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# Loss of cooperativity of secreted CD40L and increased dose–response to IL4 on CLL cell viability correlates with enhanced activation of NF-kB and STAT6

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**Chronic lymphocytic leukemia (CLL) cells fail to enter apoptosis *in vivo* as opposed to their non-malignant B-lymphocyte counterparts. The ability of CLL cells to escape apoptosis is highly dependent on their microenvironment. Compared to non-malignant B cells, CLL cells are more responsive to complex stimuli that can be reproduced *in vitro* by the addition of cytokines. To understand the molecular mechanism of the environment-dependent anti-apoptotic signaling circuitry of CLL cells, we quantified the effect of the SDF-1, BAFF, APRIL, anti-IgM, interleukin-4 (IL4) and secreted CD40L (sCD40L) on the survival of *in vitro* cultured CLL cells and found IL4 and sCD40L to be most efficient in rescuing CLL cells from apoptosis. In quantitative dose–response experiments using cell survival as readout, the binding affinity of IL4 to its receptor was similar between malignant and non-malignant cells. However, the downstream signaling in terms of the amount of STAT6 and its degree of phosphorylation was highly stimulated in CLL cells. In contrast, the response to sCD40L showed a loss of cooperative binding in CLL cells but displayed a largely increased ligand binding affinity. Although a high-throughput microscopy analysis did not reveal a significant difference in the spatial CD40 receptor organization, the downstream signaling showed an enhanced activation of the NF-kB pathway in the malignant cells. Thus, we propose that the anti-apoptotic phenotype of CLL involves a sensitized response for IL4 dependent STAT6 phosphorylation, and an activation of NF-kB signaling due to an increased affinity of sCD40L to its receptor.**



CLL (chronic lymphocytic leukemia), the most common leukemia in the western world, is characterized by the accumulation of mature $CD5^+$ B cells in the blood, bone marrow and secondary lymphoid organs in patients due to extended survival and resistance to apoptosis. Enhanced CLL cell survival is triggered by microenvironmental signals as evident by the fact that CLL cells rapidly undergo apoptosis when cultured without support *in vitro*.[1,2] This dependency can be mimicked *in vitro* by the addition of cytokines to the medium or by co-culturing with bone marrow derived stromal cell lines that lead to an increased survival time of CLL cells. However, it is currently an open question whether an enriched microenvironment is the sole cause for prolonged CLL cell survival. Alternatively, CLL cells could have a higher sensitivity to respond to survival stimuli as compared to non-malignant B cells due to deregulated intracellular signaling. Intriguingly, interactions between malignant lymphocytes and non-transformed cells are bidirectional and lead to the establishment of an abnormal microenvironment that is able to inhibit apoptosis of neoplastic B cells.[3,4] The interaction of CLL cells with non-malignant bystander cells like T lymphocytes and stromal cells supports CLL survival either directly (through cell–cell interaction) or indirectly (by secreting soluble factors into the serum).[5,6] Stromal cells, such as nurse-like cells (NLCs)[7] and mesenchymal stromal cells (MSCs),[8] protect CLL cells in coculture and are an integral part of the CLL microenvironment. Several factors are secreted by NLCs and are involved in their prosurvival capabilities: stromal

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**What's new?**

**Chronic lymphocytic leukemia cells rely on help from the microenvironment to evade apoptosis. Exactly what help are they getting? In this paper, the authors quantified how various microenvironment factors affect the survival of CLL cells. They found that IL-4 and secreted CD40L did the most to stave off CLL cell death. IL-4 stimulated the production of more STAT6 in cancer cells than in non-malignant cells, kicking off a pathway that fires up more anti-apoptosis genes. Secreted CD40L had a much stronger binding affinity in the cancer cells than in ordinary B cells, allowing the CLL cells to sidestep apoptosis by boosting NF-kB signaling.**



derived factor-1 (SDF-1)/CXCL12,[9] CXCL13,[10] BAFF (B-cell activating factor of the tumor necrosis factor family) and[11] APRIL (a proliferation inducing ligand).[11] The fact that the bone marrow in CLL patients is infiltrated with CLL cells underlines the importance of bone marrow-derived mesenchymal stromal cells (MSCs) in the pathogenesis of CLL. Furthermore, the pattern and extent of marrow infiltration correlate with clinical stage and prognosis.[12] MSCs attract CLL cells by the secretion of the chemotactic factor SDF-1/CXCL12 which binds to its receptor CXCR4 on CLL cells.[9] In addition, antigenic stimulation through the B cell receptor is strongly suggested to be one of the promoting factors that enable CLL cells to avoid apoptosis.[13]

