SUPPLEMENTARY APPENDIX

In vitro and *in vivo* evidence for uncoupling of B-cell receptor internalization and signaling in chronic lymphocytic leukemia

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Supplementary Information

Patients and samples

Peripheral blood mononuclear cells (PBMCs) from both CLL patients and normal healthy donors were isolated by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich, Gillingham, UK) and cryopreserved in 10% dimethyl sulfoxide. Cells were thawed, washed twice and rested in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich) for one hour at 37^{o}C, 5% CO_2 prior to each assay, unless otherwise stated. FNAs were only possible on patients with significant lymphadenopathy and all cases in this analysis had poor prognostic features including expression of CD38 and unmutated *IGHV* genes (Table S3). FNA was acquired from patient CLL55 prior to commencing BTKi therapy, PB both before and during treatment.

Cell surface IgM and CD79 expression

Surface IgM and IgD (sIgM, sIgD) expression was assessed using Quantum™ FITC MESF (Molecules of Equivalent Soluble Fluorochrome) microsphere kits (AbD Serotec, Kidlington, UK); surface CD79B was quantified using R-PE MESF microsphere kits. The QuickCal v. 2.3 program (www.bangslabs.com) to establish a standard curve relating channel value to fluorescence intensity in MESF units according to the manufactures recommendations. Identical cytometer settings were used for the CLL patient and control samples. PBMCs ($5x10^5$ cells) were incubated with anti-CD19-PB, anti-CD5-APC (both Biolegend) and either Fluorescein isothiocyanate (FITC)-conjugated anti-human sIgM/D or FITC-conjugated isotype control (both eBioscience) on ice, washed and prepared for flow cytometry. Approximately 5000-10000 events from gated, viable B-cells from CLL patients ($CD19^+/CD5^+$) and normal B-cells ($CD19^+$) were collected and analyzed as described above. sIgM/D and isotype control median fluorescence intensity (channel values) was converted into MESF values

using the online QuickCal program. Finally sIgM/D MESF was normalized to isotype control MESF to assign the definitive MESF value.

Calcium mobilization assay

Briefly, 5 x 10^6 cells/ml CLL PBMCs were incubated in media supplemented with 1 µM Indo1-AM for 1 h at 37°C, then washed and re-suspended in fresh medium and incubated at 37°C for 30 minutes then stored on ice before being used for experiments. For each acquisition, 3 x 10^6 PBMCs re-suspended in 1 ml of medium were used. For dead cell exclusion 5 µl of the 7-AAD Viability Staining Solution (Biolegend) was added to each sample and incubated for 10 minutes in the dark before data acquisition; cells were warmed to 37°C for 5 minutes before performing the analysis. Background or basal Ca^{2+} fluorescence was recorded followed by addition of goat $F(ab')_2$ anti-human IgM (20 µg / ml; Cambridge Biosciences) and events were recorded up to 300 seconds. Ionomycin at a concentration of 1 µM was used to produce calcium flux as a positive control. Flow cytometric data were acquired using FACS Diva software and analyzed using the kinetic application of the FlowJo software (Tree Star, Inc.). Background fluorescence threshold intensity was established at the 85^{th} percentile of basal fluorescence of unstimulated cells, as previously described. The percentage of cells responding to anti-human IgM stimulation was calculated and patients were classified as signal competent/non-anergic when the percentage of responding cells was greater than 5% or non-signal competent/anergic if responding cells were below 5% (supplementary Figure S2).

Phospho-flow cytometry

ERK1/2 phosphorylation activation was analyzed by flow cytometry in viable CD19+CD5+ CLL according to the manufactures protocol. In summary PBMCs from CLL patients were incubated with or without unlabeled $F(ab')_2$ αIgM for 10 minutes at 37°C, washed and fixed in pre-warmed fixation

buffer (Biolegend UK) for 15 minutes at 37°C. In independent experiments a pre-incubation step with agonistic αCD79B for 10mins at 37°C was included prior to αIgM stimulation to establish whether CLL B-cells that have undergone CD79B endocytosis can still signal via the BCR. Cells were then washed and permeabilized with pre-chilled True-Phos™ Perm Buffer (Biolegend UK) for 60 minutes at -20°C, washed and stained with anti-CD19, CD5 and pERK1/2 for 30 minutes at room temperature. Data were acquired and analyzed by flow cytometry. Cells stimulated with phorbol 12-myristate 13-acetate (PMA) for 10 minutes at 37°C served as positive control; ERK1/2 phosphorylation levels of untreated (baseline) and αIgM-treated (activated) B-cells were normalized to the positive control.

