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

Ruud W.J. Meijers,[1*] Alice F. Muggen,[1*] Leticia G. Leon, [1]Maaike de Bie,[1] Jacques J.M. van Dongen,[1,2] Rudi W. Hendriks[3#] and Anton W. Langerak[1#]

[1]Laboratory Medical Immunology, Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam; [2]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden and [3]Department of Pulmonary Medicine, Erasmus MC, Rotterdam, the Netherlands

**RWJM and AFM share equal responsibility and first authorship*

#RWH and AWL share equal responsibility and senior authorship

 doi:10.3324/haematol.2018.215566

Received: January 1, 2019.

Accepted: May 15, 2019.

Pre-published: May 16, 2019.

Correspondence: *ANTON W. LANGERAK* - a.langerak@erasmusmc.nl

Supplementary Methods

IGHV sequence analysis

Genomic DNA was extracted from PBMCs via spin-column kits and QIAcube (Qiagen, Valencia, CA, USA). Primers and protocols for IGHV mutation status analysis were according to the BIOMED-2 protocol and following ERIC guidelines. (1, 2) In brief, PCR products were analyzed by electrophoresis on polyacrylamide gels for monoclonality, followed by direct sequencing. Sequencing results were analyzed online by use of IMGT/V-QUEST (www.imgt.org).

Isolation of CLL cells

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll Hypaque (GE, Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. The obtained PBMC fractions were sorted via magnetic-activated cell sorting (MACS), using the B-CLL isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). For RNA sequencing, a minimum of $5x10^6$ isolated CLL cells was resuspended in RLT buffer (Qiagen) supplemented with 1% 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and stored at -80°C. To check purities, a small fraction of the isolated cells was stained with phycoerythrin (PE)-Cy7 labelled CD19, Allophycocyanin (APC)-H7 labelled CD3, together with APC-labelled CD5 (CLL cases only). All fractions had a purity of more than 95% $CD19^+$ cells.

Ca^{2+} flux assay

Intracellular Ca^{2+} fluxes were measured using Fluo-3AM and Fura Red-AM probes (both from Life Technologies, Carlsbad, CA, USA). Briefly, isolated CLL cells were incubated with 5 mM Fluo3-AM and 5 mM Fura Red-AM in loading buffer (Hank's balanced salt solution medium (HBSS) supplemented with 10 mM HEPES and 5% fetal calf serum) at 37°C for 30 min in the dark. Cells were washed and resuspended in 1 ml loading buffer. Before FACS measurements, cells were warmed to 37°C for 10 min. Basal intracellular Ca^{2+} levels were measured for 60 seconds, followed by stimulation of the BCR with 20 µg/ml anti-human IgM $F(Ab`)_2$ (Southern Biotech, Birmingham, USA) or with 20 µg/ml anti-human IgD $F(Ab`)_2$ (Southern Biotech). After stimulation cells were

acquired for 360 seconds on the LSRII flow cytometer (BD Biosciences, Erembodegem, Belgium). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

For Ca^{2+} influx analysis in TKO cells, 1×10^6 transduced TKO cells were loaded with Indo-1 (Invitrogen, Carlsbad, California, USA) with use of Pluronic (Invitrogen, Carlsbad, California, USA). Induction of ERT2–SLP65 to allow signaling was performed by the addition of 2 µM 4-hydroxy tamoxifen (4-OHT; Sigma-Aldrich, Saint Louis, Missouri, USA). As controls for BCR crosslinking 10 µg/ml Goat anti-mouse IgM $F(Ab`)_2$ (10 µg/ml; Southern Biotech) and 10 µg/ml Goat anti-mouse IgD $F(Ab`)_2$ (Southern Biotech) were used.

Phospho-flow cytometry

Isolated CLL cells with 20 µg/ml anti-human IgM $F(Ab`)_2$ (Southern Biotech) at 37°C for 1 minute to measure phosphorylation of Syk and PLCγ2. To measure phosphorylation of Akt, stimulation was done for 10 minutes. After stimulation, the cells were fixated for 10 minutes using cytofix (BD Biosciences). Before intracellular staining, cells were permeabilized by adding Perm III buffer (BD) at -20°C for 30 minutes. After washing, the cells were stained with either PE labelled pSyk (pY348; clone: I120-722), APC-Alexa fluor (AF)-647 labelled pPLCγ2 (pY759; clone: K86-689.37) or with PE-labelled pAkt (pY473; clone: M89-61). Data was acquired on the LSRII flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR, USA).

