# Survival Response to B-Cell Receptor Ligation Is Restricted to Progressive Chronic Lymphocytic Leukemia Cells Irrespective of Zap70 Expression

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## Abstract

**Despite very similar gene expression profiles, the clinical course of B-cell chronic lymphocytic leukemia (B-CLL) is heterogeneous. Immunoglobulin VH (IgVH) mutational status and expression of B-cell receptor (BCR) signaling mediators have been associated with disease progression. However, the consequences of BCR engagement on cell survival and evolution of the disease remain unclear. We show here that B-CLL cell survival is dependent on the threshold of BCR stimulation induced by immobilized antibody, in contrast to soluble anti-μ F(ab)′2 antibody, which leads to apoptosis. Measurement of metabolic activity and apoptotic response discriminated two subgroups. "Nonresponders" showed low metabolic activity and unmodified apoptotic response upon BCR stimulation. In contrast, "responders" exhibited increased metabolic activity and inhibition of spontaneous apoptosis. This survival advantage was associated to a BCR-dependent activation profile leading to induction of cyclin D2/cyclin-dependent kinase 4 (cdk4) expression and $G_1$ cell cycle progression. The ability to respond to BCR ligation correlated with an unfavorable clinical course and allowed to define an additional group of patients among IgVH-mutated cases exhibiting a risk of progression. Remarkably, we show that Zap70 expression was neither mandatory nor sufficient to generate downstream survival signals and cyclin D2/cdk4 up-regulation. In conclusion, BCR engagement has a significant effect on B-CLL cell survival, activation, and $G_1$ progression. Furthermore, our results provide new insights in the physiopathology of progressive IgVH-mutated cases.** (Cancer Res 2006; 66(14): 7158-66)

## Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of slowly dividing $CD5^+$ B lymphocytes. The clinical course of CLL is heterogeneous. Some patients progress rapidly and require early chemotherapy whereas others exhibit a stable disease over years with no need of treatment. A number of biological features have been identified that seem to have prognostic value, such as lymphocyte doubling time (LDT) measurement, serum thymidine kinase level, mutational status of the expressed immunoglobulin heavy-chain variable-region (*IgVH*) genes, and Zap70 tyrosine kinase expression (1–4).

Patients with CLL cells expressing unmutated IgVH "unmutated CLL" have a significantly more aggressive clinical course and worse outcome when compared with those expressing IgVH with >2% somatic mutation "mutated CLL" (5). These observations led to the hypothesis that B-cell receptor (BCR) activation could play a central role in the physiopathology of the disease. The use of a biased *VH* gene repertoire and the presence of nonrandom VDJ combinations further support a role for antigenic stimulation in the selection of the malignant clone (6–9). Moreover, gene profiling studies comparing mutated and unmutated CLL cases distinguished the differential expression of a small subset of genes pointing to BCR signaling pathways (10, 11).

BCR signaling is critical for B-lymphocyte development, proliferation, and survival. In animal models, a strong BCR signal is responsible for the specific expansion of $CD5^+$ B cells compared with normal B subtypes (12). Mice lacking or overexpressing various regulatory effectors of the BCR signaling pathways reproduce phenotypes with B-cell CLL (B-CLL) features. Notably, $CD19^{-/-}$, $Btk^{-/-}$, *cyclin* $D2^{-/-}$, or $vav1^{-/-}$ animals exhibit a depletion in $CD5^+$ B-cells whereas CD19 transgenic animals show an expansion of this compartment (13–16). One of the BCR signaling targeted events is the induction of cyclin D2 expression, which was shown to be specifically required in BCR-induced, but not CD40 ligand– or lipopolysaccharide-induced proliferation (17). Moreover, lack of cyclin D2 induction and subsequent cell cycle progression in response to BCR cross-linking has been associated with defects in activation pathway intermediates (15, 18, 19).

Early events in the activation of the BCR involve the recruitment and subsequent phosphorylation of various enzymes, including members of Src and Syk families of tyrosine kinases (20). Until recently, Zap70, the second member of the Syk family, was described as being specifically expressed in T cells and natural killer (NK) cells whereas its counterpart Syk was shown to function in the BCR signalosome (21). Interestingly, transcriptional studies have identified Zap70 expression in CLL cells with absence of somatic mutations "unmutated CLL" (10, 11, 22, 23). Zap70 was associated to membrane immunoglobulin/CD79b complexes on IgM ligation, which was interpreted to contribute to the enhanced response to BCR stimulation observed preferentially in unmutated CLL cases (24–26). Those results also suggested that signaling

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through BCR could differ in nature and intensity depending on the immunoglobulin mutational status (27–29).

However, it remained unclear whether antigenic stimulation may play a critical role in the progression and maintenance of the disease. In the present study, we evaluated, therefore, the responsiveness to BCR stimulation and showed that B-CLL cell survival depends on the threshold of BCR stimulation. BCR signaling promoted *in vitro* B-CLL cell survival and $G_1$ progression leading to cyclin D2/cyclin-dependent kinase 4 (cdk4) up-regulation in a subset of patients with progressive disease and unfavorable prognostic factors only. In contrast, BCR stimulation had no effect on cells from patients with stable disease. Moreover, this analysis allowed the identification of a group of progressive patients exhibiting mutated IgVH and lack of expression of Zap70 tyrosine kinase.

## Materials and Methods

**CLL samples.** Peripheral blood mononuclear cells were obtained from 58 untreated CLL patients after informed consent. The diagnosis of CLL was ascertained by both typical morphology and immunophenotype as evidenced by Royal Marsden Hospital score >4.

**Cell isolation and culture.** B lymphocytes from patients were separated by Roset Sep kit according to the recommendation of the manufacturer, followed by Ficoll-Hypaque density gradient (Stem Cell Technologies, Grenoble, France). All further experiments were done with these freshly isolated cells. Purity was verified by flow cytometry; contaminating T lymphocytes and NK cells were always <2%. B-CLL cells were cultured at $2\times 10^6$/mL during 72 hours in 96-well plates in the presence of coated rabbit anti-IgM antibody (10 μg/mL; Jackson ImmunoResearch, Baltimore, MD) or 10 μg/mL of either soluble or immobilized goat F(ab′)2 anti-μ antibody (Southern Biotech, Cambridge, United Kingdom).

