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


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

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Original data and protocols are available without restrictions. These data can be obtained by contacting of corresponding author.
Conflict-of-interest disclosure

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Efforts to combine anti-CD20 antibodies (such as rituximab or obinutuzumab) with BCR inhibitors or venetoclax lead to the necessity to better understand the largely unclear mechanisms of CD20 regulation and its function(s) (reviewed in Pavlasova and Mraz). This is underscored by the observation that in chronic lymphocytic leukemia (CLL) the combination of ibrutinib with rituximab does not provide a clinical benefit in comparison to ibrutinib alone likely because ibrutinib down-modulates CD20 levels. PI3Kδ inhibitor idelalisib has been approved in combination with rituximab or ofatumumab; however, it remains unclear if idelalisib affects CD20 levels or function(s). Here we show for the first time that single-agent idelalisib therapy in CLL leads to CD20 down-modulation \textit{in vivo} by interfering with a previously unknown mechanism of CD20 transcriptional regulation via IL4-STAT6 axis. We describe a novel mechanism of CD20 regulation in CLL B cells, which has implications for combinatorial therapy with PI3K inhibitors.

We have recently shown a concurrent upregulation of CD20 and cell-surface IgM in CLL cells from immune niches and demonstrated that this functionally serves to increase BCR signaling propensity. The SDF1 (CXCL12) chemokine induces ~30-50% upregulation of CD20, however, this cannot fully explain the ~2-fold higher CD20 levels in CLL cells from immune niches. Here we hypothesize that the same factor inducing cell-surface IgM in the CLL microenvironment, namely IL4 produced by T cells, might also be inducing CD20. Indeed, stimulating primary CLL cells with IL4 led to a significant upregulation of CD20 (mean fold-change 1.6-3.1 [24-72 hrs], n=25; Figure 1A-C) and IgM on the cell-surface (Figure S1A). IL4 also induced cell-surface CD20 in normal B cells (~2-fold induction; Figure S1B). This is in line with previous anecdotal observations suggesting a role for IL4 in CD20 regulation. We next compared the effects of SDF1 and IL4 on CD20 levels and noted that each factor independently induces CD20, and their combination has a more potent effect (Figure 1D). IgM induction was used as a control in this experiment since it is known to only be induced by IL4 and not SDF1 (Figure S1C). IL4’s effect on CD20 was transcriptional since $CD20$ mRNA (gene $MS4A1$) was induced similarly to its cell-surface levels (Figure S1D). The IL4 effect was observed irrespective of IGHV status or the
presence of chromosomal aberrations (Figure S1E-J and data not shown). It is known that
IL4 supports CLL cell viability, and to avoid any potential confounding effects from the
different viability on CD20 levels, we gated on viable cells and we also analyzed separately
CLL samples with comparable viability in control and IL4-treated cells (Figure S1K-O).
Altogether, the data show that IL4 (produced by T cells) induces CD20 in CLL cells.

We next hypothesized that CD20 induction by IL4 might be mediated by STAT6 as it
is a well-known key IL4 signaling effector. Indeed, STAT6 silencing by siRNA or a specific
STAT6 inhibitor (AS1517499) impaired the IL4-induced CD20 expression (Figure 1E-G).
SiRNA against STAT6 or STAT6 inhibitor did not have any effect on cell viability (Figure
S2A, and data not shown). Chromatin immunoprecipitation revealed that STAT6 binds to the
CD20 promoter in CLL cells (-2 nucleotides from transcription start-site [TSS]), and the
occupancy of this novel site by STAT6 was significantly increased (~2-fold) immediately after
IL4 treatment (30 minutes, Figure 1H). We have also observed a weaker STAT6 binding at
another putative binding site (-197 nucleotides from TSS; Figure 1H, S2B-C). We also noted
higher STAT6 phosphorylation in freshly obtained unstimulated CXCR4<sup>dim</sup>CD5<sup>bright</sup> CLL
subpopulation in comparison to CXCR4<sup>bright</sup>CD5<sup>dim</sup> cells using flow cytometry (Figure S2D-
E). The CXCR4<sup>dim</sup>CD5<sup>bright</sup> cells are regarded as an intraclonal CLL cell subpopulation that
has recently exited immune niches versus resting CXCR4<sup>bright</sup>CD5<sup>dim</sup> cells. However, we
could not reliably detect phosphorylated STAT6 in CLL cells by a less sensitive technic of
immunoblotting suggesting that its levels were very low in peripheral blood in general.
Altogether, these data demonstrate a novel direct role for STAT6 in transcriptional CD20
regulation upon IL4 stimulation. This is potentially a mechanism coupling regulation of two
molecules (IgM and CD20) required for BCR signaling<sup>3,7,8</sup> by both being induced by IL4
produced in immune microenvironment. This might be a part of the pathway crosstalk by
which IL4 alternates the BCR pathway, a phenomenon described by others.<sup>10</sup>

