LETTER

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Chronic lymphocytic leukaemia is driven by antigen-independent cell-autonomous signalling

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B-cell antigen receptor (BCR) expression is an important feature of chronic lymphocytic leukaemia (CLL), one of the most prevalent B-cell neoplasias in Western countries[1]. The presence of stereotyped and quasi-identical BCRs in different CLL patients suggests that recognition of specific antigens might drive CLL pathogenesis. Here we show that, in contrast to other B-cell neoplasias, CLL-derived BCRs induce antigen-independent cell-autonomous signalling, which is dependent on the heavy-chain complementarity-determining region (HCDR3) and an internal epitope of the BCR. Indeed, transferring the HCDR3 of a CLL-derived BCR provides autonomous signalling capacity to a non-autonomously active BCR, whereas mutations in the internal epitope abolish this capacity. Because BCR expression was required for the binding of secreted CLL-derived BCRs to target cells, and mutations in the internal epitope reduced this binding, our results indicate a new model for CLL pathogenesis, with cell-autonomous antigen-independent signalling as a crucial pathogenic mechanism.

CLL cases can be divided into two groups based on the mutational status of the immunoglobulin genes. Notably, in B cells from ~1% of both groups, unmutated CLL and mutated CLL cases express almost identical BCRs, and in 30% of cases the BCRs can be classified into certain stereotypes based on structural similarities and the HCDR3 (refs 2–5). This restricted BCR repertoire has been interpreted to be the result of a BCR-driven selection mechanism initiated by specific antigens that may promote the expansion of the respective CLL clone[6,7].

We have recently reported functional similarity between the pre-BCR and polyreactive BCRs, and have demonstrated that both activate cell-autonomous signalling in pre-B cells[8]. Furthermore, we have shown that the pre-BCR induces ligand-independent cell-autonomous signalling by surrogate light-chain-mediated binding to an intrinsic pre-BCR glycosylation site[9]. CLL-derived BCRs are also often polyreactive[10,11], suggesting that, in analogy to the pre-BCR, CLL BCRs might activate ligand-independent autonomous signalling through the binding of BCR-intrinsic motifs. We tested this hypothesis by comparing the signalling capacity of randomly chosen BCRs from different B-cell lymphomas in triple knockout (TKO) cells that do not express endogenous pre-BCRs or BCRs owing to inactivation of *RAG2* and *λ5* genes. An additional deficiency in SLP65 (also known as BLNK) expression makes TKO cells an ideal reconstitution system to test receptor function by transfection of receptor components and a tamoxifen-inducible ERT2–SLP65 fusion protein[12]. As shown previously[8], the pre-BCR and a polyreactive human BCR derived from an early bone marrow B cell (BCR62) activate Ca^{2+} mobilization autonomously without additional ligands, whereas a non-polyreactive human BCR derived form a selected mature B cell (BCR53) requires additional crosslinking (Supplementary Fig. 1a and data not shown). Similar to BCR53, we found that BCRs derived from multiple myeloma ($n = 4$), marginal zone lymphoma ($n = 3$), mantle cell lymphoma ($n = 4$) and follicular lymphoma ($n = 4$) required crosslinking with anti-BCR antibody in addition to SLP65 activation to induce Ca^{2+} flux (Fig. 1, Supplementary Fig. 1b, c and Supplementary Table 1; data not shown). By contrast, all tested ($n = 17$) CLL-derived BCRs, including those containing typical CLL variable heavy chain (VH) elements (for example, IGHV4-34, IGHV3-7, IGHV3-23, IGHV3-30 and IGHV1-69)[3] and stereotyped HCDR3s, induced autonomous signalling on SLP65 activation (Fig. 1, Supplementary Fig. 2a and Supplementary