B-cell receptor internalization

BCR internalization was assessed in two ways. First, we used the pH sensitive fluorescent sensor, pHrodo™ Red avidin (Life Technologies, Paisley, UK) linked to agonistic anti-IgM or IgD (pHrodo-αIgM or D) to detect uptake and retention of ligand/receptor complexes in acidified endosomes. The fluorogenic dye-avidin conjugate is non-fluorescent outside the cell at a neutral pH but dramatically increases in fluorescence as the pH of its surroundings becomes more acidic. One microgram of pHrodo™ Red Avidin was incubated with an equimolar amount of biotinylated goat $F(ab')_2$ anti-human Immunoglobulin M or D (αIgM or IgD; Cambridge Biosciences, Cambridge, UK) for 1h at room temperature. Target cells were incubated with either pHrodo-avidin-αIgM (pHrodo-αIgM or D) for 30 minutes at 4^oC and then 37^oC for 1h and stained with viability dye, anti-CD19, CD5 and CXCR4, according to the manufactures recommendations; data were acquired on a FACS Canto II flow cytometer (Becton Dickinson, Oxford, UK) and analyzed using FlowJo software (Tree Star; Ashland, OR, USA); approximately 5000-10000 events were collected from each sample gated on live cells, expressing CD19 and CD5 ($CD19^+/CD5^+$). All incubations were performed in PBS pH 7.4, so that any observed fluorescence was due to BCR internalization and trafficking of the pHrodo-αIgM to

acidified endosomes. Results are expressed as the pHrodo mean fluorescent intensity (MFI) after subtraction of the background signal (MFI of unlabelled anti-IgM).

To confirm BCR specificity, experiments were performed in the presence of a molar excess of unlabeled αIgM or D (Cambridge Biosciences). To investigate energy dependence and the role of the cytoskeleton, cells were pre-incubated with either sodium azide (0.2%; Severn Biotech Ltd, Kidderminster, UK) or cytochalasin D (10μM; Sigma-Aldrich) for 1h at 37^{o}C. In addition, a pre-incubation with agonistic αCD79B for 10mins at 37^{o}C was included to specific experiments prior to αIgM stimulation, to assess the effect of CD79B endocytosis on BCR internalization in CLL B-cells. Second, BCR internalization was also assessed directly by measuring the rate of disappearance of surface IgM following ligation by agonistic anti-IgM over the same duration of time as the pHrodo-αIgM uptake assay. As described above, normal and CLL PBMCs (5x10^{5}/tube) were incubated with biotinylated αIgM for 30 minutes at 4^{o}C to allow initial receptor binding, washed twice with ice cold PBS, then incubated at 37^{o}C for the time indicated (0, 2, 5, 10, 30, and 60 min) to allow receptor endocytosis. The cells were then washed twice with ice cold PBS and stained with CD79B FITC and streptavidin (SAV)-APC, in addition to viability dye, CD19 PB and CD5 PE-Cy7 in PBS for 30 minutes on ice. CD79B internalization was also assessed following ligation by agonistic biotinylated αCD79. Here, cells were washed and stained with anti-CD19, CD5, IgM and streptavidin (SAV)-APC. Cells were then washed twice and fixed in PBS 1% paraformaldehyde (PFA) before FACS acquisition. Maximal fluorescence (Fmax) was defined as the APC fluorescence at 4^{o}C which represents the starting amount of αIgM-APC bound to the cells without endocytosis (0 minutes at 37^{o}C). APC mean MFI was recorded at each time point (Ftp). The rate of BCR internalization or disappearance was calculated and defined as the percentage surface BCR expressed or remaining (following internalization) using the following formula: Ftp/Fmax x100.

Immunofluorescence Staining

B-cells from CLL patients and healthy controls were isolated using a human B-cell negative-selection kit without CD43 depletion (StemCell Technologies, Grenoble, France), according to the manufacturer's instructions. Briefly, $5x10^5$ purified cells were incubated with pHrodo-avidin or pHrodo-αIgM as above, washed and re-suspended in 150µl of cell culture media (supplemented with 20% FBS) and deposited onto poly-l-lysine coated glass slides by cytospin (300x g for 5 minutes). Slides were fixed with 1% PFA for 15 minutes at room temperature, washed 3 times in PBS and permeabilized in 0.5% Triton/PBS for 10 minutes. The slides were washed again 3 times and stained with CytoPainter Phalloidin-iFluor 488 reagent (1:1000 PBS with 1% BSA; Abcam, Cambridge, UK) for 30 minutes, then washed and stained with 100µl of water-based mounting medium (Life Technologies). Images were acquired on a Nikon Eclipse Ti-E inverted microscope equipped with the Nikon A1R Si confocal imaging system. Image analysis was with Nikon Elements v4.2 software.

Supplementary Figures

Figure S1.