**Measurement of serum thymidine kinase levels.** Thymidine kinase levels were measured with Prolifigen TK-Rea kit according to the instructions of the manufacturer (Diasorin, Stillwater, MN).

**Determination of VH mutational status.** Following amplification with consensus 5′ FR1c and 3′ JH primers, PCR fragments were sequenced and analyzed for the mutational status using the Ig Blast database. Sequences were considered as unmutated when exhibiting at least 98% homology with the closest germ-line *VH* genes.

**Annexin V labeling and 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay.** Metabolic activity was determined by 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay according to the instructions of the manufacturer (Promega France, Charbonnieres, France). Metabolic fold increase (%MTS) was calculated as follows: [(absorbance stimulated $t_{72}$) − (absorbance control $t_{72}$) / (absorbance control $t_{72}$)] × 100. Cell apoptosis was analyzed by flow cytometry after Annexin V-FITC and propidium iodide staining (BD PharMingen, Le Pont de Claix, France). Inhibition of apoptosis was calculated as follows: (% Annexin V–positive unstimulated cells) − (% Annexin V–positive stimulated cells).

**Cytometry and cell cycle analysis.** Cell cycle analysis was done with BrdUrd/propidium iodide staining; BrdUrd (10 μmol/L) was incubated 16 hours on day 1 and then cells were then further labeled with anti-BrdUrd antibody and propidium iodide (50 μg/mL). CD23 and CD138 membrane markers were analyzed by flow cytometry using anti-CD23-phycoerythrin and anti-CD138-phycoerythrin antibodies (BD PharMingen).

**Western blotting and immunoprecipitations.** Protein extracts were prepared in 1% NP40 lysis buffer. Total extracts were analyzed by either Western blotting (25 μg) or immunoprecipitation (400 μg) with the following antibodies: rabbit anti-cyclin D2 (C17), cdk4 (C22), p27 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-pTyr 4G10 (purified from ascitis), anti-Syk, anti-Zap70 2F3.2 monoclonal antibodies (mAb) from Upstate Biotechnology (Lake Placid, NY); and anti-Vav1 monoclonal antibody from Abcam (Cambridge, United Kingdom). Detection was done with enhanced chemiluminescence plus reagent (Amersham Biosciences Europe, Freiburg, Germany).

## Results

**Both anti-IgM and anti-μ F(ab)′2 immobilized antibodies promote B-CLL cell survival.** To investigate the effect of BCR engagement on B-CLL cell survival *in vitro*, we standardized our stimulation procedures by measurement of the metabolic mitochondrial activity using MTS assay. Freshly isolated B-CLL cells from five patients were stimulated with immobilized anti-IgM for various periods of time and compared with unstimulated cells. B-CLL cell survival, monitored at a given concentration of 10 μg/mL of antibody, indicated that the best discriminating kinetic study was achieved after 72 hours of culture (Fig. 1*A*). At this reference time, maximal antibody dose response was reached with 5 μg/mL and remained stable up to 10 μg/mL of immobilized anti-IgM (Fig. 1*B*). Consequently, all further assays were set up at 72 hours of cell culture in the presence of 10 μg/mL of immobilized anti-IgM. The effect of immobilized anti-IgM was then compared with those of soluble or immobilized anti-μ F(ab)′2 stimulations. Interestingly, soluble anti-μ F(ab)′2 antibody stimulation resulted in decreased MTS ratio in all cases, indicative of a proapoptotic process (Fig. 1*C*). In contrast, immobilized anti-μ F(ab)′2 antibody, similarly to IgG anti-μ but to a lesser extent, restored B-CLL cell survival with positive MTS ratio. Quantitation of the apoptotic process was assessed by Annexin V/propidium iodide labeling. In agreement with the above results, anti-IgM and anti-μ F(ab)′2 immobilized antibodies reduced the extent of spontaneous apoptosis (54% and 36%, respectively), in contrast to soluble anti-μ F(ab)′2 (Fig. 1*D*). These results indicate that immobilized and soluble antibodies had opposite effects on the apoptotic process. Furthermore, they suggest that immobilized antibodies likely mimicked *in vivo* conditions by promoting B-CLL cell survival.

**BCR stimulation of B-CLL cells defines two groups of patients.** Based on these conditions, BCR survival upon immobilized anti-IgM stimulation was next evaluated on a series of 58 untreated B-CLL cases (Fig. 2*A*). For all patients, the MTS ratio was increased after immobilized anti-IgM stimulation, indicating a sustained survival effect, which was, however, highly variable, ranging from 244% to 1%. To confirm that the metabolic activity was due to apoptosis protection rather than cell proliferation, BrdUrd uptake during 16 hours and propidium iodide staining were assessed at 24 hours of culture upon IgM stimulation. None of the eight patient cells tested exhibited an entry into the S phase, including those that had shown the highest MTS values (UPN 10 and 15). Furthermore, we did not find any evidence for entry in the S phase on stimulation of cells as compared with unstimulated cells (Fig. 2*B*). These results thus suggested that increased metabolic activity was associated with sustained survival rather than increased proliferative activity. Annexin V membrane exposure was also analyzed on the stimulation process for 31 of the cases. As shown in Fig. 2*C*, all the B-CLL cells exhibited a rather high but variable ratio of spontaneous apoptosis after 72 hours of *in vitro* culture, ranging from 22% to 88% of Annexin V–positive cells. Stimulation using immobilized anti-IgM revealed either a protective mechanism against spontaneous apoptosis (up to 50-60% of reduction) or no protection, depending on B-CLL cells (Fig. 2*C*). The excellent correlation between MTS and Annexin V assays allowed to distinguish two subgroups (Fig. 2*D*): group A ($n$ =