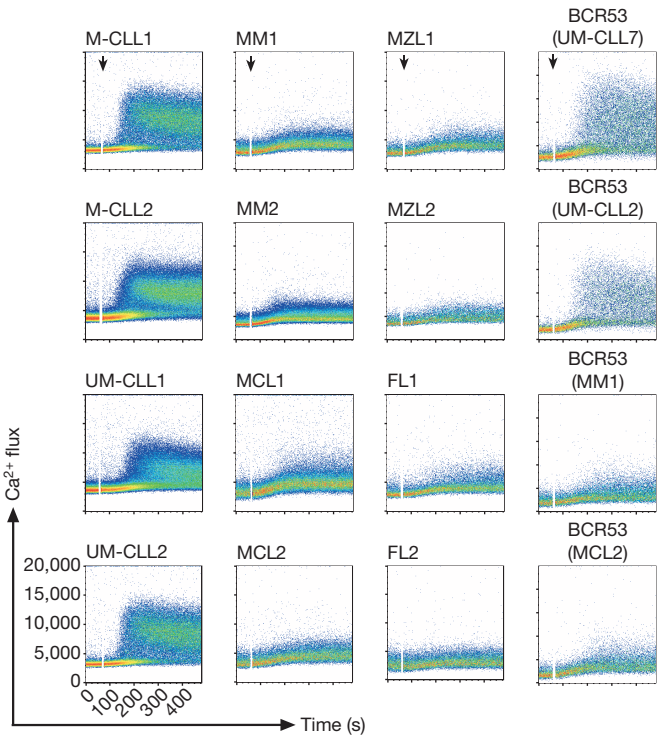

Figure 1 | CLL-derived BCRs possess autonomous signalling capacity. Fluorescence-activated cell sorting (FACS) analyses of Ca^{2+} flux after the addition of 4-hydroxytamoxifen (4-OHT) to TKO cells expressing the ERT2–SLP65 fusion protein and four representative CLL-derived BCRs (left column), two representative BCRs of multiple myeloma (MM), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL) and follicular lymphoma (FL) (middle two columns) and BCR53 variants containing different HCDR3s of representative CLL, multiple myeloma or mantle cell lymphoma receptors (right column). Addition of the stimulus is indicated by black arrows. M, mutated; UM, unmutated.

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Table 1; data not shown). No obvious difference was found between mutated ($n = 10$) and unmutated ($n = 7$) CLL BCRs (Fig. 1 and data not shown). Importantly, BCRs derived from healthy donors who possess CLL-typical VH genes such as *IGHV1-69* or *IGHV4-34* did not display autonomous signalling (Supplementary Fig. 2b).

To investigate whether autonomous signalling is also involved in lymphoproliferation in animal models of CLL, we examined TCL1 mice, which express a transgene for human T-cell leukaemia 1 (*TCL1*) under the control of the VH promotor and the Eμ enhancer[13]. TCL1 is highly expressed in unmutated CLL[14] and TCL1 transgenic mice show clonal expansion of $CD5^+$ IgM^+ B cells bearing unmutated BCRs[13,15]. To test whether transformed clones derived from TCL1 mice express autonomously active BCRs, we studied four different BCRs from TCL1 mice in the TKO reconstitution system. All four TCL1-derived BCRs showed autonomous Ca^{2+} flux similar to the human CLL BCRs and the murine pre-BCR, whereas a 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP)-specific murine BCR (B1-8) showed no autonomous signalling (Supplementary Fig. 3a, b). Similar to human B-cell CLL, TCL1-transformed murine B cells are $CD5^+$. Therefore, we tested whether autonomous signalling is a characteristic feature of murine B1a B cells that are typically $CD5^+$ and IgM^+. Interestingly, BCRs from B1a B cells did not induce autonomous signalling, indicating that this capacity is a unique feature of leukaemia-derived BCRs (Supplementary Fig. 3c). Together, our data show that, in contrast to all other B-cell lymphomas tested, BCRs derived from CLL patients and TCL1 mice possess the unique capacity to induce autonomous signalling.

The bias in VH gene usage and the high proportion of stereotyped HCDR3s in CLL suggest that HCDR3 has a critical role in the development of this disease[2,3,5]. To test whether transferring HCDR3 from a CLL-derived BCR transfers autonomous signalling capacity to a non-autonomously active BCR, we replaced the HCDR3 of BCR53 with three different CLL-derived HCDR3s. As a negative control, we replaced the HCDR3 of BCR53 with the HCDR3s of a multiple myeloma BCR and a mantle cell lymphoma BCR that contained the same VH gene as the chosen CLL BCRs. Although all modified BCR53 derivatives were expressed on the cell surface, only those containing a CLL-derived HCDR3 acquired the capacity for autonomous signalling (Fig. 1 and Supplementary Fig. 1; data not shown).

