B,

Snyderman R. Regulation of the human chemokine receptor CCR1: cross-regulation by CXCR1 and CXCR2. J Biol Chem. 2000;275:9201-9208.

33. Nieto M, Navarro F, Perez-Villar JJ, et al. Roles of chemokines and receptor polarization in NK-target cell interactions. J Immunol. 1998;161:3330-3339.
34. Gashler A, Sukhatine VP. Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. Prog Nucleic Acid Res Mol Biol. 1995;50:191-224.
35. Perlmann T, Jansson L. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. 1995; 9:769-782.
36. Postel EH, Berberich SJ, Flint SJ, Ferrone CA. Human c-myc transcription factor PuF identified as nm23-H2 nucleoside diphosphate kinase, a candidate suppressor of tumor metastasis. Science. 1993;261:478-480.
37. Braun S, Mauch C, Boukamp P, Werner S. Novel roles of NM23 proteins in skin homeostasis, repair and disease. Oncogene. 2007;26:532-542.
38. Messmer BT, Messmer D, Allen SL, et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J Clin Invest. 2005;115:755-764.
39. Longo PG, Laurenti L, Gobessi S, et al. The Akt signaling pathway determines the different proliferative capacity of chronic lymphocytic leukemia B-cells from patients with progressive and stable disease. Leukemia. 2006;21:110-120.
40. Longo PG, Laurenti L, Gobessi S, et al. The Akt/Mcl-1 pathway plays a prominent role in mediating antiapoptotic signals downstream of the B-cell receptor in chronic lymphocytic leukemia B-cells. Blood. 2008;111:846-855.
41. Peng SL, Gerth AJ, Ranger AM, Glimcher LH. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. Immunity. 2001;14:13-20.
42. Lemaire M, Froment C, Boutros R, et al. CDC25B phosphorylation by p38 and MK-2. Cell Cycle. 2006;5:1649-1653.
43. Chiles TC. Regulation and function of cyclin D2 in B lymphocyte subsets. J Immunol. 2004;173: 2901-2907.
44. Muzio M, Apollonio B, Scielzo C, et al. Constitutive activation of distinct BCR-signaling pathways in a subset of CLL patients: a molecular signature of anergy. Blood. 2008;112:188-195.
45. Mockridge CI, Potter KN, Wheatley I, et al. Reversible anergy of sIgM-mediated signalling in the two subsets of CLL defined by Vh-gene mutational status. Blood. 2007;109:4424-4431.
46. Gobessi S, Laurenti L, Longo PG, et al. ZAP-70 enhances B-cell receptor signalling in spite of absent or inefficient tyrosine kinase activation in chronic lymphocytic leukemia and lymphoma B-cells. Blood. 2007;109:2032-2039.
47. Kipps T. The B-cell receptor and ZAP-70 in chronic lymphocytic leukemia. Best Pract Res Clin Haematol. 2007;20:415-424.
48. Plas DR, Johnson R, Pingel JT, et al. Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. Science. 1996;272:1173-1176.
49. Zupo S, Isnardi L, Megna M, et al. CD38 expression distinguishes two groups of B-cell chronic lymphocytic leukemias with different responses to anti-IgM antibodies and propensity to apoptosis. Blood. 1996;88:1365-1374.
50. Zupo S, Massara R, Dono M, et al. Apoptosis or plasma cell differentiation of CD38-positive B-chronic lymphocytic leukaemia cells induced by cross-linking of surface IgM or IgD. Blood. 2000; 95:1199-1206.
51. Contri A, Brunati AM, Trentin L, et al. Chronic lymphocytic leukemia B cells contain anomalous Lyn tyrosine kinase, a putative contribution to detective apoptosis. J Clin Invest. 2005;115:369-378.
52. Chung JY, Park YC, Ye H, Wu H. All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. J Cell Sci. 2002;115:679-688.
53. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol. 2003;3:745-756.
54. Lee EG, Boone DL, Chai S, et al. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. Science. 2000; 289:2350-2354.
55. Vallat LD, Park Y, Li C, Gribben JG. Temporal genetic program following B-cell receptor cross-linking: altered balance between proliferation and death in healthy and malignant B cells. Blood. 2007;109:3989-3997.