IL4-STAT6 signaling induces CD20 in chronic lymphocytic leukemia and this axis is repressed by PI3Kδ inhibitor idelalisib

Veronika Sandova,[1,2] Gabriela Mladonicka Pavlasova,[1] Vaclav Seda,[1,2] Katerina Amruz Cerna,[1] Sonali Sharma,[1] Veronika Palusova,[1] Yvona Brychtova,[2] Sarka Pospisilova,[2] Stacey M. Fernandes,[3] Anna Panovska,[2] Michael Doubek,[2] Matthew S. Davids,[3] Jennifer R. Brown,[3] Jiri Mayer[2] and Marek Mraz[1,2]

[1]Central European Institute of Technology, Masaryk University, Brno, Czech Republic; [2]Department of Internal Medicine, Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic and [3]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

Correspondence: MAREK MRAZ - marek.mraz@email.cz

doi:10.3324/haematol.2021.278644

SUPPLEMENTARY MATERIALS

IL4-STAT6 signaling induces CD20 in chronic lymphocytic leukemia and this axis is repressed by PI3Kδ inhibitor idelalisib

Veronika Sandova,[1,2] Gabriela Mladonicka Pavlasova,[1] Vaclav Seda,[1,2] Katerina Amruz Cerna,[1] Sonali Sharma,[1] Veronika Palusova,[1] Yvona Brychtova,[2] Sarka Pospisilova,[2] Stacey M. Fernandes,[3] Anna Panovska,[2] Michael Doubek,[2] Matthew S. Davids,[3] Jennifer R. Brown,[3] Jiri Mayer,[2] Marek Mraz[1,2]*

[1] Central European Institute of Technology, Masaryk University, Brno, Czech Republic.
[2] Department of Internal Medicine, Hematology and Oncology, University Hospital Brno and Faculty of Medicine, Masaryk University, Brno, Czech Republic.
[3] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA.

