SUPPLEMENTARY APPENDIX

Toll-like receptor 9 stimulation can induce IκBζ expression and IgM secretion in chronic lymphocytic leukemia cells

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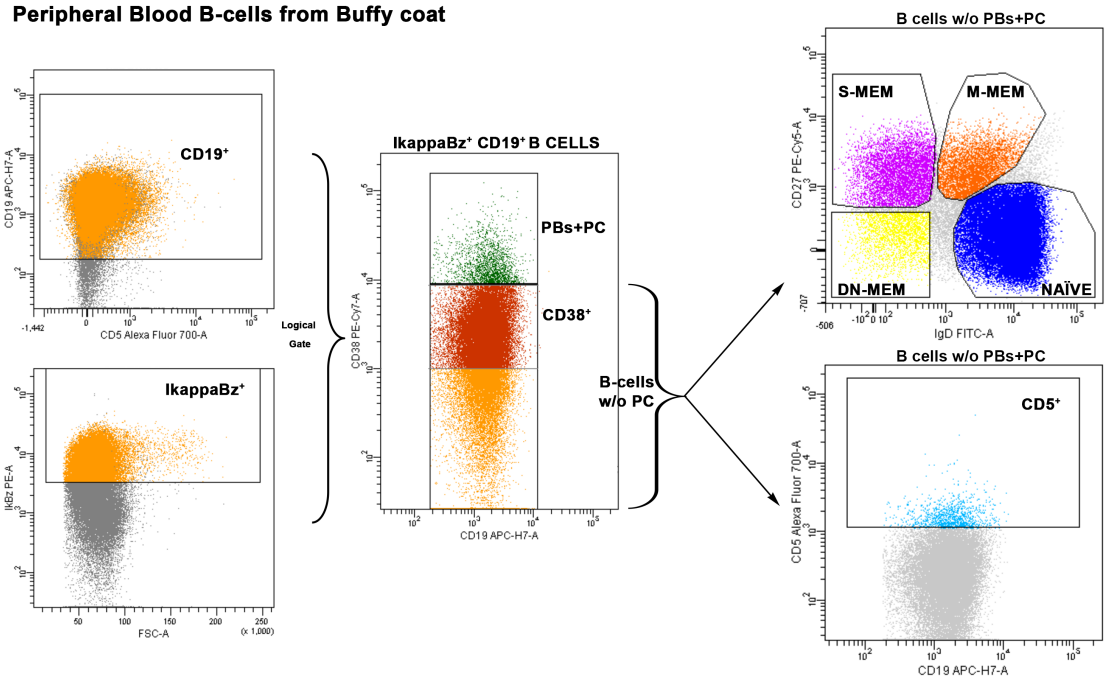
Supplementary Table 1

