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## ***In-vitro* and *in-vivo* evidence for uncoupling of BCR internalization and signaling in chronic lymphocytic leukemia**

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**Running title:** BCR internalization and signaling in CLL

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## **Abstract**

B-cell receptor activation, occurring within lymph nodes, plays a key role in the pathogenesis of chronic lymphocytic leukemia and is linked to prognosis. As well as activation of downstream signaling, receptor ligation triggers internalization, transit to acidified endosomes and degradation of ligand-receptor complexes. In the present study we investigated the relationship between these two processes in normal and leukemic B-cells. We found that leukemic B-cells, particularly anergic cases lacking the capacity to initiate downstream signaling, internalize and accumulate ligand in acidified endosomes more efficiently than normal B-cells. Furthermore, ligation of either surface CD79B, a B-cell receptor component required for downstream signaling, or surface IgM by cognate agonistic antibody, showed that the two molecules internalize independently of each other in leukemic but not normal B-cells. Since association with surface CD79B is required for surface retention of IgM, this suggests that uncoupling of B-cell receptor internalization from signaling may be due to dissociation of these two molecules in leukemic cells. Comparison of lymph node with peripheral blood cells from chronic lymphocytic leukemia patients showed that, despite recent B-cell receptor activation, lymph node B-cells expressed higher levels of surface IgM. This surprising finding suggests that the B-cell receptors of lymph node and peripheral blood derived leukemic cells might be functionally distinct. Finally, long-term therapy with the Bruton's tyrosine kinase inhibitors ibrutinib or acalabrutinib resulted in a switch to an anergic pattern of B-cell receptor function with reduced signaling capacity, surface IgM expression and more efficient internalization.

## **Introduction**

It is now clear that signaling through the BCR plays a key role in the pathogenesis of CLL and other lymphomas. Several components of this pathway including: Syk,(1) Erk,(2) Akt,(3) NFAT(4) and NFkB(5) can be constitutively activated and drugs that target BCR signaling, such as the Bruton's tyrosine kinase inhibitors (BTKi), ibrutinib and acalabrutinib, are proving extremely effective in the clinic.(6, 7) BCR responsiveness varies markedly between patients with CLL and is linked to prognosis.(8) Some cases show features of anergy,(4, 9) a pattern that is associated with lack of

ability to transduce a downstream signal in response to BCR ligation and the presence of markers of good prognosis, including low levels of CD38 and mutated immunoglobulin heavy-chain variable (*IGHV*) genes. In contrast, cases with responsive or signaling competent BCRs usually express high levels of CD38, have unmutated *IGHV* genes and a more unfavorable clinical course;(10) interestingly these patients tend to respond more rapidly to BCR antagonists than those with anergic BCRs. Although BTKi therapy is very successful in controlling CLL, it is not curative and many patients are left with low level residual disease, which regrows on discontinuation of drug or when resistance mutations develop.(11, 12) This persistent disease also suggests that, within individual patients, the tumor may not behave in a homogeneous manner.(13)

Despite the central importance of BCR signaling in CLL and the efficacy of drugs that block this pathway, there is relatively little known about BCR dynamics in leukemic B-cells. Surface levels of IgM and other BCR components are generally lower in CLL compared to normal B-cells and it has been suggested that this might be due to a failure to properly assemble the sIg  $\alpha/\beta$  subunits CD79A and CD79B.(14) Recent studies have shown that total IgM and CD79A levels are near normal in CLL but that CD79B expression, which is required for transport of BCR to the cell surface,(15) is reduced thus trapping IgM within the cell.(16) Exposure to IL4 increases CD79B expression and allows sIgM levels to increase and BCR signaling capacity to improve.(16, 17) CLL cell surface BCRs have an immature pattern of glycosylation that matures following *ex-vivo* incubation(18) or exposure to IL4(17) in keeping with accelerated BCR turnover induced by chronic activation. It has also been reported that, within the PB of individual patients with CLL, leukemic cells with the lowest sIgM expression show biochemical features of recent activation and proliferation, presumably because they have recently been released from lymphoid tissues where BCR stimulation and activation are thought to occur.(19, 20) Taken together, these previous data suggest that the reduced sIgM levels observed in CLL are due to a combination of increased turnover consequent on chronic activation coupled with defective transport to the cell surface resulting from a deficiency of CD79B.

The ability of CLL BCRs to become internalized also has implications for how the tumor interacts with other cells, such as T-cells. We, and others, have previously shown that, as in normal LNs, activated CD4+ T-cells co-localize with proliferating tumor cells and, *in-vitro*, can supply signals that cause tumor proliferation.(21-24) This process normally involves endocytosis and processing of antigen bound to BCR, however it is not known whether CLL B-cells are capable of providing this function. In the present study we investigated whether, like normal B-cells, CLL cells can internalize their BCR and to what extent this is linked to downstream signaling in both untreated patients and those receiving therapy with the Bruton's Tyrosine Kinase inhibitors ibrutinib and acalabrutinib. Our results shed new light on BCR function in CLL and have implications for understanding the mode of action of this important new class of drugs.

## **Methods**

### **Patients and samples**

Peripheral blood (PB) samples were obtained from 19 healthy volunteers, 40 untreated patients with confirmed CLL (Table S1), and an additional 15 patients receiving BTKi therapy (Table S2). Lymph node fine needle aspirate (FNA) and paired matched PB samples were derived from 7 untreated CLL patients (Table S3). Ethical approval was obtained from the National Research Ethics Service (08/H0906/94); all patients provided informed written consent. Patients were classified as having unmutated *IGHV* genes if homology with germline was >98%(25) and as CD38+ if expression levels were 7% or higher (Tables S1-3).(26)

### **Flow cytometry**

Cells were stained according to the manufacturer's recommendations using fluorochrome-coupled antibodies (Table S4). Viable CD19+CD5+ CLL and CD19+ normal B-cells were acquired on a FACS Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Surface (s)IgM and IgD expression was assessed using Quantum™ FITC MESF (Molecules of Equivalent Soluble Fluorochrome) microsphere kits and the QuickCal v. 2.3 program according to the manufacturer's recommendations. Surface CD79B was quantified using R-PE MESF microsphere kits.

BCR signaling competence was determined using the ratiometric  $\text{Ca}^{2+}$  detector Indo1-AM (Life Technologies) to measure intracellular calcium ( $\text{Ca}^{2+}$ ) levels and  $\text{Ca}^{2+}$  influx after  $\alpha$ IgM stimulation, as previously described.(4, 9)

ERK1/2 phosphorylation activation was analyzed in both treatment-naïve and BTKi-treated (collected before and during BTKi therapy) CLL patient B-cells. Cells were incubated with or without  $\alpha$ IgM, or with phorbol 12-myristate 13-acetate (PMA; served as positive control) for 10 minutes at 37°C prior to intracellular and surface staining.

### **B-cell receptor internalization in normal and CLL B-cells**

BCR internalization was assessed in two ways. The uptake and retention of ligand/receptor complexes in acidified endosomes was measured using the pH sensitive fluorescent sensor, pHrodo™ Red avidin (Life Technologies) linked to agonistic  $\alpha$ IgM or IgD (pHrodo- $\alpha$ IgM or D). Target cells were incubated with either pHrodo- $\alpha$ IgM or D for 30 minutes at 4°C and then 37°C for 1h. Results are expressed as the pHrodo mean fluorescent intensity (MFI) after subtraction of the background signal (MFI of unlabelled anti-IgM). BCR internalization in normal and CLL B-cells was also directly assessed by measuring the rate of disappearance of surface (s)IgM following ligation by agonistic  $\alpha$ IgM. Full methodology is provided in the supplementary information.

### **Immunofluorescence Staining**

CLL B-cells were isolated using a human B-cell negative-selection kit without CD43 depletion (StemCell Technologies) according to the manufacturer's instructions and purity confirmed by flow cytometry. Cells were labeled with pHrodo-avidin or pHrodo- $\alpha$ IgM, deposited onto poly-l-lysine coated glass slides by cytospin and stained with CytoPainter Phalloidin-iFluor 488 reagent. 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 (see supplementary information).