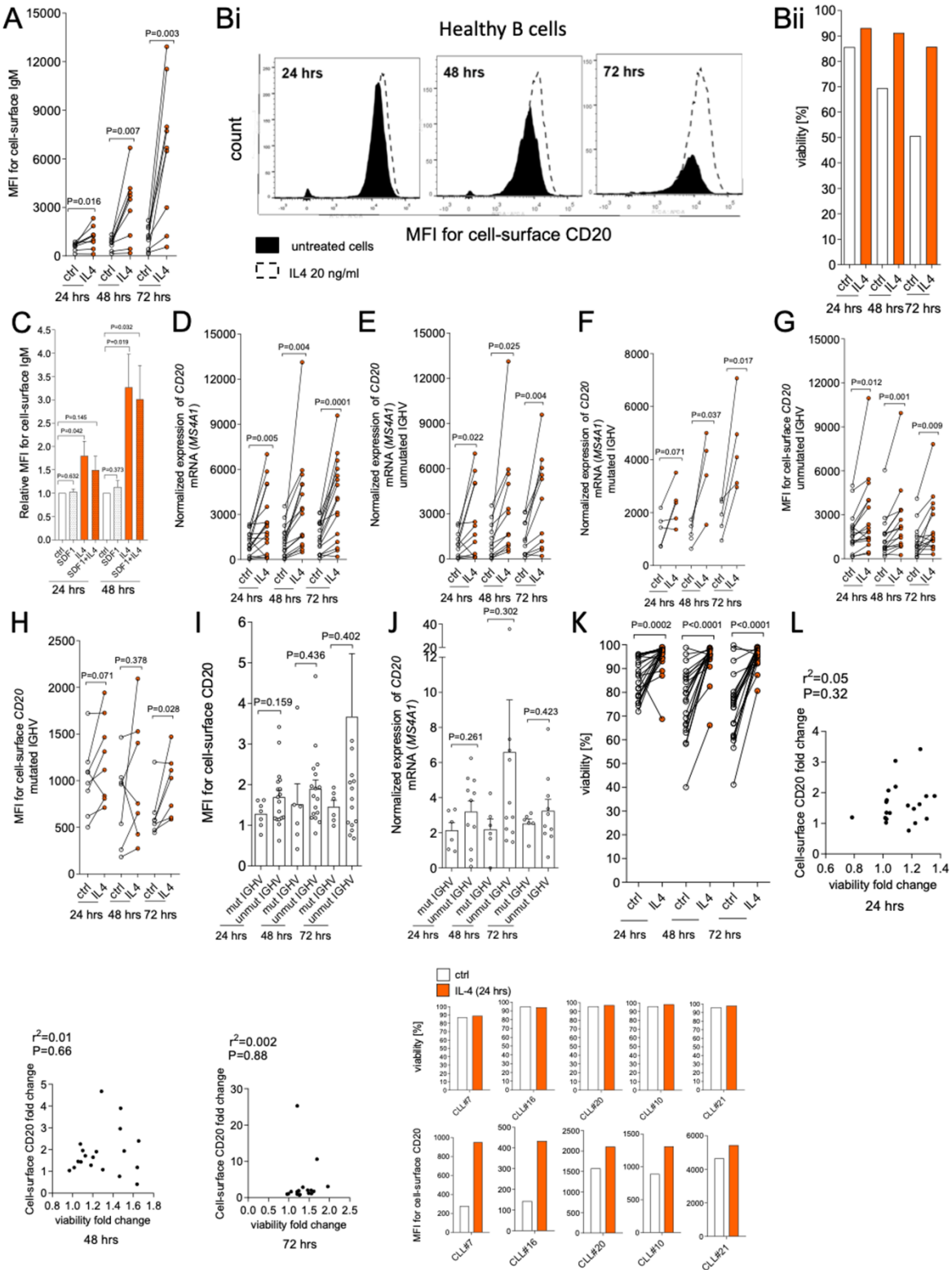
* corresponding author

Correspondence:

Marek Mraz, M.D., Ph.D.
Associate Professor of Oncology
Central European Institute of Technology, Masaryk University
Kamenice 5, 625 00 Brno, Czech Republic
E-mail: marek.mraz@email.cz
Tel.: +420 549498143