Our previous results indicated that the ability to induce autonomous Ca^{2+} flux was associated with BCR polyreactivity[8]. Because most secreted CLL-derived BCRs were reported to be polyreactive[10], we tested the chosen CLL BCRs for polyreactivity. Only three out of six randomly chosen CLL-derived BCRs—two unmutated and one mutated—showed polyreactive potential by binding to more than two antigens in enzyme-linked immunosorbent assays (ELISA) and showing cytoplasmic staining of HEp-2 slides in indirect immunofluorescence assays (Supplementary Fig. 4a, b). Furthermore, only one out of four tested TCL1 BCRs (TCL1 no. 3) showed polyreactivity in the same assays (Supplementary Fig. 4c, d). The frequent lack of polyreactivity suggests that other mechanisms induce the autonomous signalling of CLL-derived BCRs. To show that specific antigens on adjacent cells are not required for the activation of CLL BCRs, we examined autonomous Ca^{2+} signalling in isolated, single cells. We detected autonomous Ca^{2+} flux in single cells expressing a CLL-derived BCR, whereas additional stimulation with anti-BCR antibodies was required for activation of single cells expressing the BCR53 control (Fig. 2a, b).

The lack of polyreactivity suggests that antigen-independent signalling might be achieved by HCDR3-dependent binding of BCR-intrinsic motifs, suggesting mutual BCR recognition on CLL cells. Indeed, secreted CLL-derived BCRs bound to the surface of TKO cells expressing a CLL BCR or the control BCR53, although they failed to bind to TKO cells that did not express a receptor (Fig. 3a, b and Supplementary Fig. 5a). This suggests that the CLL-derived BCRs might interact with intrinsic BCR structures and that this interaction

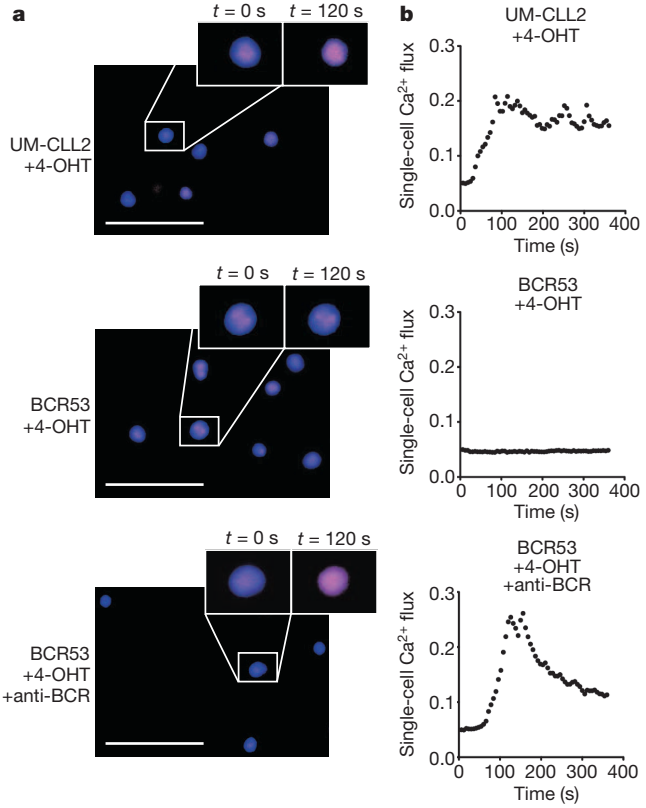

Figure 2 | CLL BCRs induce cell-autonomous signalling. **a**, Live-cell imaging of single TKO cells expressing ERT2–SLP65 and unmutated CLL2 or BCR53 after induction with 4-OHT. Images show merged channels of Indo-1 unbound (blue) and Indo-1 bound (violet). Magnification is ×40; scale bars, 25 μm. **b**, Ca^{2+} flux of representative single cells from **a**. Kinetics are representative of at least ten single cells.

