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Responsiveness of chronic lymphocytic leukemia cells to B-cell receptor stimulation is associated with low expression of regulatory molecules of the nuclear factor-κB pathway

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a disease with heterogeneous clinical and biological characteristics. Differences in Ca^{2+} levels among cases, both basal and upon B-cell receptor (BCR) stimulation, may reflect heterogeneity in the pathogenesis due to cell-intrinsic factors. Our aim was to elucidate cell-intrinsic differences between BCR-responsive and -unresponsive cases. We therefore determined BCR responsiveness *ex vivo* based on Ca^{2+} influx upon α-IgM stimulation of purified CLL cell fractions from 52 patients. Phosphorylation levels of various BCR signaling molecules, and expression of activation markers were assessed by flow cytometry. Transcription profiling of responsive (n=6) and unresponsive cases (n=6) was performed by RNA sequencing. Real-time quantitative polymerase chain reaction analysis was used to validate transcript level differences in a larger cohort. In 24 cases an α-IgM response was visible by Ca^{2+} influx which was accompanied by higher phosphorylation of PLCγ2 and Akt after α-IgM stimulation in combination with higher surface expression of IgM, IgD, CD19, CD38 and CD43 compared to the unresponsive cases (n=28). Based on RNA sequencing analysis several components of the canonical nuclear factor (NF)-κB pathway, especially those related to NF-κB inhibition, were expressed more highly in unresponsive cases. Moreover, upon α-IgM stimulation, the expression of these NF-κB pathway genes (especially genes coding for NF-κB pathway inhibitors but also NF-κB subunit *REL*) was upregulated in BCR-responsive cases while the level did not change, compared to basal level, in the unresponsive cases. These findings suggest that cells from CLL cases with enhanced NF-κB signaling have a lesser capacity to respond to BCR stimulation.

Introduction

Chronic lymphocytic leukemia (CLL) is a lymphoid malignancy that is characterized by a monoclonal expansion of mature B cells with a homogeneous morphology and a characteristic immunophenotype.[1] CLL is the most common type of leukemia in the Western world and mainly affects the elderly.[1] Based on the somatic hypermutation (SHM) status of the immunoglobulin heavy chain (IGHV) gene, CLL can be divided into unmutated CLL (U-CLL) and mutated CLL (M-CLL), with U-CLL generally being a more aggressive form of the disease and M-CLL a more indolent form.[2,3] Around 30% of all cases can be grouped into subsets based on so-called stereotypic B-cell receptors (BCR), which are identified by their restricted IGHV/IGHD/IGHJ gene usage plus similarities in length and amino acid sequence of their complementarity-determining region 3 (CDR3). [4]

BCR stereotypy would be indicative of the involvement of similar specific antigens and underlines the importance of antigenic stimulation and BCR specificity in the pathogenesis of CLL.[4] In general, most U-CLL express a BCR that is polyreactive and recognizes self- and non-self-antigens with low-affinity binding.[5-8] In addition, for some stereotypic CLL subsets the antigens recognized by their BCR have been identified.[9-13]

However, it was previously shown that the BCR from CLL cells could also be stimulated independently of external antigens, as the CDR3 regions are able to recognize an internal epitope in framework 2 (FR2) of the IGHV domain.[14] This induces a higher level of antigen-independent autonomous BCR signaling, since these cells exhibit a higher Ca^{2+} level in their cytoplasm, as demonstrated *in vitro* using a triple knockout (TKO) cell system.[14]

We previously demonstrated that primary CLL cells generally have higher basal Ca^{2+} levels compared with peripheral B cells from healthy individuals.[15] Basal Ca^{2+} levels correlated with IGHV mutational status, as we found on average higher basal Ca^{2+} levels in M-CLL than in U-CLL.[14,15] However, our data also showed large variation within the subgroups, as cases with high and low basal Ca^{2+} levels could be found in both M-CLL and U-CLL groups.[15] Since there was no correlation with BCR characteristics (e.g., Ig expression level, HCDR3 length, charge and composition) or with cytogenetic aberrations, it is conceivable that high basal Ca^{2+} levels are partly directed by the SHM status and that cell-intrinsic differences caused by cell anergy could explain the variation.[15]

Anergy is an immune state in which the cell is silenced upon low-affinity recognition of self-antigens.[16] Anergic cells remain capable of antigen binding, but have a reduced ability to respond to BCR-dependent antigenic stimulation.[16] Anergy has been linked to CLL based on low surface BCR expression, reduced responsive capability,[17,18] and increased basal Ca^{2+} levels.[15] M-CLL in particular shows these increased basal Ca^{2+} levels in combination with a poorer response to BCR stimulation[15] which is in line with other studies showing that the α-IgM response is associated with IGHV mutational status and with the surface expression of markers of prognosis, such as CD38.[18,19] Moreover, a high level of surface IgM is associated with a clinically aggressive form of the disease, which has potential implications as a diagnostic parameter for disease progression.[20]

However, Ca^{2+} levels, both basal and upon BCR stimulation, vary within the U-CLL and M-CLL groups. We hypothesized that this heterogeneity in BCR responsiveness could reflect a diverse disease pathogenesis involving cell-intrinsic differences. In this study we aimed to elucidate potential cell-intrinsic differences underlying the observed differences in Ca^{2+} levels between CLL cases.

Methods

Study population

Fifty-two patients were included of whom 30 (58%) had U-CLL and 22 (42%) had M-CLL as determined by the IGHV SHM status (*Online Supplementary Methods*). The patients' characteristics are shown in *Online Supplementary Table S1*. The majority of the included patients (n=41, 79%) were treatment-naïve. Purified CLL cells were isolated (*Online Supplementary Methods*) upon informed consent and anonymized for further use, following the guidelines of the institutional review board (METC-2015-741) and in accordance with the Declarations of Helsinki.

Flow cytometry

Flow cytometry was used to assess the responsive capacity upon α-IgM stimulation by measuring Ca^{2+} levels (*Online Supplementary Methods*) and to determine the expression of activation markers by using antibodies listed in *Online Supplementary Table S2*. Phospho-flow analysis was done to study the phosphorylation of Spleen tyrosine kinase (Syk), Phopholipase Cγ2 (PLCγ2) and Protein kinase B (Akt) upon α-IgM stimulation. (*Online Supplementary Methods*).