$CD4^+$ T cells predominate in the bone marrow and around proliferation centers called "pseudofollicles" (which contains proliferating CLL cells) in lymph nodes of CLL patients,[14] and these T cells stimulate CLL cell growth and survival by secreting cytokines, such as interleukin-4 (IL4).[15] Within proliferation centers, a significant proportion of T cells display CD40L (CD154), a member of the TNF superfamily that binds to CD40 receptor (CD40) on CLL cells, rescuing them from apoptosis.[15–17] Secreted CD40L (sCD40L) alone can induce both survival and proliferation in non-malignant B cells *via* NF-kB dependent and NF-kB independent signaling. In CLL cells, however, only survival can be induced by sCD40L.[18]

In this study, we have compared the responsiveness of CLL cells to different microenvironmental stimuli including treatments with single ligands (also added in combination) T-cell-derived cytokines IL4 and sCD40L were found to be the most efficient factors in rescuing CLL cells from apoptosis *in vitro*. Although sCD40L when used *in vitro* is likely to represent physiological secreted CD40L, it cannot be ruled-out that observed effects possibly also originate from the physiological functions of membrane-bound CD40L. CLL cells also showed a different dose dependent survival response to IL4 and sCD40L as compared to non-malignant B cells. In IL4 signaling the shape of the response curve displayed a largely increased fraction of surviving cells that could be related to enhanced STAT6 levels and phosphorylation without apparent difference in the affinity of the cytokine to its receptor. In contrast, sCD40L–receptor interactions displayed an about 30-fold higher affinity that resulted in an enhanced activation of NF-kB signaling, in the absence of changes in the spatial organization of CD40 receptor. Thus, our study identifies important features of deregulated signaling in CLL that are related to the disease phenotype in terms of prolonged survival under conditions that would normally lead to apoptosis of non-malignant B lymphocytes.

## Material and Methods

### Specimen and cell purification

After obtaining informed written consent (ethics committee approval 96/08, http://www.uni-ulm.de/ethikkommission/), peripheral blood cells were collected from CLL patients. Clinical characteristics of the CLL patients used are listed in Supporting Information Table S1. No patient in this study had received prior therapy. In the case of samples from healthy donors, peripheral blood was collected from age-matched donors after oral informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density-gradient centrifugation over Ficoll-Hypaque according to the manufacturer's protocol (Biochrom AG, Berlin, Germany). Cells were used either fresh or viably frozen in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Karlsruhe, Germany) containing 40% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO) for storage in liquid nitrogen. All cells were cultured at 37°C with 10% $CO_2$ in Dulbecco's modified eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% FCS and penicillin/streptomycin.

$CD19^+$ cells from CLL patients and healthy donors were isolated by magnetic bead-activated cell sorting (MACS) using CD19-microBeads following the instructions of the manufacturer (MiltenyiBiotec, Bergisch Gladbach, Germany). The purity of the $CD19^+$ fraction was $94 \pm 2\%$ after isolation from PBMCs of healthy donors and $98 \pm 1\%$ for isolation from PBMCs of CLL patients as measured by flow cytometry.

### Culture and stimulation of CLL and non-malignant B cells

Viably frozen CD19-sorted PBMCs from patients and healthy donors were thawed and directly cultured in DMEM containing 10% FCS at a density of $1 \times 10^6$ cells/well in a 6-well plate or in 24-well plates at a density of 2 or $5 \times 10^5$ cells/well. Cells were stimulated with ligand for the indicated periods of time and cell survival was measured by flow cytometry. Recombinant human interleukin 4 (IL4), recombinant human stromal derived factor-1 (SDF-1), recombinant human B-cell activating factor (BAFF), recombinant human a proliferation inducing ligand (APRIL) and human sCD40L

were purchased from Peprotech GmbH (Hamburg, Germany), F(ab′)2 Fragment Goat Anti-Human IgM was obtained from Jackson Immunoresearch Laboratories (Pennsylvania). Although sCD40L and CD40L have different functionality *in vivo*, it cannot be excluded that sCD40L might *in vitro* also induce effects that are part of the physiological repertoire of membrane-bound CD40L.