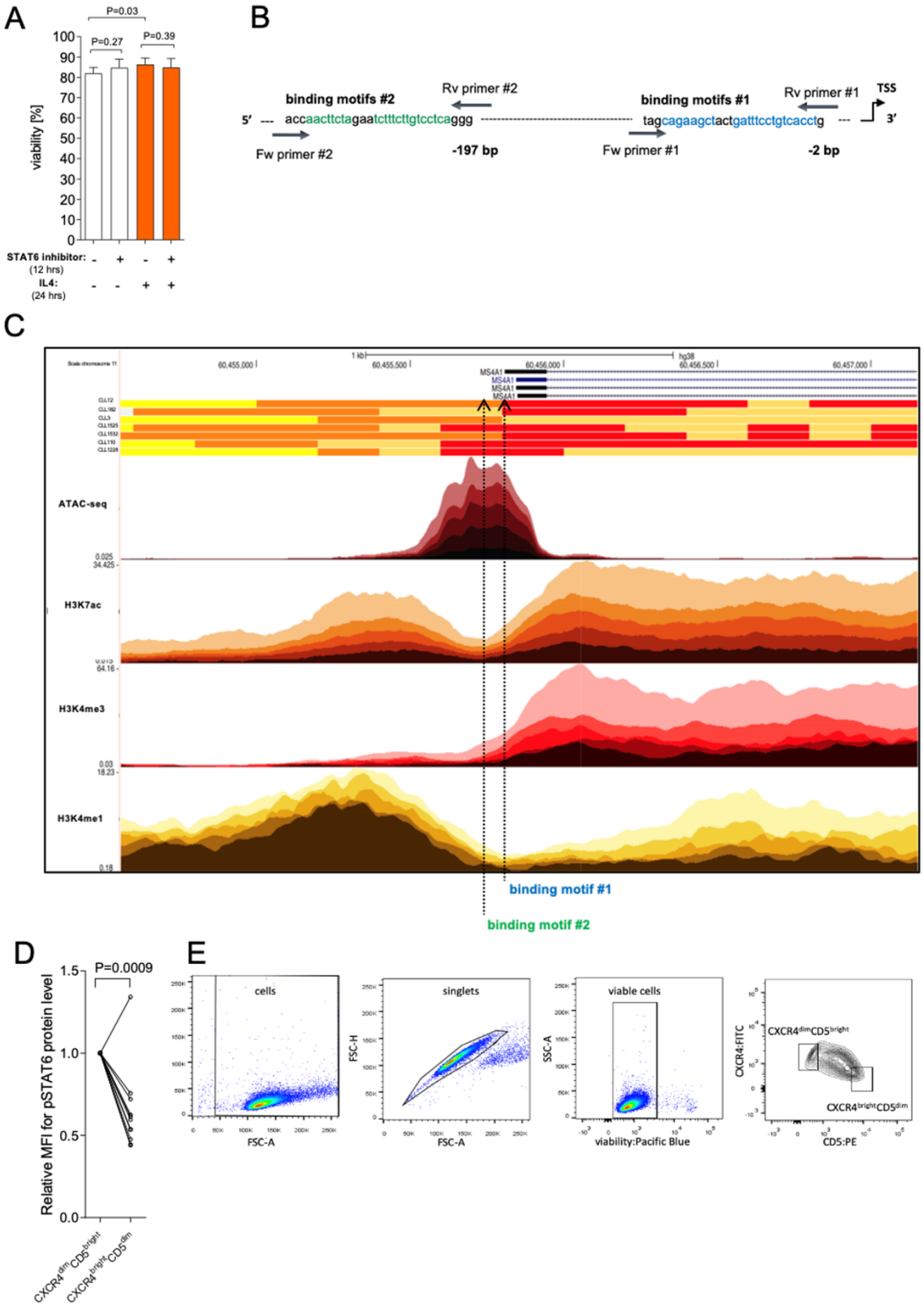
Supplementary Figures: 3

Supplementary Figure 1



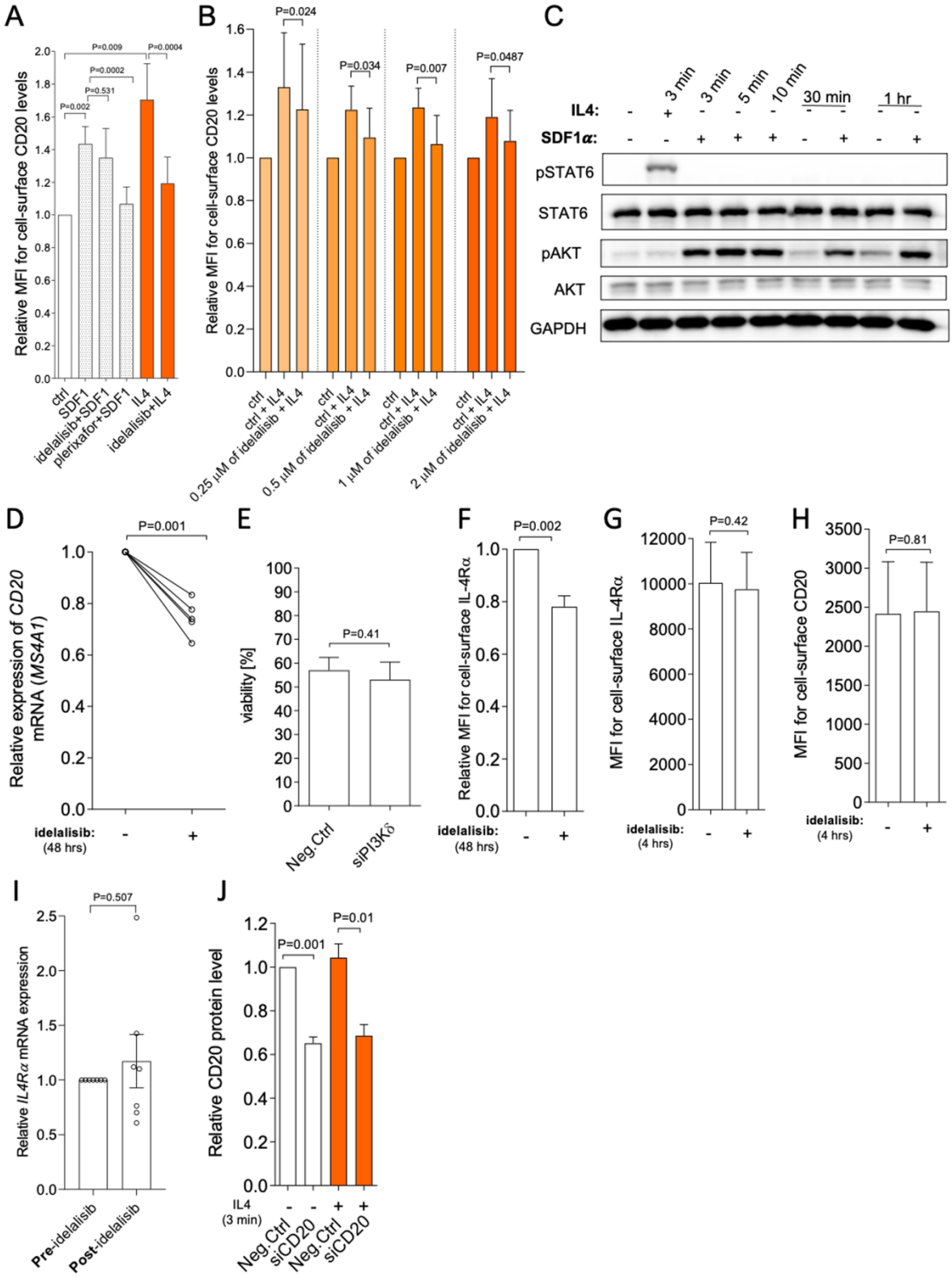
Supplementary Figure 1. The effect of IL4 on CD20 and IgM expression.
(A) Primary CLL cells were exposed to IL4 (20 ng/ml) for 24-72 hrs, or left untreated as a negative control (ctrl), and cell-surface IgM (anti-IgM, Sony Biotechnology) was measured (n=9). **(B)** Peripheral blood B lymphocytes from a healthy donor were *in vitro* stimulated with IL4 (20 ng/ml) or left untreated (ctrl), and cell-surface CD20 levels (Alexa Fluor-647 anti-CD20, Sony Biotechnology) **(B) (i)** or cell viability (PI/DiOC6**) (B) (ii)** were measured at 24-72 hrs. **(C)** Normalized cell-surface IgM levels in primary CLL cells treated by SDF1 (100 ng/ml), IL4 (20 ng/ml) or their combination (SDF1+IL4; 100 ng/ml+20 ng/ml) for 24-48 hrs (n=7). The untreated control (ctrl) was set as 1. **(D)** Normalized mRNA expression of *CD20* in CLL patients treated with IL4 20 ng/ml for 24-72 hrs (n=17). The analysis of mRNA of *CD20* (*MS4A1*) was performed with TaqMan Gene Expression Assay (Thermo Fisher Scientific) and normalized to endogenous control *HPRT* using the $2^{-\Delta