could mediate antigen-independent stimulation if it takes place within the membrane of the same cell. Using a phage-display approach, a recent study identified different peptide motifs that bind to primary CLL cells in a HCDR3-dependent manner, and one of these motifs showed specific binding to each of the tested CLL samples[16]. Through sequence analysis we found that this motif possesses homology to a conserved epitope in the framework region 2 (FR2) of VH domains (Supplementary Fig. 5c). To test whether this region contains an internal epitope important for autonomous signalling, we generated point mutations of the amino acids Val 37 and Arg 38 within this motif. As a control we mutated a conserved arginine (Arg 43) outside of the motif. Our results show that mutating Val 37 or Arg 38, but not Arg 43, abolishes autonomous signalling of CLL BCRs, whereas stimulation with anti-BCR antibodies show normal Ca^{2+} flux for all mutants (Fig. 3c, d and Supplementary Fig. 5b, d; data not shown). As the introduced mutations are located outside of the antigen-recognition site of the BCR, these data indicate that the observed autonomous signalling is not induced by BCR interaction with external antigens in the environment, because such antigens should still be able to bind to and stimulate the mutated CLL BCR. To confirm this view, we mutated Val 37 and Lys 38 in the NIP-specific B1-8 BCR and found that the resulting mutant BCRs show normal antigen-dependent stimulation after incubation with NIP–bovine serum albumin (Fig. 3c and Supplementary Fig. 5b). Thus, these data indicate that the loss of autonomous signalling was caused by the inability of the CLL BCR to bind to the mutated intrinsic motif. In agreement with this, the binding of secreted CLL BCRs to TKO cells was reduced when the cells expressed a BCR with a mutated intrinsic motif (Fig. 3e). Together, our results suggest that an intrinsic BCR motif and HCDR3 mediate autonomous BCR stimulation in CLL-derived BCRs.

