Mimicking the tumour microenvironment of chronic lymphocytic leukaemia in vitro critically depends on the type of B-cell receptor stimulation

Ans Rombout1, Sofie Lust2, Fritz Offner2, Evelien Naessens1, Bruno Verhasselt1 and Jan Philippe*,1

1Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium and 2Department of Internal Medicine, Hematology, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

Background: The B-cell receptor (BCR) has a key role in the cross-talk between chronic lymphocytic leukaemia (CLL) cells and the tissue microenvironment, which favours disease progression by promoting proliferation and drug resistance. In vitro studies on downstream signalling and functional effects of CLL BCR ligation often report contradictory results, in part owing to the lack of a standardised stimulation protocol. Our aim was to define a biologically relevant and robust in vitro stimulation method with regard to cellular phenotypic and transcriptional responses.

Methods: We evaluated mRNA (FOS, MYC, LPL) and protein (CD54, CD19, CD62L, CD184) expression of genes modulated by BCR triggering in immunoglobulin heavy-chain variable region genes (IGHV)-mutated and -unmutated CLL cells, after stimulation using soluble or immobilised anti-IgM antibodies from different suppliers.

Results: The effect of BCR stimulation on gene and protein expression was comparable in all CLL patients, irrespective of IGHV mutation status. However, immobilised anti-IgM stimulation elicited clear and robust changes in gene and protein expression, whereas the response to soluble anti-IgM was far less obvious.

Conclusions: These data indicate that the method of BCR stimulation is of major importance regarding responsiveness of CLL cells in the context of the tumour microenvironment, whereas genetic differences in the BCR pathway are less critical.

Chronic lymphocytic leukaemia (CLL) is characterised by the accumulation of clonal CD5+/CD23+ B lymphocytes in peripheral blood (> 5.0 × 10⁹/µl⁻¹), bone marrow and lymphoid organs. The clinical course of CLL patients is highly variable: Some patients live for decades after diagnosis, whereas others progress rapidly despite aggressive treatment (Chiorazzi et al, 2005). Major progress has been made in the identification of genetic and cellular markers, which predict disease progression. Several prognostic factors in CLL, such as mutation status of the immunoglobulin heavy-chain variable region genes (IGHV) (Damle et al, 1999; Hamblin et al, 1999), zeta-chain-associated protein kinase 70 (ZAP70) (Crespo et al, 2003; Wiestner et al, 2003), lipoprotein lipase (LPL) (Heintel et al, 2005; van’t Veer et al, 2006) and chemokine ligand 3 and 4 (CCL3/4) expression (Burger et al, 2009; Sivina et al, 2011) are associated with B-cell receptor (BCR) activation, suggesting that BCR signalling has a critical role in CLL pathogenesis. Also, the biased VH gene repertoire over CLL cases supports the idea of antigen-driven selection and expansion of CLL clones via antigens present in the microenvironment (Capello et al, 2004; Messmer et al, 2004). Activation of the BCR of normal B cells predominantly occurs in the lymphatic tissues and triggers a cascade of signalling events that cause B-cell selection.
proliferation, differentiation and antibody production (Niïro and Clark, 2002). In CLL and other B-cell malignancies, the leukaemic cells critically depend on interactions with bystander cells in the tissue microenvironment to acquire survival and growth signals. The BCR has a central role in this cross-talk with the tumour microenvironment, involving critical downstream kinases such as Lyn, Syk and Btk. In addition, CLL cells secrete cytokines and chemokines upon BCR activation, which attract accessory cells, such as T cells, which further contribute to the maintenance and expansion of the leukaemic clone (Burger et al, 2009; Herishanu et al, 2011; Burger and Gribben, 2014). Promising results obtained in clinical trials, evaluating new inhibitors targeting this CLL microenvironment cross-talk, further emphasise the central role for BCR signalling in CLL (Quiroga et al, 2009; Hoellenriegel et al, 2011; de Rooij et al, 2012).