Cell culture and retroviral transduction of triple knockout cells

TKO cells, derived from a signaling-competent mouse pre-B-cell line lacking the expression of endogenous pre-BCR due to inactivation of *RAG2* and *λ5* genes,[21] and Phoenix cells (ATCC CRL-3214) were both cultured as described by Meixlsperger *et al.*[21] The protocol used for the transduction of TKO cells was also documented before by Meixlsperger *et al.*[21]

RNA sequencing

Twelve cases from our cohort were selected based on their responsiveness to α-IgM stimulation (6 responsive, 6 unresponsive) and their RNA was sequenced. The RNA was extracted using Allprep DNA/RNA/miRNA Universal (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA sequencing was performed on a TruSeq platform (Illumina, San Diego, CA, USA) at the Human Genome Facility (Erasmus Medical Center, Rotterdam, the Netherlands). Reads were extracted from the raw sequencing data using CASAVA 1.8.2 (Illumina) and aligned to the human reference genome (UCSC's hg19) using the STAR (2.5.0c) splice aware aligner with gencode v19 transcriptome annotations as an additional template. The BAM files were processed using various tools from the picard software suite (v1.90), as well as tools from the Genome Analysis ToolKit (GATK, v3.5). Quality control metrics were collected at various steps using picard and evaluated, along with coverage metrics using GATK. Read counts per exon/gene were then determined by the featureCounts function of the subread package (v1.4.6-p1) using the gencode v19 annotation as markers. The raw read counts were normalized through the fragments per kilobase of exon model per million reads mapped (FPKM) methodology, normalizing for library yield and gene size.

For classification analysis, the calculated Spearman correlation as a distance (1/similarity) measurement and Ward.D2 for the unsupervised clustering were applied to the samples used. R packages (version 3.4.4) weres used for differential expression analysis and to create plots for visualization. We analyzed the sample fitting with edgeR, the gene-wise negative binomial generalized linear models for contrast.

To validate transcript level differences in a larger cohort, RNA was synthesized to cDNA and real-time quantitative polymerase chain reaction (RQ-PCR) was performed (*Online Supplementary Methods* and *Online Supplementary Table S2*).

Results

Unmutated cases of chronic lymphocytic leukemia are generally more responsive than mutated cases to α-IgM stimulation

To determine whether high basal Ca^{2+} levels are BCR-dependent or caused by cell-intrinsic factors, we selected

a small series of CLL samples with known high (n=3) or low (n=6) basal Ca^{2+} levels from our previous study in 2015,[15] and cloned their BCR into TKO cells as described by Dühren-von Minden *et al.*[14]

Even though we could detect Ca^{2+} signaling by the BCR in TKO cells for all analyzed CLL-derived BCR expressed as IgM, we did not detect any correlation (R^2=0.014, *P*=0.764) between the Ca^{2+} signal in CLL and that in TKO cells (Figure 1B) indicating that the high basal Ca^{2+} levels seen in some CLL samples would result from cell-intrinsic changes rather than from BCR-dependent autonomous signaling.

To determine which cell-intrinsic differences might cause the heterogeneity in Ca^{2+} signaling in basal conditions and upon BCR stimulation, we established a new cohort of patients (n=52, *Online Supplementary Table S1*). CLL cells were isolated from peripheral blood and immediately used for further analysis. First, basal Ca^{2+} levels were assessed (Figure 1B). Similar to the previous study, basal Ca^{2+} levels were heterogeneous in both U-CLL and M-CLL cases.[15]

Next, we examined the responsive capacity of the CLL samples upon BCR stimulation. Figure 1D shows two flow cytometric examples. In line with our previous study,[15] we found that U-CLL cells in general responded significantly (*P*=0.049) better upon α-IgM stimulation compared with M-CLL cells (Figure 1E). Although no differences were found in the response after α-IgD stimulation (Figure 1F), there was a strong correlation between the relative response to α-IgM and α-IgD stimulation (R^2=0.508, *P*<0.0001) (Figure 1G). Based on this, we further defined CLL subgroups based on BCR responsiveness upon α-IgM stimulation. Twenty-four cases were classified as responsive (median fluorescence intensity ratio, response/basal signal: 1.1-6.5; n=17 U-CLL and n=7 M-CLL) and 28 cases were unresponsive (median fluorescence intensity ratio, response/basal signal: <1.1; n=13 U-CLL and n=15 M-CLL).

Higher phosphorylation of PLCγ2 and Akt in chronic lymphocytic leukemia correlated with responsiveness upon B-cell receptor stimulation

In order to gain a better understanding of BCR responsive capacity, as defined by Ca^{2+} influx, we examined phosphorylation of Syk, PLCγ2 and Akt upon α-IgM stimulation. First, we evaluated differences in basal phosphorylation levels of Syk (pSyk), PLCγ2 (pPLCγ2) and Akt (pAkt) (Figure 2A). The responsive cases showed a significantly (*P*=0.0013) higher basal pPLCγ2 level than unresponsive cases but no differences were found in basal pSyk and pAKT levels (Figure 2B). Next we examined the relative response of kinase phosphorylation upon BCR stimulation. Even though no difference in relative response of pSYK after α-IgM stimulation was found, the responsive patients had a higher relative response of pPLCγ2 and pAkt upon α-IgM stimulation (Figure 2C).

Taken together, the α-IgM response as determined by Ca^{2+} influx, is consistent with greater phosphorylation of pPLCy2 and pAkt upon α-IgM stimulation

Chronic lymphocytic leukemia cases showing good B-cell receptor responsiveness have a more activated phenotype

Next we examined whether the expression of activation markers is associated with the response to α-IgM. As expected, CLL cells from responsive cases displayed a significantly (*P*=0.0002) higher expression of surface IgM compared to the unresponsive cases; likewise, IgD (*P*=0.036), CD19 (*P*=0.029), CD38 (*P*=0.035), and CD43 (*P*=0.047) expression levels were also higher in responsive cases than in unresponsive cases (Figure 3A). No differences were found in CD20, CD21, CD27, CD69, CD80, CD86 and CXCR4 expression (*Online Supplementary Figure S1*).

To determine whether the α-IgM responsiveness within the responsive cases correlates with the expression level of these markers, we compared surface expression and relative response. The relative response did correlate with surface IgM (R^2=0.322, *P*=0.0038) and CD21 (R^2=0.469, *P*=0.0002) expression levels (Figure 3B).

IκB-related genes in particular are differentially expressed between B-cell receptor-responsive and -unresponsive cases

Twelve cases from our cohort were selected to evaluate cell-intrinsic differences based on RNA sequencing of total RNA from MACS-purified (>95%) CLL cells. Six patients were classified based on Ca^{2+} levels as responders upon α-IgM stimulation and were compared to another six patients who were unresponsive. (*Online Supplementary Table S3*) First, RNA expression profiles of the 12 cases were compared to each other via Spearman correlation (Figure 4A). Based on these results the patients could be divided into three major clusters, which did not correlate with BCR responsiveness or SHM status. In addition, when comparing the variation in total gene expression levels between the samples, as shown by Z-scores in a heat map (*Online Supplementary Figure S2*), no clear division of responsive and unresponsive cases was found either, probably reflecting the biological heterogeneity of CLL samples, even when classified as BCR-responsive and -unresponsive.