N°	IkBz expression (CpG-Ctrl)		Sex	Rai	Binet	Surface CD38 result	Progression result	IGHV identity
	(%)	MFI				(%)		(%)
1	16,3	2,06	F	0	A	72,35	progressive	96,94
2	72,5	3,47	M	0	A	36,40	stable	100,00
3	51,2	2,25	M	2	NA	1,00	progressive	93,15
4	56,6	2,69	M	1	A	50,81	stable	ND
5	49,3	2,73	M	1	A	97,04	stable	97,22
6	60,2	2,6	M	0	A	29,81	progressive	100,00
7	47,5	2,66	F	0	A	54,98	stable	100,00
8	47,3	2,66	F	0	A	4,00	stable	96,50
9	27,7	1,08	F	0	A	0,04	stable	88,54
10	6,0	0,33	M	0	A	0,61	stable	96,88
11	15,6	0,52	F	0	A	0,10	stable	ND
12	64,5	2,17	F	0	A	71,76	progressive	100,00
13	22,8	1,72	F	0	A	0,57	stable	92,36
14	36,7	0,72	M	0	A	19,10	stable	95,14
15	84,2	5,57	F	0	A	4,60	stable	100,00
16	49,2	2,66	F	NA	NA	0,64	progressive	92,98
17	45	3,25	F	0	A	42,42	stable	ND
18	1	0	F	NA	NA	NA	stable	87,72
19	9	1,3	M	0	A	0,07	stable	88,42
20	37,7	1,4	M	0	A	14,90	progressive	92,01
21	48,4	1,75	F	0	A	5,82	stable	100,00
22	14,1	1,13	F	0	A	0,00	stable	89,24
23	48	2,7	M	0	A	57,70	stable	100,00
24	29,7	0,44	F	0	A	93,40	stable	94,79
25	39,7	1,73	F	0	A	0,43	stable	100,00
26	13,6	0,5	M	0	A	0,02	stable	92,98
27	46,8	2,85	M	2	NA	0,40	progressive	96,14
28	25,2	1,23	M	1	A	91,64	stable	100,00
29	32,4	1,49	M	1	A	0,18	stable	91,23
30	32	1,27	M	0	A	0,22	stable	100,00
31	17,5	0,86	M	0	A	0,13	stable	92,71
32	22	1,08	M	0	A	0,50	stable	Troublesome
33	61,2	3,08	F	0	A	27,40	progressive	100,00
34	8	0,92	M	0	A	0,30	stable	NA
35	42	4,28	F	1	B	80,68	stable	92,98
36	78,7	4,33	M	2	B	NA	stable	100,00
37	43,1	1,67	F	0	A	0,58	stable	96,53
38	4,3	0,33	M	0	A	0,16	stable	92,10
39	1,3	0,2	F	0	A	10,42	stable	91,67
40	13,4	0,47	F	0	A	1,30	stable	94,79
41	20,6	0,97	M	0	A	1,20	stable	100,00
42	25,9	1,39	M	1	A	17,72	stable	97,57
43	9,4	0,6	F	1	B	0,08	progressive	90,48
44	19,2	1,27	M	0	A	0,11	stable	93,68
45	14,6	0,54	M	0	A	1,90	progressive	100,00
46	0,1	-0,21	M	NA	NA	NA	progressive	100,00
47	5,6	0,51	M	0	A	0,02	stable	92,98
48	56,5	4,44	M	0	A	69,30	Stable	100,00

49	19,9	2	M	0	A	0,00	Stable	95,09
50	0,4	0,2	F	0	A	1,10	Stable	NA
51	20,1	1,1	F	0	A	3,00	progressive	88,80
52	27,8	1,8	F	0	A	0,40	Stable	93,75
53	13,9	0,7	F	0	A	17,80	stable	99,66
54	6,6	0,67	M	0	A	2,60	stable	93,70
55	1,5	0,2	M	1	A	0,20	stable	NA
56	26,5	1,8	F	0	A	9,30	progressive	98,64
57	29,8	1,6	F	2	A	26,30	stable	100,00
58	16,2	0,7	F	4	C	97,41	progressive	NA
59	47,1	2,5	M	1	A	49,60	progressive	100,00
60	30,8	1,5	M	1	B	0,90	progressive	100,00
61	6,1	0,6	F	0	A	0,90	stable	NA
62	2,2	0,7	M	0	A	0,04	stable	NA
63	0,2	0,58	M	0	A	0,10	stable	91,67
64	1,6	0,1	M	0	A	2,30	stable	92,28
65	13,7	1	M	NA	NA	19,10	progressive	100,00
66	21,3	0,8	M	NA	NA	3,79	stable	90,28
67	14,1	0,5	F	0	A	0,16	stable	94,39
68	2,8	0	M	NA	NA	75,40	progressive	NA
69	59,3	3,7	M	0	A	11,00	stable	NA
70	9,3	-0,5	M	1	A	1,14	stable	93,40
71	3	0,1	F	0	A	0,58	stable	93,40
72	68,2	4,17	F	NA	A	59,70	progressive	100,00
73	13,4	0,8	M	0	A	0,40	stable	91,67
74	13,6	0,8	M	0	A	3,43	stable	92,71
75	14	1,3	M	0	A	0,24	stable	92,71