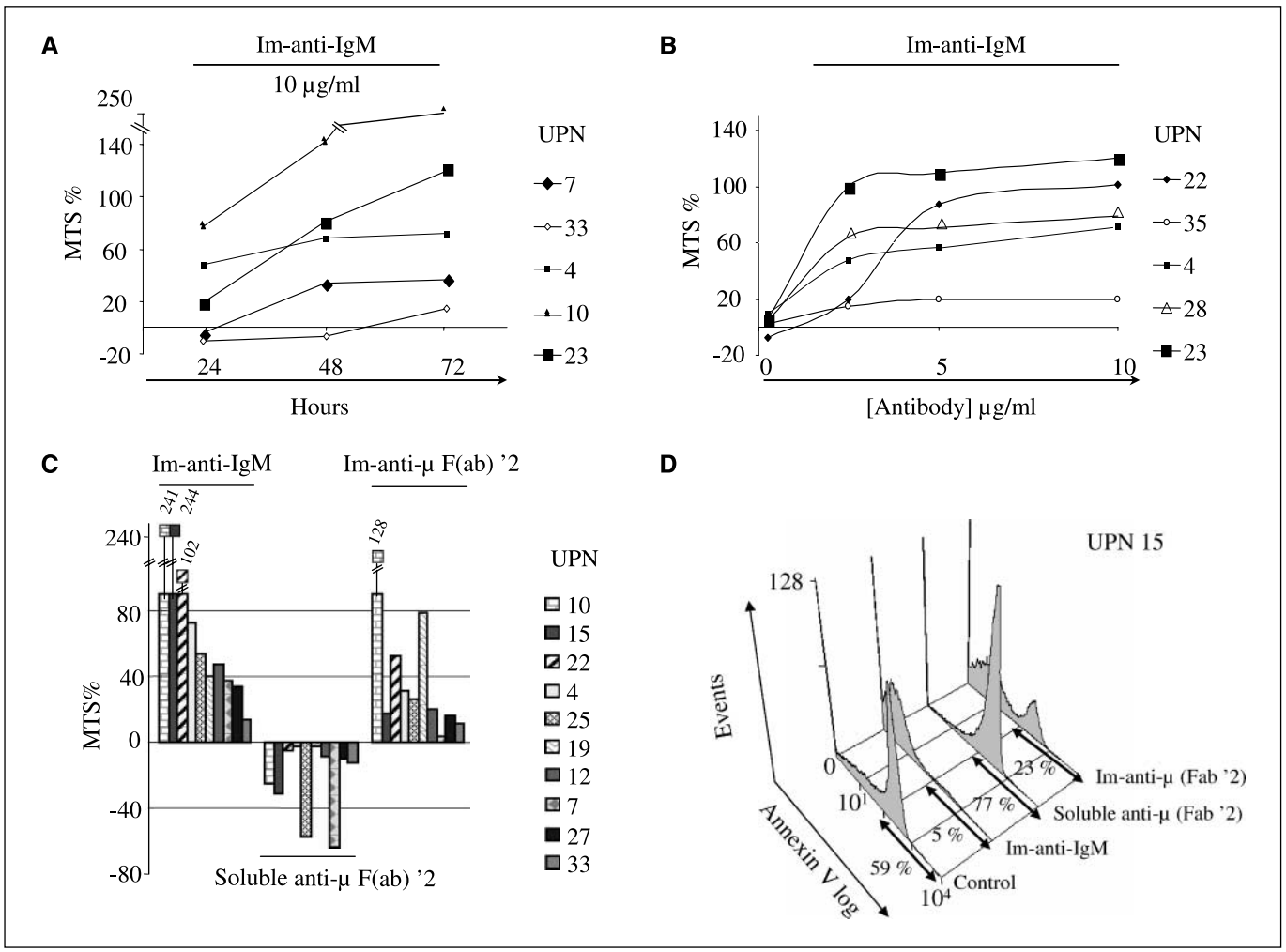


**Figure 1.** Effect of BCR engagement on B-CLL cell activation. Freshly isolated B-CLL cells ($2 \times 10^6$/mL) were incubated in the presence or absence (control) of immobilized (*Im*) anti-IgM or anti-μ F(ab)′2 or soluble anti-μ F(ab)′2 antibodies as indicated. Kinetic and antibody dose-response MTS analyses were done after stimulation with 10 μg/mL of immobilized anti-IgM for the indicated period of time (*A*) or after 72-hour stimulation with variable concentrations of immobilized anti-IgM (*B*). *C,* cells from 10 patients (UPN) were stimulated for 72 hours in the presence of the indicated antibodies (10 μg/mL) and subjected to MTS assay. Percentage of viable metabolic activity (%MTS) was calculated as follows: [(absorbance stimulated − absorbance nonstimulated) / (absorbance nonstimulated)] × 100. *D,* Annexin V/propidium iodide staining for one representative B-CLL case (UPN 15) stimulated with either immobilized anti-IgM or soluble or immobilized F(ab)′2 anti-μ antibodies (10 μg/mL) for 72 hours. Percentage of apoptosis was calculated as the percentage of Annexin V–positive cells.

19) with significant protection against apoptosis after BCR ligation, referred to as "responders" (MTS >25%; inhibition of apoptosis ≥10%), and group B ($n$ = 12) with no significant effect of BCR ligation on cell survival (MTS <25%; inhibition of apoptosis <10%), referred to as "nonresponders." Extrapolation of these criteria to all MTS patients attributed 38 and 20 cases to groups A and B, respectively (Table 1).

**Downstream targets to BCR signaling segregate the two groups.** Because the level of the response to BCR stimulation was able to discriminate between the two groups, we next investigated whether survival could be attributed to a stronger activation of the signaling cascade in the responder group. Upon BCR stimulation, cells from group A (responder cases), but not from group B, revealed a significant increase in size ($P$ = 0.0007, Wilcoxon test) and granularity (SSC, $P$ = 0.007) as shown by fluorescence-activated cell sorting (FACS) and box plot analysis (Fig. 3*A* and *B*). Morphologically, increased metabolic activity also resulted in cytoplasmic expansion (Fig. 3*C*), all three variables being characteristic features of activated B cells. This response to BCR stimulation was further supported by the induction of specific early B-cell activation antigens, such as CD23, in group A only. However, this increase did not lead to a differentiation process toward plasma cells as evidenced by unmodified CD138 surface labeling in both groups during stimulation (Fig. 3*D*; ref. 30).