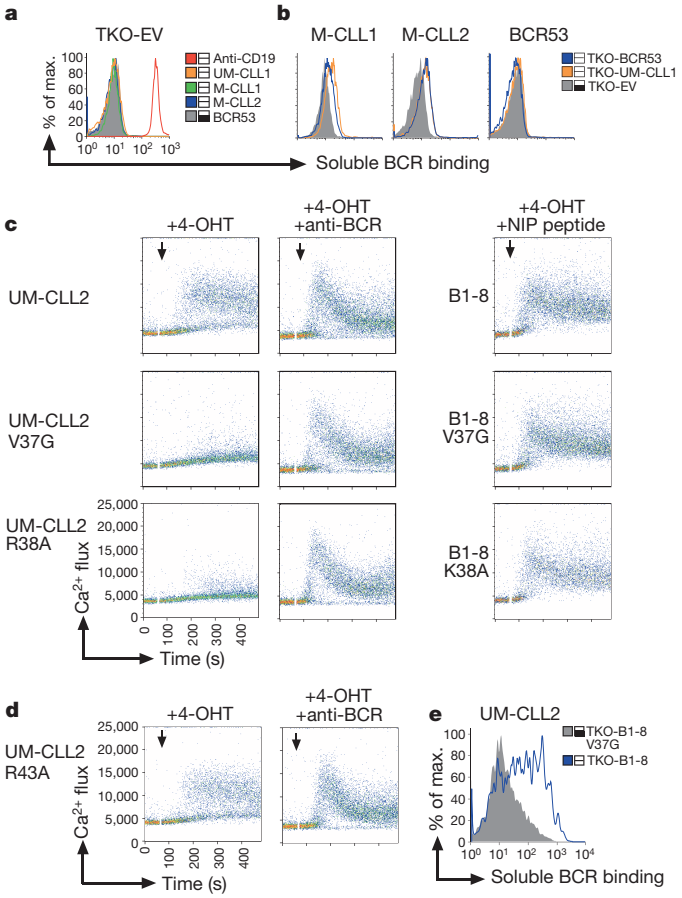

Figure 3 | Autonomous signalling of CLL-derived BCRs is mediated by the recognition of a BCR-intrinsic epitope. **a**, Surface-binding profiles of different secreted CLL BCRs on TKO cells expressing empty vector (EV). Secreted BCR53 and anti-CD19 antibody were used as negative and positive controls, respectively. The amount of secreted BCR used was >100-fold higher than for the anti-CD19 antibody. **b**, Surface-binding profiles of secreted M-CLL1, M-CLL2 and BCR53 on TKO cells expressing the indicated BCRs. **c**, **d**, FACS analysis for autonomous Ca^{2+} flux after addition of the indicated stimuli in TKO cells expressing the ERT2–SLP65 fusion protein. **c**, The UM-CLL2 mutants V37G and R38A (left) or the corresponding B1-8 mutants (right). **d**, The UM-CLL2 R43A mutant. Arrows indicate addition of the stimuli. **e**, Surface-binding profile of UM-CLL2 on TKO cells expressing B1-8 or B1-8 V37G.

To show that autonomous signalling can also be detected in primary CLL samples, we monitored Ca^{2+} flux in primary B cells from CLL patients and compared them to primary peripheral B cells from healthy donors or patients with other B-cell neoplasias. Because SLP65 function is constitutively active in primary cells, a synchronous start of autonomous signalling as in the TKO cells cannot be achieved in primary cells. However, constitutive autonomous signalling suggests that a proportion of the primary cells show increased Ca^{2+} flux. In fact, our experiments show that primary CLL samples show elevated Ca^{2+} mobilization compared with the control cells. Moreover, this signalling capacity required BCR function, as inhibition of the protein tyrosine kinase SYK, which transmits BCR signals[17], prevented this activity (Fig. 4 and Supplementary Fig. 6a). Similarly, monitoring Ca^{2+} mobilization in single cells showed periodic SYK-dependent signals in isolated primary CLL B cells but not in control cells (Fig. 4 and Supplementary Fig. 6b). As survival of CLL B cells is associated with nuclear-factor κB (NF-κB) activation[18], we investigated the expression of NF-κB-regulated survival genes[18] in primary CLL cells and found that inhibition of autonomous BCR signalling by inhibition of SYK led to a reduction in the expression of MYC and XIAP (Supplementary Fig. 6c).

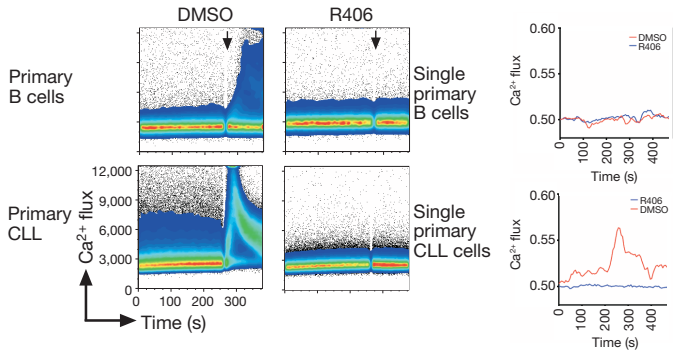

Figure 4 | Cell-autonomous Ca^{2+} signalling is elevated in primary CLL B cells. Ca^{2+} flux in primary peripheral B cells from a healthy donor or a CLL patient with or without R406 treatment (left panels; addition of an anti-BCR stimulus as a control for cell viability and Indo loading is indicated by a black arrow) and Ca^{2+} flux in single cells from a healthy donor or a CLL patient (right panels). DMSO, dimethylsulphoxide.

BCR expression is commonly used as a diagnostic marker for the characterization of CLL patients. The restricted BCR repertoire of both mutated and unmutated CLL suggests a potentially important role for BCR signalling in the transformation and expansion of CLL B cells. The restricted BCR repertoire and the observed polyautoreactivity suggested that CLL BCRs might recognize extrinsic autoantigens, thereby inducing the selective expansion of the respective B cells. Therefore, blocking such autoantigens or blocking BCR function by inhibiting downstream signalling factors, such as the tyrosine kinase SYK, are considered as potential therapies for CLL[19–22].