Gene mutation sequencing

To examine a previously reported 4-bp deletion in the first exon of the *NFKBIE* gene, as described by Mansouri et al. (3), this region was sequenced. (3) In brief, first DNA (10 ng/µl) was amplified by hot-start PCR (35 cycles, Tm=60°C) by addition of Gold buffer (10x; Thermo Fisher), $MgCl_2$ (25 mM; Applied Biosystems Life Technologies), dNTPs (20 mM; GE Healthcare), Taq Gold polymerase (5U/µl; Thermo Fisher), forward primer (10 pmol/µl; 5'-CCTCAAAAGTGGGCTGAG-3') and reverse primer (10 pmol/µl; 5'-CAAGGAACCACAGGAGAAGG-3'). Two µl of the amplified DNA was used and mixed with Big Dye® Terminator (BDT; Thermo Fisher), 5x sequencing buffer (Thermo Fisher) together with the forward and reverse primers (separately). After amplification, the sequence run was performed on an ABI Prism® 3130*xl* Genetic Analyzer (Thermo Fisher). Analysis was done using the program CLC Main Workbench 7 (Qiagen).

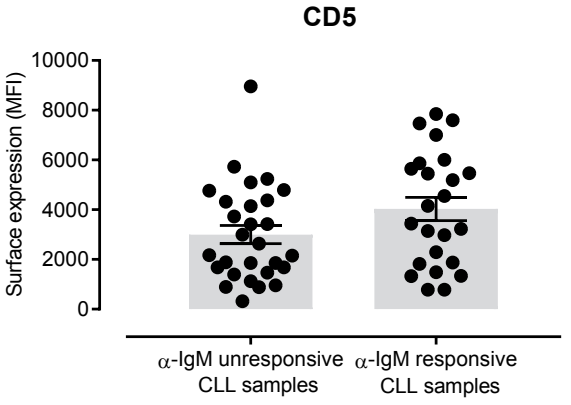
cDNA synthesis and Real-time Quantitative PCR (RQ-PCR)

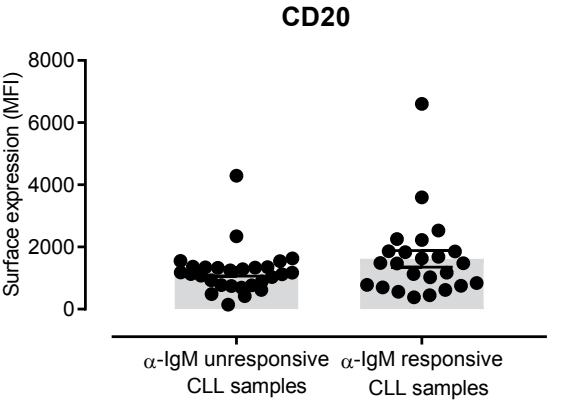
cDNA was synthesized from isolated RNA by adding reverse transcriptase Superscript II (Invitrogen Life Technologies, Waltham, MA, USA), 10x CA buffer (0.2 M Tris pH 8.3, 0.5 M KCl), dNTP (GE Healthcare, Cleveland, OH, USA), dithiotreitol (Invitrogen Life Technologies), $MgCl_2$ (Applied Biosystems Life Technologies), recombinant RNAsin (Promega, Fitchburg, WI, USA) and random primers (Invitrogen Life Technologies). The reaction mixture was incubated for 45 minutes at 42°C and the reaction was stopped by a 2 minute incubation at 99°C.

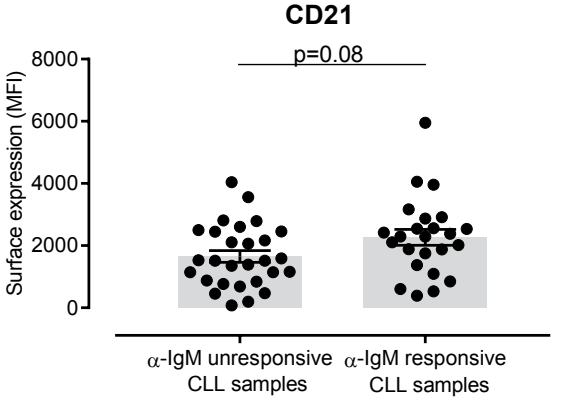
Real-time Quantitative PCR (RQ-PCR) assays were designed with the Roche Universal Probe Library (Roche, Basel, Switzerland) (Supplementary Table 4). TaqMan Universal PCR master mix (2x; Applied Biosystems) was used for the reaction mix and the run was performed on the StepOne Plus instrument (Thermo Fisher, Waltham, MA, USA). Ct values of the samples of each target gene were normalized to the Ct value of the ABL housekeeping gene (ΔCT) and the relative quantification ($2^{-\Delta CT}$) was performed. (4)

