Supplemental figure legends:

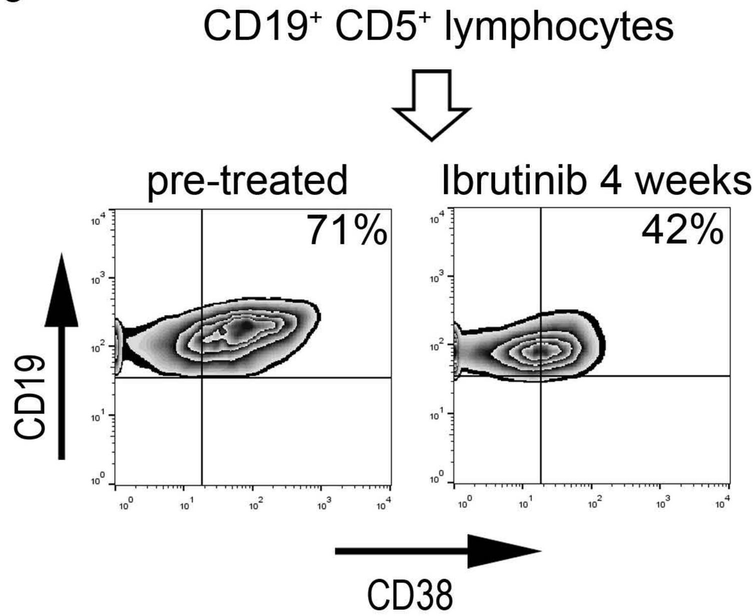
Supplementary Figure 1. Cytometric studies for proliferative fractions (PF) and single parameter detection of CD38, CD86, IgM/IgG and CXCR4/CD5 cell markers in leukemic cells of CLL patients treated with ibrutinib *in vivo*. Criteria used for cytometry gating of all PF evaluated. Results are shown as representative dot plots for CD38 expression **(i)** CD86 **(ii)**, IgM/IgG **(iii),** and CXCR4/CD5 **(iv)**, from where the group data depicted in Figure 1A was taken. For evaluating the effect of ibrutinib treatment in the percentage of $CXCR4^{low}CD5^{high}$ cells, we followed the described criteria to identify such population and fixed a gate in the pre-treated condition for each patient. Next, we acquired in the same experimental conditions the tubes for 1 and 4 weeks after treatment and measured the percentage in those previously fixed gating conditions **(iv)**.

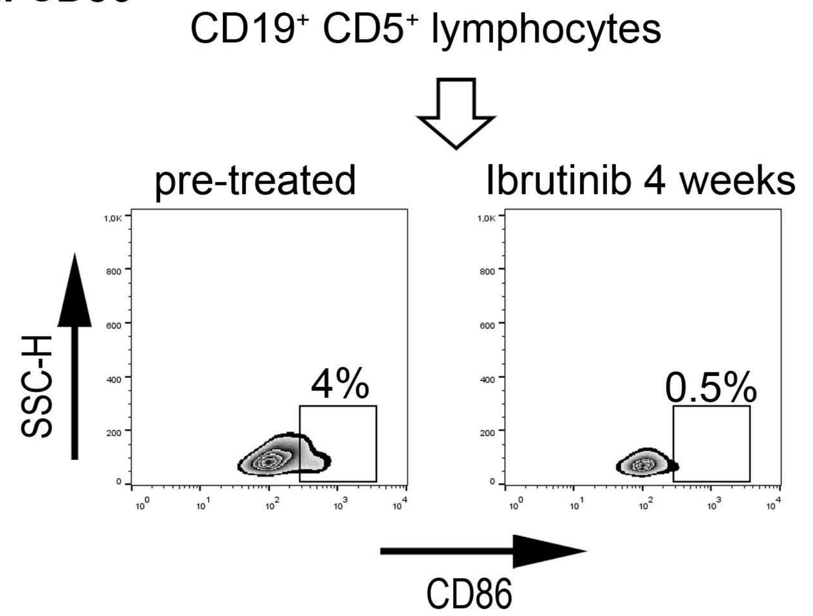
Supplementary Figure 2. Positive controls and cytometric criteria for intracellular detection of AID. In order to set the experimental conditions for AID detection by flow cytometry, peripheral blood samples from 3 untreated CLL patients were centrifuged in Ficoll gradient, and total mononuclear cells obtained were cultured in presence of IL-4 over a HeLa CD40L-expressing cell line layer for 5 days or over HeLa cells alone as control. AID labeling was developed as detailed in Methods section. Shown are representative histograms for a single CLL patient incubated with irrelevant or anti-AID antibody, for the control condition (upper graphics) or the CD40L+IL-4 stimulated condition (lower graphics). Percentage of AID positive cells in the activated condition for the three patients ranged from 18 to 55%. Concerning AID expression in the whole CLL only significant differences were observed comparing pre- and Wk4, 1.8 % vs. 1.1, respectively (mean difference = 0.72, CI 95% = 0.14-1.30; *p=0.016;* n = 10, One way ANOVA, multiple comparisons test).