### **Statistical analysis**

Statistical analyses were performed using GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA). Shapiro–Wilk test, *t*-test, Mann–Whitney and/or Wilcoxon’s test were used where indicated. *P*-values of <0.05 were considered significant.

## **Results**

### **Internalization of normal and CLL BCRs.**

The uptake and retention of pHrodo- $\alpha$ IgM labeled BCRs in acidified endosomes was similar in normal and CLL B-cells (mean fluorescence intensity [MFI],  $\pm$  standard deviation: normal control: 147.5 $\pm$ 16.8, n=19, p=0.825, CLL: 151.3 $\pm$ 12.0, n=40; figure 1A) and correlated with sIgM expression ( $r^2=0.405$ , p=0.0001, n=40; figure 1B). Confocal immunofluorescence microscopy confirmed that the labeled BCRs accumulate in an intracellular compartment (supplementary figure S1A) and pre-incubation with excess unlabeled anti-IgM, sodium azide and cytochalasin-D showed the process to be specific and dependent on energy and the cytoskeleton (supplementary figures S1B, S1C and S1D). Repeated measurements confirmed the reproducibility of the assay within individual CLL cases ( $r^2=0.874$ , p<0.0001, n=30; supplementary figure S1E). It has previously been reported that sIgM expression is lower in CLL compared to normal B-cells and this was also the case in our patients (figure 1C). Since BCR uptake by normal and CLL B-cells is similar and dependent on sIgM expression, this suggested that the process might be more efficient in CLL compared to normal B-cells. Correction of the pHrodo- $\alpha$ IgM uptake value for the number of surface IgM molecules (uptake index) confirmed this to be the case (figure 1D). After correcting for the level of surface IgM expression, the pHrodo- $\alpha$ IgM signal, which reflects accumulation in acidified endosomes, was 3.04 times more efficient in CLL compared to normal B-cells (Figure 1D, p<0.0001). The uptake index varied considerably between patients but was significantly higher in those whose BCRs lacked the

ability to mobilize calcium in response to BCR ligation and cases with low CD38 expression (see supplementary figure S2, table S1).

Prolonged *ex-vivo* incubation has previously been shown to reverse features of anergy, namely, re-expression of sIgM and restoration of BCR responsiveness.(4, 9) We therefore examined BCR expression and internalization after 24 hours *ex-vivo* incubation however results were heterogeneous with no significant recovery in sIgM expression (supplementary figure S3A,  $p=0.83$ ,  $n=7$ ) or change in BCR internalization efficiency (supplementary figure S3B,  $p=0.88$ ,  $n=7$ ). It was not possible to assess internalization efficiency at later time points as the assay critically depends on metabolic integrity of the cells, which was not consistently maintained after 24 hours.

Since the pHrodo- $\alpha$ IgM signal reflects both uptake into and retention within acidic endosomes, we also assessed the BCR internalization rate more directly by measuring loss from the cell surface following ligation with agonistic antibody. This showed that all cases of CLL internalize their BCRs more rapidly than normal B-cells (figure 1E;  $p=0.04$ , 2 minute time point). More subtle differences were observed between anergic and signal competent cases of CLL with more rapid initial loss from the surface in the former (figure 1E;  $p=0.02$  at 2 minute time point).

We also measured sIgD expression and the levels of pHrodo- $\alpha$ IgD uptake using the same assays. All CLL B-cells internalized pHrodo- $\alpha$ IgD and pre-incubation with unlabeled  $\alpha$ IgD reduced the level of uptake (supplementary figure S4A;  $n=18$ ;  $p=0.001$ ). No significant correlation between pHrodo- $\alpha$ IgD uptake and surface IgD expression was observed (supplementary figure S4B) and there was no difference in the pHrodo- $\alpha$ IgD uptake index between CD38<sup>hi</sup> and low, anergic/non anergic and IGHV mutated and unmutated patients (supplementary figures S4C). This suggests that uptake and retention mechanisms differ between IgD and IgM; this was not addressed further in the present study.

### **Mechanism of dissociation of BCR signaling and internalization**



We next investigated the role of surface (s)CD79B, a molecule that is closely associated with the BCR and essential for signal transduction, in BCR internalization. As previously reported(27) CLL B-cells express lower levels of sCD79B compared to normal B-cells with particularly reduced expression in anergic compared to signaling competent cases (Normal B-cell MFI=2561.2±400.9, anergic CLL MFI=318.2±63.4, signaling competent MFI=397.5±71.1). We also found that sIgM expression correlates with sCD79B both between (figure 2A) and within a patient with CLL (figures 2B & C). Despite these findings and the fact that sIgM and sCD79B are known to be associated during BCR signaling, no significant reduction in sCD79B expression occurred following ligation and downregulation of sIgM with agonistic antibody in both anergic and signaling competent cases of CLL (figure 2D). In normal B-cells, a slight but significant reduction in sCD79B expression was observed 30 and 60 minutes after BCR ligation with  $\alpha$ IgM. Similarly, downregulation of CD79B with an agonistic anti-CD79B had no effect on the level of sIgM in anergic and signaling competent CLL cells and normal B-cells (figure 2E & F). Thus, although both normal and CLL B-cells are capable of internalizing IgM and CD79B following ligation with cognate agonistic antibody, the two molecules do not co-internalize and thus cannot be closely associated during membrane trafficking.

Having established that sCD79B is not required for sIgM internalization we next assessed its role in BCR signaling by measuring the effect of prior sCD79B downregulation on  $\alpha$ IgM ERK phosphorylation in CLL cells. As expected, prior sCD79b downregulation reduced but did not abolish  $\alpha$ IgM induced ERK phosphorylation (figure 2G). In contrast, depletion of sCD79B was without effect on pHrodo- $\alpha$ IgM uptake (figure 2H).

### ***In-vivo* relationship between BCR signaling and internalization**

We next investigated the relationship between BCR internalization and signaling *in-vivo* in CLL by studying subsets of peripheral blood (PB) cells, those from the lymph node (LN) where BCR activation and proliferation is thought to take place and longitudinal samples obtained from patients being treated with BTK inhibitors ibrutinib and acalabrutinib.

### *Recently proliferated and quiescent subsets*

It has previously been shown that recently proliferated LN emigrant express low levels of CXCR4 and high levels of CD5 (CXCR4<sup>dim</sup>CD5<sup>bright</sup>).<sup>(28)</sup> Since proliferation within LNs is linked to BCR signaling, we compared BCR expression and internalization efficiency on CXCR4<sup>dim</sup>CD5<sup>bright</sup> and CXCR4<sup>bright</sup>CD5<sup>dim</sup> subsets. Since the BCR internalization assay involves incubation with agonistic  $\alpha$ IgM, we first investigated whether 1-hour incubation with pHrodo- $\alpha$ IgM altered the expression of these markers. As expected, CXCR4 expression was reduced and CD5 increased following ligation of BCR, however, over the 1h assay period, the magnitude of change was small (CXCR4 mean fold change =  $0.93 \pm 0.02$  and CD5 mean fold change  $1.24 \pm 0.04$ , data not shown). Since the MFI of the top and bottom decade of CXCR4 and CD5 expression differed by  $11.8 \pm 5.4$  and  $8.7 \pm 6.7$  fold respectively, assay induced changes could not have changed the composition of these subsets. Since the CXCR4<sup>dim</sup>CD5<sup>bright</sup> subset are thought to have recently undergone BCR activation within the LN, we expected to find downregulation of sIgM in this fraction, however, this was not the case and levels were higher than in the CXCR4<sup>dim</sup>CD5<sup>bright</sup> subset (figure 3A,  $p < 0.0001$ ). In keeping with post-activation anergy, in the majority of patients (14/21) there was more efficient uptake of pHrodo- $\alpha$ IgM in the CXCR4<sup>dim</sup>CD5<sup>bright</sup> subset however there was significant variability and, in the whole population, this did not reach statistical significance (figure 3B;  $n=21$ ;  $p=0.281$ ).