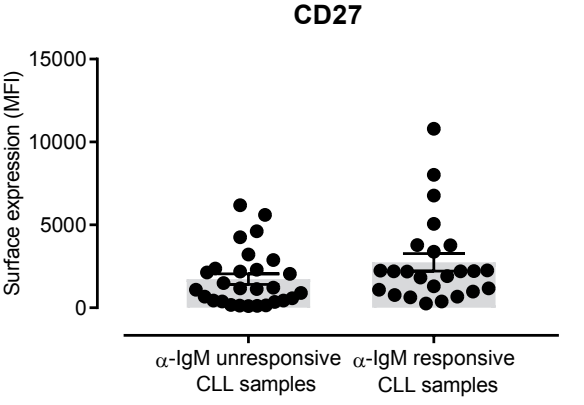
1. Langerak AW, Davi F, Ghia P, et al. Immunoglobulin sequence analysis and prognostication in CLL: guidelines from the ERIC review board for reliable interpretation of problematic cases. Leukemia. 2011 Jun;25(6):979-84.
2. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003 Dec;17(12):2257-317.
3. Mansouri L, Sutton LA, Ljungstrom V, et al. Functional loss of IkappaBepsilon leads to NF-kappaB deregulation in aggressive chronic lymphocytic leukemia. J Exp Med. 2015 Jun 1;212(6):833-43.
4. Beillard E, Pallisgaard N, van der Velden VH, et al. Evaluation of candidate control genes for diagnosis and residual disease detection in leukemic patients using 'real-time' quantitative reverse-transcriptase polymerase chain reaction (RQ-PCR) - a Europe against cancer program. Leukemia. 2003 Dec;17(12):2474-86.