Ibrutinib leads to a significant reduction of CD20 levels in CLL by interfering with
SDF1 signaling.<sup>1,3,4</sup> Notably, in vivo therapy with idelalisib as a single-agent prominently
reduced CD20 mRNA and protein levels within several weeks (Figure 2A-C). Next, we
tested if idelalisib interferes with the IL4- or SDF1-dependent induction of CD20. Idelalisib clearly inhibited CD20 induction by IL4, but not by SDF1 (Figure 2D, S3A). The repression of CD20 induction by idelalisib was observed with doses ≥ 0.5 μM (Figure S3B). Therefore, we hypothesized that PI3K is involved in STAT6 activation, while CXCR4-SDF1 axis does not include STAT6. Indeed, CXCR4 signaling does not induce any STAT6 phosphorylation (Figure S3C), while idelalisib treatment in vitro (48 hours) significantly impaired STAT6 phosphorylation and CD20 induction by IL4 (Figure 2E). To exclude secondary effects of idelalisib or decreased cell viability, we also performed a short pre-treatment of cells by idelalisib (4 hours) and observed an identical decrease in pSTAT6 levels (Figure 2F and data not shown; idelalisib had no effect on cell viability at 4 hrs and minor effect at 48 hrs). In vitro, the treatment of CLL cells with idelalisib (1 or 2 μM) also led to a reduction of CD20 levels prior to IL4 exposure (Figure S3D, 2Eiii-iv, and data not shown), however, this was less pronounced than during IL4 stimulation. This suggests that idelalisib might affect other CD20 regulators besides STAT6 or low-level basal STAT6 activity influences also “basal” CD20 transcription. Indeed, we detected some STAT6 phosphorylation in unstimulated CLL cells (see above).

The silencing of PI3Kδ by siRNA decreased the STAT6 phosphorylation after IL4 (Figure 2G, S3E), indicating a direct role of PI3Kδ isoform in IL4-induced CD20 expression and a specific on-target effect of idelalisib leading to CD20 down-modulation. This is in line with studies describing the involvement of PI3Kδ in IL4 signaling of normal B cells. However, idelalisib does not impair IL4-induced increase in CLL cell viability in vitro, suggesting that IL4’s pro-survival effect is not dependent on STAT6 or that weaker STAT6 phosphorylation is sufficient to provide a pro-survival signal. Altogether, our data demonstrate that PI3Kδ inhibition interferes with CD20’s transcriptional activation by the IL4-STAT6 axis.

Besides the effects of idelalisib on STAT6 phosphorylation and CD20 levels, we have also noticed a minor decrease in cell-surface IL4 receptor (IL4Rα) levels after 48 hours
of idelalisib treatment in vitro (Figure S3F). However, this is not responsible for the reduced responsiveness to IL4, since incubation of CLL cells with idelalisib for 4 hours also impaired STAT6 phosphorylation after IL4 (Figure 2F), but IL4Rα or CD20 levels remained unchanged during these short-term experiments (Figure S3G-H). Moreover, we did not observe significant down-modulation of IL4Rα levels during idelalisib therapy in vivo (Figure S3I). This supports a direct role of PI3K in STAT6 signaling and the on-target effect of idelalisib.