### *BCR expression and internalization in the PB and LN of CLL patients.*

Since our results suggested that BCR internalization is influenced by the capacity to initiate downstream signaling, which occur within LNs, we went on to directly compare sIgM levels and uptake of pHrodo- $\alpha$ IgM by LN CLL cells to those derived from simultaneously obtained PB from the same patients. As we have previously shown,<sup>(29)</sup> LN CLL cells expressed higher levels of CD5 than those derived from the PB, in keeping with BCR activation at these sites (data not shown). As was the case for the recently proliferated CXCR4<sup>dim</sup>CD5<sup>bright</sup> subset, sIgM levels were significantly higher on cells derived from the LN compared to PB (figure 3C;  $p=0.03$ ,  $n=7$ ; supplementary figure S5).

Again, there was great variability in pHrodo- $\alpha$ IgM uptake efficiency with higher levels in the LN than PB in 5 of 7 patients, but no significant difference overall (figure 3D;  $p=0.218$ ,  $n=7$ ).

#### *BTKi-treated CLL B-cells display features of anergy*

We next investigated the effect of BCR pathway blockade on BCR expression and function. Peripheral blood samples were collected over time from BTKi-treated patients (ibrutinib and acalabrutinib) with a prolonged lymphocytosis for a minimum of 12 months (Table S2). On comparison of CD19+CD5+ CLL B-cells before and after 1 month of treatment, most cases showed an initial increase in sIgM expression (Figure 3E;  $p=0.02$ ,  $n=7$ ) followed by a decrease in sIgM levels after at least 12 months of treatment (Figure 3F;  $p=0.008$ ,  $n=7$ ). After 12 months or more BTKi therapy, CLL B-cells exhibited more efficient BCR internalization (Figure 3G;  $p=0.023$ ,  $n=7$ ) and had reduced ability to activate ERK following BCR stimulation (Figure 3H;  $p=0.024$ ,  $n=12$ ).

## **Discussion**

In this study we investigated the capacity of normal and CLL B-cells to internalize ligands that bind to the BCR. Using two complementary techniques, we showed that BCR internalization and transit to acidified endosomes occurs in both normal and CLL B-cells. When corrected for the level of sIgM expression, we found that BCR internalization and accumulation in endosomes is 3 times more efficient in CLL than normal B-cells and is highest of all in cases with anergic BCRs. Using agonistic antibodies to sIgM and sCD79B we also showed that internalization of sIgM is not accompanied by internalization of CD79B and vice-versa. Comparison of LN with PB CLL-cells and PB CXCR4<sup>dim</sup>/CD5<sup>bright</sup> cells, representing recently proliferated LN emigrants,(28) with the CXCR4<sup>bright</sup>/CD5<sup>dim</sup> subset showed that both the LN and recent emigrants express higher levels of sIgM, however, no consistent difference in BCR internalization efficiency was observed. This was a surprising finding given that BCR activation takes place in the LN and should result in downregulation sIgM. Finally, long term *in-vivo* inhibition of BCR signaling using BTKis resulted in an increase in the

number of CLL cells exhibiting features of anergy including a reduction in sIgM expression and signaling competence as well as an increase in the efficiency of BCR internalization.

These results have a number of implications. First, it is clear that in CLL, BCR internalization is uncoupled from downstream signaling, since anergic CLL B-cells internalize their BCRs more efficiently than non-anergic cases and normal B-cells. These findings are in keeping with previous murine studies which showed that reduced surface BCR expression and uncoupling of signaling in normal anergic B-cells is due to rapid internalization and retention in endosomes.(30) Since enhanced accumulation of BCR in endosomes was observed in all patients studied, the present results show that, at least in this respect, all cases of CLL show some features of anergy.

Second, although CD79B is known to associate with sIgM and is essential for export of IgM to the cell surface and downstream BCR signaling, our data strongly suggest that in CLL B-cells, they internalize independently and thus cannot be associated during endocytosis. In normal B-cells a small proportion of sCD79B and sIgM co-internalized, again suggesting minimal association of the two during endocytosis. A plausible explanation for these observations is that BCRs exist in two configurations in CLL (figure 4); the first in which sIgM and sCD79B are not and do not become associated following ligand binding and a second in which sIgM and sCD79B are already colocalized or are induced to associate following activation. The first configuration could not transduce downstream signals but, because retention of sIgM at the surface requires association with CD79B, would internalize efficiently. On the other hand, BCRs in which sIgM and CD79B are associated could initiate signaling, however, the association with CD79B would favor retention at the cell surface, at least until the subunits undergo phosphorylation induced dissociation.(31) Such a model has previously been proposed for normal B-cells(32) and is thought to favor signaling in response to low-affinity ligands such as autoantigens of the type recognized by CLL BCRs.(33)

It is currently believed that BCR activation takes place in LNs and that this, combined with other signals from the microenvironment, leads to tumor proliferation. In other receptor systems, ligand

binding is generally accompanied by downregulation of cell-surface receptor(34) and our observation that sIgM levels are actually higher in the LN than PB and in recently proliferated CXCR4<sup>dim</sup>CD5<sup>bright</sup> compared to CXCR4<sup>bright</sup>CD5<sup>dim</sup> PB CLL cells was therefore unexpected. As recently suggested by others, it is possible that the elevated sIgM levels observed within the LN and CXCR4<sup>dim</sup>CD5<sup>bright</sup> subsets are due to IL4 induced upregulation of CD79B within lymphoid tissues.(16, 17)

Our findings also shed further light on the mechanism of action of BTKis. As noted by others,(13, 35) a persistent low-level lymphocytosis is frequently seen in such patients and complete remissions are rare. In the early period following the commencement of BTKi therapy, we observed an increase in sIgM expression in PB CLL cells. Since we have shown that cells in the LNs express higher levels of sIgM than those in the PB, this most likely reflects the previously documented redistribution of tumor from the former compartment to the later.(35) Over the longer term, however, the opposite is the case with a significant reduction in sIgM that is accompanied by an increase in BCR internalization efficiency and a reduced capacity to phosphorylate ERK. These findings indicate that long term BTKi therapy causes the emergence of a population of neoplastic B-cells with anergic phenotypic and functional properties. This may either occur through reprogramming of the tumor into a more anergic state or because there is subclonal heterogeneity within the tumor and selection of cells with anergic features by BTKi therapy. The latter possibility is supported by *in-vitro* studies of leukemic B-cell migration(29) and *in vivo* observations using heavy water or glucose labeling(36) that suggest the existence of subclonal heterogeneity at a functional level in CLL.

Finally, since BCR internalization into endosomes is the first event in antigen processing, our data support the theory that, under some circumstances, CLL B-cells might act as antigen presenting cells. We have recently show that LN derived CLL B-cells express higher levels of costimulatory molecules, form immune synapses and stimulate an allogeneic mixed lymphocyte reaction more efficiently than those from the PB.(29) In addition, elution of peptides from CLL MHC class II reveals presentation of a range of autologous peptides(37) with evidence for expansion of cognate T-cell clones in CLL but

not normal PB. Furthermore, a number of groups have documented the presence of T-cells in CLL patients that are capable of responding to a range of other molecules including Rh antigen,(38) tumor idiotype(23) immunoglobulin framework,(39) or CDR3 motifs(40) as well as broader responses against tumor lysates(41) or intact leukemic cells.(42) It is thus plausible that the abnormal phenotype, repertoire and function of CLL T-cells might be a consequence of excessive and aberrant antigen presentation occurring within lymphoid tissues.

In summary, we have shown that, as in normal B-cell anergy, CLL B-cells internalize ligands that bind to the BCR more efficiently than normal. This process is uncoupled from downstream signaling and does not involve association with CD79B. We demonstrate that BTKi therapy induces or selects for cells with anergic properties that persist in the long term. Understanding how this occurs will be important in order to optimize the efficacy of this important new class of drugs.