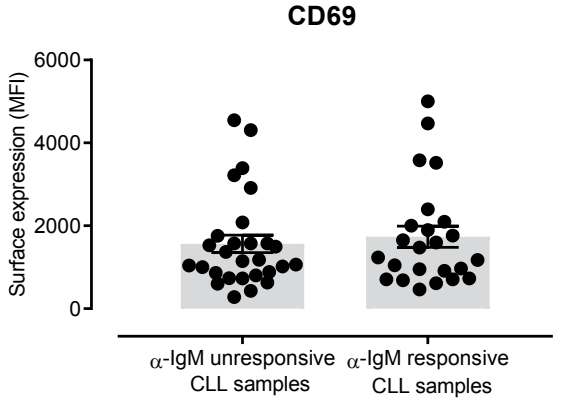
Supplementary Figure 1

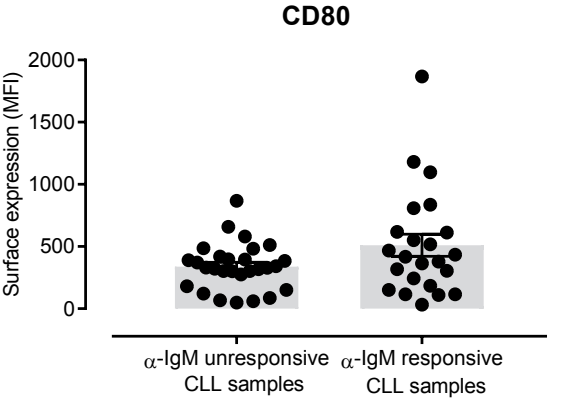


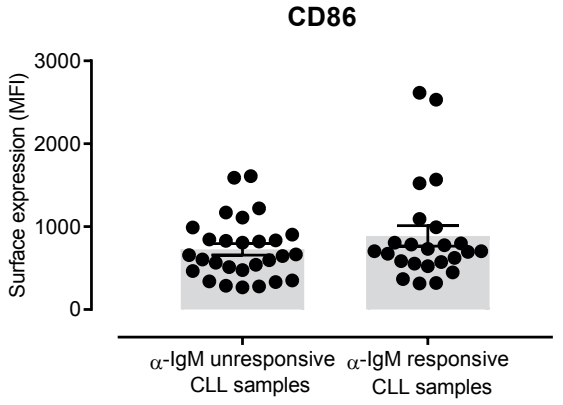


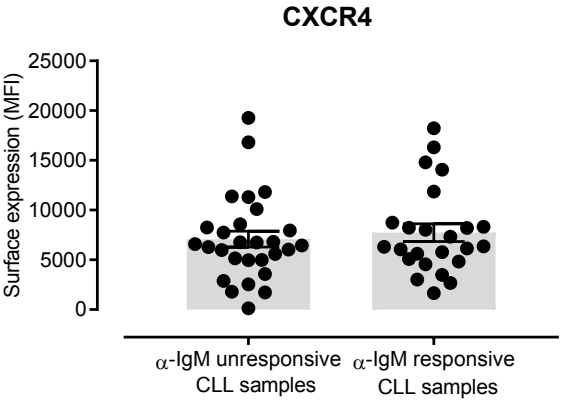