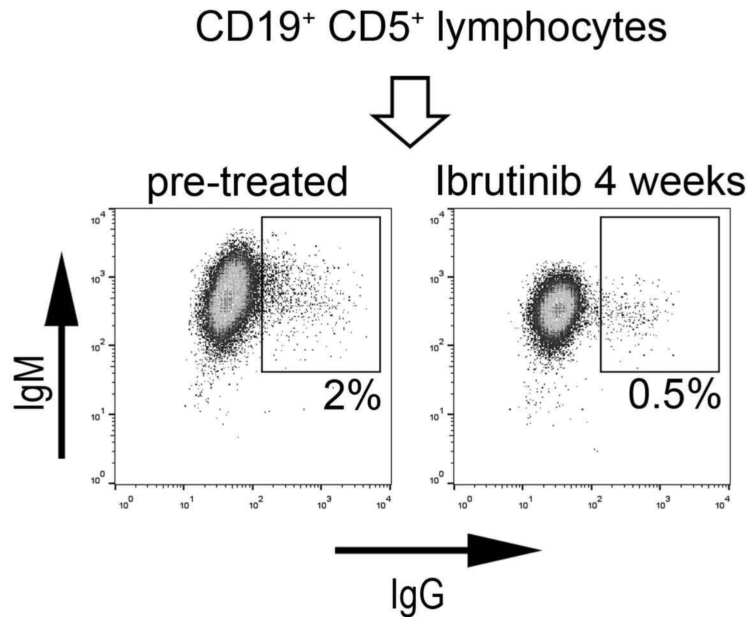
Supplementary Data 1. Extended data of Material and methods section

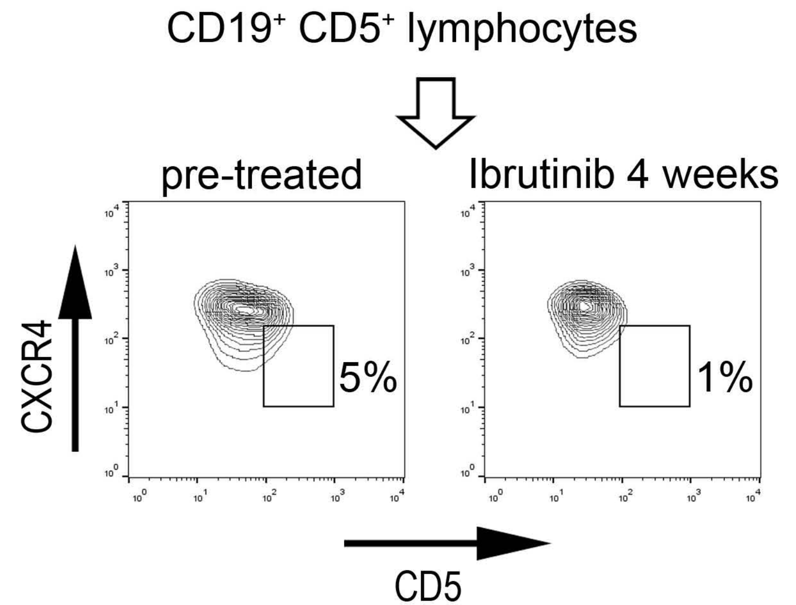
Supplementary Data 2. **Analysis of the phospho-protein profiling in purified CD5^{high}/CXCR4^{low} leukemic cells from *in-vivo* ibrutinib-treated patients. (A)** Fold change of the protein levels obtained for the AKT phospho-signaling array by Full Moon Bio Systems. The list includes all the sites evaluated, either phosphorylated or not, shown as the ratio between the signal obtained in the samples 4 weeks post-ibrutinib treatment over the signal obtained in the pre-treated condition, for each site of each protein in each of the three patients studied (BL3, BL6 and BL36, respectively). **(B)** Shown are the ratios obtained when studying the fold change in the phosphorylation status of each specific site in each protein (dephosphorylated over phosphorylated) taking into account the 4-week treated sample over the pre-treated sample. This is the ratio of the ratio that is shown in the Heat Map of Figure 2B used to build the statistics from Figure 2A. Each p value obtained is detailed in column.