NA: not available

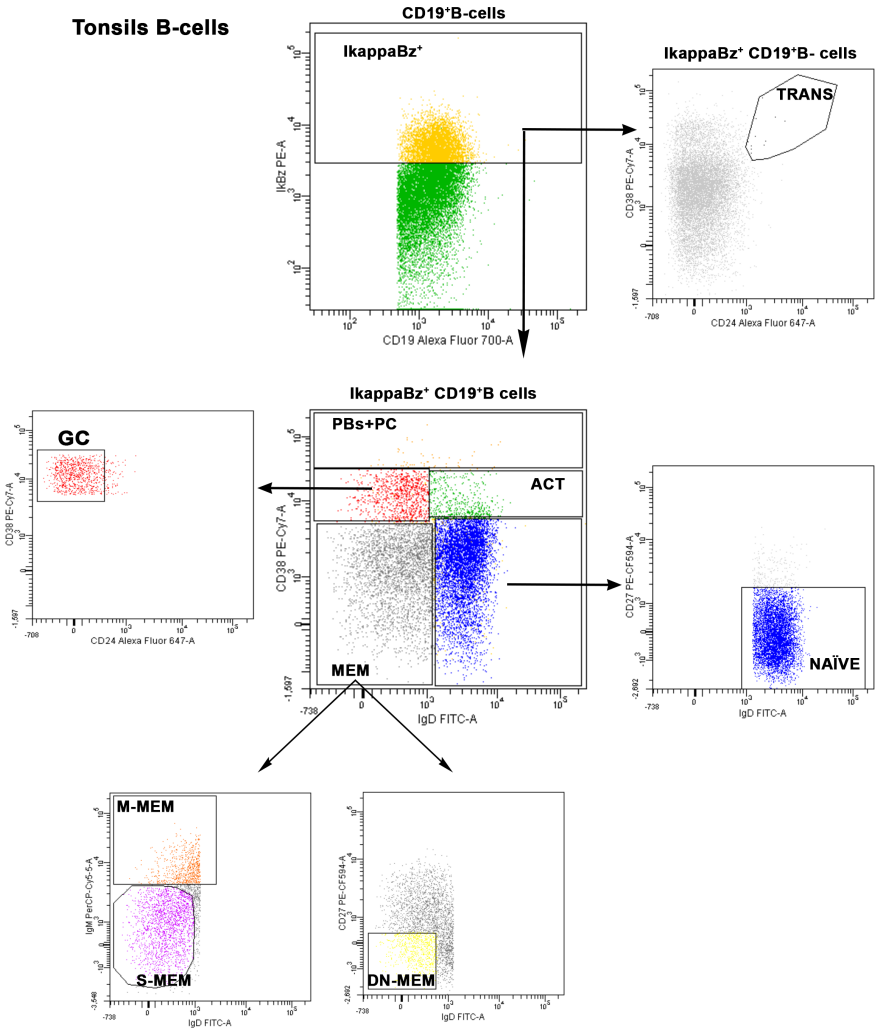
Supplementary Figure 1



Supplementary Figure 1. Multiparametric flow cytometry analysis of peripheral blood B cells subpopulations from buffy coats.

A representative buffy coat sample (#3) in which enriched B cells were stimulated 4-hours with CpG and stained for CD19, CD38, IgM, IgD, CD24, CD27, CD5 surface markers and IkappaBzeta intracellular marker. A logical gate was first drawn from $CD19^{+}$ and IkappaBzeta gates (IkappaBzeta-positive B-cells were gated using unstimulated cells as negative control). Distribution of IkappaBzeta in the different B cells subsets was then analyzed. Plasmablasts and plasma cells (PBs+PC) were identified by gating $CD38^{bright}$ cells and CD38-positive B-cells ($CD38^{+}$) by gating $CD19^{+}$ B cells with CD38 intermediate expression; from gate on $CD19^{+}$ B cells excluding PC were identified naïve B cells (IgD^{bright} $CD27^{-}$), IgM memory (M-MEM) B cells ($IgD^{-/low}$ $CD27^{+}$), switched memory B cells (S-MEM) ($IgD^{-}CD27^{+}$), double negative memory ($IgD^{-}CD27^{-}$) (DN-MEM) and CD5-positive (CD5+) B cells. Total memory (MEM) B cells ($IgD^{-/low}CD27^{-/+}$) were identified by a logical gate which comprises M-MEM, S-MEM, and DN-MEM subsets.