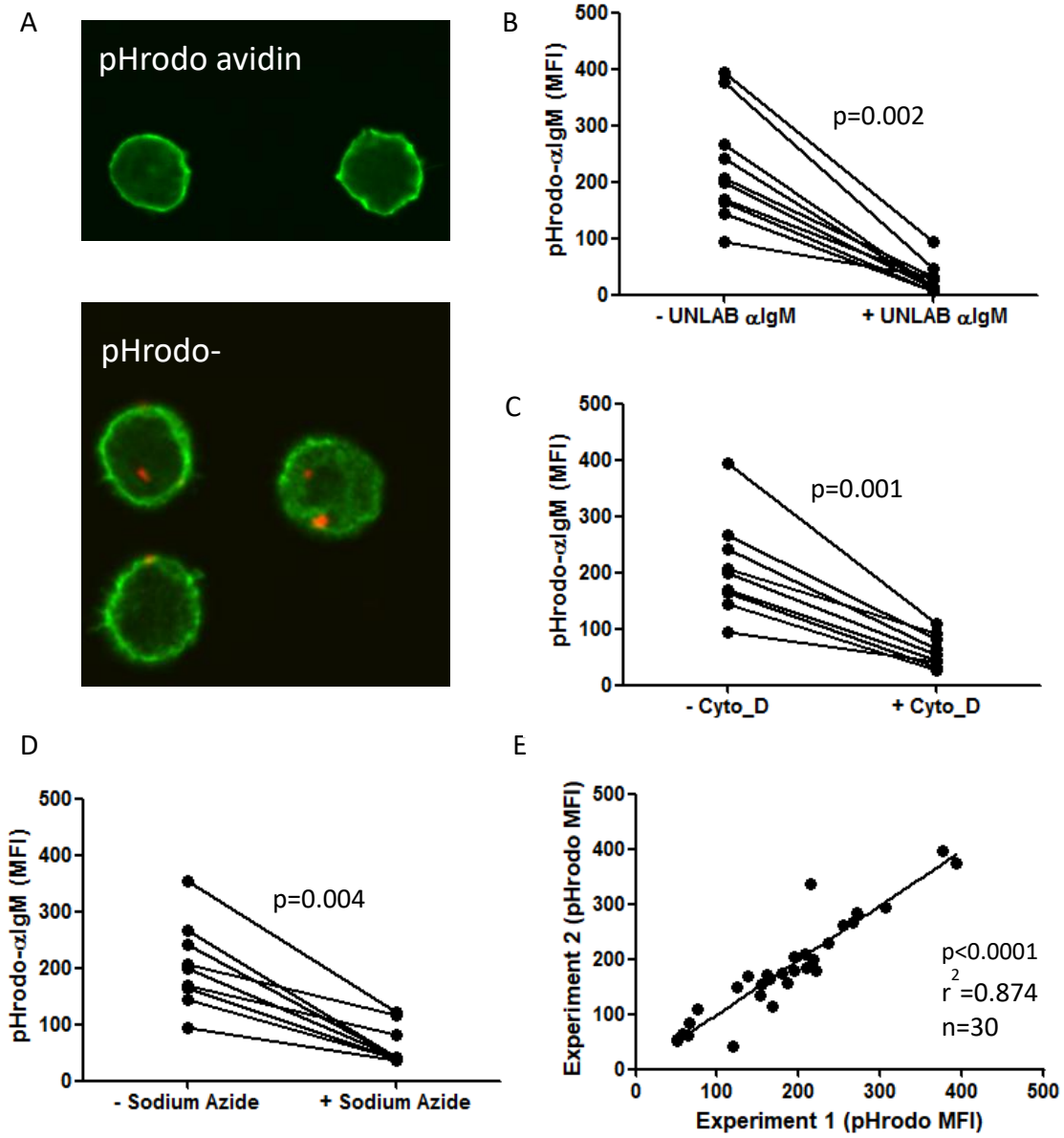

Figure S1. BCR-pHrodo-αIgM internalization: intra-patient variability and specificity in CLL B-cells

(A) A representative image of B-cells from an unmutated CLL patient post-internalization of pHrodo-αIgM. CLL B-cells were selected from PBMCs (negative selection) and incubated with control (pHrodo-avidin) or pHrodo avidin conjugated to biotinylated F(ab')$_2$ anti-IgM (pHrodo-αIgM; red) for the time specified. Cells were fixed and stained with phalloidin (green) and evaluated by

immunofluorescence (original magnification, X100). The effect of unlabeled $F(ab')_2$ anti-IgM (UNLAB-αIgM; n=9; B), cytochalasin D (cyto D; n=9; C) and sodium azide (n=9; D) on BCR-pHrodo-αIgM internalization in CLL patients was determined. CLL cells were pre-incubated for the times specified prior to pHrodo-αIgM incubation. Internalization was measured by flow cytometry and mean fluorescence intensity (MFI) values recorded. Statistical analysis was performed by use of paired t-test. (E) A correlation plot measuring experimental reproducibility within each individual CLL case (Pearson's correlation).

Figure S2.