As BCR signalling is assumed to have such a crucial role in CLL pathogenesis, stimulation of the BCR in vitro is relevant to unravel the biology of the CLL cell and its surrounding microenvironment. However, controversial data have been published concerning the change in gene expression and functional consequences upon BCR stimulation. Furthermore, some studies show that cross-linking the surface IgM receptor results in a heterogeneous response among CLL cases, correlating with prognostic indicators of progressive disease (Deglesne et al, 2006; Guarini et al, 2008; Quiroga et al, 2009; Vlad et al, 2009). We and others did, however, not observe a differential response upon BCR stimulation between prognostic classes of CLL (Deglesne et al, 2006; Pallasch et al, 2008; Pede et al, 2013). In a previous study, we investigated the transcriptional response of CLL cells upon IgM stimulation of the BCR using anti-IgM-coated polyacrylamide beads and found that both IgHV-mutated and -unmutated CLL cells responded with increased expression of FOS, MYC and other BCR activation-associated genes (Pede et al, 2013). Here, we wanted to evaluate whether the observed response was limited to the use of this particular reagent, and if we could reach out for a standardised protocol of BCR stimulation. We compared different stimulation protocols and found a marked difference in respect to immobilised vs soluble triggers of the BCR. We could confirm our previously published data with different reagents and propose a BCR stimulation protocol, to mimic what CLL cells experience in the lymphoid microenvironment.

**Materials and Methods**

**Ethics statement.** This study was approved by the Ghent University Hospital Ethics Committee and conducted according to the principles expressed in the Declaration of Helsinki. Patient samples were obtained after written informed consent.

**Patients sample collection and characterisation.** Blood samples were collected from patients diagnosed with CLL at our institution, which were either untreated or had not received treatment for at least 6 months prior to the time of blood collection. Peripheral blood mononuclear cells (PBMC) were isolated on a Lymphoprep (Nycomed, Oslo, Norway) layer, and used fresh or frozen viably in fetal bovine serum (FBS, Hyclone, Thermotifer Scientifc, Waltham, MA, USA) supplemented with 10% dimethylsulphoxide (Sigma-Aldrich (SA), Diegem, Belgium) for storage in liquid nitrogen. Cryopreservation did not compromise functional experiments after thawing (Pede et al, 2013). Characteristics of the patients included are summarised in Table 1. Determination of IgHV mutation status, ZAP70 protein expression and cytogenetic analysis were performed as previously described (Van Bockstaele et al, 2007). CD38 expression was evaluated by flow cytometric staining according to van Dongen et al (2012). Measurements were performed on a FACSCanto II flow cytometer (BD Biosciences, Erembodegem, Belgium). CD38 expression was deemed positive if 30% of the cells stained positive with a cutoff set on the T-cell population.

**Cell culture and BCR stimulation.** After isolation or thawing, PBMCs were cultured overnight at a density of $1.0 \times 10^7$ cells ml$^{-1}$ in Iscove’s modified Dulbecco’s medium (IMDM, Invitrogen, Merelbeke, Belgium) supplemented with penicillin (100 U ml$^{-1}$), streptomycin (100$\mu$g ml$^{-1}$; Invitrogen) and 10% FBS in a humidified atmosphere of 37°C containing 7% (v/v) CO$_2$. After overnight incubation, CD19$^+$ cells were selected by negative depletion using EasySep technology (Stem Cell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. Purity was assessed by flow cytometry and was at least 95.8% (data not shown).

Cells were cultured at a density of $1.0 \times 10^7$ cells ml$^{-1}$ in complete IMDM medium in 96-well plates in a humidified atmosphere of 37°C containing 7% (v/v) CO$_2$. BCR stimulation was performed with 10$\mu$g ml$^{-1}$ or 100$\mu$g ml$^{-1}$ of rabbit anti-human IgM polyclonal immunoad bindant (IgM, Sigma-Aldrich (SA), Diegem, Belgium) or with either soluble or immobilised rabbit anti-human IgM antibody (Jackson Immunoresearch (JI), Suffolk, UK); or goat F(ab)'; antibody-human IgM (MP Biomedicals (MB), Santa Ana, CA, USA; or SA). Rabbit anti-human IgA polyclonal immunoad bindant (IgA, Sigma-Aldrich (SA), Diegem, Belgium) served as negative controls. Untreated (UT) cells kept in culture in IMDM complete without addition of any stimulation or control reagent were used as a reference population. After 24 h of culture or stimulation, cells were harvested for flow cytometric staining of cell surface markers and Qiaxolysis for mRNA isolation. Annexin V and 7-Aminoactinomycin D (7-AAD) labelling was performed after 24, 48 and 72 h of stimulation.