We next asked if CD20 down-modulation by idelalisib might affect CD20’s function(s) in some signaling pathways other than BCR. To our surprise, CD20 silencing by siRNA significantly impaired response to IL4 in MEC1 and primary CLL cells (Figure 2H-I, S3J), and had a minor effect on the phosphorylation of IKK after CpG or CD40L (data not shown). This suggests that CD20 is embedded in several receptor-associated pathways, including the regulation of IL4 signaling propensity. IL4 maintains CD20 levels via STAT6 activation, and CD20 increases responsiveness of CLL cells to IL4 via a yet unclear mechanism. We noted that IL4 treatment in CLL cells does not lead to internalization of IL4 receptor (data not shown), allowing cells to respond to IL4 continuously. These observations of CD20’s role in T cell interactions are in line with studies of CD20 in normal B cells, since CD20 knockout in mice or CD20 loss in humans leads to defects in T-cell dependent immunity. However, a full understanding of this phenomenon will require further insight into CD20 functions and interaction partners, which is a long-standing question in the field.

Altogether, here we describe a novel CD20 regulatory axis and reveal for the first time that T cell interactions via IL4 induce CD20 transcription by STAT6 binding to its promotor (summarized in Figure 3). The PI3Kδ is involved in CD20 induction by IL4-STAT6 axis, and consequently, idelalisib therapy represses CD20 in CLL. Idelalisib has been approved in combination with anti-CD20 antibodies without comparison to single-agent idelalisib, and is currently being mainly used in therapy of relapsed/refractory disease and/or in cases of a BTK inhibitor intolerance. Down-modulation of CD20 by idelalisib likely
reduces the rituximab-induced apoptosis and CDC, since complement fragment deposition is closely dependent on cell-surface CD20 levels, and might impair ADCC/ADCP since these are also facilitated by opsonization of target cells with complement. Indeed, idelalisib inhibited \textit{in vitro} the immune cell-mediated mechanisms induced by anti-CD20 antibodies, but this requires further investigation and might also include idelalisib's effects on effector cells. Based on our data the benefit of rituximab addition should be tested in a clinical trial since this might fail to show improvement in PFS/OS, similarly to the lack of benefit for ibrutinib plus rituximab combination versus ibrutinib alone. It would be interesting to also test the combination of idelalisib/PI3K\(\gamma\) inhibitor with anti-CD20 antibodies whose efficacy is less dependent on CD20 levels such as obinutuzumab. In conclusion, any clinically used inhibitor blocking PI3K\(\gamma\) or interfering with the IL4-STAT6 pathway will reduce CD20 expression with potential consequences for combinatorial therapy.
References


**Figure legends**

**Figure 1. IL4 up-regulates CD20 expression via STAT6.**

(A) Cell-surface level of CD20 after IL4 treatment (20 ng/ml, PeproTech) for 24 hrs (n=25), 48 hrs (n=23), or 72 hrs (n=22) in comparison to untreated control cells (ctrl). (B) Representative immunoblot of CD20 and pSTAT6 (Tyr 641) protein levels in CLL cells after IL4 stimulation (24-72 hrs). (C) Densitometric quantification of CD20 protein levels for independent replicates of the experiment described in [B] (n=17; 24-72 hrs). Untreated control (ctrl) without IL4 was set as 1 and compared to the other samples. (D) Normalized cell-surface CD20 levels in primary CLL cells treated by SDF1α (100 ng/ml, PeproTech), IL4 (20 ng/ml), or their combination (SDF1+IL4) for 24-48 hrs (n=7). The untreated control (ctrl) was set as 1. (E) Peripheral blood CLL cells were electroporated with siRNA against STAT6 (siSTAT6, 500 nM; Dharmacon) or negative control (Neg.Ctrl). IL4 (20 ng/ml) was added 48 hrs after transfection and cells were cultured for another 24 hrs. For the immunoblot, β-actin was used as a loading control and pSTAT6 (Tyr 641)/tSTAT6 levels were assessed. (F) Densitometric quantification of CD20 protein levels for independent replicates (n=4) of the experiment described in [E]. Neg.Ctrl without IL4 was set as 1 and compared to the other samples. (G) Peripheral blood CLL cells were pre-treated with pSTAT6 inhibitor (AS1517499, 1 μM, Selleckchem) for 12 hrs. Subsequently, IL4 (20 ng/ml) was added to the media, and cells were cultured for another 24 hrs. CD20 expression was determined by real-time qPCR (TaqMan, ABI), and the expression of CD20 was normalized to endogenous control HPRT (n=6). (H) Chromatin immunoprecipitation analysis of samples that were immunoprecipitated with anti-STAT6 antibody in comparison with IgG antibody before (-) and after (+) IL4 stimulation (n=5; 40 ng/ml, 30 minutes). IgG antibody was used as an isotype control. For all in vitro experiments in Figure 1 and 2 CLL cells were purified by RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies) to obtain purity ≥95% of CD5+CD19+ cells. For immunoblots, β-actin was used as a loading controls. In all
experiments, the statistical difference was tested using a paired t-test, and the error bars indicate SEM.