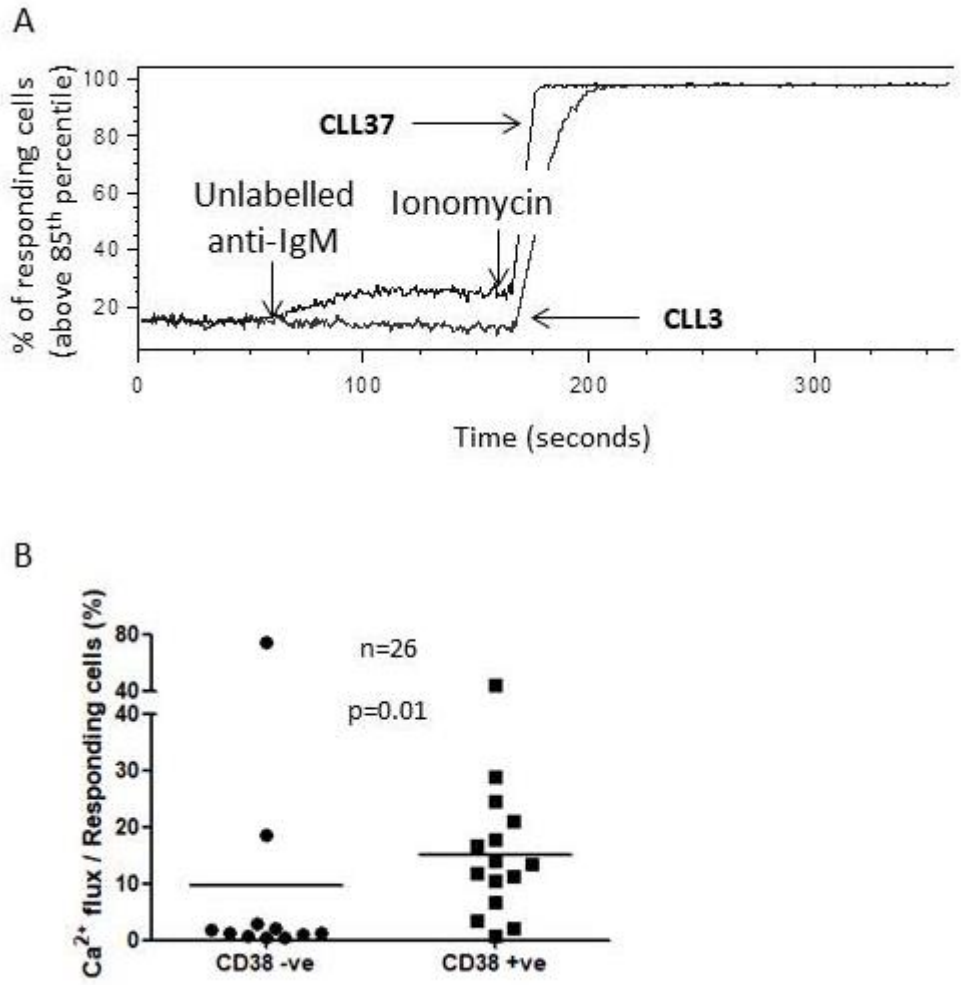

Figure S2. Determining sIgM-mediated signaling responses in CLL patients.

Leukemic cells from 26 patients were labeled with the calcium-sensitive dye, Indo1-AM, and analyzed by flow cytometry before and after addition of $F(ab')_2$ anti-IgM. (A) Representative trace measuring intracellular calcium levels before and after (indicated by arrow) BCR ligation with unlabeled $F(ab')_2$ anti-IgM in two CLL patients. CLL37 was identified as signaling competent, due to detection of increased intracellular calcium levels above baseline or unstimulated threshold (>5% of cells were able to increase the calcium-associated fluorescence following BCR stimulation). CLL3 was unresponsive to anti-IgM stimulus, and was therefore considered a non-signaler, or anergic. Both

patients produced a complete response when the non-specific stimulant, ionomycin was added to cell culture (indicated by arrow). (B) CD38 status and calcium flux. Leukemic cells were grouped on the basis of CD38 status, a marker with prognostic impact. Mean calcium flux was lower in CD38 negative (-ve) than CD38 positive (+ve) CLL B-cells (mean % responding cells: CD38-ve = 9.5±6.7, CD38+ve = 15.1±2.9; p=0.01), with 9/11 CD38-ve patient samples below the threshold cut off and BCRs deemed unresponsive (no statistical difference in mean % responding cells was detected between mutated and unmutated CLL B-cells; p=0.06, data not shown). Graph shows individual data point and means (horizontal line). Data were analyzed by using Mann-Whitney *U* test (*P* value is indicated).

Figure S3.