**Flow cytometry.** The expression of surface molecules was analysed by flow cytometry, using the following monoclonal antibodies (mAbs): CD5-PECy7 (clone UCHT2, ebioscience, Vienna, Austria), CD19-allophycocyanin (APC) (clone LT19 Miltenyi Biotec, Leiden, The Netherlands), CD54 (ICAM-1)-phycoerythrin (PE) (clone LB-2, BD Biosciences), CD62L (L-selectin)-fluorescein isothiocyanate (FITC) (clone DREG-56, ebioscience), CD184 (CXCR4)-PE (clone 12G5, BD Biosciences), CD19-allophycocyanin (APC) (clone LT19 Miltenyi Biotec, Leiden, The Netherlands), CD54 (ICAM-1)-phycoerythrin (PE) (clone LB-2, BD Biosciences), CD62L (L-selectin)-fluorescein isothiocyanate (FITC) (clone DREG-56, ebioscience), CD184 (CXCR4)-PE (clone 12G5, BD Biosciences). A total of $10^6$ CD19$^+$ cells were incubated for $30 \text{ min}$ at $4^\circ\text{C}$ in the dark with saturating concentrations of mAbs. Data acquisition was performed with a LSRII flow cytometer (BD Biosciences) and data analysis was performed using BD FACSDiva software.

**Measurement of intracellular calcium flux.** Calcium flux measurement was essentially performed as described by Gergely et al (1997). CD19$^+$ cells were resuspended at $1 \times 10^6$ cells ml$^{-1}$ in IMDM medium and incubated for $30 \text{ min}$ at $37^\circ\text{C}$ in the dark with $1 \mu\text{M}$ Fluo-4-AM (Invitrogen). Subsequently, an equal amount of IMDM medium supplemented with 10% FBS was added and cells were further incubated for 10 min. Finally, cells were washed twice in IMDM supplemented with 5% FBS and once in Hanks balanced salt solution (HBSS) flux buffer (Invitrogen) supplemented with 5% FBS, 1.5$\mu$M CaCl$_2$ (SA) and buffered to pH 7.5 with 10$\mu$M HEPES (SA). Cells were resuspended in HBSS flux buffer at a concentration of $1 \times 10^6$ cells ml$^{-1}$ and kept at $37^\circ\text{C}$ until analysis. Aliquots of $1 \times 10^6$ cells ($100 \mu\text{l}$) were transferred into 200$\mu\text{l}$ pre-warmed HBSS flux buffer in $12 \times 75 \text{ mm}$ polystyrene tubes. Each tube was acquired for $30 \text{ s}$ to measure background fluorescence. Subsequently, BCR stimulation was performed by addition of 100$\mu$g ml$^{-1}$ anti-human IgM polyclonal immunoad bindant (IgM) or soluble rabbit anti-human IgM antibody (IgM) and acquisition was continued for a total of $140 \text{ s}$. Treatment with...
5 μg ml⁻¹ ionomycin served as positive control for calcium flux measurement.

**Intracellular LPL staining.** Flow cytometric analysis of intracellular LPL expression was performed using the protocol for intracellular ZAP70 staining described by Van Bockstaele et al (2006) optimised for LPL staining. In brief, 10⁵ CD19⁺ cells were fixed and permeabilised using the Fix and Perm kit (ImTec Diagnostics, Antwerp, Belgium). cDNA was synthesised from total cellular RNA with the Superscript III reverse transcriptase kit (Life Technologies). mRNA was amplified using either published primers and probe for MYC (Mestdagh et al, 2007) or an Assay-on-Demand gene expression assay for MYC (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. Prior to Annexin V/7-AAD staining, cells were labelled with surface markers CD19-APC and CD5-PECy7. Cells treated with 2.5 μM staurosporine (Merck Millipore, Billerica, MA, USA) were used as a positive control for apoptosis.

**RNA isolation, cDNA synthesis and real-time quantitative PCR.** Total cellular RNA was extracted with the miRNeasy mini kit (Qiagen, Hilden, Germany) according to the supplier’s instructions. Contaminating DNA was removed through DNase treatment using the DNase I, Amplification Grade, kit (Life Technologies). cDNA was synthesised from 1 μg RNA with the Superscript III reverse transcriptase kit (Life Technologies). LPL mRNA expression levels were measured with qPCR as published before (Van Bockstaele et al, 2007). All other genes were measured using assays that were either SybrGreen based with primers described before for MYC (Mestdagh et al, 2010) or probe hydrolysis based with either published primers and probe for ABL1 (Beillard et al, 2003) or an Assay-on-Demand gene expression assay for FOS (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. All reactions were performed in duplicate on a LightCycler 480 (Roche, Basel, Switzerland) and each PCR run included non-template controls and a calibration