Next, we therefore focused on genes involved in BCR signaling using Qiagen's Ingenuity Pathway Analysis (IPA). As illustrated by the volcano plot, responsive cases demonstrated significantly higher expression of *EBF1*, *FCGR2A*, *SYK* and *FYN* (positive $\log_{FC}$ values), whereas the non-responders showed significantly higher expression of *NFKBID*, *NFKB2*, *CAM2KA*, *NFKBIE*, *RAF1*, *NFKBIB*, *NFKB1*, *RPS6K1*, *PLCG1* and *BCL3* (negative $\log_{FC}$ values) (Figure 4B and *Online Supplementary Figure S2*). Interestingly, the *NFKBIB*, *NFKBID* and *NFKBIE* genes all encode inhibitors of NF-κB (IκB), while *NFKB1*, *NFKB2* and *BCL3* are genes coding for NF-κB components that are associated with inhibition.[22]

B-cell receptor-unresponsive cases have higher expression of genes expressing regulatory molecules of nuclear factor-κB signaling

Additional samples were selected (n=13 unresponsive, n=15 responsive) to validate the differences in transcript levels of NF-κB genes (*NFKB1*, *NFKB2*, *BCL3*, *NFKBIB*, *NFKBID* and *NFKBIE*) using RQ-PCR. RQ-PCR results (displayed as $2^{-deltaCT}$ values) indeed confirmed that responding cases had significantly lower expression of *NFKB1* and *NFKB2* (Figure 5A) *NFKBIB* and *NFKBIE* (Figure 5B). Furthermore, we found a trend towards lower *NFKBID* expression, but no difference in *BCL3* expression between the subgroups (Figure 5B).

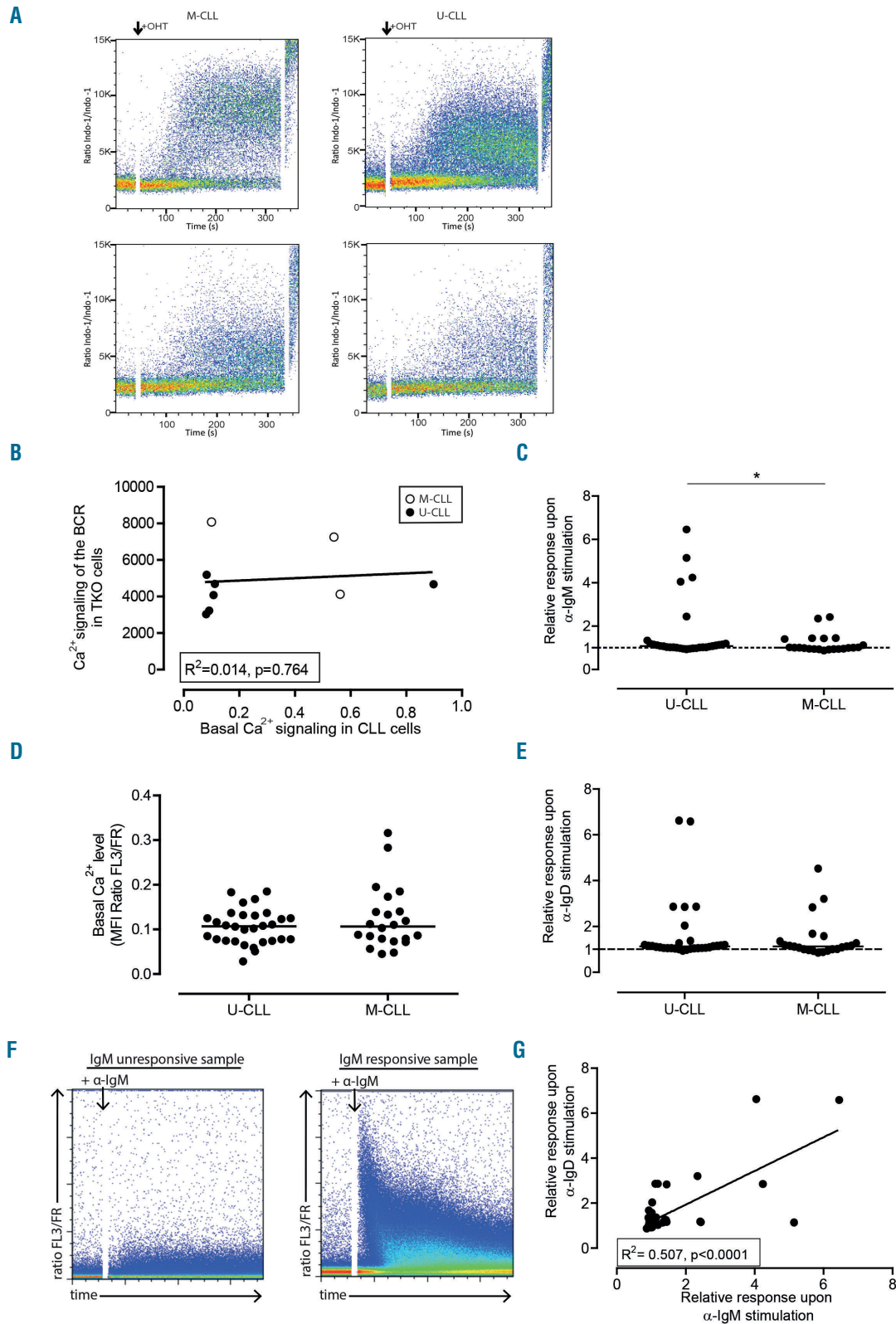In addition, we investigated whether the transcription-