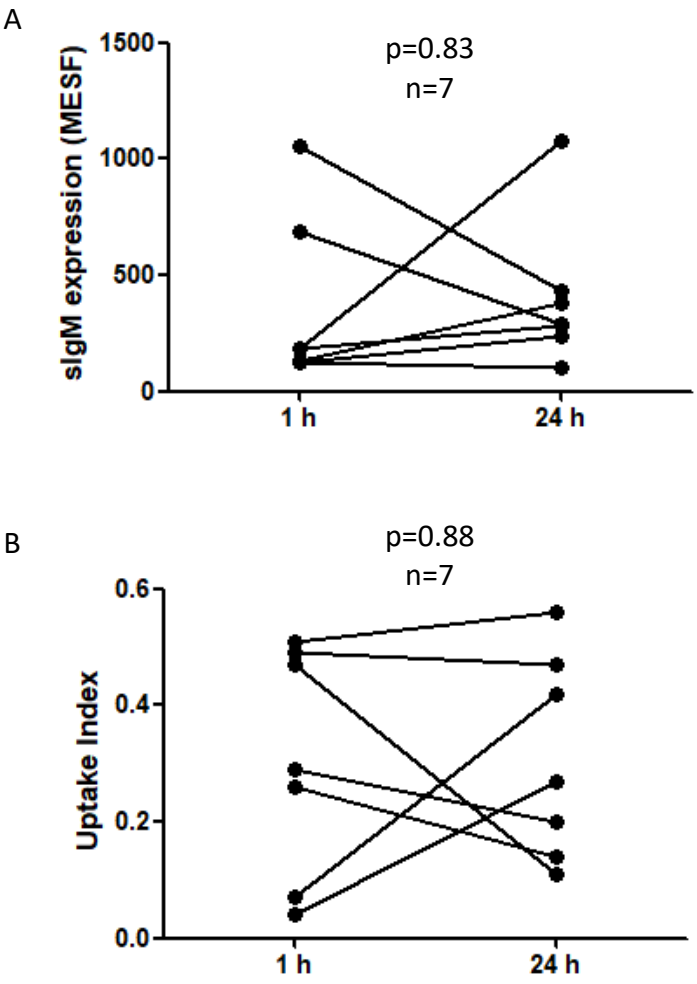

Figure S3. CLL B-cells maintain features of anergy following 24h *in-vitro* culture.

CLL B-cells (n=7 patients) were incubated for 1 hour or cultured for 24 hours in media prior to measuring sIgM expression (A) and BCR internalization efficiency (B; uptake index). Data were analyzed using the Wilcoxon match pairs test.

Figure S4.