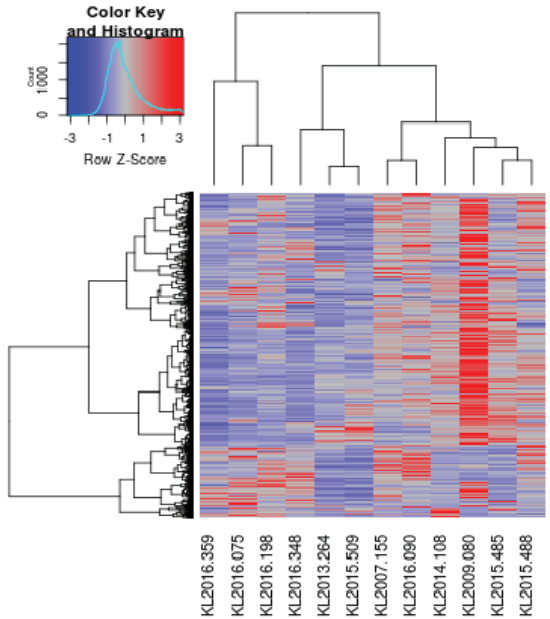




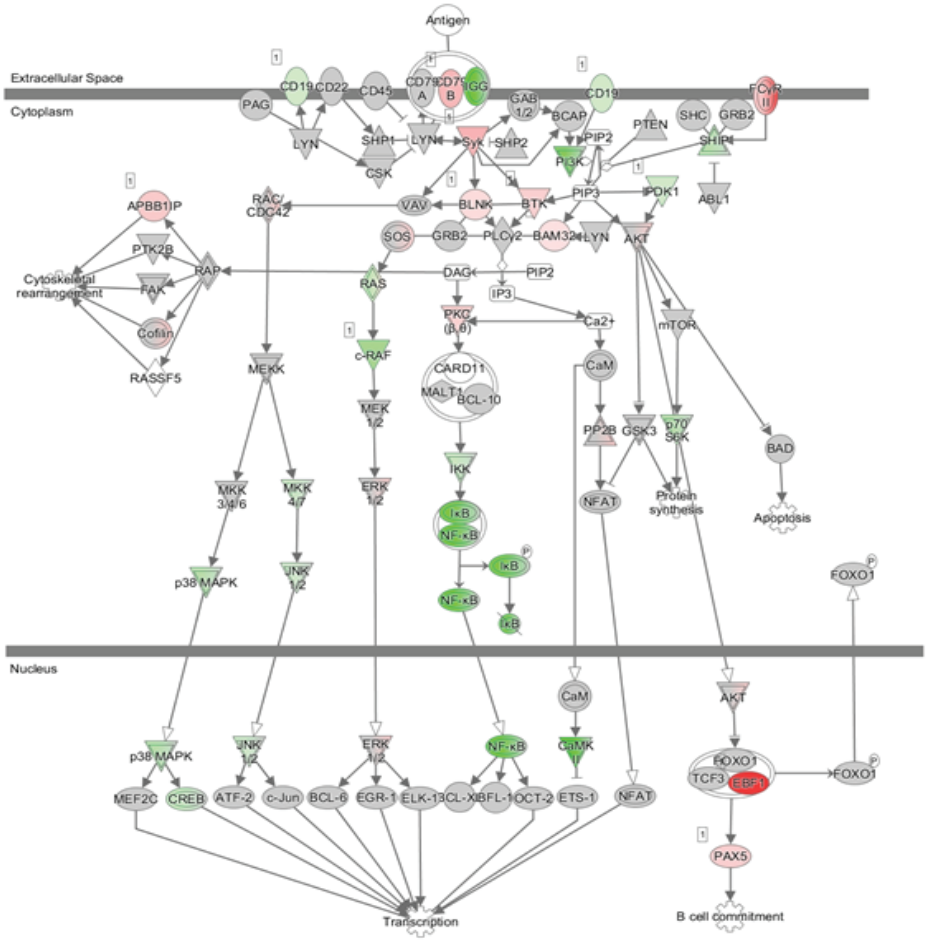


Supplementary Figure 2

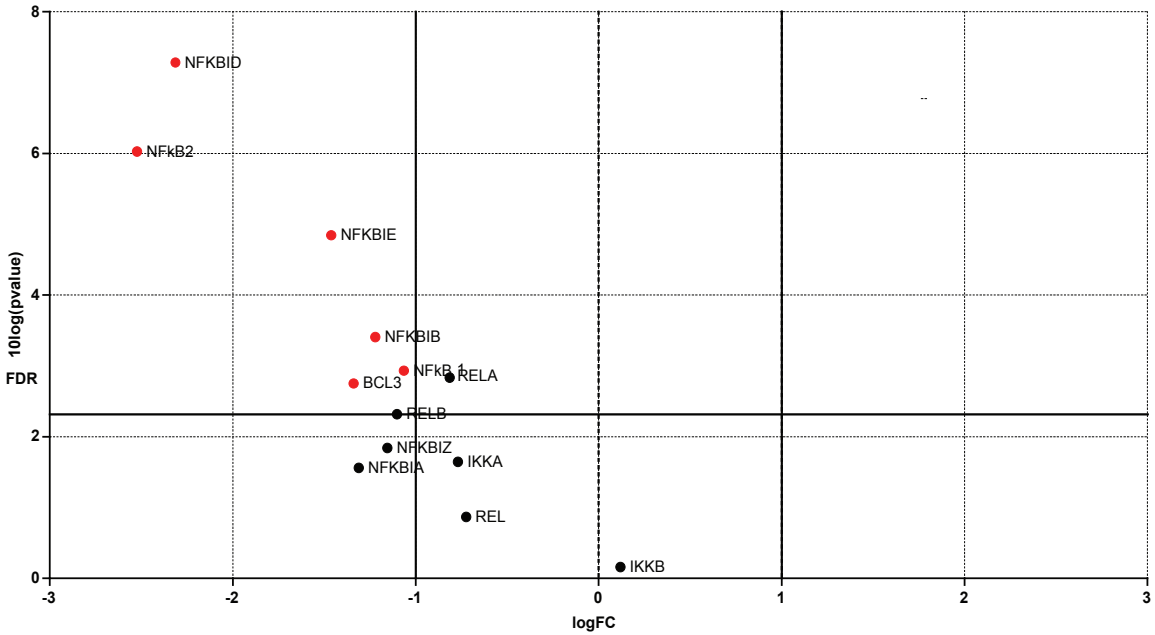
A.



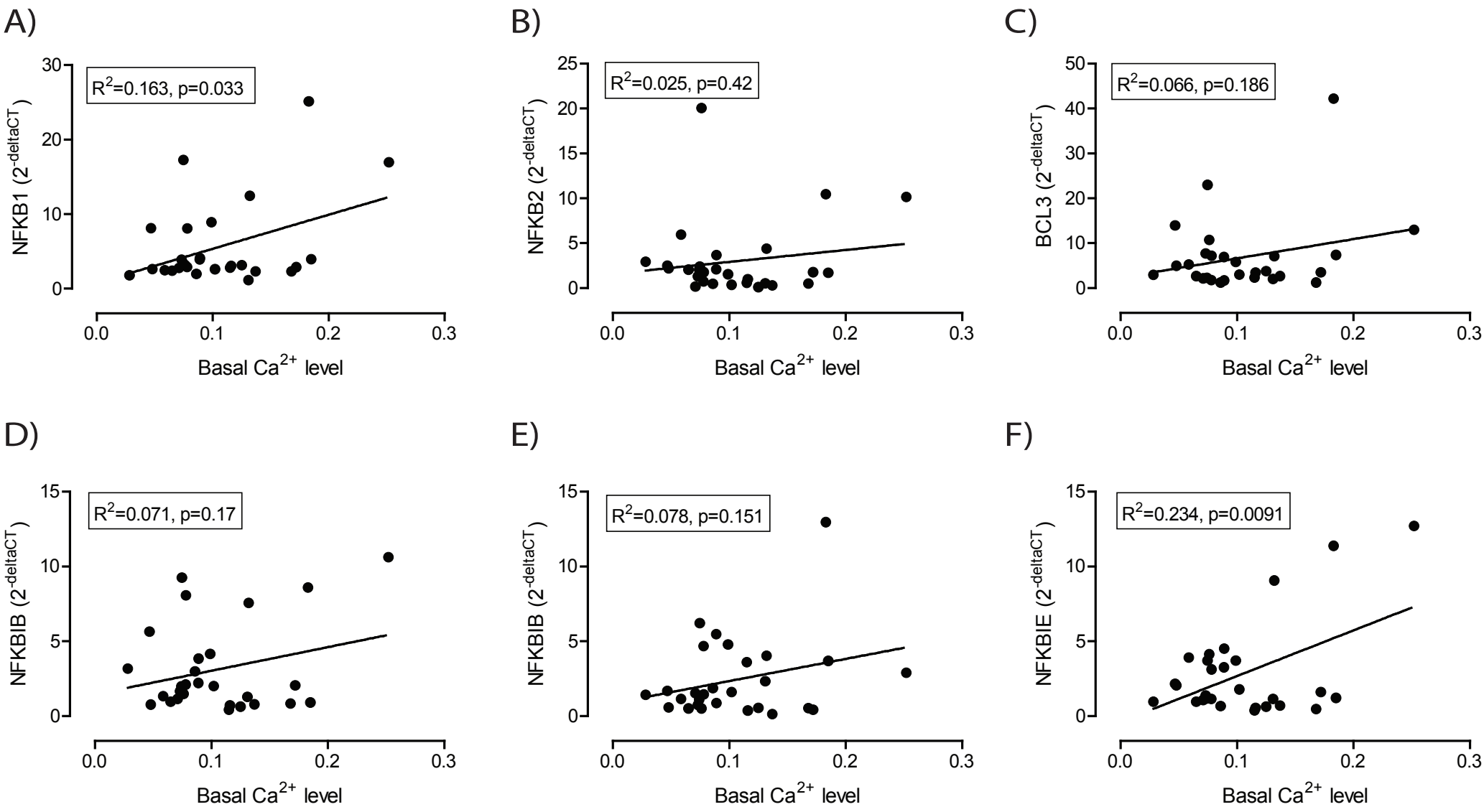
B.