**Table 1. Patient characteristics**

| Patient ID | Gender | Age at diagnosis | CLL score | Rai | Binet | Cytogenetic analysis | VH gene usage | % Homology | Mutation status | ZAP70 | CD38 | LPL |
|------------|--------|-----------------|-----------|-----|-------|---------------------|---------------|------------|----------------|-------|------|-----|
| CLL_1      | M      | 46              | 5         | 0   | A     | No aberr            | 1–3           | 94.8       | M              | ND    | N    | P   |
| CLL_2      | M      | 69              | 5         | 2   | A     | No aberr            | 2–5           | 95.0       | M              | ND    | N    | N   |
| CLL_3      | M      | 47              | 5         | 0   | A     | No aberr            | 4–34          | 90.6       | M              | N     | N    | N   |
| CLL_4      | M      | 52              | 5         | 1   | A     | del13q             | 3–7           | 96.2       | M              | N     | N    | N   |
| CLL_5      | M      | 63              | 4         | 4   | C     | del13q             | 3–30          | 81.3       | M              | N     | N    | N   |
| CLL_6      | M      | 64              | 5         | 4   | C     | del11q             | 3–30          | 95.7       | M              | N     | N    | N   |
| CLL_7      | M      | 55              | 5         | 4   | C     | del13q             | 3–33          | 95.8       | M              | N     | N    | N   |
| CLL_8      | F      | 42              | 4         | 0   | A     | tris12, del13q     | 1–3           | 99.7       | U              | P     | P    | P   |
| CLL_9      | M      | 64              | 4         | 1   | A     | del13q             | 1–18          | 99.7       | U              | P     | ND   | P   |
| CLL_10     | M      | 51              | 5         | 4   | C     | No aberr            | 2–70          | 99.0       | U              | ND    | ND   | N   |
| CLL_11     | M      | 67              | 5         | 2   | B     | No aberr            | 3–48          | 100        | U              | N     | P    | P   |
| CLL_12     | M      | 51              | 5         | 4   | C     | del13q             | 4–34          | 100        | U              | P     | N    | P   |
| CLL_13     | M      | 52              | 5         | 0   | A     | tris12             | 1–69/4–30–2   | 100/100     | BC(U/U)       | ND    | ND   | P   |
| CLL_14     | F      | 57              | 5         | 0   | A     | del13q             | 3–33          | 90.0       | M              | N     | N    | N   |
| CLL_15     | M      | 66              | 3         | 0   | A     | tris12             | 3–23          | 94.2       | M              | P     | P    | P   |
| CLL_16     | F      | 49              | 3         | 1   | A     | No aberr            | 3–30          | 93.8       | M              | N     | ND   | N   |
| CLL_17     | M      | 68              | 4         | 4   | C     | del13q             | 4–34          | 95.8       | M              | P     | N    | N   |
| CLL_18     | F      | 72              | 5         | 0   | A     | del13q             | 3–07          | 94.0       | M              | N     | ND   | N   |
| CLL_19     | M      | 63              | 5         | 0   | A     | del13q             | 3–30–3        | 94.2       | M              | N     | N    | N   |
| CLL_20     | M      | 63              | 5         | 0   | A     | No aberr            | 4–34          | 95.4       | M              | N     | N    | N   |
| CLL_21     | F      | 58              | 4         | 0   | A     | del13q             | 2–70          | 100        | U              | N     | P    | P   |
| CLL_22     | F      | 74              | 5         | 0   | A     | del17p             | 3–23          | 100        | U              | N     | N    | P   |
| CLL_23     | M      | 61              | 5         | 1   | A     | No aberr            | 3–30          | 100        | U              | N     | P    | P   |
| CLL_24     | M      | 49              | 5         | 0   | A     | del13q             | 2–70          | 99.0       | U              | N     | N    | P   |
| CLL_25     | M      | 75              | 5         | 1   | B     | del13q             | 1–8           | 100        | U              | P     | ND   | P   |
| CLL_26     | M      | 69              | 5         | 1   | A     | del13q             | 1–03          | 100        | U              | P     | N    | P   |
| CLL_27     | M      | 46              | 5         | 0   | A     | del11q             | 5–01          | 99.5       | U              | N     | P    | P   |
| CLL_28     | M      | 75              | 5         | 0   | A     | del17p             | 3–23/4–30–4   | 96.2/93.4   | BC(M/M)       | N     | N    | N   |