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## Figure legends

### Figure 1: B-cell receptor expression and internalization in CLL patients and healthy controls.

(A) PBMCs from CLL patients (n=40) and healthy controls (n=19) were incubated with pHrodo- $\alpha$ IgM and the MFI of cells internalizing pHrodo-labelled avidin was measured within the CD19+5+ (CLL) and CD19+ (normal) B-cell population by flow cytometry. (B) B-cell receptor (BCR) internalization correlates with sIgM expression (Pearson's correlation); correlation plot comparing the relationship between pHrodo- $\alpha$ IgM uptake and surface IgM (sIgM) expression in CD19+5+ cells derived from 40 CLL patients. (C) Although the level of pHrodo- $\alpha$ IgM uptake was comparable between CLL patients and healthy controls, marked differences in surface expression (sIgM) were observed ( $p=0.0001$ ). (D) After correcting the level of uptake per molecule of sIgM, the uptake index was measured in both CLL patients and healthy controls. Within the CLL patient subsets, CLL B-cells from CD38 negative, anergic and mutated patients were identified as having a greater uptake index than their counterparts, although mutational status was not significant ( $p=0.05$ ,  $p=0.05$  and  $p=0.436$ , respectively; unpaired t-test). (E) To assess the rate of BCR internalization more directly, the disappearance of surface (s)IgM following ligation of agonistic  $\alpha$ IgM was measured. Accelerated BCR endocytosis was detected (2 min time point) in anergic B-cells (n=6) compared to signaling competent (n=6) and normal (n=6) B-cells ( $p=0.02$  and  $p=0.01$ , respectively\*; Mann-Whitney test).

### Figure 2: Dissociation of BCR signaling and internalization in CLL cells.

(A) A correlation was detected between the expression levels of surface (s)IgM and sCD79B on CD19<sup>+</sup>5<sup>+</sup> cells derived from 21 CLL patients (both anergic and signaling competent B-cells, Pearson's correlation). Values are expressed as molecules of equivalent soluble fluorochrome (MESF) and derived from mean fluorescence intensity [MFI] values). (B) To examine the relationship within an individual patient, fifteen subsets of CD19<sup>+</sup>5<sup>+</sup> cells were created, as defined by increasing sIgM expression, and mean sCD79B MFI values were recorded within the same gate. (C) A representative

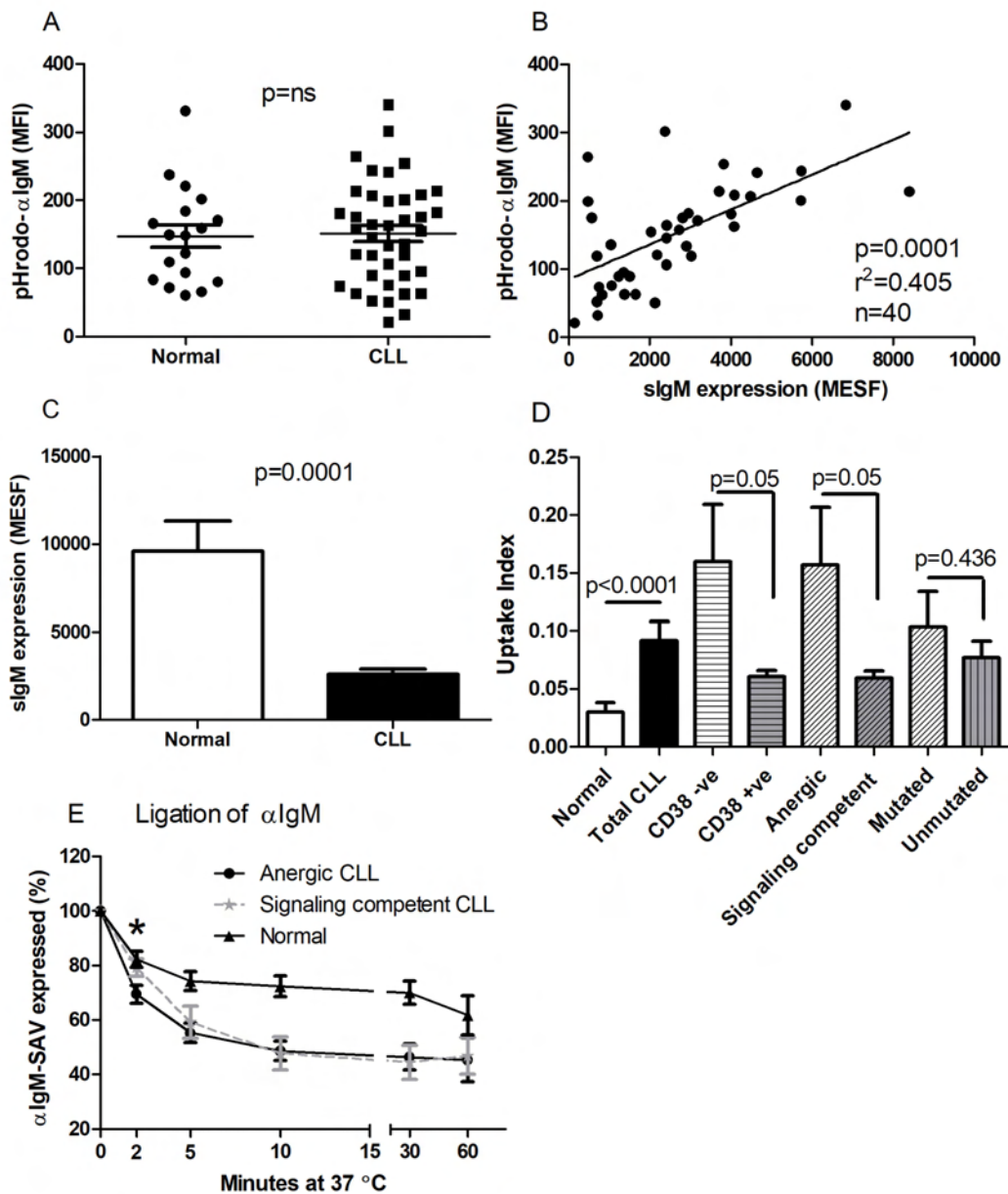
patient demonstrating a correlation between sIgM and sCD79B. (D) The percentage of sCD79B receptor expression was measured on CLL and normal B-cells following agonistic  $\alpha$ IgM ligation; a gradual reduction and internalization of sCD79B on normal CD19+ B-cells was detected after 60 min incubation (n=6; p=0.03\*; Wilcoxon match pairs test). In addition, CD79B internalization (E) and sIgM receptor expression (F) was measured upon  $\alpha$ CD79B ligation, and compared between anergic, signaling competent and normal B-cells. CD79B internalization occurred more rapidly (2 min time point) in anergic B-cells compared to signaling competent and normal B-cells (p=0.01 and p=0.001, respectively\*; Mann-Whitney test), however the percentage of sIgM receptor expression remained unchanged in all CLL cases. Finally, signaling competent CLL B-cells were pre-incubated with agonistic  $\alpha$ CD79B for 10mins at 37°C prior  $\alpha$ IgM stimulation to determine the effect of CD79B internalization on  $\alpha$ IgM-induced pERK activation (G: n=9; pERK levels were normalized to the positive control), as well as pHrodo- $\alpha$ IgM uptake (H: n=9). Statistical analysis was performed by use of Wilcoxon match pairs test.

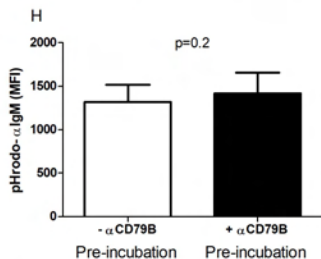
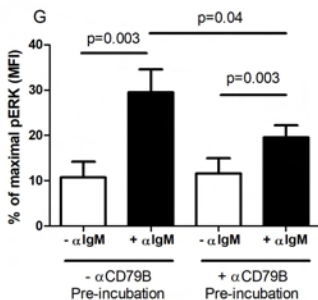
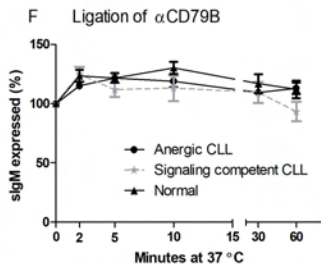
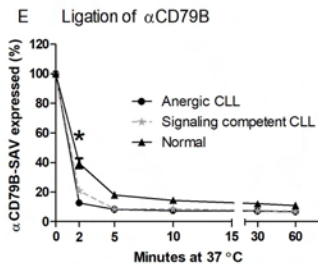
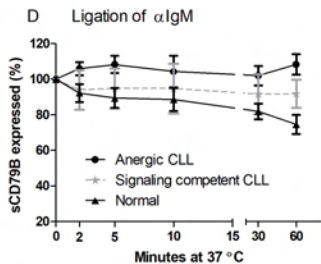
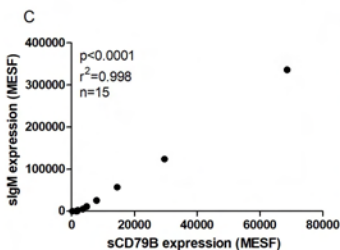
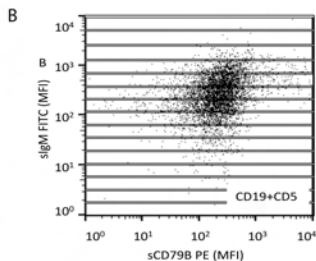
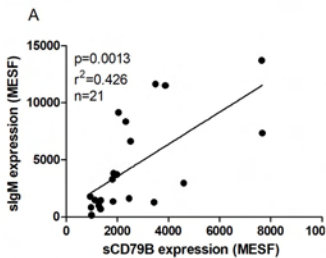
**Figure 3: *In-vivo* relationship between BCR signaling and internalization in CLL cells.**