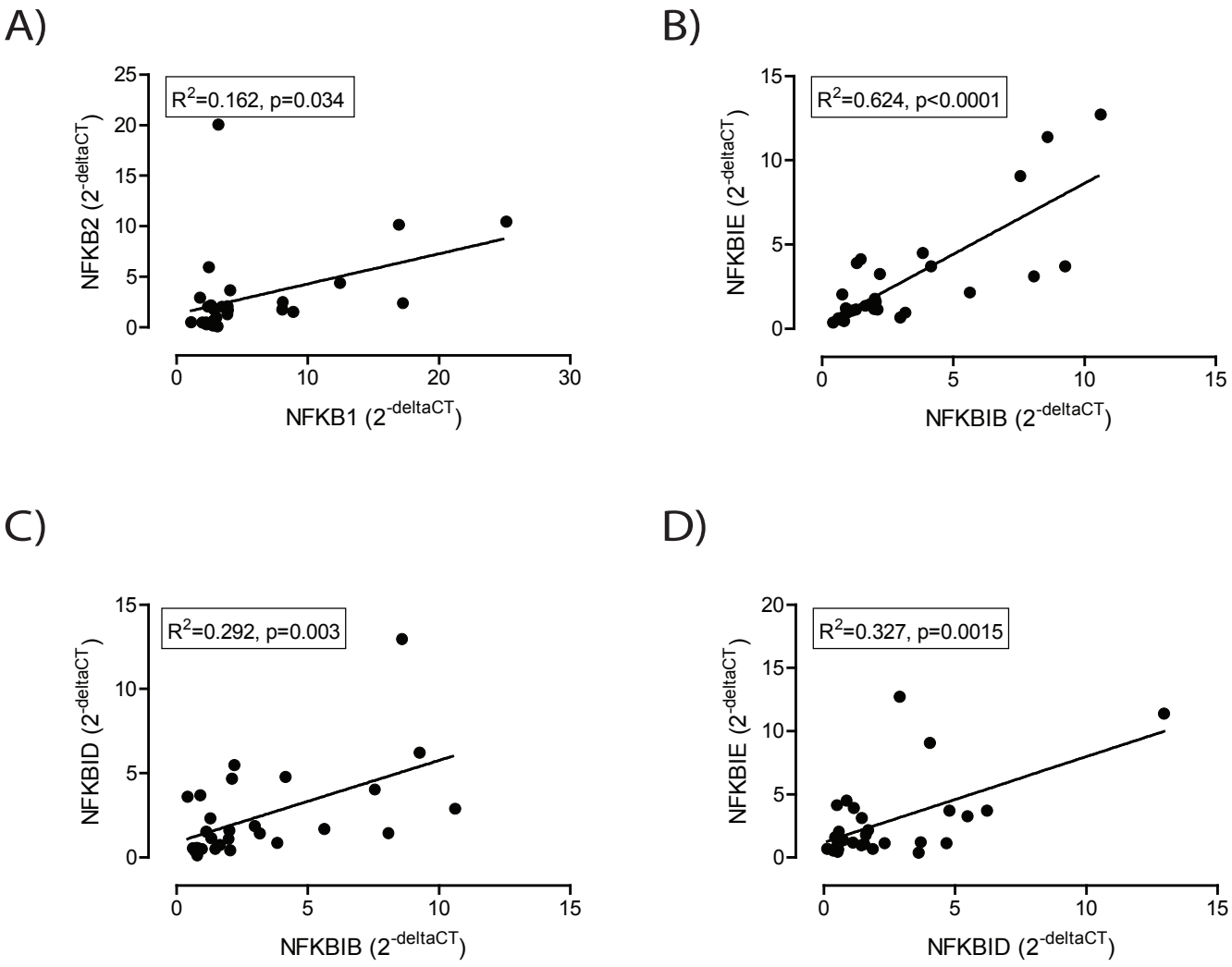
C.