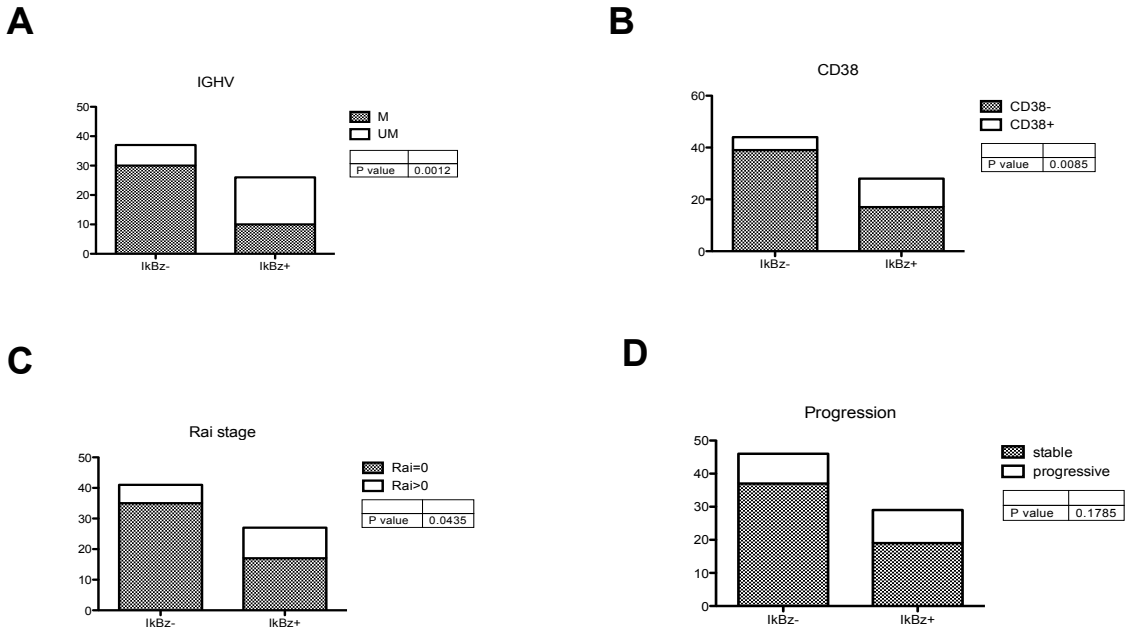
Supplementary Figure 2



Supplementary Figure 2. Multiparametric flow cytometry analysis of tonsils B cells subpopulations.

A representative tonsil sample (#16) in which purified B cells were stimulated 4-hours with CpG and stained for CD19, CD38, IgM, IgD, CD24, CD27, surface markers and IkappaBzeta intracellular marker. First B cells were gated by CD19, a characteristic mature B cells marker and subsequently IkBz-positive B-cells were gated using unstimulated cells as negative control. Distribution of IkappaBzeta in the different B cells subsets was then analyzed identifying transitional B cells (TRANS) by gating $CD38^{+}CD24^{+}$ cells, Plasmablasts and plasma cells (PBs +PC) by gating $CD38^{bright}$ cells, memory (MEM) B cells by gating $IgD^{-/low}CD38^{-}$ cells, activated B cells by gating $IgD^{-}CD38^{+}$, germinal center (GC) B cells first by gating $IgD^{-}CD38^{+}$ and then $CD24^{-}$ cells, naïve B cells first by gating $IgD^{bright}CD38^{-}$ and then $CD27^{-}$ cells. Memory B-cell subsets were obtained from MEM subset by gating $IgM^{bright}IgD^{-/low}CD27^{+}$cells (IgM memory, M-MEM), $IgM^{-}IgD^{-}CD27^{+}$cells (switched memory, S-MEM) and $IgD^{-}CD27^{-}$ cells double negative memory (DN-MEM).