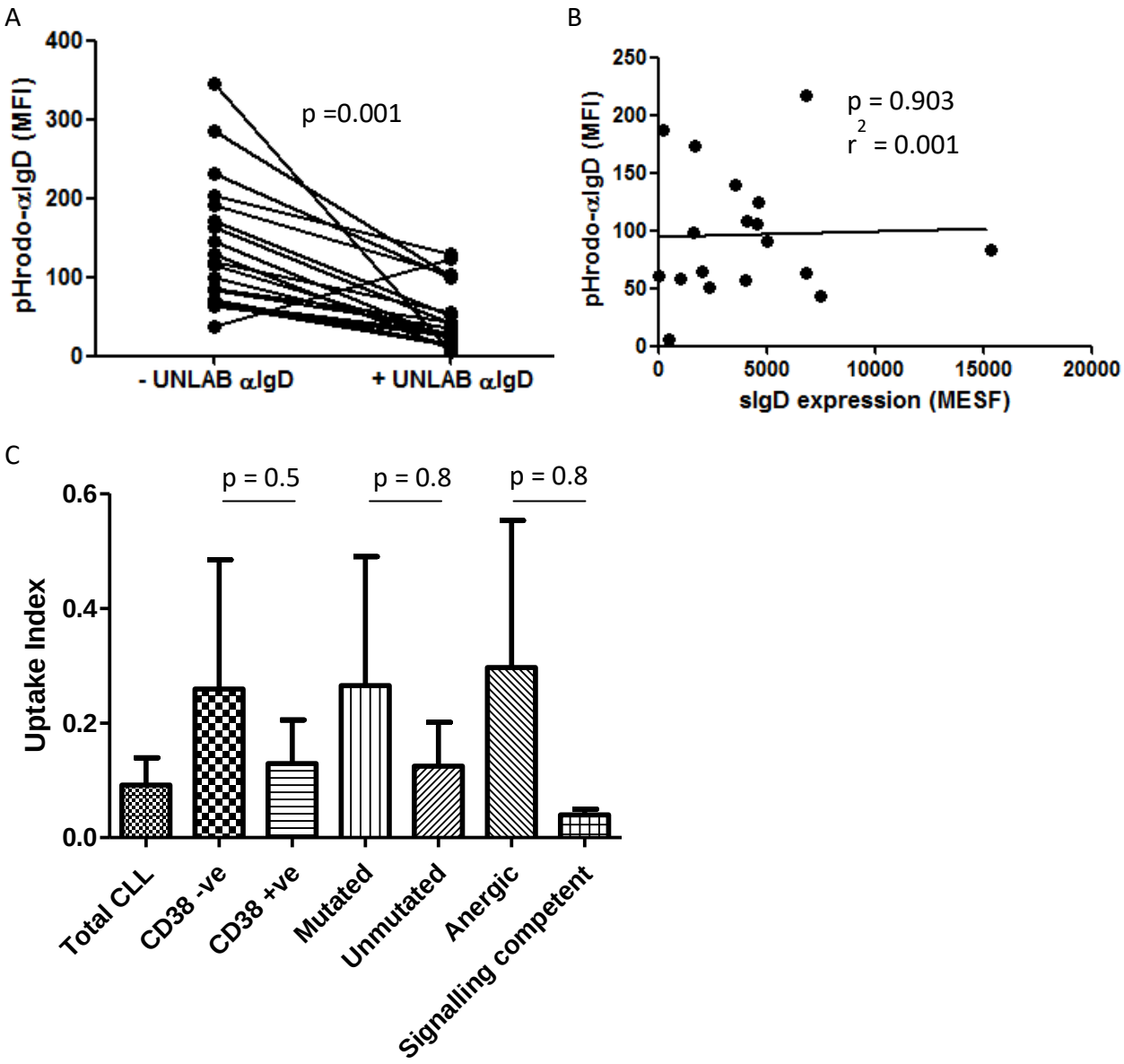

Figure S4. Levels of IgD expression and internalization do not correlate with prognostic factors in CLL.

The effect of unlabeled unlabeled αIgD on the levels of pHrodo-αIgD uptake in CLL B-cells was determined (n=18). PBMCs from CLL patients were pre-incubated for the times specified prior to pHrodo-αIgD incubation. Internalization was measured within the CD19+5+ B-cell population by flow cytometry and mean fluorescence intensity (MFI) values recorded. (B) Correlation plot exploring the

relationship between pHrodo-αIgD uptake and surface IgD (sIgD) expression. (C) Comparing the pHrodo-αIgD uptake index between subgroups that determine good or poor prognosis. Statistical analysis was performed by use of unpaired T-test.

Figure S5.