Figure 1. Ca²⁺ signaling in chronic lymphocytic leukemia cells. (A) Flow cytometric analysis of Ca²⁺ flux (ratio Indo-1/Indo-1) after the addition of 4-hydroxytamoxifen (4-OHT) to triple knockout (TKO) cells expressing the B cell receptor (BCR) from two representative samples of mutated chronic lymphocytic leukemia (M-CLL) (left) and two unmutated samples (U-CLL) (right). (B) From nine CLL cases (6 U-CLL, black dots and 3 M-CLL, open dots) in whom the basal Ca²⁺ level (x-axis) had been assessed earlier, the BCR was cloned into TKO cells to determine the autonomous Ca²⁺ signal (y-axis). Linear regression was performed and the R² and *P*-value are shown. (C) Basal Ca²⁺ level [median fluorescence intensity (MFI) ratio FI3/FR] was determined in a new cohort of 52 CLL samples (freshly isolated) consisting of 30 U-CLL and 22 M-CLL cases. (D) Responsive capacity upon BCR stimulation. Flow cytometric analysis of a representative CLL sample showing no Ca²⁺ influx (ratio FL3/FR) upon α-IgM stimulation (left) and a representative CLL sample with an increase in Ca²⁺ influx (ratio FL3/FR) upon α-IgM stimulation (right). Based on this analysis the responsive capacity upon α-IgM stimulation (E) and α-IgD stimulation (F) was determined in the 30 U-CLL and 22 M-CLL cases. Individual plots and medians (gray bars) are shown. The Mann-Whitney U-test was performed for statistical analysis between the groups of patients (*P<0.05). (G) Linear regression analysis between the relative response after α-IgM and α-IgD stimulation: R² and *P* values are shown.
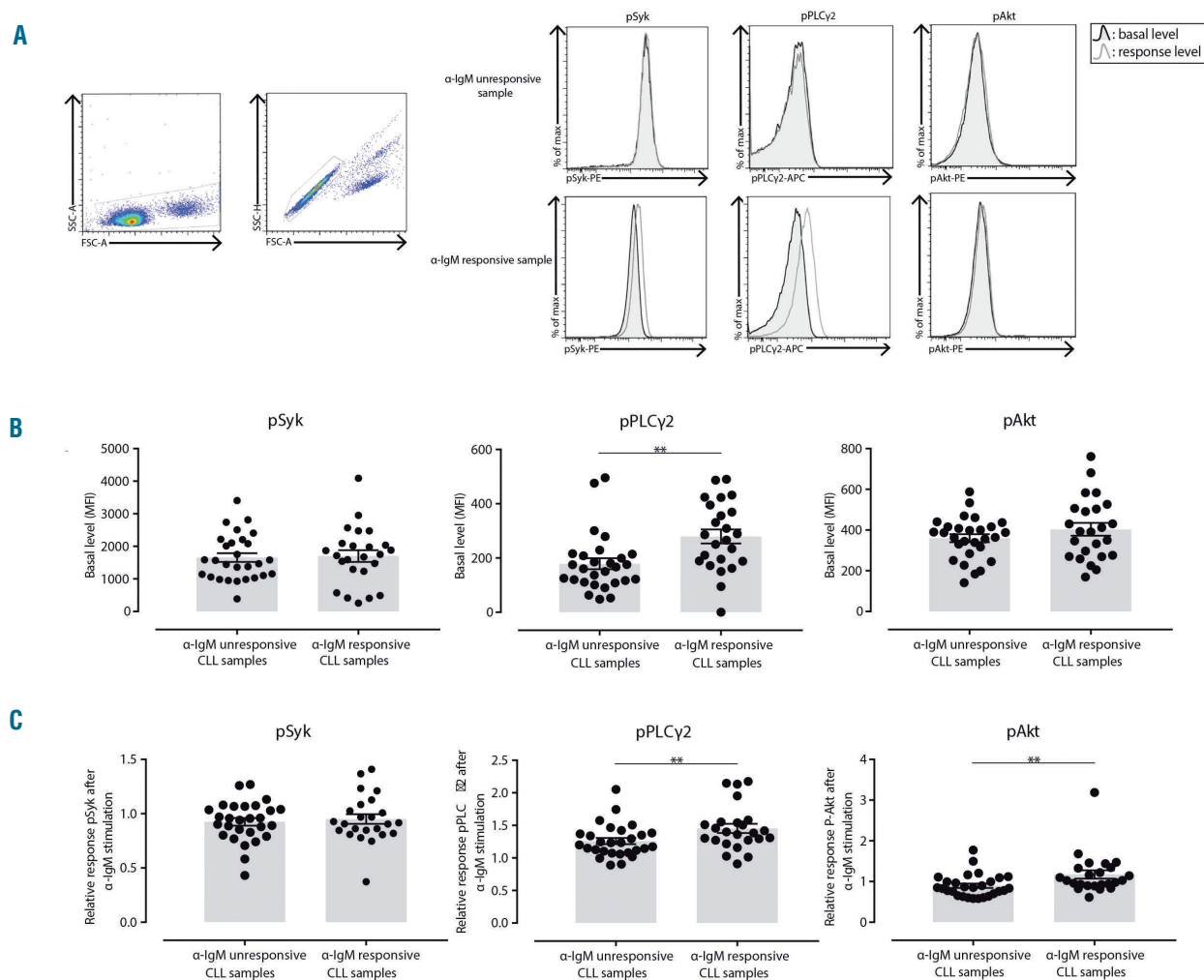
Figure 2. Phosphorylation of Syk, PLCγ2 and Akt. (A) The levels of pSyk, pPLCγ2 and pAkt were determined at baseline and upon stimulation with α-IgM and correlated with the α-IgM response as determined by Ca²⁺ signaling. Representative examples of the analysis in a case of unresponsive chronic lymphocytic leukemia (CLL) (upper histograms) and a responsive CLL case (lower histograms) are shown. After single viable cell selection, the levels of pSyk, pPLCγ2 and pAkt were determined, both at baseline (black line) and upon stimulation with α-IgM (gray line). (B) Differences in basal levels of pSYK, pPLCγ2 and pAKT (x-axis) between α-IgM unresponsive and responsive samples. (C) The relative response after α-IgM stimulation for pSYK, pPLCγ2 and pAKT (x-axis) in the two groups of patients. Individual plots and medians (gray bars) are shown. The Mann-Whitney U-test was performed for statistical analysis between the groups of patients (**P<0.01).

al levels of these NF-κB pathway genes also correlated with basal Ca²⁺ levels (*Online Supplementary Figure S3*). A significant correlation could only be found between basal Ca²⁺ levels and *NFKB1* (R²=0.163, *P*=0.033) and *NFKBIE* (R²=0.234, *P*=0.0091) transcript levels (*Online Supplementary Figure S3*).

Since loss of IκBε (encoded by *NFKBIE* as caused by an identical 4-bp frameshift deletion in the first exon), has been associated with a progressive form of CLL,[23] we determined whether patients in our cohort with low *NFKBIE* expression carried this identical deletion. Upon sequencing of the first exon of *NFKBIE*, this 4-bp deletion was not observed (*data not shown*).

Expression levels of genes coding for NF-κB regulators (*NFKB1* and *NFKB2*) and coding for IκB that were expressed at lower levels in responsive cases appeared to correlate with each other (*Online Supplementary Figure S4*), implying that unresponsive patients show higher expression of multiple NF-κB inhibitors. Even though we could

not detect statistically significant differences in expression levels of genes coding for the NF-κB subunits *RELA*, *RELB* and *REL* between the two subgroups (*data not shown*), we did observe clear correlations between expression levels of genes associated with NF-κB inhibition and expression levels of *RELA* and *REL* (*Online Supplementary Figure S5*), both involved in the canonical NF-κB. No correlations between inhibitor levels and levels of the non-canonical NF-κB subunit *RELB* were found (*data not shown*).