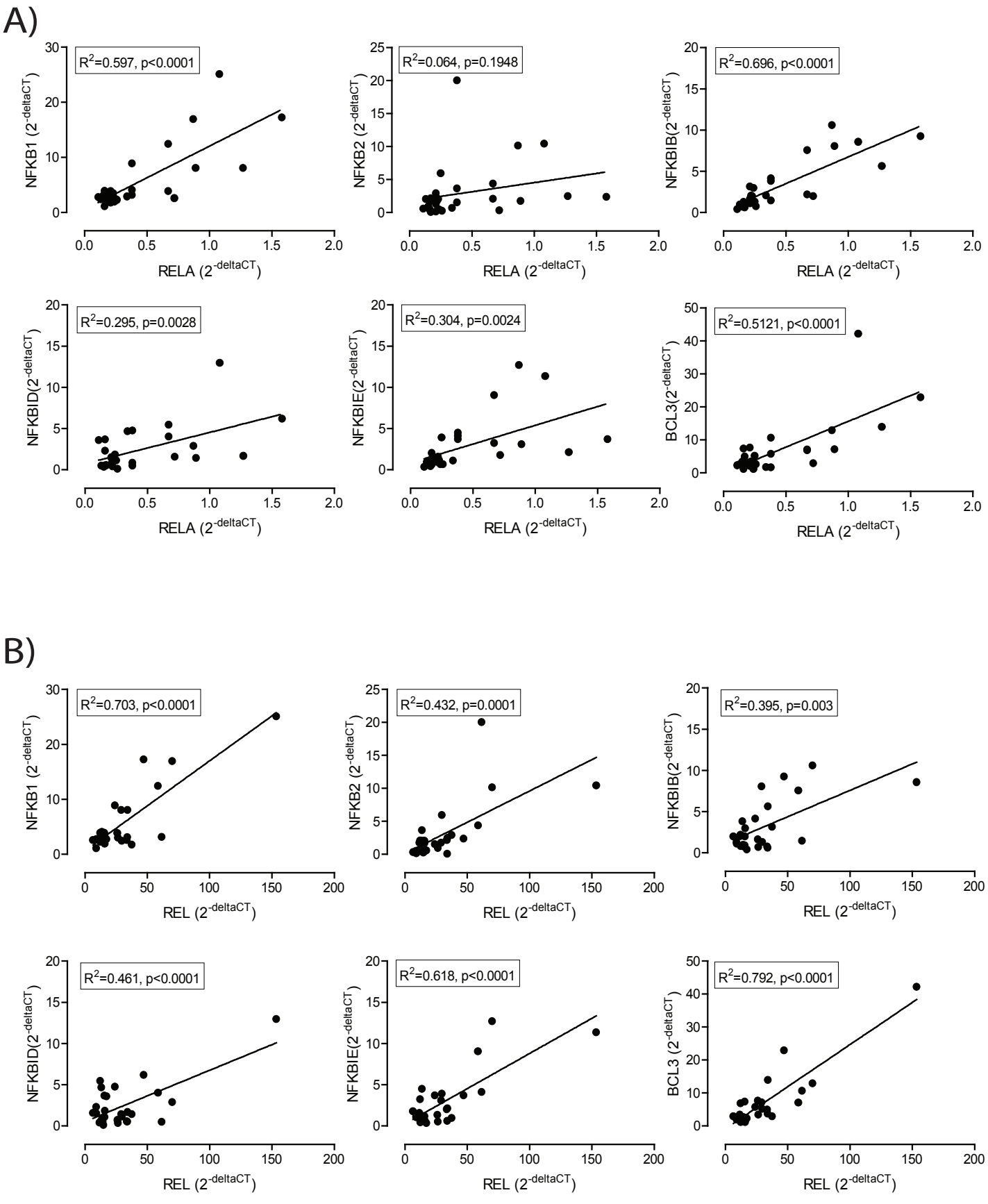
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table 1: Patient characteristics