Ct}$ method. **(E-F)** *CD20* mRNA levels after IL4 treatment in samples stratified according to unmutated IGHV **(E)** or mutated IGHV **(F)**. **(G-H)** CD20´s cell-surface levels after CLL cell stimulation with IL4 in samples with unmutated IGHV **(G)** or mutated IGHV **(H). (I-J)** Comparison of induction of cell-surface CD20 (**H**) and mRNA *CD20* (**I**) between samples with mutated IGHV (mut IGHV) and unmutated IGHV (unmut IGHV) after IL4 stimulation. **(K)** Viability of CLL cells stimulated with IL4 (20 ng/ml; 24-72 hrs) or left untreated (ctrl). Cell viability was measured for each time point 24 hrs (n=25), 48 hrs (n=23) and 72 hrs (n=22). **(L-N)** Analysis of correlation between fold change for CD20 and viability after IL4 treatment described in [K]. **(O)** Representative examples (n=5 selected CLL samples) that had similar viability with IL4 stimulation (20 ng/ml; 24 hrs) or without stimulation (ctrl). The top figures represent viability, and the bottom figures represent the cell-surface CD20 levels. In all experiments, the statistical difference was tested using a paired t-test, and the error bars indicate SEM (GraphPad Prism Software version 8; GraphPad Software).