Besides the IκB genes, we also found a difference in expression of tumor necrosis factor-α induced protein 3 (*TNFAIP3*; log$_{FC}$=-1.70, 10log(Pvalue)=2.24) based on RNA sequencing analysis. *TNFAIP3* encodes for protein A20 that is induced by TNF-α and functions as a negative regulator through inhibition of NF-κB signaling.[24] In addition, RQ-PCR showed significantly (*P*=0.017) higher expression of *TNFAIP3* in unresponsive cases than in responsive ones (Figure 5C).
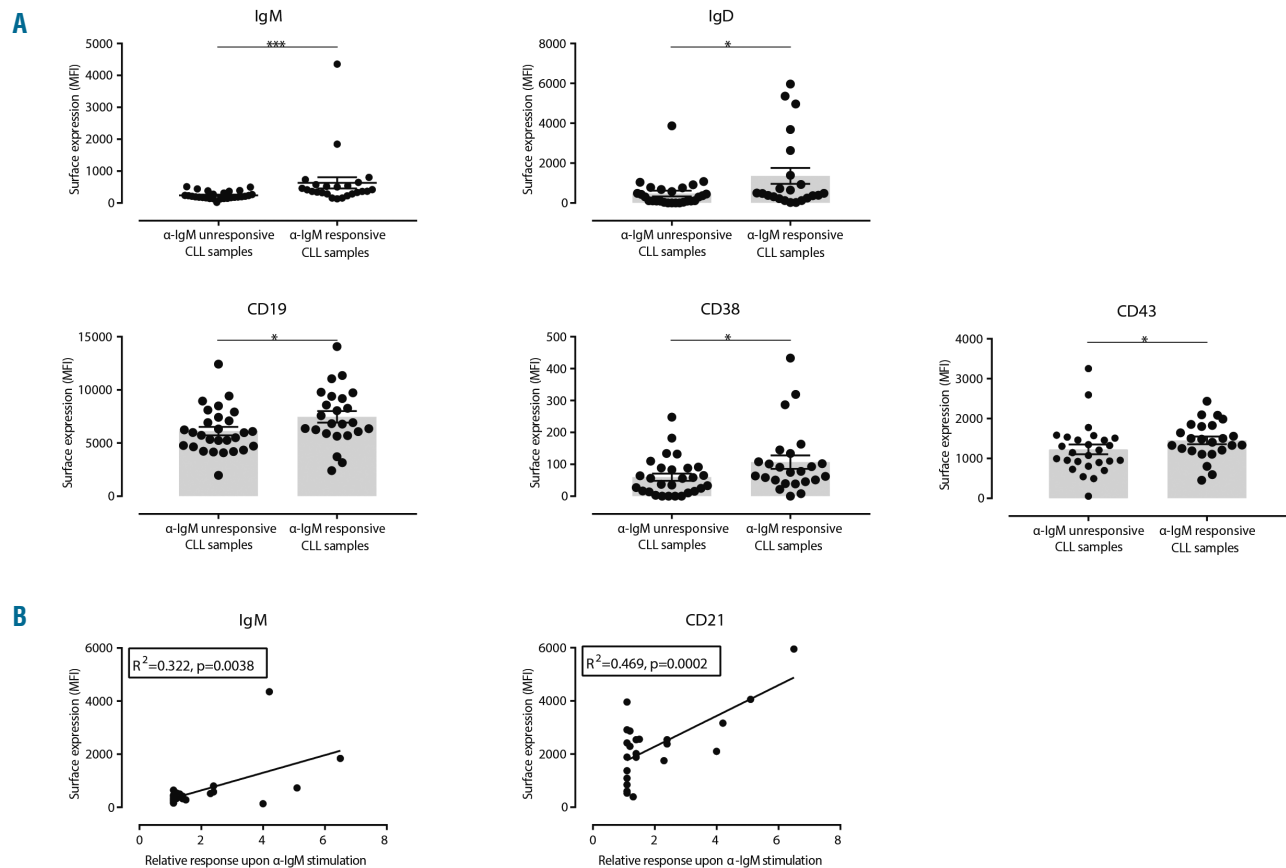
Figure 3. Surface expression of activation-associated markers. The surface expression of several activation-associated markers was measured in 52 patients with chronic lymphocytic leukemia (CLL). (A) Surface expression levels (x-axis) of IgM, IgD, CD19, CD38 and CD43 in α-IgM-unresponsive and α-IgM-responsive cases. Individual plots and medians (gray bars) are shown. The Mann-Whitney U-test was performed for statistical analysis between the groups of patients (*$P<0.05$). (B) Relative response upon α-IgM stimulation (x-axis) is plotted against the surface expression [median fluorescence intensity (MFI)] expression level of IgM and CD21. Linear regression analysis was performed and R^2 and P values are shown.

Collectively, these results illustrate that unresponsive cases have higher basal gene expression of several regulatory molecules of canonical NF-κB pathway signaling.

Upregulation of nuclear factor-κB pathway genes upon α-IgM stimulation in B-cell receptor-responsive cases

To further study expression of the NF-κB genes upon stimulation, frozen peripheral blood mononuclear cells from 21 cases (unresponsive CLL; n=11 and responsive CLL; n=10) were thawed, after which CLL cells were MACS-isolated and stimulated for 2.5 h with α-IgM (optimal stimulation was defined using normal B cells; *data not shown*). The $2^{-deltaCT}$ values obtained after incubation (α-IgM-stimulated and -unstimulated) were normalized by subtraction of the basal $2^{-deltaCT}$ value to calculate the fold differences in expression between the groups of patients (Figure 6). BCR-responsive cases showed significant upregulation of *NFKB2, REL, NFKBID, NFKBIE* and *TNFAIP3* after stimulation compared with unresponsive cases for which the expression of the NF-κB genes remained roughly equal.

In summary, α-IgM-unresponsive cases had high basal transcription of especially NF-κB inhibitory components, whereas the responsive cases showed clear upregulation of NF-κB inhibitory components, including *TNFAIP3* and NF-κB subunit *REL*, upon stimulation.

Discussion

Here we aimed to study cell-intrinsic differences between unresponsive and responsive CLL, which may underlie differences in Ca^{2+} levels upon α-IgM stimulation. Based on RNA sequencing analysis several components of the canonical NF-κB pathway, especially related to NF-κB inhibition, were expressed more highly in unresponsive cases. Besides these inhibitors, the TNFα-induced NF-κB inhibitor A20 was also significantly more highly expressed in the BCR-unresponsive cases. Lastly we showed that upon α-IgM stimulation, the expression of these NF-κB pathway genes (especially genes coding for NF-κB pathway inhibitors but also NF-κB component *REL*) is upregulated in BCR-responsive cases while for the unresponsive cases the transcriptional level did not change compared to basal levels, indicating that NF-κB signaling is an important pathway for CLL cells in their ability to respond upon BCR stimulation.