To further support the observed differential activation between the two groups, expression of late BCR signaling targets critical for $CD5^+$ B-cell development, such as cyclin D2, was analyzed. Cyclin D2 protein expression was strongly increased at 24 hours and maintained over 72 hours of stimulation in responder cases exclusively (Fig. 4*A* and *B*). Moreover, the expression of its catalytic partner, the cyclin dependent kinase cdk4, was induced to the same extent in this group. In contrast, even after 3 days of culture, no significant increase of either cyclin D2 or cdk4 protein could be seen in nonresponder cells. These results suggested that, in addition to the attenuation of apoptosis, BCR ligation could potentially induce in the responder group a limited $G_1$ cell cycle progression relevant to a competent signaling cascade downstream of the receptor. However, in both subgroups, levels of the cell cycle

inhibitor p27kip-1 remained highly expressed after IgM engagement. These results may account for the absence of $G_1$-S progression (Figs. 2*B* and 4*B*).

**Response to BCR ligation is not restricted to Zap70 kinase expression.** Syk has been described as the unique member of the tyrosine kinase family expressed in B cells (21). Recently, ectopic expression of Zap70 in B-CLL cells has been associated with an increased signaling after BCR stimulation (25, 26). In our series, several responder B-CLL cases able to induce cyclin D2 expression did not express significant levels of Zap70 protein. Conversely, two nonresponder cases expressed Zap70 protein (Fig. 4*B* and *C*; Table 1). Thus, these results showed that Zap70 kinase expression was neither obligatory nor sufficient to generate downstream survival signals and cyclin D2/cdk4 up-regulation.

Therefore, we investigated whether a constitutive phosphorylation of Syk/Zap70 kinases might account for the differences observed between the two groups. In freshly purified B-CLL cells, analysis of phosphotyrosine profile in cell lysates indicated variable levels among patient cells irrespective of the group (Fig. 4*C*). The level of expression of Syk protein was comparable between the two groups. Furthermore, we did not evidence a relationship between the presence or absence of Zap70 and the level of Syk expression. This observation showed the absence of a compensatory mechanism regulating the expression of the two members of the family. In contrast, Syk seemed to be highly tyrosine phosphorylated in the responder group as compared with the nonresponder group (mean of the ratio pSyk/Syk: 0.35 versus 0.08). Syk phosphorylation occurred independently of Zap70 expression or Zap70 phosphorylation status (Fig. 4*C*). Altogether, these results suggested that BCR proximal signaling effectors were present in both groups but showed a higher constitutive activation in responder B-CLL cases. These results also indicate that response to BCR signaling is not restricted to an ectopic expression of Zap70.

**Response to BCR ligation is representative of the clinical outcome.** Finally, we investigated the clinical and biological status of our two groups of patients (Table 1). We observed that the nonresponder group (MTS <25%) was homogeneous; all patients were Binet stage A and exhibited criteria of a stable disease: long