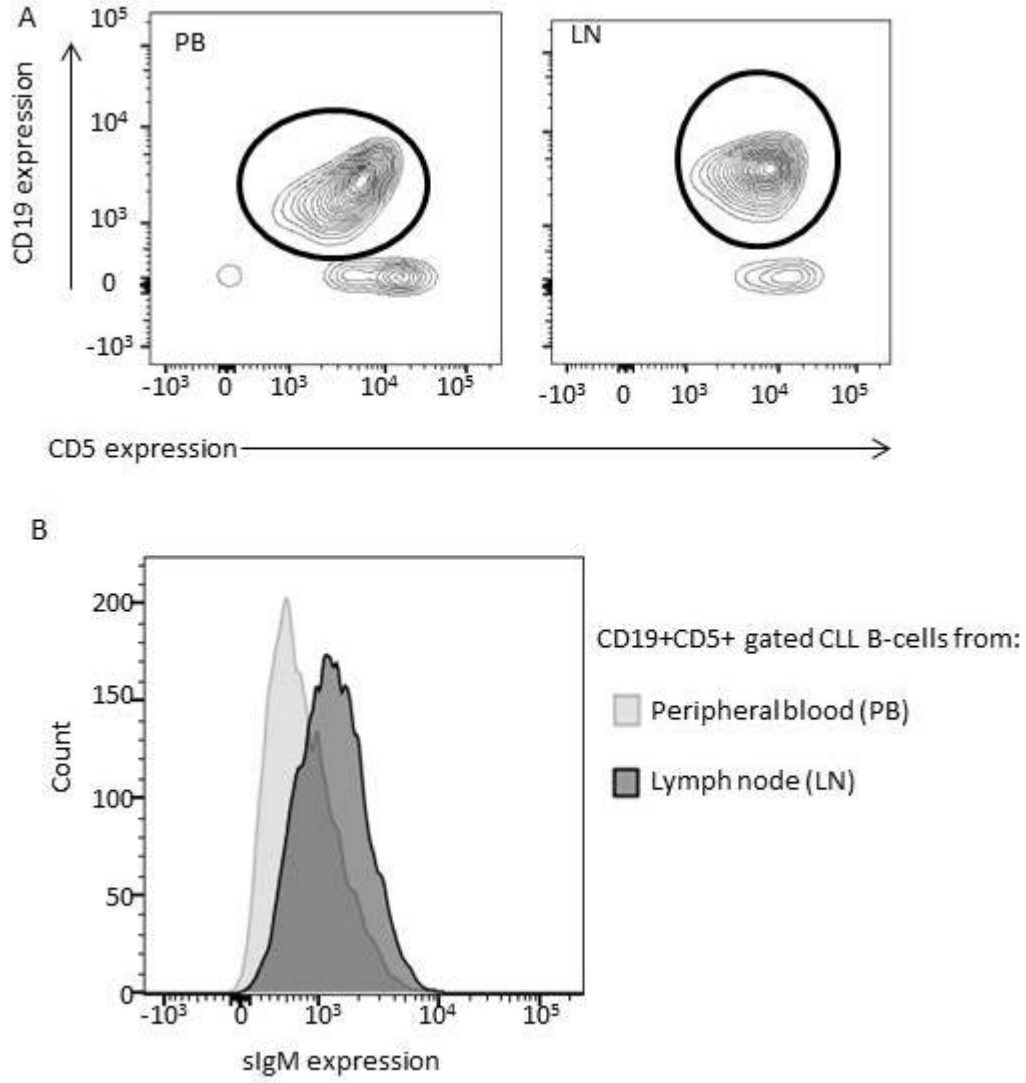

Figure S5. Comparing sIgM expression in matched PB and LN CLL B-cells.

(A) Representative contour plots showing matched PB and LN CD19+CD5+ (circled) CLL cells from a single patient (CLL55). (B) The gated CD19+CD5+ cells were co-stained with sIgM FITC and presented as a histogram of fluorescence intensity.

Supplementary Tables

Table S1: Characteristics and clinical features of CLL patients

PATIENT ID	GENDER (M/F) / AGE (YEARS)	CD38 (%) EXPRESSION [1]	IGVH STATUS [2]	FISH	BINET STAGE	SIGNALING COMPETENT BCR [3]
CLL1	F / 74	0	MUTATED	Normal	A	N
CLL2	M / 62	1	MUTATED	Negative for 11q and 17p	A	N
CLL3	F / NA	2	MUTATED	NA	A	N
CLL4	M / 80	3	MUTATED	TP53 - , 13q -	A	Y
CLL5	M / 73	3	MUTATED	Negative for 11q and 17p	A	Y
CLL6	M / 53	3	MUTATED	13q-	B	N
CLL7	F / 53	3	MUTATED	Negative for 11q and 17p	A	NA
CLL8	F / 35	4	MUTATED	Normal	A	N
CLL9	F / 71	5	MUTATED	NA	A	N
CLL10	F / 49	6	MUTATED	Normal	A	N
CLL11	M / NA	10	MUTATED	13q-	A	NA
CLL12	M / NA	15	MUTATED	13q-	C	N
CLL13	M / 66	18	MUTATED	13q-	B	NA
CLL14	F / 64	36	MUTATED	NA	A	NA
CLL15	M / 60	42	MUTATED	13q- , Partial deletion of IGH	A	Y
CLL16	F / 63	44	MUTATED	13q-	A	NA
CLL17	M / 69	49	MUTATED	deletion of one copy of IGH variable region	A	NA
CLL18	M / 54	77	MUTATED	13q-	A	NA
CLL19	M / NA	88	MUTATED	13q- , ATM -	B	NA
CLL20	M / 68	91	MUTATED	13q-	C	N
CLL21	F / 67	1	UNMUTATED	13q- , TP53 -	A	N
CLL22	M / 84	5	UNMUTATED	13q- , Trisomy 12 Loss of one copy of IGH@ variable region	B	N
CLL23	NA / 59	15	UNMUTATED	11q -	NA	Y
CLL24	F / 72	16	UNMUTATED	TP53 -	B	Y
CLL25	M / 46	18	UNMUTATED	deletion of both copies of IGH@ variable region	NA	Y
CLL26	M / 58	19	UNMUTATED	13q-	C	NA
CLL27	M / 33	19	UNMUTATED	Normal	A	NA
CLL28	M / 52	23	UNMUTATED	13q- , TP53 - deletion of one copy of IGH@ variable region	B	Y
CLL29	F / NA	30	UNMUTATED	ATM -	B	NA
CLL30	F / 39	34	UNMUTATED	Normal	B	NA
CLL31	M / 50	51	UNMUTATED	Loss of IGH@ variable region, ATM -	B	Y
CLL32	M / 75	57	UNMUTATED	deletion of one copy of IGH@ variable region	C	Y
CLL33	M / 68	61	UNMUTATED	13q- , ATM -, deletion of both copies of IGH variable region	B	N
CLL34	M / 74	66	UNMUTATED	13q - , TP53 -	B	NA
CLL35	M / 75	83	UNMUTATED	Trisomy12	C	Y
CLL36	M / 74	84	UNMUTATED	13q - , 11q -	C	Y
CLL37	F / 75	94	UNMUTATED	Trisomy12, 13q- , ATM -, TP53 -	C	Y

CLL38	M / 66	97	UNMUTATED	Normal	B	Y
CLL39	M / 64	98	UNMUTATED	Trisomy12, ATM -	C	NA
CLL40	F / 52	99	UNMUTATED	Trisomy12, partial deletion of IGH@ variable region	A	Y

(1) CD38 positive (+) indicates ≥7% of CLL cells express CD38 above control.

(2) Unmutated indicates <2% change in IGVH gene sequence compared to germline.