Figure 2. CD20 is down-modulated by idelalisib and embedded in IL4 pathway.

**A** Cell-surface CD20 levels and **B** relative expression of \( CD20 \) mRNA in paired samples before (Pre) and after 5 weeks (idelalisib-week 5) and 9 weeks (idelalisib-week 9) of idelalisib therapy *in vivo* (cell surface CD20: \( n=1 \) week 4, \( n=6 \) week 5, \( n=6 \) week 9; \( CD20 \) mRNA: \( n=6 \) week 5 and week 9). CLL cells were isolated by density centrifugation (Ficoll-Paque) followed by magnetic anti-CD3 MicroBeads separation (Miltenyi Biotec) or in some cases negative selection with RosetteSep Human B Cell Enrichment Cocktail (Stemcell Technologies) was used to obtain purity of \( \geq 95\% \) of CD5+19+ cells. **C** Representative examples (\( n=3 \)) of CD20 protein levels in CLL cells obtained before (Pre) and during idelalisib therapy *in vivo* (week 4/5 and 9). **D** CLL cells (purity \( \geq 95\% \)) were pre-treated with idelalisib (2 \( \mu \text{M}, \) Selleckchem) for 4 hrs or plerixafor (5 \( \mu \text{g/ml}, \) Selleckchem) for 4 hrs and then SDF1 (100 ng/ml, CXCR4 ligand) or IL4 (20 ng/ml) were added into the media for 24 hrs (for result of 48 hrs treatment with SDF1/IL4 see Figure S3A). Cell-surface CD20 levels were measured and the results are visualized as a fold change to untreated control (ctrl) (\( n=12 \)). Viable CLL cells were gated for assessment of cell-surface CD20 levels. The pre-treatment of CLL cells by idelalisib or plerixafor (CXCR4 inhibitor) for 4 hrs was performed to ensure a full inhibition of the pathways before exposure to the receptor ligands. **E (i)** Representative immunoblot of CLL cells treated *in vitro* with idelalisib (2 \( \mu \text{M}; \) 48 hrs) and subsequently stimulated by IL4 (40 ng/ml; 3 min). **E (ii)** Densitometric quantification of pSTAT6 (Tyr 641) protein levels for independent replicates (\( n=7 \)) of the experiment described in [Ei]. **E (iii)** Representative immunoblot of CLL cells treated *in vitro* with idelalisib (2 \( \mu \text{M}; \) 48 hrs) then washed twice with clean culture media and stimulated by IL4 in full media (20 ng/ml; 24 hrs). **E (iv)** Densitometric quantification of CD20 protein levels for independent replicates of the experiment described in [Eiii] (\( n=7 \)). **F (i)** Representative immunoblot of pSTAT6 (Tyr 641) down-modulation after pre-treatment of CLL cells with
idelalisib (2 μM; 4 hrs) followed by IL4 stimulation (40 ng/ml; 3 and 5 min). (F) (ii) Densitometric quantification of pSTAT6 (Tyr 641) protein level for independent replicates (n=5) of the experiment described in [Fi]. (G) Representative immunoblot of CLL cells transfected with siRNA against PI3Kδ isoform (siPI3Kδ, 500 nM, Dharmaco) or a negative control (Neg.Ctrl). Seventy-two hours after transfection, cells were stimulated by IL4 (40 ng/ml; 3 min) and STAT6 phosphorylation (Tyr 641) was assessed. Phosphorylation of AKT (Ser 473) was used as a surrogated marker for PI3Kδ down-modulation after siPI3Kδ since we were not able to detect PI3Kδ protein due to issues with anti-PI3Kδ primary antibody. (H) (i) Representative immunoblot of MEC1 cells transfected with siRNAs against CD20 (siCD20, 500 nM, Thermo Fisher Scientific) or negative control (Neg.Ctrl). Forty-eight hours after the transfection, cells were stimulated by IL4 (40 ng/ml; 3 min). (H) (ii) Densitometric quantification of pSTAT6 (Tyr 641) protein level for independent replicates (n=4) of the experiment described in [Hi]. Neg.Ctrl without IL4 was set as 1 and compared to the other samples. (I) Representative immunoblot of primary CLL cells transfected with siRNA against CD20 (siCD20) or negative control (Neg.Ctrl), cultured for 48 hrs, and then stimulated by IL4 (40 ng/ml; 3 min). For immunoblots, β-actin or GAPDH were used as a loading controls. In all experiments, the statistical difference was tested using a paired t-test, and the error bars indicate SEM.