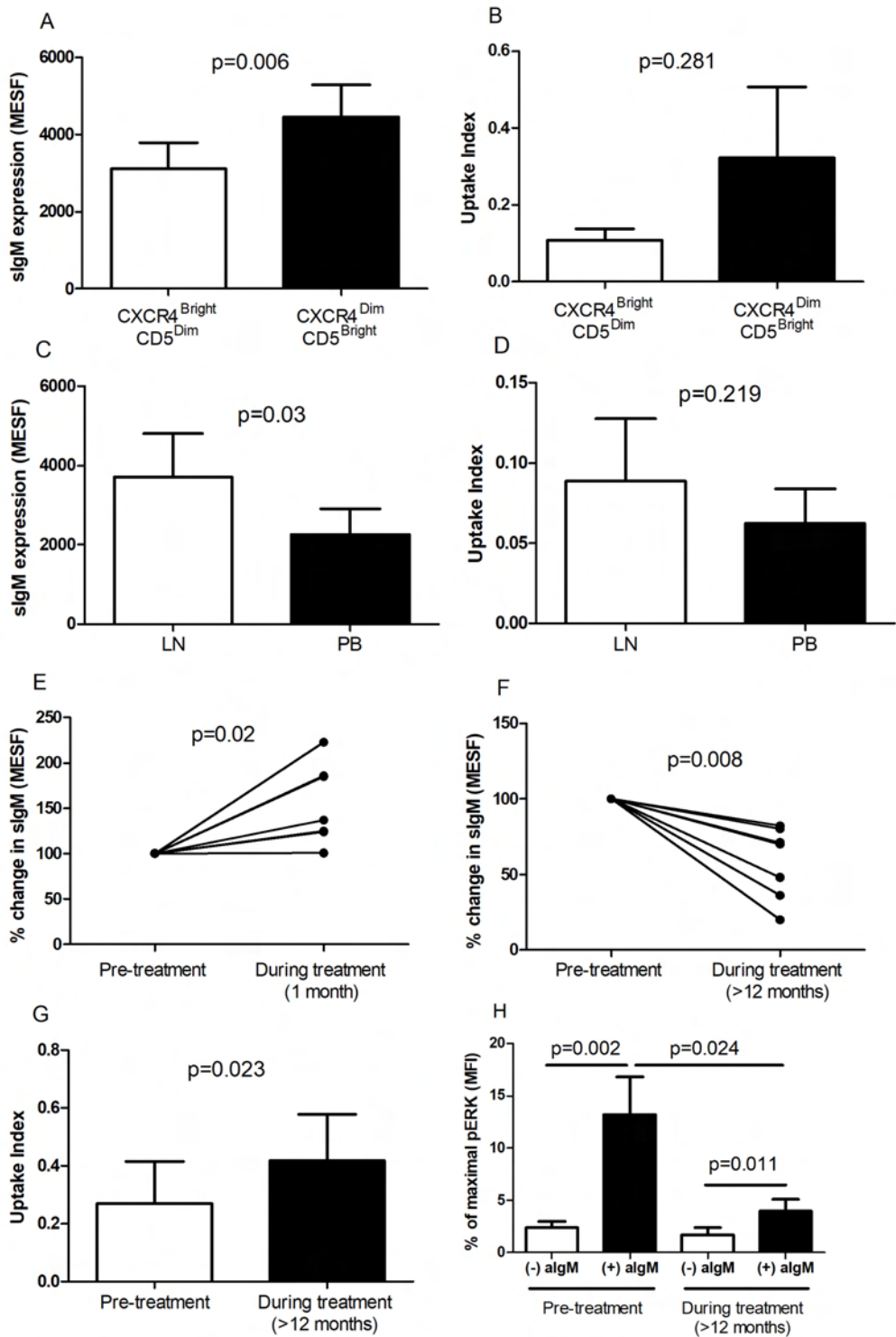
BCR expression and uptake index were investigated firstly in subsets of peripheral blood (PB) cells (CXCR4<sup>bright</sup>CD5<sup>dim</sup> and CXCR4<sup>dim</sup>CD5<sup>bright</sup> expressing CLL cells, A-B) and secondly, CD19+CD5+ cells from the matched lymph node (LN) and PB samples from 7 unmutated CLL patients (C-D). Thirdly, sIgM expression was measured in CD19+CD5+ peripheral blood (PB) cells from CLL patients before, 1 month and at least 12 months after commencing Bruton's tyrosine kinase inhibitor (BTKi) treatment (ibrutinib, n=6 or acalabrutinib, n=1; presented as a percentage change in sIgM expression, E-F). Uptake index (G) and the capacity to induce pERK activation following BCR stimulation (H: pERK levels normalized to PMA/positive control) was also examined and compared in CLL B-cells, pre- and during treatment. Statistical analysis was performed by use of Wilcoxon match pairs test.

**Figure 4. Proposed mechanism for the dissociation of BCR signaling and internalization in CLL.**

Two alternative configurations of the BCR are proposed. Type 1 in which CD79B and sIgM are already closely associated or become associated following ligand binding. This form of the receptor can transduce downstream signals but is not internalized at least until the subunits dissociate. Type 2 BCRs do not contain CD79B either before or after ligand binding. These receptors therefore cannot signal but can become internalized. Possible BCR compositions in anergic and non anergic CLL are illustrated. In anergic CLL, both sIgM and sCD79B levels are low and there is little potential for association between the two either before or after ligand binding. In this scenario, downstream BCR signaling is minimal but internalization is efficient. In non-anergic cases of CLL, both sIgM and sCD79B levels are higher and there is a greater likelihood of the two to becoming associated. Signaling is thus relatively more efficient but the capacity for internalization is less.