Suplementary Figure 1

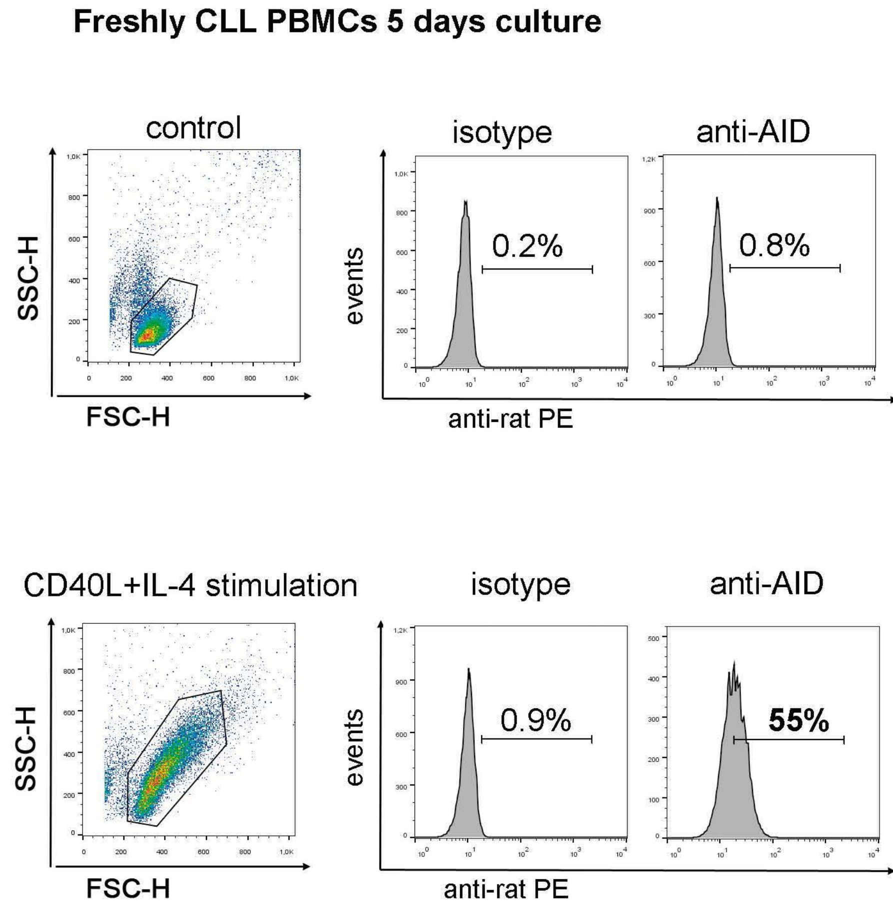


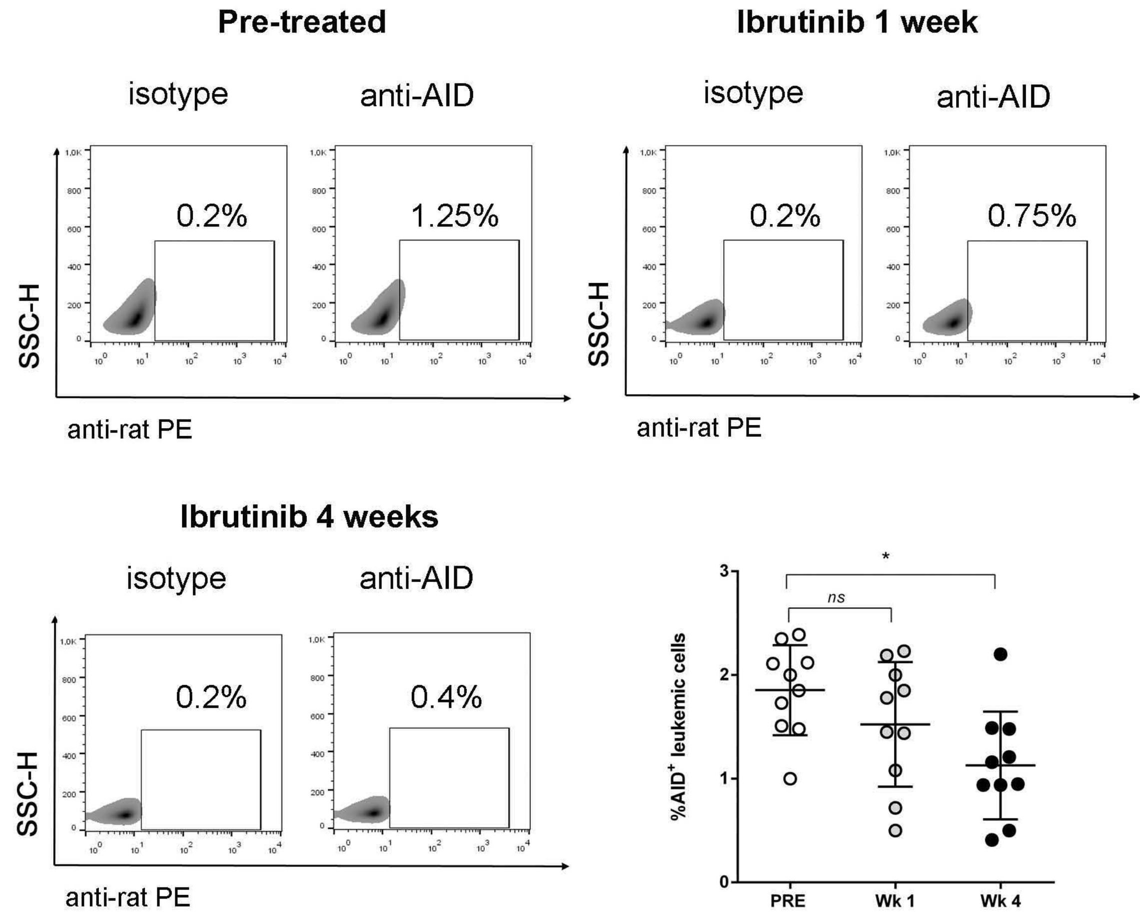






Supplementary Figure 2





Supplementary data 1: Extended Material and methods

Flow cytometry analysis:

Phenotypic surface analysis of leukemic B cells was performed using the following antibodies: anti-CD5 FITC (clone UCHT2), anti–CXCR4 PE or APC (clone 12G5), anti-CD86 FITC (clone 2331 FUN-1) and anti-CD38 APC (clone HIT2, all from BD Biosciences, Franklin Lake, NJ); anti-CD19 PerCp Cy5.5 (clone HIB19 from BioLegend, San Diego, CA); anti–human μ chains $F(ab')_2$ PE, and anti–human γ chains $F(ab')_2$ FITC (Catalog 709-116-073 and 709-546-098 respectively, from Jackson ImmunoResearch, West Grove, PA). Briefly, 10^6 blood purified B cells were incubated with the corresponding mixture of antibodies or isotype controls for 30 minutes at 4°C in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO). Cells were then washed twice in PBS 0.5% BSA before acquisition in a flow cytometer instrument. For intracellular protein detection, after surface labeling, 10^6 PBMCs were fixed in PBS 4% paraformaldehyde, washed twice in PBS and then permeabilized in PBS containing 0.5% saponin and 5% FBS (PermB) following the addition of the specific antibody. For Ki-67 staining, anti–human Ki-67 Alexa Fluor 647 (Santa Cruz Biotechnology, Dallas, TX) was incubated for 30 minutes on ice, and then cells were washed three times in PermB, suspended in PBS with subsequent flow cytometry acquisition. For intracellular staining of AID, after permeabilization, rat anti-human AID clone EK2 5G9 (Cell Signaling Technology, Danvers, MA) was incubated 1:500 in PermB for 1 hour on ice, washed three times using PermB, and incubated for 30 min with secondary PE labeled anti-rat Antibody (Thermo Fisher Cat A10544, Waltham, MA). As primary antibody isotype control, rat IgG2a mixture (Catalog number 02-9288, Thermo Fisher) was used for unspecific binding in the same experimental conditions. Cells were washed three times in PermB and suspended in PBS before acquisition. Positive controls of AID protein staining were performed on fresh CLL cells from 3 different patients incubated for four days on HeLa-CD40L transfected cells monolayer with the addition of IL-4 (1000 U/mL), or on HeLa Wild type cells monolayer alone as control. Flow cytometry data was acquired using a FACSCalibur (BD Biosciences), and analysis was performed using FlowJo v10.1 (TreeStar, Ashland, OR) and Summit v4.3 software (Dako, Santa Clara, CA).

Western-Blot and immunodetection

Protein samples were obtained from frozen PBMC or *in vitro* cultures. They were quantified using BCA Kit (Pierce, Thermo Fisher) and mixed with SDS-loading buffer (60mM Tris-HCl pH 6.8, 3.3% SDS, 20mM Dithiothreitol (DTT), 0.01% bromophenol blue). After electrophoresis separation, proteins were transferred to a nitrocellulose membrane (General Electric, Boston, MA), then membranes were blocked with 5% non-fat dry milk in 0.1% PBS-Tween for 30 min and incubated with AID primary antibody diluted 1:1.000 (clone EK2 5G9 Cell Signaling Technology, Danvers, MA), Anti-STAT6 1:1000 (Cat ab32520, abcam), Anti-STAT6-phospho Y641, 1:1000 (Cat ab188080, abcam) and GAPDH (Cat G9545, Sigma) as loading control. Chemiluminescence detection was performed with the SuperSignal West Femto Maximum Sensitivity kit (Thermo Fisher).

Quantitative PCR and primers.

For gene expression analyses of AID, we used Corbette Rotor Gene 6000 Real-Time PCR and the SYBR Green I dye. Primers used in this study were the same that for RT-PCR analysis. One µg of total RNA was isolated from $5x10^6$ B-cells and retro-transcribed as above described. One µl from 20µl cDNA reaction were used for AID and GAPDH amplification in a PCR reaction including 40 cycles of amplification (95 °C 20 sec; 60°C 30 sec; 72°C 30 sec.). Positive calibrator values were obtained from CD40L and IL-4 activated CLL B-cells. Primer used were: AID-Forward, 5′-GAG GCA AGA AGA CAC TCT GG-3′, and AID-Reverse, 5′-CTA CTT CTG TGA GGA CCG C-3′; AID/RQ-PCR-Forward 5´- GCA ATA AGA ACG GCT GCC AC-3´ and AID/RQ-PCR-Reverse 5´- ACA TGT CGG GCA CAG TCG TA-3´. GAPDH-Forward, 5′-TGT TCC TAC CCC CAA TGT GT-3′ and GAPDH-Reverse, 5′-CAC TGA GCA TCT CCC TCA CA-3'.