Supplementary Figure 2



Supplementary Figure 2. STAT6 induces *CD20* expression.
(A) CLL cells were treated by STAT6 inhibitor (1 μM) for 12 hrs, then IL4 (20 ng/ml) was added directly into the media, and cells were cultured for another 24 hrs. After the indicated time, the viability of CLL cells was measured (n=6). **(B-C)** The scheme of predicted binding motifs for STAT6 near to the *CD20* transcription start site (TSS). **(B)** We have identified 2 potential binding sites with STAT6 motifs in closer proximity to the transcription start site (TSS) of *CD20 (MS4A1)* gene (binding motif 1, indicated in blue; binding motif 2 indicated in green). Identification of these binding sites was based on prediction made in online tool ConTra v3.[1] Binding of STAT6 was verified by ChIP where precleaned chromatin was incubated with ChIP grade antibodies of anti-STAT6 (#5397; 1.5 μg; Cell Signaling) and anti-IgG (# 2729; 1.5 μg; Cell Signaling) overnight at 4°C and concurrently section of chromatin was kept without any antibodies as an input. Following sequences of ChIP primers were used for STAT6 binding motifs in promoter of *CD20*: Fw primer for motif 1: 5´-GCCCTAAAAGTGAAGCCAGA-3´; Rv primer for motif 1:5´-AAGCAGGTGTGGATGGTAGG-3´; Fw primer for motif 2: 5´-CCAGAAGGTAAAAGTCAGTGCTA-3´; Rv primer for motif 2: 5´-CCCACCCTATAAGCAGGTGT-3´. ChIP data are presented as a percentage of input counted as $100 \times 2^{(\text{average Ct of input-LOG(dilution factor, 2))} - \text{Ct of antibody of interest})}$. **(C)** The chromatin landscape analysis of epigenetic and transcription marks in *CD20 (MS4A1)* promoter region (extracted from publicly available data [2]). We have visualized ATAC-seq, H3K7ac, H3K4me3 and H3K3me1 data for the *CD20* containing region (n=7 CLL samples[2]), and indicated the position of STAT6 binding sites (horizontal lines with arrow). The data are represented as overlay histograms with 5^{th}, 25^{th}, 50^{th}, 75^{th} and 95^{th} percentiles of signals intensity. The lighter color represents 95^{th} percentile, and the darker color corresponds to the 5^{th} percentile. **(D-E)** Levels of phosphorylated STAT6 (Tyr 641) in $CXCR4^{dim}CD5^{bright}$ versus $CXCR4^{bright}CD5^{dim}$ CLL cell subpopulations. **(D)** Protein level of pSTAT6 (Tyr 641) in CXCR4/CD5 cell subpopulations (n=11) quantified by intracellular staining and flow cytometry. For detection of intracellular pSTAT6 (Tyr 641) in CXCR4/CD5 subpopulations, CLL cells were stained with FITC anti-CXCR4 (Sony Biotechnology), PE anti-CD5 (Sony Biotechnology) and with fixable viability dye eFluor e450 (Thermo Fisher Scientific), fixed and permeabilized, and pSTAT6 (Tyr 641) (#9361; 1:100; Cell Signaling) protein levels were measured. The level of pSTAT6 (Tyr 641) is visualized as relative MFI compared to isotype control Alexa-647 (#4414S; 1:500; Cell Signaling) in each subpopulation. **(E)** Representative gating strategy for CXCR4/CD5 subpopulations. Detailed gating strategy used for determination of intracellular pSTAT6 (Tyr 641) levels in intraclonal CXCR4/CD5 subpopulations is described in Sharma *et al*.[3] In all experiments, the statistical difference was tested using a paired t-test, and the error bars indicate SEM (GraphPad Prism Software version 8, GraphPad Software).

Supplementary Figure 3



Supplementary Figure 3. The effect of idelalisib on *CD20/IL4Rα* expression and STAT6 activation.