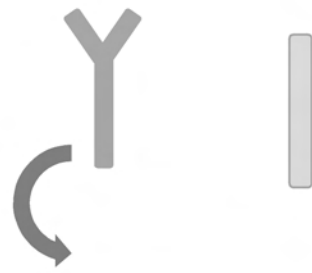






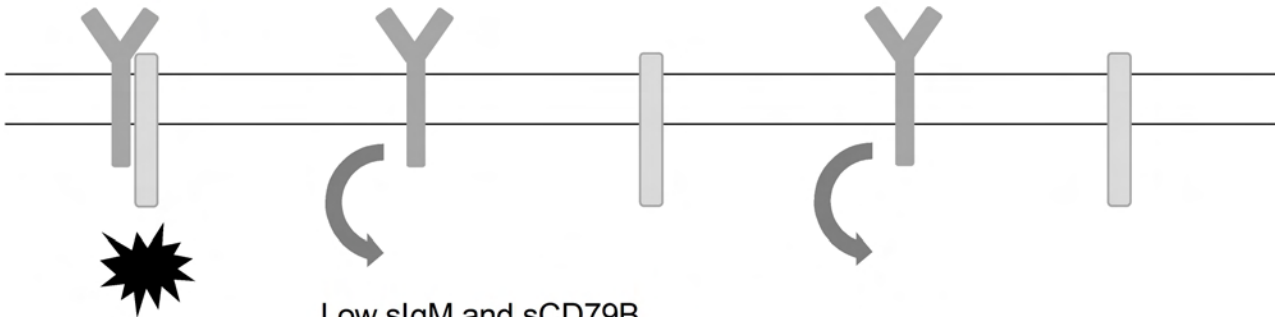


Type 1 BCR  
Can signal but not internalize



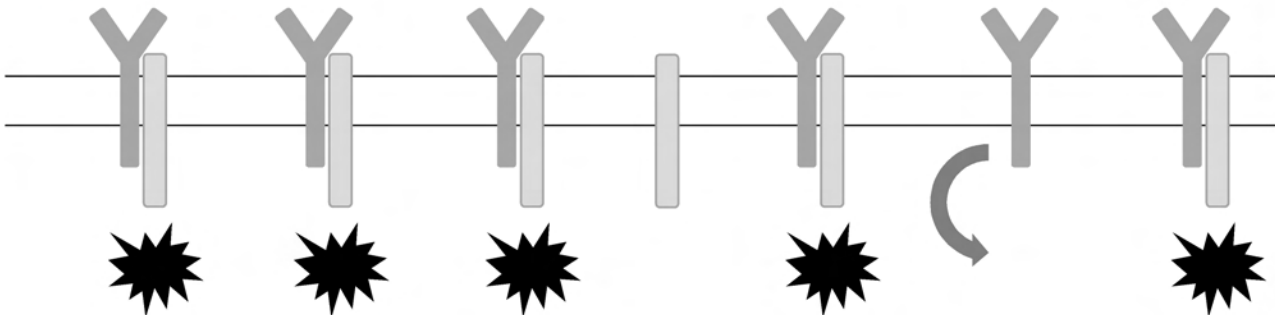
Type 2 BCR  
Can internalize but not signal

### Anergic CLL



Low sIgM and sCD79B  
Efficient internalization but inefficient signaling

### Non-anergic CLL



Higher sIgM and sCD79B  
Efficient signaling but inefficient internalization

## 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°C, 5% CO<sub>2</sub> 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](http://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 ( $5 \times 10^5$  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<sup>+</sup>/CD5<sup>+</sup>) and normal B-cells (CD19<sup>+</sup>) 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,  $5 \times 10^6$  cells/ml CLL PBMCs were incubated in media supplemented with  $1 \mu\text{M}$  Indo1-AM for 1 h at  $37^\circ\text{C}$ , then washed and re-suspended in fresh medium and incubated at  $37^\circ\text{C}$  for 30 minutes then stored on ice before being used for experiments. For each acquisition,  $3 \times 10^6$  PBMCs re-suspended in 1 ml of medium were used. For dead cell exclusion  $5 \mu\text{l}$  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  $37^\circ\text{C}$  for 5 minutes before performing the analysis. Background or basal  $\text{Ca}^{2+}$  fluorescence was recorded followed by addition of goat  $\text{F(ab')}_2$  anti-human IgM ( $20 \mu\text{g} / \text{ml}$ ; Cambridge Biosciences) and events were recorded up to 300 seconds. Ionomycin at a concentration of  $1 \mu\text{M}$  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<sup>th</sup> 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-nergic when the percentage of responding cells was greater than 5% or non-signal competent/nergic 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  $\text{F(ab')}_2$   $\alpha\text{IgM}$  for 10 minutes at  $37^\circ\text{C}$ , 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  $\alpha$ CD79B for 10mins at 37°C was included prior to  $\alpha$ IgM 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  $\alpha$ IgM-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- $\alpha$ IgM 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')<sub>2</sub> anti-human Immunoglobulin M or D ( $\alpha$ IgM or IgD; Cambridge Biosciences, Cambridge, UK) for 1h at room temperature. Target cells were incubated with either pHrodo-avidin- $\alpha$ IgM (pHrodo- $\alpha$ IgM or D) for 30 minutes at 4°C and then 37°C 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<sup>+</sup>/CD5<sup>+</sup>). All incubations were performed in PBS pH 7.4, so that any observed fluorescence was due to BCR internalization and trafficking of the pHrodo- $\alpha$ IgM to

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

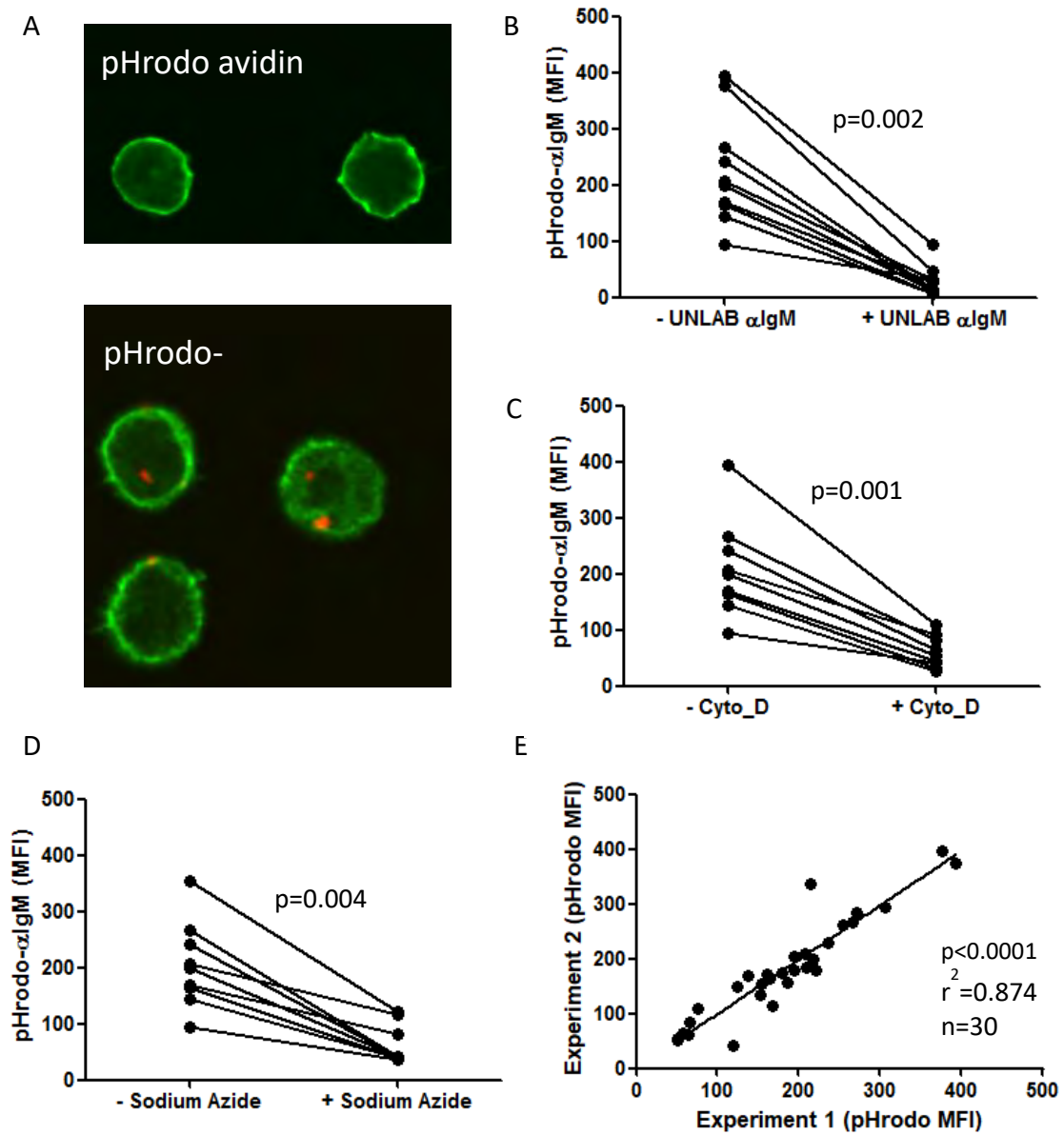
To confirm BCR specificity, experiments were performed in the presence of a molar excess of unlabeled  $\alpha$ IgM or D (Cambridge Biosciences). To investigate energy dependence and the role of the cytoskeleton, cells were pre-incubated with either sodium azide (0.2%; Severn Biotech Ltd, Kidderminster, UK) or cytochalasin D (10 $\mu$ M; Sigma-Aldrich) for 1h at 37°C. In addition, a pre-incubation with agonistic  $\alpha$ CD79B for 10mins at 37°C was included to specific experiments prior to  $\alpha$ IgM 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- $\alpha$ IgM uptake assay. As described above, normal and CLL PBMCs (5x10<sup>5</sup>/tube) were incubated with biotinylated  $\alpha$ IgM for 30 minutes at 4°C to allow initial receptor binding, washed twice with ice cold PBS, then incubated at 37°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  $\alpha$ CD79. 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 (F<sub>max</sub>) was defined as the APC fluorescence at 4°C which represents the starting amount of  $\alpha$ IgM-APC bound to the cells without endocytosis (0 minutes at 37°C). APC mean MFI was recorded at each time point (F<sub>tp</sub>). 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: F<sub>tp</sub>/F<sub>max</sub> 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,  $5 \times 10^5$  purified cells were incubated with pHrodo-avidin or pHrodo- $\alpha$ IgM as above, washed and re-suspended in 150  $\mu$ l of cell culture media (supplemented with 20% FBS) and deposited onto poly-l-lysine coated glass slides by cytopspin (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 100  $\mu$ l 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- $\alpha$ IgM 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- $\alpha$ IgM. CLL B-cells were selected from PBMCs (negative selection) and incubated with control (pHrodo-avidin) or pHrodo avidin conjugated to biotinylated F(ab')<sub>2</sub> anti-IgM (pHrodo- $\alpha$ IgM; 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')<sub>2</sub> anti-IgM (UNLAB- $\alpha$ IgM; n=9; B), cytochalasin D (cyto D; n=9; C) and sodium azide (n=9; D) on BCR-pHrodo- $\alpha$ IgM internalization in CLL patients was determined. CLL cells were pre-incubated for the times specified prior to pHrodo- $\alpha$ IgM 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.