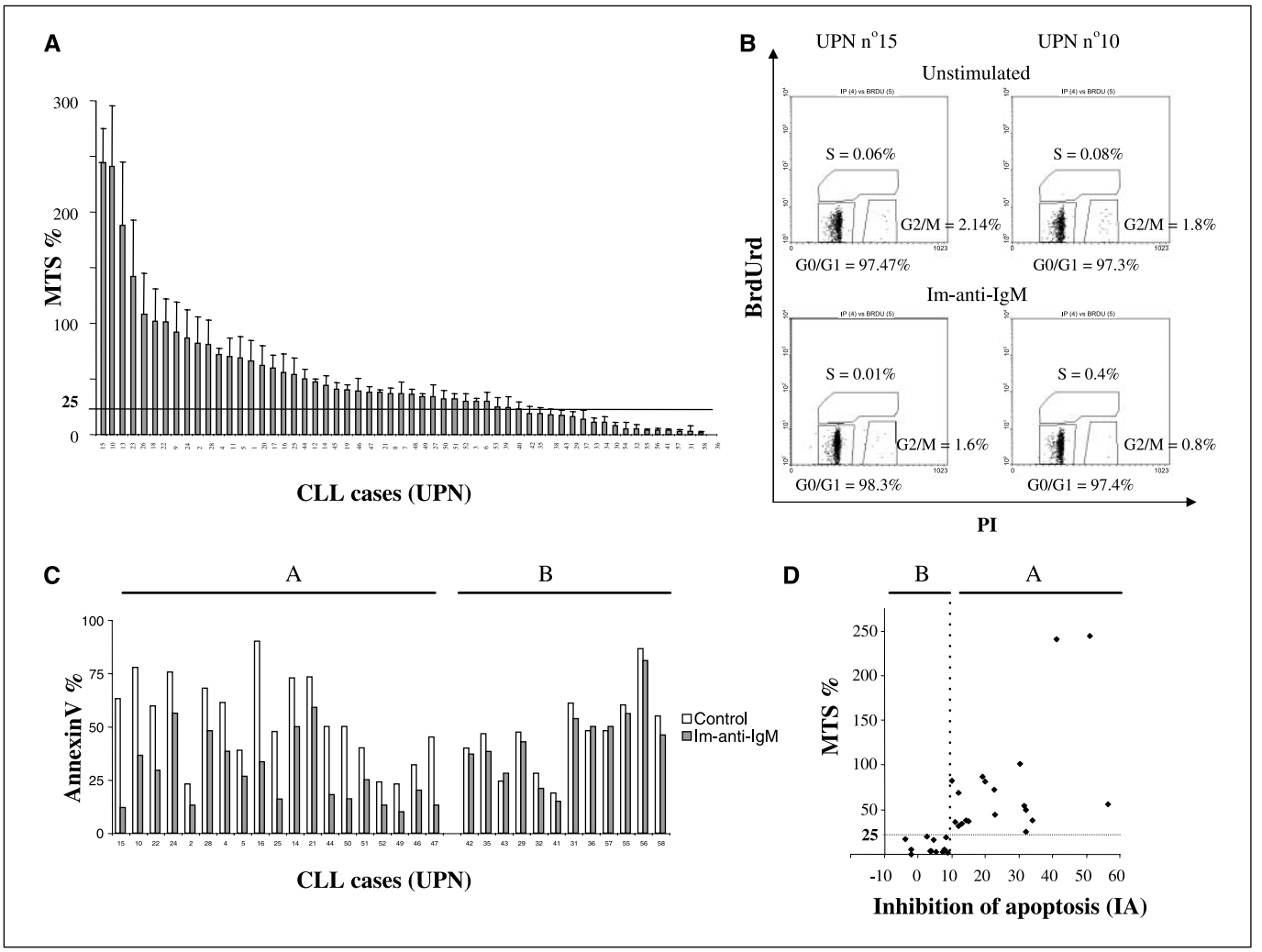


**Figure 2.** Metabolic activity measurement and Annexin V exposure are discriminative following IgM stimulation. *A,* response to BCR engagement was measured by MTS assay on 58 B-CLL cases after 72 hours of IgM stimulation. *B,* cell cycle progression with BrdUrd and propidium iodide incorporation. Cells either stimulated for 24 hours Im-anti-IgM or left unstimulated were incubated with BrdUrd for 16 hours. FACS analysis of two representative cases. Relative percentage of cells in the different cell cycle phases is also indicated. *C,* the percentage of Annexin V–positive cells was analyzed after 72 hours in stimulated (immobilized anti-IgM) and unstimulated cells (control) for 31 cases. *D,* a correlative diagram between inhibition of apoptosis, calculated as (% Annexin V control) − (% Annexin V stimulated), and MTS response. Two groups of cases are delineated: *A,* responders (inhibition of apoptosis >10%; MTS >25%); *B,* nonresponders.

**Table 1.** Clinical and biological variables of the 58 B-CLL cases (UPN)

| UPN | %MTS* | Binet stage † | LDT ‡ | TK§ | VH‖ | IgVH mutational status¶ | ZAP-70** |
|---|---|---|---|---|---|---|---|
| Group A (38 responders) | | | | | | | |
| 15 | 244 | B | Sup | 8.3 | V4,61 | 86 | Neg |
| 10 | 241 | C | Inf | 40 | V1,2 | 99 | Pos |
| 13 | 188 | A | Sup | 5.1 | V4,39 | 99 | — |
| 23 | 142 | A | Sup | — | V3,9 | 88 | Neg |
| 26 | 108 | A | Sup | 4.8 | V2,5 | 94 | Neg |
| 18 | 102 | B | Sup | 9.2 | V3,7 | 95 | Pos |
| 22 | 101 | A | Sup | 18 | V4,59 | 94 | Neg |
| 9 | 92 | B | Inf | 25 | V4,34 | 99 | Pos |
| 24 | 87 | A | Sup | — | V4,59 | 95 | Neg |
| 2 | 82 | B | Inf | 45 | V1,69 | 99 | — |
| 28 | 81 | A | Inf | 21 | V4,59 | 82 | Neg |
| 4 | 72 | B | Inf | 22 | V3,21 | 98 | Pos |
| 11 | 70 | B | Inf | 29 | V1,69 | 99 | Pos |
| 5 | 69 | C | Inf | 33 | V1,69 | 99 | — |
| 1 | 66 | B | Inf | — | V1,3 | 98 | — |
| 20 | 62 | B | Sup | 3.7 | V3,30 | 95 | Neg |
| 17 | 60 | C | Inf | 6.2 | V3,33 | 90 | Pos |
| 16 | 56 | B | Sup | 6.7 | V3,9 | 94 | Neg |
| 25 | 54 | A | Sup | 5.7 | V3,20 | 94 | Pos |
| 44 | 50 | A | Sup | 16 | V3,49 | 100 | Pos |
| 12 | 47 | B | Inf | 18 | * | * | Pos |
| 14 | 44 | C | Inf | 48 | V3,33 | 99 | Pos |
| 45 | 40 | A | Sup | 3.6 | V3,74 | 91 | Neg |
| 19 | 40 | B | Inf | — | V3,48 | 93 | Pos |
| 46 | 39 | A | Inf | 19 | V1,69 | 98 | Pos |
| 47 | 38 | A | Sup | 15 | V4,44 | 99 | Pos |
| 21 | 38 | B | Inf | 14 | V4,61 | 95 | Neg |
| 8 | 37 | B | Inf | 74 | V1,69 | 98 | — |
| 7 | 37 | C | Inf | 35 | V1,69 | 98 | Pos |
| 48 | 36 | A | Sup | 4.6 | V4,34 | 95 | Neg |
| 49 | 34 | B | Inf | 8.8 | V1,69 | 100 | Pos |
| 27 | 34 | C | Sup | 5.2 | V3,53 | 90 | Neg |
| 50 | 32 | B | Sup | 18 | V1,18 | 94 | Neg |
| 51 | 32 | B | Inf | 7.1 | V3,9 | 100 | Pos |
| 52 | 30 | A | Sup | 4.9 | V3,33 | 87 | Neg |
| 3 | 30 | B | Sup | 16 | V3,30 | 98 | — |
| 6 | 30 | B | Inf | 40 | V1,69 | 98 | Pos |
| 53 | 25 | A | Inf | 14 | V4,b | 100 | Pos |
| Group B (20 nonresponders) | | | | | | | |
| 39 | 24 | A | Sup | 8.3 | V3,48 | 90 | Neg |
| 40 | 23 | A | Sup | 8.6 | V3,30 | 89 | — |
| 42 | 20 | A | Sup | 4.7 | V6,1 | 96 | Neg |
| 35 | 19 | A | Sup | 5.4 | V3,48 | 91 | Neg |
| 38 | 18 | A | Sup | — | V3,15 | 88 | — |
| 43 | 17 | A | Sup | 4.3 | V1,69 | 88 | Neg |
| 29 | 16 | A | Sup | 8.5 | V3,7 | 92 | Neg |
| 37 | 14 | A | Sup | 6.6 | V3,74 | 90 | — |
| 33 | 11 | A | Inf | 26 | V3,48 | 95 | Pos |
| 34 | 11 | A | Sup | — | V4,34 | 92 | — |
| 30 | 8 | A | Sup | 10 | V4,34 | 91 | Pos |
| 54 | 5 | A | Sup | 3.5 | V1,69 | 96 | Neg |
| 32 | 5 | A | Sup | 8 | V3,30 | 92 | Neg |
| 55 | 4 | A | Sup | 2.7 | V3,13 | 94 | Neg |

(*Continued on the following page*)