Abbreviations: F = female; M = male; M = mutated; N = negative; ND = not determined; No aberr = no aberration; P = positive; U = unmutated. CLL score: flow cytometric Moreau-Matutes-Catovsky scoring of the presence of surface markers diagnostic for CLL; Rai and Binet refer to clinical scoring (Rai et al, 1981; Rai et al, 1975); VH gene usage: family of variable region of immunoglobulin variable heavy-chain genes; % homology compared with germ line sequence; 98% cutoff between mutated and unmutated IGHV; ZAP70: based on 20% cutoff, CD38: based on 30% cutoff, LPL: based on the optimal cutoff value determined by ROC curve analysis.
B-cell receptor stimulation in CLL

CLL cells respond differentially to soluble and immobilised anti-IgM stimulation, irrespective of IGHV mutational status. The discrepancies found in literature concerning changes in gene expression and functional consequences upon BCR stimulation can, at least partially, be attributed to the lack of a standardised protocol to trigger the CLL BCR in vitro. We measured mRNA and protein expression of genes modulated by stimulation of the BCR in CLL, to assess the efficiency of different in vitro stimulation methods. Purified CD19⁺ B cells from 13 CLL patients (7 IGHV mutated (M) and 6 IGHV unmutated (U) CLL’s) were stimulated with soluble or immobilised anti-IgM antibodies or F(ab’)² fragments at a concentration of 10 or 100 μg ml⁻¹. UC were used as a reference, cells treated with anti-IgA or anti-IgG served as negative controls. After 24 h of BCR stimulation we measured the cell surface expression of proteins previously reported to be modulated by stimulation of the BCR in CLL (Quiroga et al, 2009; Vlad et al, 2009); CD54 (ICAM-1), CD19, CD62L (L-selectin) and CD184 (CXCR4) (Figure 1). Of all 13 CLL patients included in this study, one M-CLL case hardly responded to in vitro BCR stimulation and was excluded from further analysis. Comparing the mean fluorescence intensity (MFI) ratios of immobilised anti-IgM stimulated samples with control (anti-IgG or anti-IgA) samples, we noticed a clear upregulation of CD54 (1A) and a firm downregulation of CD19 (1B) expression both at 10 and 100 μg ml⁻¹, whereas the expression of both proteins was nearly unaffected by soluble anti-IgM even in the highest concentration. Expression of CD62L (1C) was decreased after immobilised anti-IgM stimulation at both concentrations and after soluble anti-IgM at 100 μg ml⁻¹. Comparing anti-IgM with control (anti-IgA/G) samples, a statistically significant (P<0.05) change in CD54, CD19 and CD62L expression was induced by immobilised anti-IgM in all samples, whereas only in some conditions soluble anti-IgM induced significant effects. CD184 protein expression (1D) was significantly downregulated when immobilised anti-IgM was used at 100 μg ml⁻¹, but at the lower concentration the effect was not significant in all conditions. When using soluble stimulation, only one source of anti-IgM (MB) induced a significant downregulation of CD184 at 100 μg ml⁻¹. After 24 h of stimulation, we also measured mRNA expression levels for FOS, MYC and LPL, which were among the genes significantly upregulated by BCR stimulation in our previous study (Pede et al, 2013) (Figure 2). The expression profiles for FOS (2A) and MYC (2B) closely resemble the ones for CD54 and CD19. Indeed, soluble anti-IgM stimulation had no or only a very minor effect on FOS and MYC mRNA expression, whereas a considerable upregulation of both genes was observed after stimulation using immobilised anti-IgM. Five out of the six U-CLL cases included were considered LPL positive, measured by qPCR as described before (Van Bockstaele et al, 2007). The BCR induced LPL mRNA expression profile of these endogenously LPL-positive CLL patient samples measured after 24 h (2C), resembles the ones obtained for FOS and MYC and shows a clear upregulation only after 24 h of immobilised anti-IgM stimulation. The majority of M-CLL patient samples (n = 6) was, as expected, LPL negative and no robust upregulation of LPL mRNA expression levels after stimulation was observed (data not shown). These data were confirmed in an additional series of seven M-CLL and seven U-CLL cases stimulated with 100 μg ml⁻¹ anti-human IgM immunobeads, or either immobilised or soluble anti-human IgM antibody (Supplementary Figures 1 and 2).