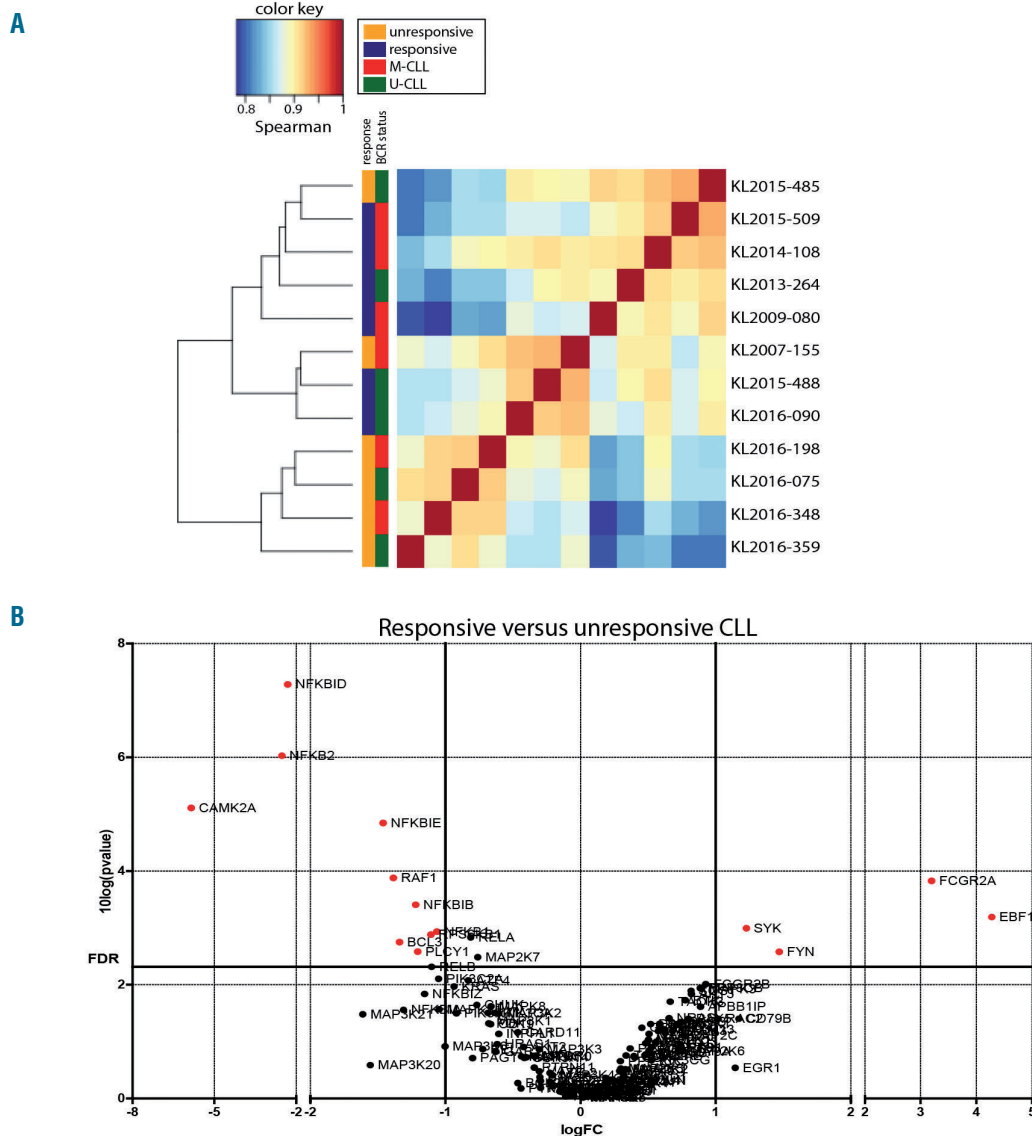Based on the lack of correlation between basal Ca^{2+} lev-

A

B

Figure 4. Differential expression analysis based on RNA sequencing data. (A) Results of the Spearman correlation of the RNA expression analysis in different chronic lymphocytic leukemia (CLL) samples (n=6 responsive *vs.* n=6 unresponsive CLL cases). The color scale indicates the degree of correlation varying from blue (low correlation) to red (high correlation). The two panels at the left end indicate the responsiveness (orange = unresponsive, blue = responsive) and the IGHV somatic hypermutation status [red=mutated (M)-CLL, green=unmutated (U)-CLL] of the selected CLL cases. (B) Volcano plot showing differences in transcript levels of genes involved in the B-cell receptor signaling pathway as determined using Qiagen's Ingenuity Pathway Analysis (IPA). A negative $\log_{FC}$ value indicates higher expression of certain genes in unresponsive CLL cases, while a positive $\log_{FC}$ value is indicative of higher expression in responsive CLL cases. The $\log_{FC}$ value was plotted against the $10\log(P\text{value})$. False discovery rate (FDR) was calculated and transcriptional differences of genes with a $\log_{FC}$ value of 1 or -1 in combination with a $10\log(P\text{value})$ above the FDR are indicated in red.

els and autonomous signaling in TKO cells,[14] we aimed to gain more insight into possible cell-intrinsic differences, although we cannot formally exclude that Ca^{2+} levels could also (partly) have been high due to previous antigenic stimulation in our *ex-vivo* samples. Using a new cohort, Ca^{2+} signaling was determined in freshly isolated cells instead of thawed cells, which on average resulted in lower basal Ca^{2+} levels (*data not shown*). This might be, in combination with the heterogeneity in basal Ca^{2+} levels, an underlying explanation for the fact that in this cohort the basal Ca^{2+} levels were not different between M-CLL and U-CLL cases. Further building on the study of Mockridge *et al.*,[18] who also showed differences in responsiveness to BCR stimulation between CLL cases,

we therefore divided our cohort of patients based on their responsive capacity to BCR stimulation. In both the M-CLL and U-CLL groups, there were cases showing a clear α-IgM response based on Ca^{2+} influx, while others did not show such a response, indicating that the level of anergy is independent of the IGHV SHM status of the BCR.