**Figure 3. Schematic overview for CD20 down-modulation by idelalisib via impaired IL4-STAT6 axis.**

In CLL, IL4-STAT6 axis up-regulates CD20 gene expression through the STAT6 phosphorylation and its direct binding to CD20 (MS4A1) promoter. The IL4-STAT6-CD20 axis is inhibited by PI3Kδ inhibitor idelalisib. NFkB and FoxO1 represent other two known regulators of CD20 transcription in CLL (NFkB is a positive regulator, and FoxO1 is an indirect negative regulator). This figure was created with tools as BioRender.com.
In the extracellular space, IL4 binds to the IL4R receptor on the surface of the T cell. This activation leads to the phosphorylation of STAT6, which then translocates to the cytoplasm. In the cytoplasm, STAT6 is further phosphorylated by idelalisib, leading to the activation of FoxO1 and NFκB, which together regulate the expression of the MS4A1 (CD20) gene in the nucleus.
IL4-STAT6 signaling induces CD20 in chronic lymphocytic leukemia and this axis is repressed by PI3Kδ inhibitor idelalisib

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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^-ΔΔCT 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

A

B

C

D

E

Supplementary Figure 2

A

B

C

D

E
**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 precleared 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′-GCCCTAAAGTGGAAGCCAG-3′; Rv primer for motif 1: 5′-AAGCAGGTGGATGGTAGG-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 x 2^[(average Ct of input-LOG(dilution factor, 2))] – 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 samples2), and indicated the position of STAT6 binding sites (horizontal lines with arrow). The data are represented as overlay histograms with 5th, 25th, 50th, 75th and 95th percentiles of signals intensity. The lighter color represents 95th percentile, and the darker color corresponds to the 5th percentile. (D-E) Levels of phosphorylated STAT6 (Tyr 641) in CXCR4dimCD5bright versus CXCR4brightCD5dim CLL cell subpopulations. (D) Protein level of pSTAT6 (Tyr 641) in CXCR4/C5D5 cell subpopulations (n=11) quantified by intracellular staining and flow cytometry. For detection of intracellular pSTAT6 (Tyr 641) in CXCR4/C5D5 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; "-"-). \( CD20 \) expression was determined using real-time PCR and normalized to endogenous control \( HPRT \) (n=5). (G) 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; "-"-). (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) 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 IL4Rα 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