(3) BCR signaling responses are analysed by calcium mobilization. The percentage of cells responding to anti-human IgM stimulation was calculated and patients were classified as signal competent/ non anergic when the percentage of responding cells was greater than the 5% (Y) or non-signal competent/ anergic if responding cells were below the 5% cut off (N).

NA, not available; - , deletion; M, Male; F, Female.

Table S2: Characteristics and clinical features of CLL patient undergoing ibrutinib or acalabrutinib treatment.

PATIENT ID	GENDER (M/F) / AGE (YEARS)	IBRUTINIB / ACALABRUTINIB TREATMENT	DURATION OF TREATMENT (MONTHS)	CD38 (%) EXPRESSION (1)	IGVH STATUS (2)	FISH	BINET STAGE
CLL41	F / 58	IBRUTINIB	17.0	0	MUTATED	13q -	C
CLL42	M / 84	IBRUTINIB	30.3	5	UNMUTATED	13q- , Trisomy 12 Loss of one copy of IGH@ variable region	B
CLL43	M / 59	ACALABRUTINIB	17	30	NA	Trisomy 12	C
CLL44	F / 59	IBRUTINIB	26	7	UNMUTATED	13q- , Trisomy 12 Loss of one copy of IGH@ variable region	B
CLL45	M / 63	IBRUTINIB	31	1	MUTATED	11q -	B
CLL46	M / 61	IBRUTINIB	36	37	MUTATED	13q -	C
CLL47	M / 57	IBRUTINIB	25	6	MUTATED	13q- , Loss of one copy of IGH@ variable region	C
CLL48	F / 71	IBRUTINIB	12	2	MUTATED	17p - , Trisomy 12	B
CLL49	NA / 61	IBRUTINIB	12	17	NA	17p -	C
CLL50	NA / 61	IBRUTINIB	12	59	UNMUTATED	Trisomy 12	B
CLL51	F / 68	IBRUTINIB	12	4	NA	11q - , Trisomy 12	C
CLL52	M / 67	IBRUTINIB	13	86	MUTATED	13q -	C
CLL53	M / 57	ACALABRUTINIB	22	2	UNMUTATED	13q - , 17p -	A
CLL54	NA / 55	ACALABRUTINIB	20	75	UNMUTATED	17p - , Trisomy 12	C
CLL55	M / 51	IBRUTINIB	12	31	UNMUTATED	ATM - , Trisomy12	Relapsed disease

(1) CD38 positive (+) indicates ≥7% of CLL cells express CD38 above control.

(2) Unmutated indicates <2% change in IGVH gene sequence compared to germline.

NA, not available; - , deletion; M, Male; F, Female.

Table S3: Characteristics and clinical features of CLL patient samples used for matched LN and PB experiments.

PATIENT ID	GENDER (M/F) / AGE (YEARS)	CD38 (%) EXPRESSION (1)	IGVH STATUS (2)	FISH	BINET STAGE
CLL55	M / 51	31	UNMUTATED	ATM - , Trisomy12	Relapsed disease
CLL56	M / 58	5	UNMUTATED	13q-	C
CLL57	M / 62	78	UNMUTATED	ATM - , 13q -	B
CLL58	M / 56	50	UNMUTATED	13q -	B
CLL59	F / 65	85	UNMUTATED	13q -	B
CLL60	M / 70	9	UNMUTATED	13q -	A
CLL61	M / 65	8	NA	TP53 - , 13q -	C

(1) CD38 positive (+) indicates ≥7% of CLL cells express CD38 above control.

(2) Unmutated indicates <2% change in IGVH gene sequence compared to germline.

NA, not available; - , deletion; M, Male; F, Female.

Table S4: Flow cytometry antibody list

ANTIBODY/DYE	FLUOROCHROME / LABEL	CLONE	ISOTYPE	COMPANY
CD5	APC	UCHT2	Mouse IgG1, κ	Biolegend
CD5	PE-CY7	UCHT2	Mouse IgG1, κ	Biolegend
CD19	PB	HIB19	Mouse IgG1, κ	Biolegend
CD79B	PE	CB3-1	Mouse IgG1, κ	Biolegend
CXCR4	APC	12G5	Mouse IgG2a, κ	Biolegend
anti-ERK1/2 Phospho (Thr202/Tyr204)	PE	6B8B69	Mouse IgG2a, κ	Biolegend
sIgM	FITC	SA-DA4	Mouse IgG1, κ	eBiosciences
sIgD	FITC	IA6-2	Mouse IgG2a, κ	Biolegend
αIgM	Biotin	-	-	SouthernBiotech
αIgD	Biotin	-	-	SouthernBiotech
αCD79B	Biotin	SN8	Mouse IgG1, κ	Ancell
Streptavidin	APC	-	-	Biolegend
Fixable viability dye	efluor780	-	-	eBiosciences
Viability staining solution	7AAD	-	-	Biolegend