**Table 1.** Clinical and biological variables of the 58 B-CLL cases (UPN) (Cont'd)

| UPN | %MTS* | Binet stage† | LDT‡ | TK§ | VH‖ | IgVH mutational status¶ | ZAP-70** |
|---|---|---|---|---|---|---|---|
| 56 | 4 | A | Sup | 5.3 | V4,34 | 95 | Neg |
| 41 | 4 | A | Sup | — | V3,23 | 89 | Neg |
| 57 | 3 | A | Sup | 8.6 | V3,23 | 94 | Neg |
| 31 | 3 | A | Sup | 3.4 | V1,2 | 90 | — |
| 58 | 2 | A | Sup | 4.7 | V3,34 | 96 | Neg |
| 36 | 1 | A | Sup | 5.1 | V3,7 | 96 | Neg |

NOTE: UPN 12 presented a hypermutated IgVH status. Shaded boxes represent unfavorable factors.
*MTS responses appear in decreasing order.
†Binet stage of the patients.
‡LDT lymphocyte doubling time (sup >1 year; inf <1 year).
§Serum thymidine kinase level relative to proliferation index.
‖VH sequence.
¶IgVH mutational status (% homology to the closest germ line *VH* gene).
**Presence (pos) or absence (neg) of Zap70 verified both by reverse transcription-PCR using specific primers on purified B-CLL cells and by Western blotting (R. Letestu, unpublished data).

lymphocyte doubling time (LDT >1 year; 19 of 20 patients) and low levels of serum thymidine kinase (thymidine kinase <10 units; 16 of 17 tested). Conversely, 32 of 38 (84%) responder patients (MTS >25%) exhibited at least one clinical (Binet stage B or C) or biological criterion of an evolutive disease with proliferation markers such as short lymphocyte doubling time (LDT <1 year; 20 of 38 patients) or high levels of serum thymidine kinase (thymidine kinase >10 units; 20 of 34 tested). The metabolic activity in response to BCR ligation was significantly associated with Binet status, lymphocyte doubling time, and serum level of thymidine kinase ($P = 3.4e^{-6}$, $P = 0.0003$, and $P = 0.0006$, respectively, $\chi^2$ test).

The absence of IgVH mutation (≥98% of homology to the closest germ line *VH* gene) has defined a subset of cases with worse clinical outcome. Comparison of the survival response upon BCR ligation and the IgVH mutational status showed a significant association between the two criteria ($P = 0.0001$, $\chi^2$ test). All the nonresponder cases were IgVH mutated (20 of 20; Table 1). Similarly, all unmutated cases were included into the responder group (19 of 38). However, the second half of the responder group (19 of 38) exhibited mutated IgVH sequences. Remarkably, regardless of IgVH mutational status, therapy was indicated, according to National Cancer Institute criteria, for 10 of these 19 mutated cases, pointing out to the high incidence of an evolutive disease in this group. Therefore, the ability to respond to BCR ligation identified a larger group of patients with a potential risk of disease progression than IgVH mutational analysis alone.

Finally, ectopic expression of Zap70 has been shown to correlate with unmutated IgVH status (10). In our study, all unmutated IgVH cases were associated with expression of Zap70 when tested. Overexpression of Zap70 has also been associated with increased BCR signaling in CLL (24). Indeed, our results indicate that 18 of 20 (90%) Zap70-expressing cases belong to the responder group. Association between MTS response and Zap70 expression was significant in our cohort of patients ($P = 0.003$, $\chi^2$ test). However, the *in vitro* survival response was also shown in 44% of the cases that did not express Zap70 (14 of 32 tested) and two Zap70-positive cases were nonresponders and clinically stable. These results indicate that Zap70 was dispensable (44%) and not sufficient (UPN 30 and 33) to induce a BCR-mediated activation profile. Thus, the ability to respond to BCR ligation identified a larger group of patients with potential risk of disease progression than Zap70 expression.

Altogether, this analysis allowed the identification of a group of clinically progressive patients exhibiting mutated IgVH and lack of Zap70 expression.

## Discussion

In this study, we provide novel evidence that BCR engagement has a significant effect on B-CLL cell survival, activation, and $G_1$ progression and is associated with the physiopathology of progressive cases.

We established experimental *in vitro* conditions in which immobilized antibodies seem to mimic *in vivo* conditions by promoting B-CLL cell survival and activation. We showed that resistance to spontaneous apoptosis in culture was maintained upon BCR ligation with immobilized antibodies over a period of 3 days. Our results support the data of Bernal et al. (31) who first showed similar apoptosis inhibition and induction of Bcl2 and Mcl-1 at somewhat earlier time points. In their study, this was achieved with soluble antibodies whereas, in the present study, immobilized antibodies were necessary. This might reflect some differences between the various sources used and, thus, a more transient activation with our soluble antibody as compared with a sustained signaling on immobilized anti-μ stimulation (27, 31). Difference in responses depending on how the receptor is targeted has also been observed in other immunoreceptor tyrosine-based activation motif–dependent receptors (32). Independently of these considerations, these results show that induction of BCR signaling with significant strength inhibits apoptosis. Thus, these biological settings allowed us to further analyze the effect of BCR engagement among patients with either indolent or more aggressive B-CLL and to identify two groups based on their survival response to BCR stimulation.

Progression into the $G_1$ phase on BCR engagement might reflect, for some of these cells, a higher capacity to proliferate

*in vivo*. Cyclin D2, a specific target of BCR, but not of CD40 or lipopolysaccharide signaling cascades, has been shown to be critical for $CD5^+$ B-cell development (17, 19). $CD5^+$ B cells, when stimulated with anti-IgM antibodies, rely specifically on the induction of cyclin D2 but not cyclin D1 or D3 expression (16, 18). In this study, we observed a strong induction of cyclin D2 and cdk4 expression upon BCR engagement in the responder group exclusively. In contrast, neither cyclin D2 nor cdk4 was induced in any of our nonresponder cases despite a significant expression at the basal level, as we have previously described in B-CLL cells (33). However, in both subgroups, the cell cycle inhibitor p27kip-1 remained highly expressed after IgM engagement, probably accounting for the lack of $G_1$-S progression (34). Further cell cycle progression into S phase and p27 down-regulation may require additional costimulatory signals [interleukin-4 (IL-4) and CD40 ligand] provided by T lymphocytes and stromal cells *in vivo* (35). Indeed, studies using *cyclin D2*$^{-/-}$ mice have suggested that costimulatory activation, such as signaling through the CD40 receptor or IL-4, might be necessary for B-CLL to overpass the blockade and allow cell cycle progression (18, 31).