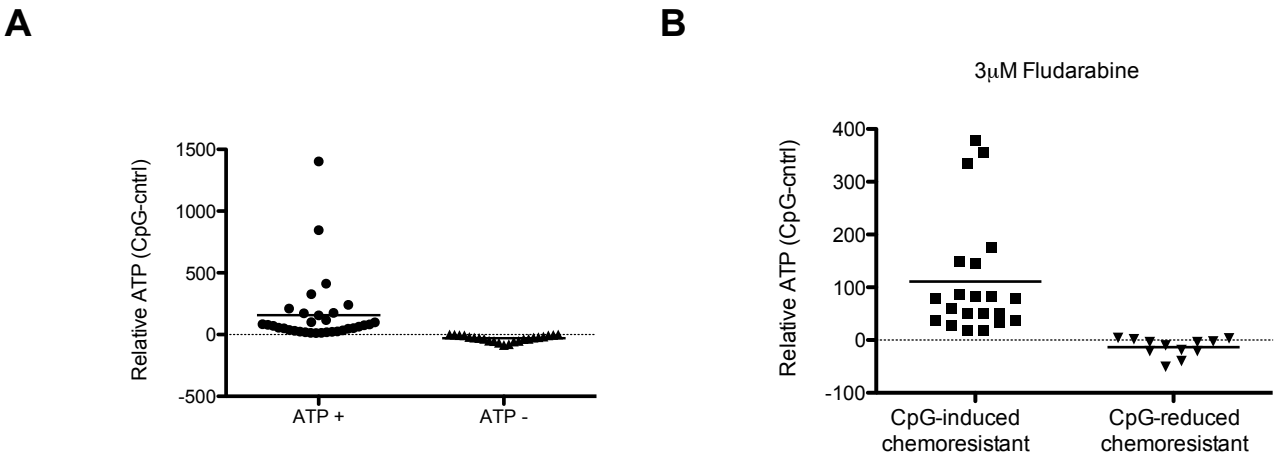
Supplementary Figure 3



Supplementary Figure 3. Comparison of IkappaBzeta positive and IkappaBzeta negative CLL cases among different groups of patients.

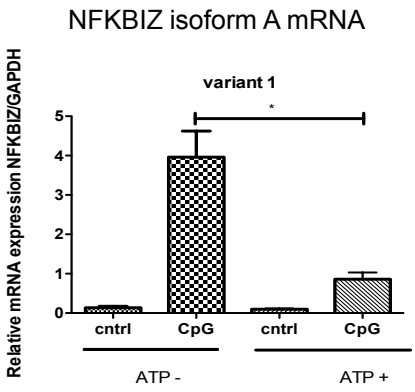
Based on a cut-off of 29.75% of increase of IkBz positive cells after CpG treatment, each CLL case was assigned as IkappaBzeta-positive or IkappaBzeta-negative. Fisher's exact test shows a significant increase of IkappaBzeta-positive cases among Unmutated IGHV (UM in **panel A**) and CD38 positive (**panel B**) patients. A decrease of IkappaBzeta-positive cases was observed in patients with a Rai disease stage=0 (**panel C**), but no difference was observed among stable and progressive disease (**panel D**).

Supplementary Figure 4



Supplementary Figure 4. Classification of "ATP+" and "ATP-" cases.
Metabolic cell activation was measured by using CellTiter-Glo Luminescent Cell Viability Assay before and after CpG treatment of the cells; increase over untreated cells was calculated for each case. Based on the relative ATP increase, 33 cases was assigned as "ATP+" and 23 as "ATP-" (**panel A**). Treatment of the cells with 3 mM Fludarabine before the measurement of metabolic cell activation allowed to assign CLL cases as "CpG-induced chemoresistant" (n=21) or "CpG-reduced chemoresistant" (n=12) (**panel B**) as previously described (Fonte et al Clin Cancer Res. 2013 Jan 15;19(2):367-79).