However, our data indicate that CLL BCRs induce cell-autonomous signalling independent of extrinsic antigens and identify an intrinsic motif that might provide new targets for the therapy of CLL by interfering with autonomous BCR stimulation. It is conceivable that additional intrinsic epitopes exist that activate the autonomous signalling of distinct CLL BCRs. In this scenario, the HCDR3-driven interaction with distinct epitopes on nearby BCRs is sufficient for the induction of autonomous signalling on CLL cells. In agreement with this, HCDR3 sequences derived from CLL BCRs were able to transfer the autonomous signalling capacity to other receptors in our experiments. Presumably, only certain HCDR3 sequences have this unique capacity, which might explain the restricted repertoire and the requirement for certain HCDR3 regions in CLL. Thus, the presence of distinct intrinsic epitopes may explain the observed structural similarities of CLL BCRs, which are probably selected on the basis of the ability of HCDR3 to interact with such intrinsic structures. It should be noted, however, that the presence of intrinsic epitopes and their role in autonomous signalling does not rule out that for certain cases, extrinsic antigens are involved in the pathogenesis of CLL. This may further potentiate the basal activity of CLL BCRs. Our study presents a new mechanism for the pathogenesis of CLL and suggests that investigating intrinsic BCR-associated epitopes may lead to the development of new therapeutic approaches based on the inhibition of the autonomous BCR activation in CLL cells.

METHODS SUMMARY

Details of lymphoma material, clinical data, mice strain data, cells and cell-culture conditions, polyreactivity assays, plasmids and retroviral transduction, flow cytometry and Ca^{2+} flux analysis, single-cell Ca^{2+} flux analysis, NF-κB-activity assays, reverse-transcriptase PCR and statistical analysis are provided in Methods.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.D.-v.M., R.Ü., D.S., F.K. and M.P.B. conducted cloning of BCRs. M.D.-v.M., R.Ü. and D.S. performed measurements of BCR surface expression and calcium flux and performed soluble BCR-binding assays. M.D.-v.M. and T.W. performed quantitative reverse-transcriptase PCR experiments. M.B. performed the NF-κB activity ELISA. E.S. analysed V_H region sequences from patient samples. M.D.-v.M. and M.F. performed single-cell Ca^{2+} measurements. D.H. and H.W. tested the polyreactivity of CLL and TCL1 receptors. K.Z. and H.V. conducted the characterization of lymphoma patients and provided samples. M.D.-v.M., T.W., F.K. and H.J. wrote the manuscript. H.J. designed experiments. All authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to H.J. (jumaa@immunbio.mpg.de).

METHODS

Lymphoma material and clinical data. CLL B-cell samples were obtained from the peripheral blood of patients at the University Medical Center Freiburg. Data for IGVH status, ZAP70 expression and time to first treatment were taken from the clinical records. The local ethics committee approved patient sampling and all patients gave informed consent.

Mice. Eμ-*TCL1* mice (provided by C. Croce) were described previously[13]. Mice were bred in the animal facility of the Max Planck Institute of Immunobiology and Epigenetics under specific pathogen-free conditions. Animal experiments were done in compliance with the guidelines of the German law.

Cells and cell-culture conditions. HEK293T, Phoenix and TKO cells were cultured in Iscove's Medium (Biochrom AG) containing 5% FCS (PAN-Biotech), 10 mM L-glutamine (Gibco) and 100 U ml^{-1} penicillin/streptomycin (Gibco). For TKO cultures, the medium was supplemented with 50 mM 2-mercaptoethanol (Gibco) and supernatant of J558L mouse plasmacytoma cells stably transfected with a murine IL-7 expression vector.

Primary peripheral B cells from CLL patients and healthy donors were cultured in RPMI containing 10% FCS, 10 mM L-glutamine and 100 U ml^{-1} penicillin/streptomycin for 24 h. For the inhibition of SYK, cells were then treated with 4 μM R406 (Rigel) for 1 h.