Total number of CLL patients	52
- U-CLL	30 (58%)
- M-CLL	22 (42%)
Heterogeneous CLL	44 (85%)
Stereotypic CLL	8 (15%)
- U-CLL #1	2
- U-CLL #3	1
- U-CLL #5	1
- U-CLL #6	1
- U-CLL #64B	2
- M-CLL #2	1
Cytogenic abberations	
- none	9 (17%)
- trisomy 12	8 (15%)
- del 11q (including partial)	8 (15%)
- del 17p	5 (10%)
- del13q isolated	15 (29%)
- del13q combined	11 (21%)
- unknown	5 (10%)
Age in years	66.3 (38-88)[#]
Female	20 (38%)
Male	32 (62%)

= mean with range

Supplementary Table 2: Antibodies used for phenotyping

Antibody	Clone	Conjugate	Supplier
CD3	Sk7	APC-H7	BD Biosciences
CD5	UCHT2	APC	BD Biosciences
CD19	J3-119	PC7	Beckman Coulter
CD20	2H7	BV606	BioLegend
CD21	B-ly4	BV711	BD Biosciences
CD27	M-T271	BV421	BD Biosciences
CD38	HB7	AF750	BD Biosciences
CD43	IG10	FITC	BD Biosciences
CD69	FN50	BV711	BD Biosciences
CD80	L307.4	PE	BD Biosciences
CD86	HA5.2B7	PE	Beckman Coulter
CD184 (CXCR4)	12G5	APC	BD Biosciences
IgD	1A6-2	PE-CF594	BD Biosciences
IgM	MHM-88	BV-510	BD Biosciences

Supplementary Table 3. Selected CLL cases for RNA sequencing

	Patient ID	SHM status	α-IgM response (FL3/FR ratio)
Unresponsive CLL cases	KL2007-155	M-CLL	0.95
	KL2016-348	M-CLL	0.94
	KL2016-198	M-CLL	1.00
	KL2016-359	U-CLL	0.98
	KL2015-485	U-CLL	0.98
	KL2016-075	U-CLL	0.96
Responsive CLL cases	KL2014-108	M-CLL	1.40
	KL2009-080	M-CLL	1.43
	KL2015-509	M-CLL	1.44
	KL2016-090	U-CLL	5.15
	KL2013-264	U-CLL	6.46
	KL2015-488	U-CLL	4.24

Supplementary Table 4. Primers and probes from Roche Universal Probe Library for RQ-PCR design.

Gene	Sequence forward primer	Sequence reverse primer[1]	Probe[2]
NFKB1	CCTGCTCCTTCCAAAACACT	CGGTGTAGCCCATTTGTCTC	22
NFKB2	CTCGTGTCTGTCCACCTGTC	GAGCATCTGCGAGCATACAG	74
BCL3	ACATCCTGAGGGGGAAGG	TGATGCGGAGAGAAGACCA	38
NFKBIB	CCCTTCATTTGGCAGTGG	CTGCCCTCAGGAGAAGCTC	27
NFKBID	ATTTCTCCCTCCCCCAGA	GAACCCACAGTCTCCGAGTG	90
NFKBIE	TGCTGTGTACCGACTGAAGC	CCAGACTGGCTCTCTTCCAC	63
REL	TGAACATGGTAATTTGACGACTG	ACACGACAAATCCTTAATTCTGC	69
RELA	CTGGGAATCCAGTGTGTGAA	AAGGGGTTGTTGTTGGTCTG	1
RELB	GCTCTACTTGCTCTGCGACA	GGCCTGGGAGAAGTCAGC	79
TNFAIP3	AATTTCCTCCAGGTCACCTAAAC	GGGGACCTTGGTTCTAGCTT	68
TNFα	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	40

[1.] *Reverse complementary primers.*

[2.] *Probe numbers according to the Roche Universal Probe Library.*