Our results show that albeit BCR proximal signaling machinery is present in both groups, high constitutive activation is only observed in responder B-CLL cases. These data showed the capability of the cells to generate signaling despite the ominously low level of surface immunoglobulins in B-CLL cells (36). The higher constitutive phosphorylation of early signaling intermediates, such as Syk or Zap70 tyrosine kinases, in responder B-CLL cases might also reflect an *in vivo* antigenic stimulation (7). This agrees also with a study using the EBV protein LMP2A as a constitutively active BCR surrogate, which showed that the development of a mouse B1 subset was dependent on a strong and prolonged BCR stimulation (12).

The ectopic expression of Zap70, absent from mature normal B cells, was considered as being responsible for a stronger response

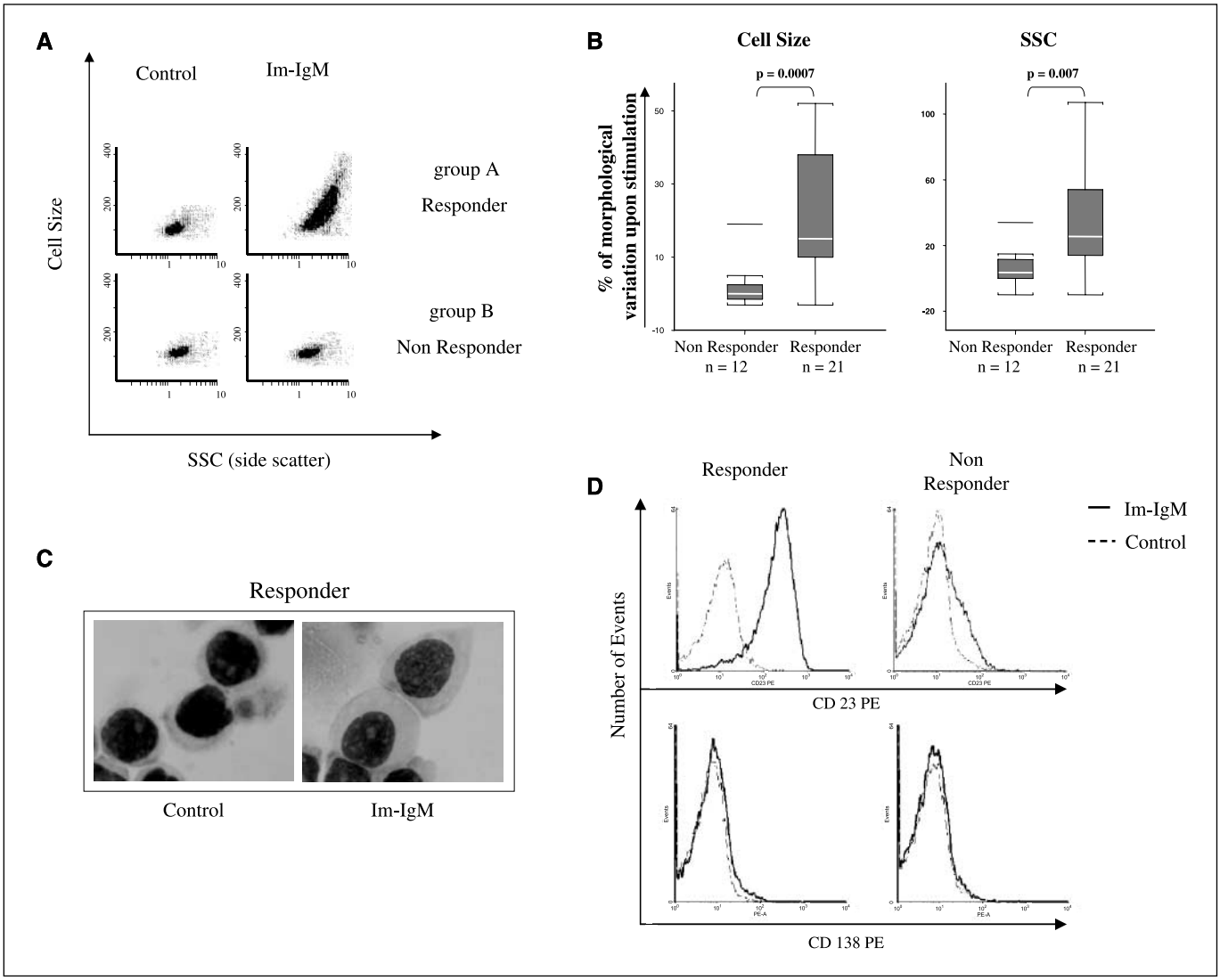


**Figure 3.** Morphologic and differentiation characterization upon BCR signaling within responder/nonresponder groups. Analyses were done on $CD19^+$ and $CD5^+$ cells left unstimulated (control) or stimulated for 72 hours [immobilized anti-IgM (*Im-IgM*)]. *A,* representative data of morphologic analysis by flow cytometry (size/SSC). *B,* box plot with percentage of size and SSC variations between the two groups upon stimulation was caculated as follows: [(mean size or SSC stimulated − mean size or SSC nonstimulated) / (mean size or SSC nonstimulated)] × 100. The difference between the two groups was significant ($P = 0.0007$ and $P = 0.007$, respectively, Wilcoxon rank-sum test). *C,* May-Grünwald-Giemsa staining. *D,* expression of CD23 and CD138 antigens.