In general, immobilised BCR triggering using anti-IgM coupled beads at a concentration of 100 μg ml⁻¹ induced the largest responses. At the lower concentration, other immobilised anti-IgM regimes were more effective. Remarkably, only minor differences were found in the response to 10 or 100 μg ml⁻¹ immobilised anti-IgM, suggesting saturation at 10 μg ml⁻¹, whereas soluble anti-IgM was usually more effective when used at a concentration of 100 μg ml⁻¹. The specificity of binding of the different sources of anti-IgM was confirmed by including anti-IgA or anti-IgG-treated samples. Compared with UC, only MB (MB) anti-IgG affected the outcome compared with non-treated cells in some cases. Other anti-IgA or anti-IgG controls acted like untreated samples and can be used as reliable negative controls.

Although the gene expression profiles of CLL cells were found to be homogenous among IGHV M-CLL and U-CLL cases (Klein et al, 2001; Rosenwald et al, 2001), previous studies are inconclusive regarding responsiveness of M-CLL and U-CLL cells to BCR ligation in vitro (Petlickovskii et al, 2005; Deglesne et al, 2006; Guarini et al, 2008; Vlad et al, 2009; Herishanu et al, 2011; Krysov et al, 2012). We previously reported that M-CLL and U-CLL cases are equally efficiently stimulated with anti-IgM beads, evaluated by FOS and MYC mRNA expression (Pede et al, 2013). In the current study, we aimed at validating these results with alternative stimulating agents. Although bead-based stimulation elicited the largest response at the level of protein and mRNA expression, we could show that both M-CLL and U-CLL cells responded to BCR triggering in vitro using other stimulation regimens as well. Indeed, the protein and mRNA expression profiles presented in Figures 1 and 2, respectively, show a similar pattern in M-CLL and U-CLL cases. When the ratios of protein and mRNA expression levels in anti-IgM and control (A/G) stimulated samples (100 μg ml⁻¹) were compared in M-CLL and U-CLL cases, significant differences in fold changes were observed only in very few conditions using immobilised anti-IgM triggering (Supplementary Figure 3).

Next to protein and mRNA expression, we measured induction of calcium flux as a direct read-out of BCR triggering in three M-CLL and three U-CLL using either 100 μg ml⁻¹ anti-IgM immunobeads or soluble anti-IgM (Supplementary Figure 4). Stimulation using anti-IgM antibody coated to culture plates was not compatible with this assay. Both M-CLL and U-CLL cases were able to mount a calcium flux upon robust BCR stimulation using bead-based immobilised anti-IgM, thereby confirming again our observations on protein and mRNA level. Soluble anti-IgM induced a weaker response that faded quickly.

Evaluation of LPL protein expression in IgM-stimulated CLL cells by flow cytometry. As mRNA levels of LPL were clearly induced in U-CLL, we asked whether this resulted in increased protein expression. To do so, we optimised an intracellular LPL protein staining protocol for flow cytometry in CLL cells. For validation, we used CLL cells overexpressing LPL protein after electroporation with IVT LPL mRNA (Figure 3A). This flow cytometric staining procedure was not sensitive enough to detect LPL protein in endogenously LPL positive CLL cells and consequently did not allow distinguishing them from LPL-negative CLL cells (data not shown). Only when CLL cells were robustly stimulated using anti-IgM-coated beads, we managed to stain LPL.

www.bjcancer.com | DOI:10.1038/bjc.2016.35
protein. As shown in Figure 3, both M-CLL (3B) and U-CLL (3C) cells showed a clear shift in LPL MFI after 24 h of anti-IgM stimulation compared with unstimulated or anti-IgA-stimulated conditions. Stimulating CLL cells with any other anti-IgM reagent, either immobilised or in solution, did not result in detectable LPL protein by intracellular staining (data not shown).

Effect of soluble and immobilised anti-IgM on CLL cell survival. In addition, we investigated the functional effects of BCR stimulation in CLL. As expected, after 24 h of culture we were not able to show induction of proliferation in a selection of samples measured by intracellular Ki67 staining, even after strong stimulation using anti-IgM beads (data not shown). Some studies also report that BCR stimulation influences CLL cell viability.