The anergic nature of unresponsive CLL was partly confirmed by the marker profile. IgM responders co-express higher levels of surface IgM and IgD, which explains the response to α-IgM as well as α-IgD stimulation. The higher expression of the prognostic marker CD38 by the responsive cases is also in line with findings of Mockridge *et al.*[18] suggesting that responsive patients in general have a poor prognosis.[2] The strong correlation
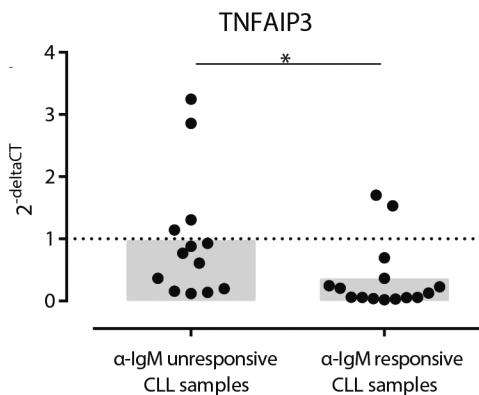
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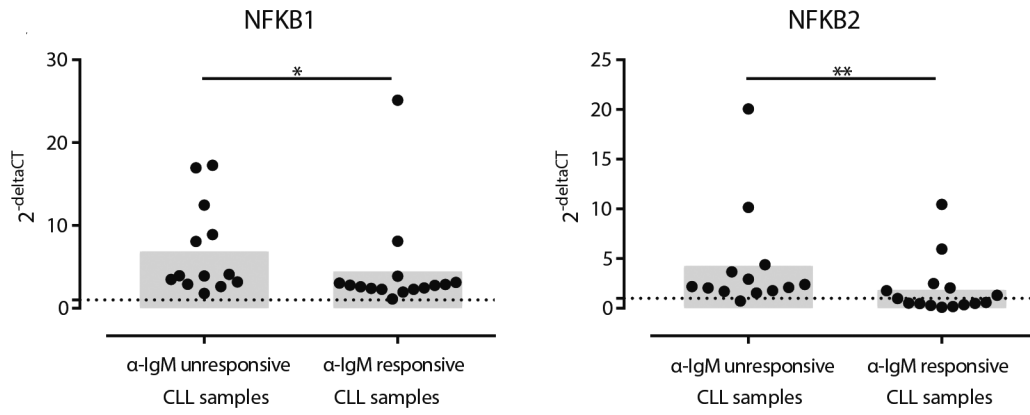
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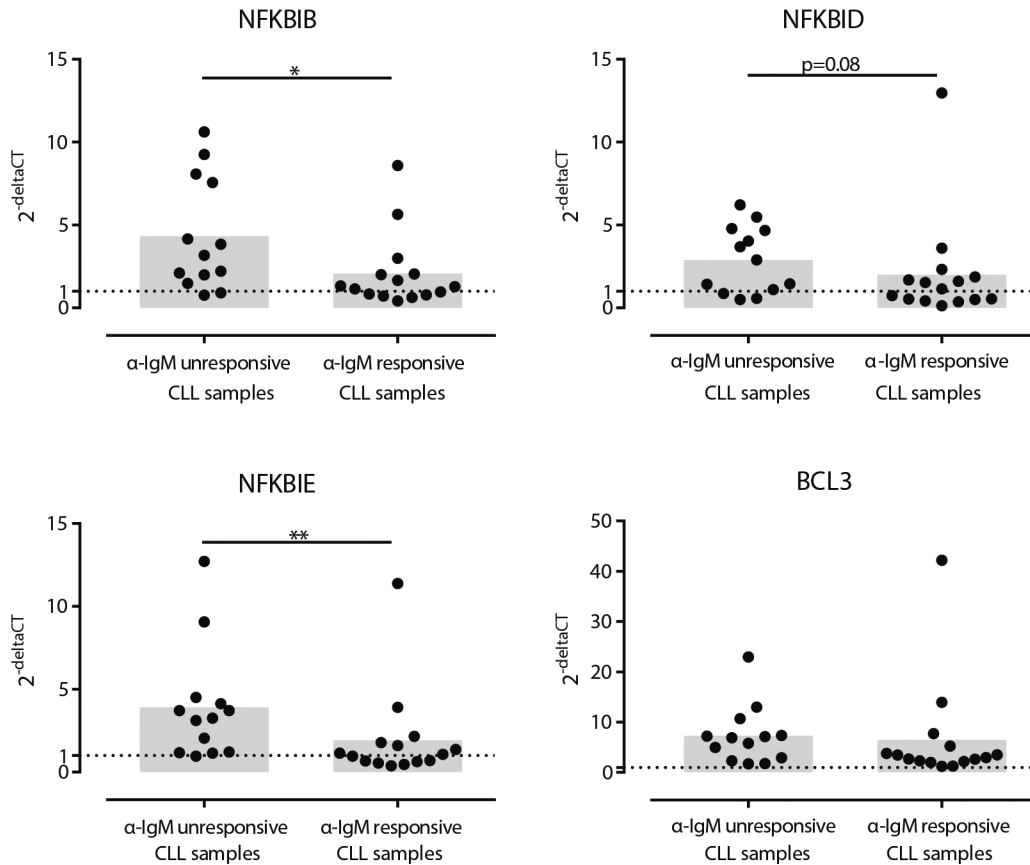
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Figure 5. Validation of transcriptional differences of nuclear factor-κB-related genes. (A-C) Real-time quantitative polymerase chain reaction validation of *NFKB1*, *NFKB2* (A), *NFKBIB*, *NFKBID*, *NFKBIE*, *BCL3* (B) and *TNFAIP3* (C) expression in an extended cohort of unresponsive (n=13) and responsive (n=15) cases of chronic lymphocytic leukemia (CLL). $2^{-deltaCT}$ values were determined for each sample and individual data plots and the medians are shown. The comparisons between the two groups were done using the Mann-Whitney U-test (*$P<0.05$, **$P<0.01$).
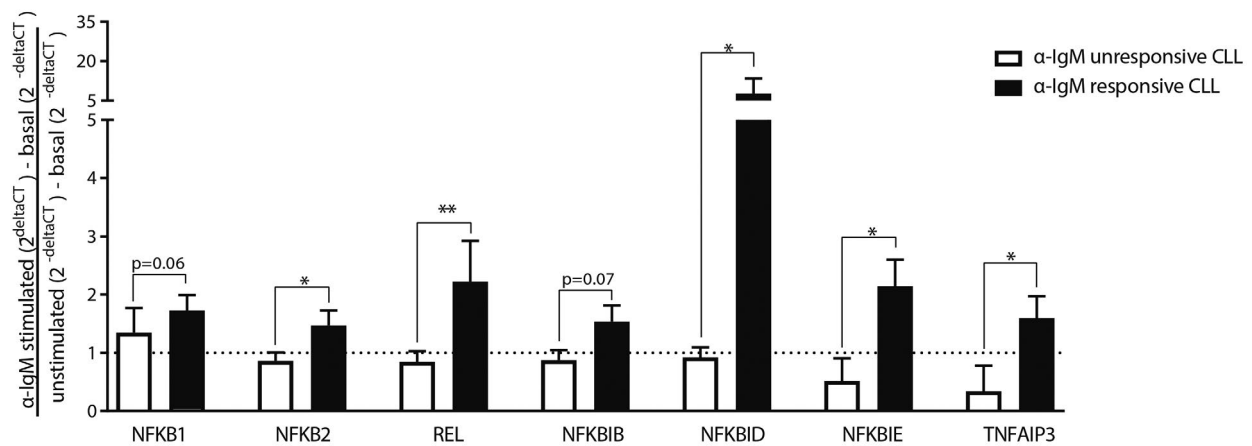
Figure 6. Transcriptional levels of nuclear factor-κB related genes upon α-IgM stimulation. Upon α-IgM stimulation, real-time quantitative polymerase chain reactions were performed to examine the transcriptional levels of multiple nuclear factor (NF)-κB pathway genes (*NFKB1*, *NFKB2*, *REL*, *NFKBIB*, *NFKBID*, *NFKBIE* and *TNFAIP3*) that were differently expressed at baseline between responsive and unresponsive cases of chronic lymphocytic leukemia (CLL). The $2^{-deltaCT}$ value upon α-IgM stimulation was subtracted from the $2^{-deltaCT}$ value at baseline and divided by the $2^{-deltaCT}$ value of the unstimulated condition corrected by the $2^{-deltaCT}$ value at baseline to calculate the net increase or decrease upon α-IgM stimulation. α-IgM unresponsive CLL cases (n=11, white bars) were compared to α-IgM responsive CLL cases (n=10, black bars) and statistical analysis was performed using the Mann-Whitney U-test (*$P<0.05$, **$P<0.01$).