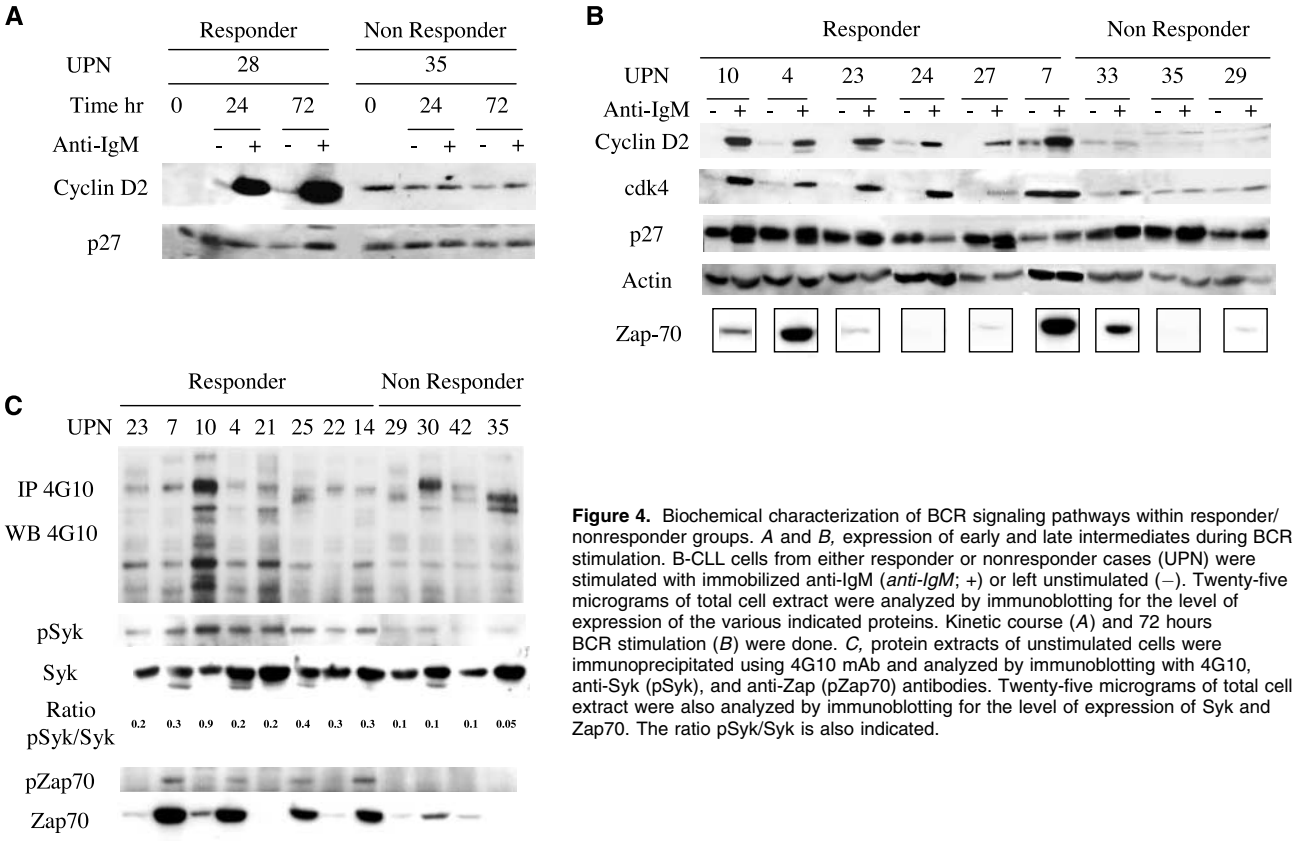


**Figure 4.** Biochemical characterization of BCR signaling pathways within responder/nonresponder groups. *A* and *B,* expression of early and late intermediates during BCR stimulation. B-CLL cells from either responder or nonresponder cases (UPN) were stimulated with immobilized anti-IgM (*anti-IgM*; +) or left unstimulated (−). Twenty-five micrograms of total cell extract were analyzed by immunoblotting for the level of expression of the various indicated proteins. Kinetic course (*A*) and 72 hours BCR stimulation (*B*) were done. *C,* protein extracts of unstimulated cells were immunoprecipitated using 4G10 mAb and analyzed by immunoblotting with 4G10, anti-Syk (pSyk), and anti-Zap (pZap70) antibodies. Twenty-five micrograms of total cell extract were also analyzed by immunoblotting for the level of expression of Syk and Zap70. The ratio pSyk/Syk is also indicated.

to BCR engagement and increased survival (23). Furthermore, in murine animal models, Zap70 kinase was shown to be expressed during early B-cell development (21). Gene profiling reports indicated the presence of Zap70 in B-CLL cells as a new prognostic marker for an evolutive form of the disease (3, 37). Although most Zap70 cases seem to be among our responder cases, it is noteworthy that the BCR signaling response was not restricted to an ectopic presence of Zap70. Thus, we found that in patients without significant amount of Zap70, cells were equally able to generate signaling toward cell survival. We did not detect in these latter cases a compensatory expression or a stronger phosphorylation of Syk. Remarkably, these results show that Zap70 kinase expression is not mandatory for the generation of downstream survival signals. Conversely, it may suggest a synergistic signaling effect of Zap70 with Syk when expressed in B-CLL cells, as described in a recent study using ectopic overexpression of Zap70 (24).

The capacity to induce late responses upon BCR engagement, together with a substantial metabolic activity (MTS >25%) and a reversal of spontaneous apoptosis, allowed the discrimination of a larger group of patients with a risk of disease progression than any of the other proposed prognostic variables. Undoubtedly, IgVH-unmutated, Zap70-positive cases were all among responders whereas nonresponders corresponded to stable, IgVH-mutated, mostly Zap70-negative, Binet stage A cases. However, neither mutational status nor Zap70 expression allowed the identification of the entire possibly progressive population. A substantial number of responder cases (19 of 38) with a strong response to BCR engagement exhibited mutated IgVH sequences. Most of those cases (84%) had at least one clinical or biological factor of adverse prognosis (Binet clinical stage, thymidine kinase level, or lymphocyte doubling time). Therefore, our responder group is representative of clinical practice showing that some patients with mutated IgVH and/or no expression of Zap70 protein experience disease progression and that survival response to BCR engagement reflects an *in vivo* proliferative potential. Ability to respond to BCR ligation by increased survival, more than IgVH mutational status, or Zap 70 expression alone seems responsible for the different clinical course of B-CLL patients (24). This is also in agreement with an early work pointing out to the importance of antigen stimulation in the pathogenesis of CLL (6). Analysis of the various cellular intermediates differentially involved in this response on BCR engagement should lead to a better understanding of the mechanisms of disease evolution and ultimately to the identification of new therapeutic targets.

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# Survival Response to B-Cell Receptor Ligation Is Restricted to Progressive Chronic Lymphocytic Leukemia Cells Irrespective of Zap70 Expression

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