Whether in vitro BCR triggering induces apoptosis or protects CLL cells from undergoing apoptosis is however not clear. The effect of BCR triggering on CLL cell survival in vitro was assessed by Annexin V/7-AAD labelling after 24 h of stimulation (Supplementary Figure 5A and B). The levels of apoptotic cells varied widely among the different CLL samples (range 8.6–58.9%). Although not always significant, we observed a trend of reduced apoptotic levels only in M-CLL cells when immobilised anti-IgM was used. For soluble stimulation, the effect was not clear and rather reagent dependent. In U-CLL cells the apoptotic levels fluctuated over all conditions without an obvious trend. In an independent series of seven M-CLL and seven U-CLL cases, levels of apoptotic cells varied among the different CLL samples (range 8.6–58.9%).
The protective effect was observed in case of immobilised anti-IgM stimulation after 24 h and both with immobilised and soluble anti-IgM after 48 and 72 h. However, in unmutated CLL, significantly lower levels of apoptotic cells were only observed after 48 h with anti-IgM coated beads, and after 72 h with anti-IgM coated to culture plates as well.

The degree of protection from apoptosis differed significantly between M-CLL and U-CLL cases only when the BCR was stimulated with anti-IgM coated to culture plates (100 μg ml⁻¹) for 48 and 72 h (Supplementary Figure 6).

**DISCUSSION**

In this study, we evaluated the efficiency of soluble and immobilised anti-IgM to stimulate the CLL BCR in vitro and found that immobilised anti-IgM triggers induce more powerful BCR stimulation compared with soluble anti-IgM, regardless of IGHV mutation status. As a read-out for responsiveness of CLL cells to stimulation, we measured protein and mRNA expression of genes modulated upon BCR triggering. The observed down-regulation of CD62L and CD184 surface expression was observed before by Vlad et al (2009), but is discordant with the findings of Quiroga et al, 2009) who showed that anti-IgM increases the expression of several adhesion molecules, among which CD62L. Next to this, both groups do not agree on the effect of BCR triggering upon CXCL12-induced migration of CLL cells. According to Quiroga et al these discrepancies can be attributed to the fact that they used soluble anti-IgM, whereas Vlad et al used immobilised anti-IgM. We found a clear downregulation of CD62L using both soluble and immobilised anti-IgM at a concentration of 100 μg ml⁻¹. At a concentration of 10 μg ml⁻¹, which was used by Quiroga et al and Vlad et al, the down-regulation by soluble anti-IgM was less obvious; however, we certainly did not find an upregulation as Quiroga et al reported. Differences in antibody specificity, affinity or avidity are not responsible for these contrasting results either, as we observed the same results using four different sources of anti-IgM, among them the one used by Quiroga et al and Vlad et al. Next to CD62L, Quiroga et al also reported BCR-induced CD54 upregulation, which was confirmed by our data; however, only when immobilised anti-IgM was used. The same holds true for CD19. Indeed, we did not find any change in cell surface expression of CD54 and CD19 in response to soluble anti-IgM. However, significant responses were observed after immobilised BCR triggering. Also for CD62L and CD184, significant responses were induced mainly with immobilised anti-IgM. Overall, the strongest effects were obtained with anti-IgM-coupled beads, representing immobilised anti-IgM antibodies. These beads were used in a...
previous study where we investigated the transcriptional response upon BCR triggering and found both in M-CLL and U-CLL cases an increased expression of genes associated with BCR activation, including FOS and MYC (Pede et al., 2013). In the present study we were able to confirm this BCR-driven induction of FOS and MYC mRNA expression, only when immobilised anti-IgM was used, thereby confirming our protein expression data. Considering the fact that the anti-IgM (and anti-IgA) beads are no longer manufactured, we show here that valuable alternatives for bead-based BCR stimulation are available, that are proven to be very efficient even at a lower concentration.

The upregulation of MYC expression after BCR stimulation was reported by others before, by using both immobilised or soluble anti-IgM (Petlickovski et al., 2005; Krysov et al., 2012). However, Krysov et al, who used soluble anti-IgM, looked at MYC protein expression only at 3 and 6 h after stimulation and did not include later time points. The differences in response following soluble and immobilised BCR triggering may indeed be attributed to the rapid internalisation of soluble anti-IgM by endocytosis, inducing a rather transient response, whereas immobilised anti-IgM induces a more sustained triggering, resulting in a more complete and stable response. In this view, cell surface protein and mRNA expression might be modulated by soluble anti-IgM more rapidly. We could, however, not confirm this by calcium flux measurement upon BCR triggering.