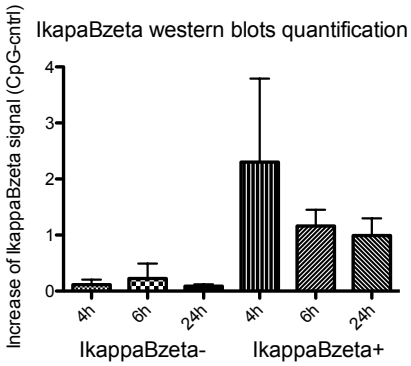
Supplementary Figure 5



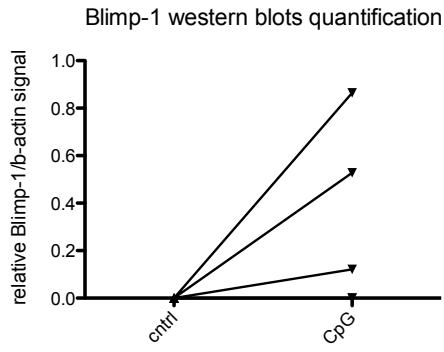
Supplementary Figure 5. Long isoform of NFKBIZ.
Real time PCR analysis of the long isoform of NFKBIZ mRNA (isoform A; 4 samples analyzed for each group); samples were incubated for 4 hours with or without CpG.

Supplementary Figure 6

A

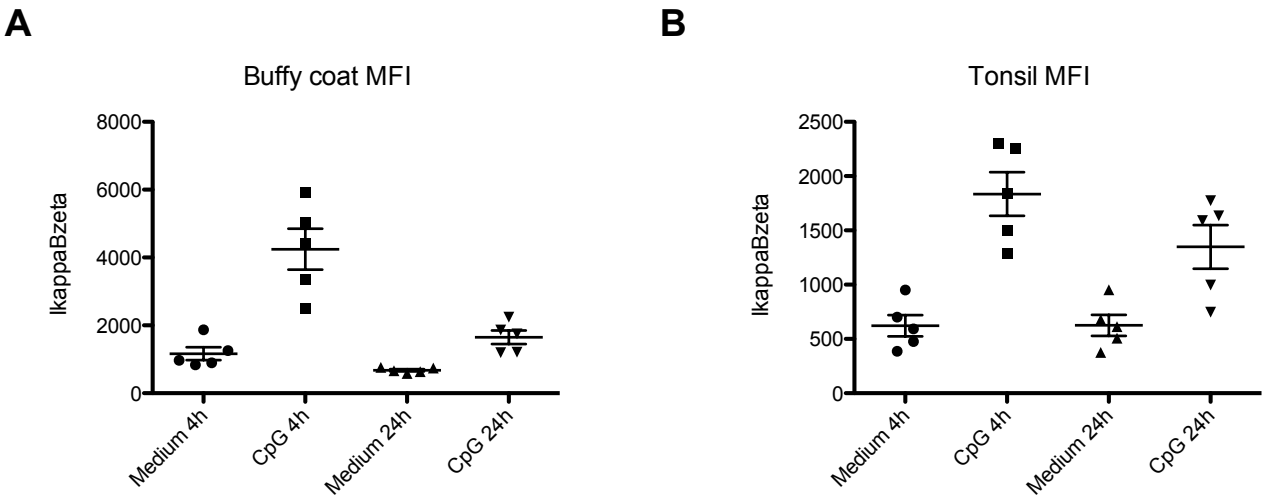


B



Supplementary Figure 6. Densitometric analysis of Western blot analyses. A) 3 IkappaBzeta+ and 3 IkappaBzeta- samples were analyzed at different time points after CpG stimulation and IkappaBzeta protein was detected by western blot analysis and quantified as relative IkappaBzeta/ beta-actin signal. Relative increase of signal is reported. **B)** 5 IkappaBzeta positive samples were analyzed before and after CpG stimulation of BLIMP-1 expression by western blot and signals quantified. Data are expressed as relative BLIMP-1/beta-actin expression.