(A) CLL cells were pre-treated with idelalisib (2 μM; 4 hrs) or plerixafor (5 μg/ml; 4 hrs) and then SDF1 (100 ng/ml) or IL4 (20 ng/ml) were added into the media for 48 hrs (for 24 hrs see Figure 2D). Cell-surface CD20 levels were measured and the results are visualized as a fold change to untreated control (ctrl) (n=12). The pre-treatment of CLL cells by idelalisib or plerixafor (CXCR4 inhibitor) for 4 hrs was performed to ensure a full inhibition of the pathway before exposure to receptor ligands. **(B)** CLL cells were pre-treated with idelalisib (0.25-2 μM) or vehicle for 4 hrs and then IL4 (20 ng/ml) was added into the media for 48 hrs. Cell-surface CD20 levels were measured, and the results are visualized as a fold change to untreated control cells (ctrl) (n=5). Viable CLL cells were gated for assessment of MFI values of cell-surface CD20 levels. The pre-treatment of CLL cells by idelalisib for 4 hrs was performed to ensure a full inhibition of the pathway before exposure to IL4. **(C)** CXCR4 activation by SDF1 does not lead to STAT6 (Tyr 641) phosphorylation. Representative immunoblot of CLL cells stimulated with SDF1 (100 ng/ml) or IL4 (40 ng/ml) for 3/5/10/30 minutes or 1 hour. STAT6 (Tyr 641) phosphorylation after IL4 was used as a positive control. The induction of AKT (Ser 473) phosphorylation after SDF1 serves as a positive control for activation of CXCR4/SDF1 axis. **(D)** Idelalisib down-modulates *CD20* expression *in vitro*. Relative expression of *CD20* after idelalisib treatment (2 μM; 48 hrs; "+") *in vitro* in comparison with a negative control (DMSO; "-"). *CD20* expression was determined using real-time PCR and normalized to endogenous control *HPRT* (n=5). **(E)** Viability of CLL cells 72 hrs after transfection with siRNA against *PI3Kδ* (siPI3Kδ) or a negative control (Neg.Ctrl) (n=4). **(F-H)** Idelalisib down-regulates cell-surface IL4Rα (anti-human IL4Rα, Sony Biotechnology) in long-term treatment but does not affect cell-surface IL4 receptor and CD20 levels in short-term treatment. **(F)** CLL cells were treated *in vitro* with idelalisib (2 μM; 48 hrs; "+") or with DMSO as control (n=7; "-") and cell-surface levels of IL4 receptor (IL4Rα) were measured. **(G-H)** CLL cells were treated *in vitro* with idelalisib (2 μM; 4 hrs; "+") or with DMSO as control (n=7; "-") and cell-surface levels of IL4 receptor (IL4Rα **(G)**) or CD20 **(H)** were measured. **(I)** Idelalisib does not affect *IL4R*α mRNA *in vivo*. The *IL4Rα* mRNA levels were analyzed in 7 paired CLL samples obtained before (Pre-idelalisib) and during (Post-idelalisib) idelalisib therapy as a single agent. The samples were taken day before first administration of idelalisib and then at week 5-6 after idelalisib treatment (n=6 week 5, n=1 week 6); depending on sample availability. Samples before therapy (Pre-idelalisib) were set as 1. **(J)** Down-modulation of CD20 protein levels after siRNA. Densitometric quantification of CD20 protein level for independent replicates (n=4) of the experiment described in [Figure 2Hi]. For immunoblots, β-actin or GAPDH were used as a loading controls. The statistical difference was tested using a paired t-test. In all experiments, the statistical difference was tested using a paired t-test, and the error bars indicate SEM (GraphPad Prism Software version 8, GraphPad Software).

Supplementary References

1. Kreft Ł, Soete A, Hulpiau P, Botzki A, Saeys Y, Bleser PD. ConTra v3: a tool to identify transcription factor binding sites across species, update 2017. In: Nucleic Acids Res 2017.
2. Beekman R, Chapaprieta V, Russinol N, et al. The reference epigenome and regulatory chromatin landscape of chronic lymphocytic leukemia. *Nature Medicine.* 2018;24(6):868-880.
3. Sharma S, Mladonicka Pavlasova G, Seda V, Amruz Cerna K, Vojackova E, Filip D, et al. miR-29 Modulates CD40 Signaling in Chronic Lymphocytic Leukemia by Targeting TRAF4: an Axis Affected by BCR inhibitors. Blood. 2021;137(18):2481-2494.