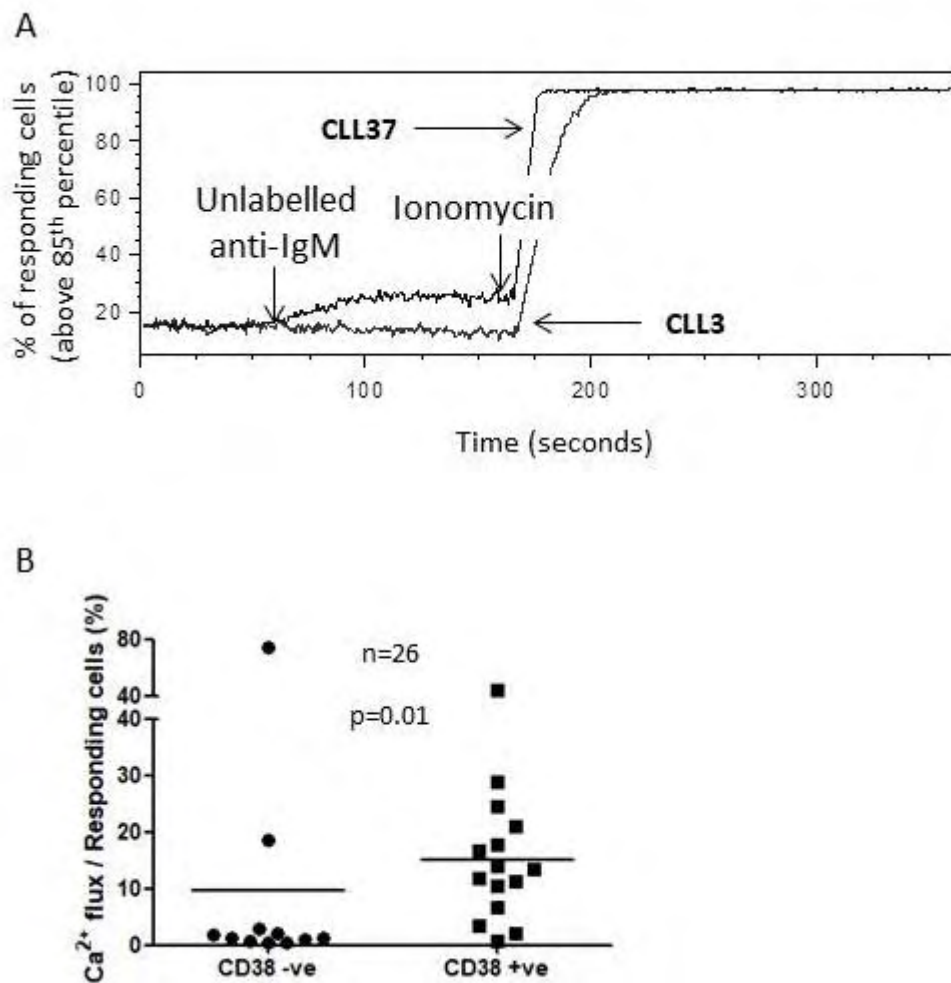


Figure S2. Determining sIgM-mediated signaling responses in CLL patients.

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')<sub>2</sub> anti-IgM. (A) Representative trace measuring intracellular calcium levels before and after (indicated by arrow) BCR ligation with unlabeled F(ab')<sub>2</sub> 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 =  $9.5 \pm 6.7$ , CD38+ve =  $15.1 \pm 2.9$ ;  $p=0.01$ ), 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.

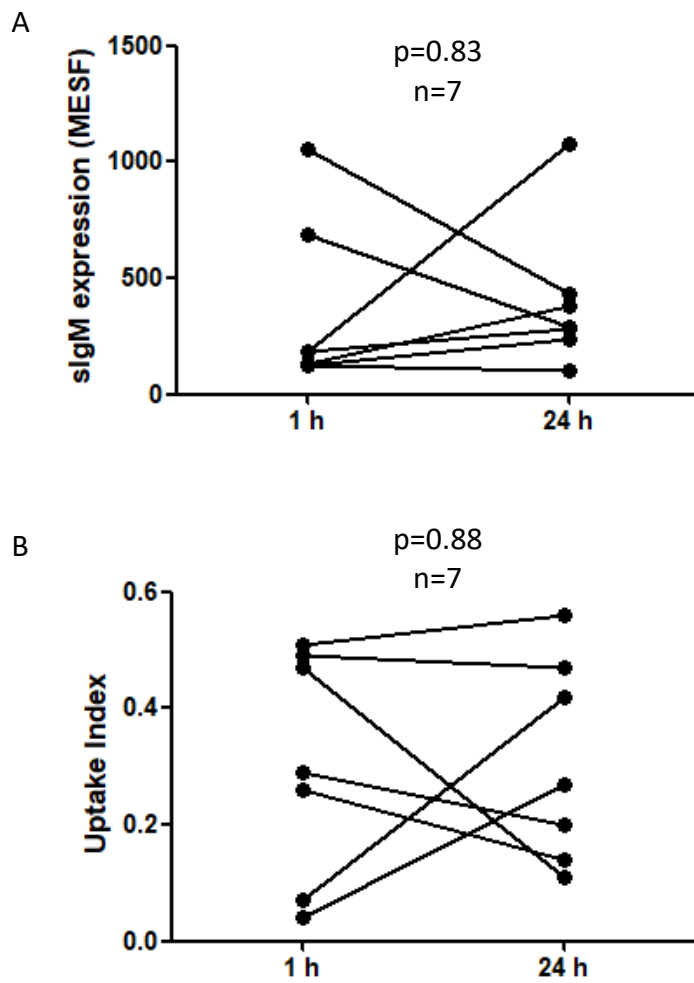


Figure S3. CLL B-cells maintain features of anergy following 24h *in-vitro* culture.

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

Figure S4.