ELISA and indirect immunofluorescence assays. ELISA assays were performed using microtitre plates coated with 10 μg ml^{-1} of single-stranded DNA, double-stranded DNA (ORGENTEC), lipopolysaccharide or human insulin (Sigma-Aldrich). CLL antibodies were produced as described previously[23] and purified antibodies were used at 1 mg ml^{-1} and three serial dilutions of 1:4 in PBS. All ELISA assays were developed with horseradish-peroxidase-labelled goat anti-human IgG (BioSource). $D_{405\,nm}$ was measured using a microtitre plate reader (SpectraMax 190).

Indirect immunofluorescence assays were done as previously described[24]. In brief, diagnostic HEp-2 slides were incubated with 15 μl purified CLL antibodies (1 mg ml^{-1}). After washing, a Cy3-conjugated anti-human IgG1 antibody (Jackson ImmunoResearch Laboratories) was used to detect binding. Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI).

Plasmids and retroviral transduction. ERT2–SLP65 (ref. 12) was expressed using a vector containing tdTomato as expression control (a gift from R. Y. Tsien). Immunoglobulin heavy chain and light chain were expressed using the biomolecular fluorescence complementation vector system described previously[8].

CLL-derived BCRs were amplified from patient samples by anchor-PCR using poly-G-tailed complementary DNA and a poly-C-containing primer as described[25,26]. Human VH domains were fused to the murine constant region (μCH) by PCR, whereas the complete human light chains were used. Other lymphoma-derived BCRs were amplified from sequencing vectors containing heavy-chain or light-chain sequences of multiple myeloma, mantle cell lymphoma, marginal zone lymphoma and follicular lymphoma (CellGenix). For virus production, phoenix cells were transfected using FuGeneHD as recommended by the manufacturer's protocol. Supernatants were collected 48 h after transfection and used for transduction of TKO cells as described[27].

Flow cytometry and Ca^{2+} flux analysis. For FACS analyses, TKO cells were stained with goat anti-mouse IgM (Southern Biotech), goat anti-human κ light chain and goat anti-human λ light chain (Southern Biotech).

Ca^{2+} analyses were performed as described[28]. In brief, 1×10^6 freshly transduced TKO cells expressing ERT2–SLP65 were loaded with Indo-1 (Invitrogen) using Pluronic (Invitrogen). Induction of ERT2–SLP65 was performed by the addition of 2 μM 4-OHT (Sigma-Aldrich). Goat anti-mouse IgM (10 μg ml^{-1}; Southern Biotech) was used for crosslinking of the BCR. For the inhibition of SYK, cells were pre-treated with 4 μM R406 (Rigel) for 1 h.

Single-cell Ca^{2+} flux analysis. TKO cells expressing ERT2–SLP65 and BCR53 or unmutated CLL2 were prepared as described[28] and seeded onto coated eight-well μ-Slides (ibidi) using BD Cell-Tak (BD Biosciences). Live-cell imaging of single cells was performed using a ScaṅR screening station on an IX81 inverse microscope (Olympus). Measurements were taken at 37 °C and at time intervals of 6 min after induction with 4-OHT or 4-OHT and goat anti-mouse IgM (Southern Biotech) or goat anti-human IgM. For the inhibition of SYK, cells were pre-treated with 4 μM R406 (Rigel) for 1 h.

NF-κB activity assay. NF-κB activity was measured using the TransAM NF-κB p50 transcription factor assay kit (Active Motif). In brief, peripheral blood B cells from CLL patients were treated with 4 μM R406 for 1 h or were left untreated. Total cellular lysates were applied to 96-well plates coated with oligonucleotides containing the NF-κB consensus sequence (5′-GGGACTTTCC-3′) and NF-κB activity was calculated by measuring p50 binding using a specific antibody.

Quantitative reverse-transcriptase PCR. Total RNA was extracted, reverse-transcribed using poly-dT primers and amplified using MYC-specific primers and XIAP-specific primers designed by Universal ProbeLibrary Assay Design Center (Roche Applied Science). In addition, the amount of actin transcripts was quantified in all samples as an internal control. SYBR green was used in the detection method.

Statistical analysis. For statistical analysis, GraphPad Prism software was used.

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