between CD21 expression and the responsive capacity upon α-IgM stimulation is striking. In other immune-related diseases, such as rheumatoid arthritis, common variable immunodeficiency[25] and Sjögren syndrome,[26] patients had increased populations of CD21^{low} B cells compared to healthy individuals.[25] These CD21^{low} B cells were found to represent unresponsive cells expressing autoreactive BCR which failed to respond, as determined from Ca^{2+} levels upon BCR stimulation.[25,26] CD21^{low} CLL cells were not found to be autoreactive and are associated with a poor prognosis.[27] Unfortunately we had no access to patients' longitudinal data and we were therefore unable to evaluate progression of the CLL.

RNA sequencing analysis showed that especially genes coding for regulatory molecules involved in NF-κB inhibition are differentially expressed between BCR-responsive and -unresponsive cases. Several studies have shown that CLL cells have higher basal NF-κB levels compared to normal B cells and that they are continuously activated.[28] In addition, it has been shown that NF-κB signaling is important for preventing apoptosis by multiple mechanisms, including CD40L-mediated signaling.[28-30]

We found that the unresponsive cases had higher basal gene expression of several components of the canonical NF-κB pathway, especially those involved in inhibition. Genes coding for the p105/p50 (*NFKB1*) and p100/p52 (*NFKB2*) subunits were expressed more highly in unresponsive CLL. Both are potential inhibitors and allow functional NF-κB activation in which p105/p50 is involved in the canonical NF-κB pathway and p100/p52 in the alternative (non-canonical) NF-κB pathway.[31] In addition, we found that genes coding for IκB were more highly expressed in unresponsive cases. IκBε (coded by *NFKBIE*), which is an important regulator of B-cell proliferation,[32] was found to be mutated in patients with CLL.[23,33] In particular, a recurrent 4-basepair frameshift deletion resulting in functional loss of IκBε and leading to continuous NF-κB activation was detected in progressive forms of CLL [23] as well as in other B-cell malignancies.[34] However, we could not identify this identical deletion as

a possible cause for the lower *NFKBIE* gene expression in the responsive cases.

Besides BCR stimulation, the canonical NF-κB pathway can be activated by TNF receptor stimulation.[31] It might thus be that NF-κB signaling in BCR-unresponsive cases is more dependent on TNF-mediated activation. Higher *TNFAIP3* expression, a negative feedback regulator of NF-κB signaling induced by TNFα, as we noted in unresponsive cases, provides a basis for this theory. From B-cell lymphoma patients it is known that increased and sustained NF-κB activation of especially the proto-oncogene c-REL promotes TNFα-induced cell survival.[35] Through this feedback loop mechanism, secretion and uptake of TNFα might result in NF-κB-induced survival of (anergic) CLL cells, independently of BCR signaling. Foa *et al.*[36] reported that CLL cells continuously produce TNFα, especially cells from patients with an indolent form of the disease compared to patients with a progressive form.[36]

Genomic aberrations in the *TNFAIP3* gene resulting in the loss of A20 are linked with autoimmune disease with a humoral component as well as several B-cell lymphomas.[37] In B cells from aged mice it was demonstrated that selective loss of A20 increases the activation threshold and enhances proliferation and survival of B cells causing an inflammatory condition and inducing autoimmunity.[38] Such a loss of A20 caused by genetic aberrations of *TNAIP3* has not been associated with human CLL.[39]

Even though the focus of our study was mostly on those genes that were expressed at higher levels in unresponsive cases, multiple genes, including *SYK*, were found to be expressed more highly in responsive cases. Although *SYK* was differently expressed based on the RNA sequencing analysis between the two groups of patients in the extended cohort of patients with CLL, we did not find a difference in SYK protein level (by phospho-flow analysis; *data not shown*). Another gene of interest that emerged from our analysis is Early B-cell Factor 1 (*EBF1*), a transcription factor important in B-cell differentiation, which was expressed at higher levels by the responsive cases.[40] Seifert *et al.* had earlier shown that

EBF1 was significantly downregulated in patients with CLL compared to conventional B cells.[41] It was suggested that the low expression of *EBF1* might result in reduced levels of B-cell signaling and might contribute to an anergic phenotype of CLL cells.[41] Our results showing a lower level of *EBF1* transcripts in unresponsive cases would support this theory. Future studies are required to elucidate the importance of *EBF1* in CLL pathogenesis.

In summary, our results indicate that responsive CLL cases, irrespective of IGHV SHM status, have a more activated phenotype and reduced basal expression of several regulatory molecules of the canonical NF-κB pathway including those associated with NF-κB inhibition. Upon α-IgM stimulation these responsive cases showed upregulation of NF-κB, including NF-κB inhibitors, whereas transcriptional levels of NF-κB signaling pathway components remained unaltered in unresponsive cases. Our findings suggest that enhanced basal NF-κB inhibition may be strongly associated with a lower capacity of CLL cells to respond to BCR stimulation and the survival of anergic CLL cells.

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