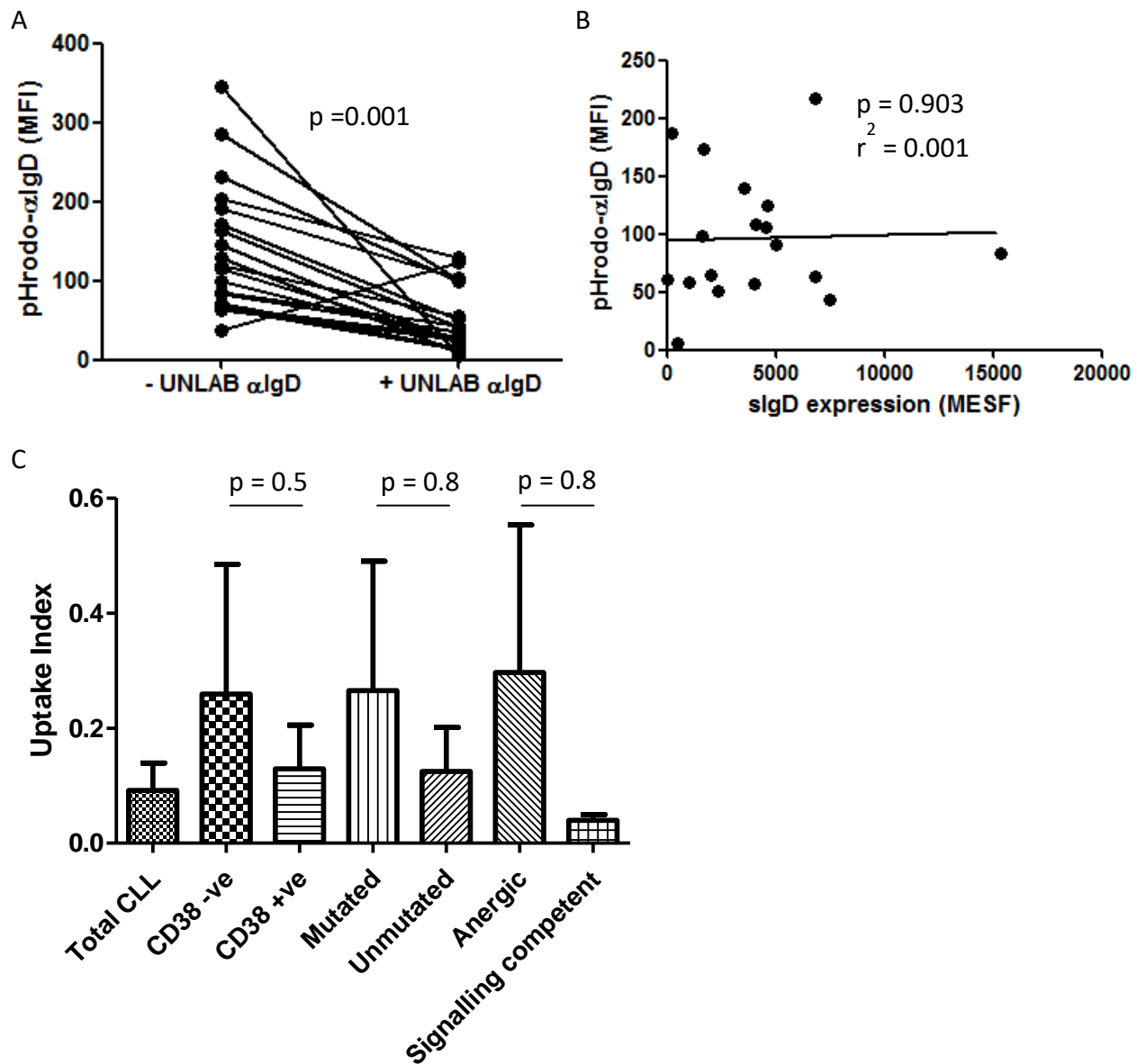


Figure S4. Levels of IgD expression and internalization do not correlate with prognostic factors in CLL.

The effect of unlabeled unlabeled  $\alpha$ IgD on the levels of pHrodo- $\alpha$ IgD uptake in CLL B-cells was determined (n=18). PBMCs from CLL patients were pre-incubated for the times specified prior to pHrodo- $\alpha$ IgD 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- $\alpha$ IgD uptake and surface IgD (sIgD) expression. (C) Comparing the pHrodo- $\alpha$ IgD uptake index between subgroups that determine good or poor prognosis. Statistical analysis was performed by use of unpaired T-test.

Figure S5.

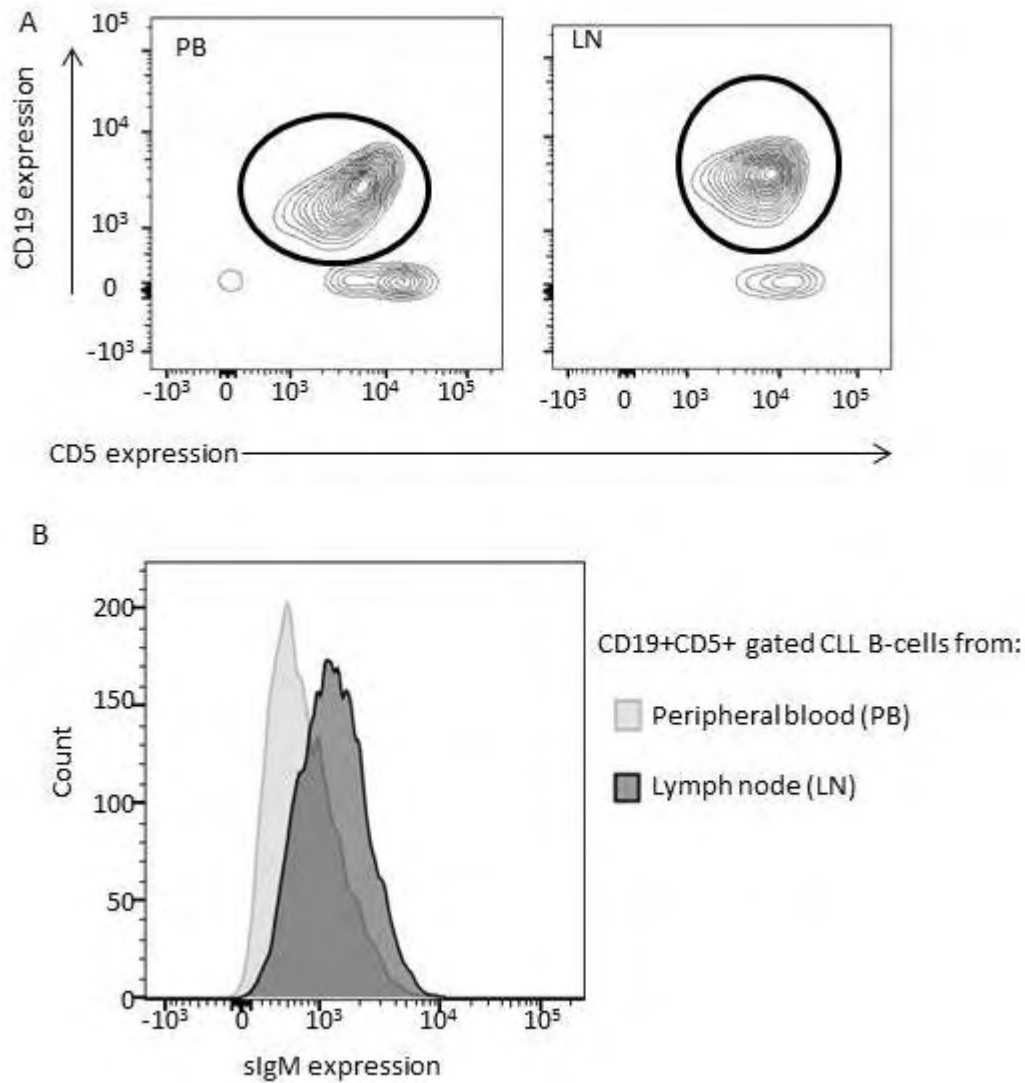


Figure S5. Comparing sIgM expression in matched PB and LN CLL B-cells.

(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 <sup>(1)</sup>	IGVH STATUS <sup>(2)</sup>	FISH	BINET STAGE	SIGNALING COMPETENT BCR <sup>(3)</sup>
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  $\geq 7\%$  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 <sup>(1)</sup>	IGVH STATUS <sup>(2)</sup>	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  $\geq 7\%$  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 <sup>(1)</sup>	IGVH STATUS <sup>(2)</sup>	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  $\geq 7\%$  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