Supplementary Figure 7



Supplementary Figure 7. Kinetic analysis of IkappaBzeta protein induction in normal B-cells. Cells were treated or not with CpG for the indicated time points, and analyzed as described in Figure 7.

Fonte et al. "TLR9 stimulation can induce IkappaBzeta expression and IgM secretion in chronic lymphocytic leukemia cells".

Supplementary Methods

Antibodies and reagents for Western blot and Flow cytometry

Antibodies used for Western Blot analysis: anti-IkappaBzeta (Cell Signaling Technology), anti-Blimp-1 isoform 2 (Cell Signaling Technology), anti-LC3 (Novus Biologicals) and anti-β-actin (Sigma). IRAK1/4 inhibitor (Sigma) was used at a concentration of 10μM.

Reagents for intracellular flow cytometry staining: for IgM detection, cells were blocked with a BSA solution for 30 minutes at 37°C and stained with anti-IgM (Southern Biotech) with or without permeabilization (BD bioscience perm/fix reagents) to differentiate surface vs intracellular expression. For IkappaBzeta detection, cells were permeabilized with a transcription factor Buffer Set (BD Pharmingen) and stained with anti-IkappaBzeta (eBioscience). Flow cytometry analyses were performed by FC500 (Beckman Coulter).

Cell culture and analysis

B lymphocytes from CLL patients and healthy donors were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Bovine Serum, 2 mM L-glutamine and 15 μg/ml gentamicin (Euroclone) at a concentration of $3x10^6$ cells/mL. CLL cells were either left unstimulated or stimulated with CpG

oligonucleotide type B-Human (ODN 2006; Invivogen), a specific ligand for TLR9, at the concentration of 2,5 µg/mL.

Metabolic cell activation was measured by using CellTiter-Glo Luminescent Cell Viability Assay according to manufacturer's protocol (Promega).

Real time PCR

RNA was purified with a QIAGEN mini kit following the manufacturer's instructions, and the cDNA was obtained by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Expression *NFKBIZ* mRNA was analyzed by using a specific Taqman gene expression assay: assays used for all the *NFKBIZ* isoforms or the long *NFKBIZ* isoform were Hs00230071 and Hs00944734 respectively (Applied Biosystems). The experiments were performed in triplicate and data were normalized with GAPDH expression for each condition. Results are expressed as relative expression levels ($2^{-\Delta Ct}$).

Transfection

CLL cells were electroporated with 30nM of either a scrambled siRNA or a siRNA targeting IkappaBzeta mRNA (Applied Biosystems). After 16 hours, cells were stimulated with 2,5 µg/ml of CpG and followed treated with 3µM of Fludarabine for additional 24 hours.

Western Blot analysis

Total cell lysates were resolved by SDS-PAGE and proteins from gel were electron-transferred onto nitrocellulose membranes and incubated over night at 4°C with indicated antibodies. Immunoreactivity was revealed by incubation

with HRP-anti-rabbit Ig or HRP-anti-mouse Ig (GE-Healthcare), followed by ECL reaction (Pierce) film exposure or digital scanner (c-Digit Blot scanner, LI-COR).

ELISA assay

Cell culture supernatants were collected starting at 48 hours after TLR stimulation, and the amount of soluble IgM was quantified by using a specific ELISA assay following the manufacturer's instructions (Human anti-IgM, Bethyl).

Immunofluorescence

CLL cells were stimulated with CpG, and attached to the slides by using poly-L-Ornithine (Sigma) for 4 hours. They were fixed with 4% of paraformaldehyde (PAF) and permeabilized using a Triton-X-100 solution and stained with anti-IkappaBzeta, overnight at 4°C. Secondary Antibodies as well as DAPI were incubated for 1 hour at RT. In focus images were obtained with a confocal microscope (Leica).