Although conflicting results are published, we show that both M-CLL and U-CLL respond to (soluble and immobilised) anti-IgM stimulation. The protein and mRNA expression profiles of M-CLL cases closely resemble the ones observed in U-CLL cases, showing that both have the ability to respond to in vitro BCR stimulation. We do not exclude that some patients respond better to anti-IgM stimulation than others, but the ability to be stimulated does not necessarily depend on IGHV mutation status. It was shown before that CLL patients express variable levels of surface IgM (sIgM), with circulating U-CLL cells showing a higher sIgM expression than M-CLL, giving rise to different degrees of responsiveness among both subgroups.

In addition to FOS and MYC, we also evaluated the levels of LPL mRNA expression. LPL is an established poor prognostic marker in CLL and was shown to be induced by BCR triggering (Pallasch et al., 2008; Herishanu et al., 2011; Pede et al., 2013). We observed a significant upregulation of LPL mRNA after immobilised anti-IgM stimulation in endogenously LPL-positive CLL samples, thereby confirming again the robustness of immobilised BCR triggering. Again, the strongest upregulation of LPL mRNA expression was induced by bead-based stimulation of the BCR, which also increased LPL protein expression to levels detectable through intracellular flow cytometric staining using our optimised protocol. This staining procedure was, however, not sensitive enough to distinguish endogenously expressed LPL-positive from LPL-negative patient samples and will thus not allow determining a CLL patient’s status for this prognostic marker as is the case for ZAP70.

Next to the effect on gene expression, there is no consensus on the functional consequences of in vitro BCR activation in CLL cells. It is believed that in vivo BCR signalling contributes to the survival and proliferation of the malignant clone. Here, we could not show BCR related induction of proliferation, although we reported active proliferation in anti-IgM-stimulated samples before (Pede et al., 2013). This discrepancy is probably due to the use of a different read-out and a different time point of analysis. Indeed, before we analysed samples after 48 h of stimulation using DRAQ5 staining, whereas in the present study we stained for Ki67 already after 24 h of stimulation, which is probably too early to observe a significant effect of stimulation upon proliferation. Protection from spontaneous apoptosis in vitro after immobilised BCR triggering was reported repeatedly before. When soluble anti-IgM was used, however, both induction and inhibition of spontaneous apoptosis after stimulation have been observed (Petlickovski et al., 2005; Deglesne et al., 2006; Quiroga et al., 2009). We did not observe a clear difference between soluble or immobilised BCR stimulation regarding apoptosis after 24 h. At later time points we found that both immobilised and soluble anti-IgM protected CLL cells from spontaneous apoptosis, presumably mimicking the effect of in vivo BCR triggering. Although the effect was more pronounced in case of immobilised BCR stimulation, induction of apoptosis was not observed in any of the conditions tested.

The protective effect of BCR stimulation was seen both in M-CLL and U-CLL, but in M-CLL it was induced more rapidly and more strongly. Even in case of soluble anti-IgM significantly lower levels of apoptotic cells could be observed in M-CLL, in contrast to U-CLL who only showed a significant response in case of immobilised BCR triggering. This is probably because of the fact that U-CLL cells are more resistant to spontaneous apoptosis compared with M-CLL cells and longer incubation times and/or more robust triggering of the BCR are needed before a significant response can be observed.

In the present study we show that immobilised BCR triggering induces a more robust response compared with soluble triggers. The use of immobilised anti-IgM is preferred over soluble anti-IgM when trying to unravel the role of BCR triggering in the CLL pathogenesis. Indeed, immobilised BCR-triggering reflects better...
what CLL cells experience \textit{in vivo} versus in vitro anti-IgM BCR stimulation, the differences in gene expression profiles previously observed in M-CLL versus U-CLL might not be due to cell intrinsic differences, but rather a reflection of differential in vivo BCR triggering.

Unmutated BCRs might have a higher specificity for antigens inducing more repetitive or sustained signalling comparable to immobilised anti-IgM, or U-CLL cells might reside more in areas with a higher chance of encountering such antigens \textit{in vivo}, ultimately resulting in stronger BCR signalling and distinct clinical and biological behaviour compared with M-CLL.

**ACKNOWLEDGEMENTS**

We thank the patients for sample donation. This work was supported by grants from the Research Foundation Flanders (FWO) to JP and BV. AR is a PhD fellow and BV is a Senior Clinical Investigator of the FWO.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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