### Flow cytometry

Apoptotic cell death of CLL and B lymphocytes was measured by flow cytometry using staining with 7-aminoactinomycin (7-AAD; Sigma, Steinheim, Germany) and PE-Annexin (Becton Dickinson, Heidelberg, Germany, #556421). CD45-APC staining was used to specifically select the lymphocytic population for analysis by gating as stromal cells are negative for this marker. Cells were harvested and resuspended 1% PBS-BSA containing saturating amounts of 7-AAD and CD45-APC (monoclonal, mouse anti-human antibody from BD Biosciences, San Jose, CA). After an incubation time of 20 min at 4°C, stained cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences) by gating on CD19-purified lymphocytes.

### Image-based flow cytometry

Population measurements were performed using the ImageStream X.[19] Following treatments and stainings as indicated in the figure legends, 2,800 cells were imaged at 60× resolution and analyzed using IDEAS software (Amnis). From single in-focus cells the segmented widefield image was eroded, and segmentation masks for plasma membrane and intracellular region (center) were calculated. All images were analyzed for (*i*) receptor clustering at the plasma membrane, and (*ii*) the ratio for receptor intensity within the cell, an index for internalization. Examples of acquired images and processing are shown in Supporting Information Figure S2. Receptors were detected with FITC Mouse Anti-Human CD40 (BD Bioscience/BD Pharmingen, Heidelberg, Germany, #555588) and PE Mouse Anti-Human CD124 (IL-4 receptor alpha chain, BD Bioscience/BD Pharmingen, Heidelberg, Germany, #552178).

### Analysis of cytokine dose–response curves

The dependence of cell survival on the concentration of a given ligand was analyzed by assuming that the percentage of surviving cells $s$ is linearly related to the fractional saturation $\theta$ of receptors with ligand in steady according to Eq. (1). The baseline $b$ corresponds to the percentage of surviving cells present in the absence of ligand and $c$ is the proportionality constant.

$$s = c \cdot \theta + b. \quad (1)$$

The parameter $\theta$ is calculated from the steady state binding equilibrium given by Eq. (2) with a dissociation constant $K_d$ and cooperativity factor $\alpha$.

$$\theta = \frac{[\text{Ligand}]^{\alpha}}{K_d^{\alpha} + [\text{Ligand}]^{\alpha}}. \quad (2)$$

### Immunohistochemistry

After stimulation with IL4 and sCD40L, CLL cells and non-malignant B cells that attached by sedimentation to a cover slide were fixed in 4% paraformaldehyde solution for 10 min at room temperature. The slides were washed in PBS and the cells permeabilized with PBS containing 0.5% Triton X-100. Cells were then incubated for 15 min in goat serum and for 1 hr with both anti-IL4R (PE labeled, BD Pharmingen) and anti-CD40 (FITC labeled, BD Pharmingen) antibodies. At the end of the incubation, nuclei were stained for 5 min using a Hoechst stain (Life Sciences). After PBS wash, the slides were mounted overnight in mowiol as described in Ref. 20.

## Results

### IL4 and soluble sCD40L have the strongest prosurvival effect on CLL cells

To identify which soluble survival factors are most efficient in rescuing CLL cells from spontaneous apoptosis *in vitro*, CLL cells were incubated with different ligands. The optimal concentration of ligand was determined where the full survival effect was observed, that is, where saturation in the dose–response curve occurred (Fig. 1*a*). Using these optimal ligand concentrations, CLL cells were simultaneously treated with ligands that were combined according to their cellular origin and occurrence in the CLL microenvironment *in vivo*: (*i*) sCD40L and IL4 originating from T cells, (*ii*) SDF-1, APRIL and BAFF secreted by stromal cells and (*iii*) the F(ab′)2 fragment of the IgM portion of anti-IgM simulating antigenic stimulation through the BCR. After 2 days of culture, IL4 and sCD40L showed the strongest survival effect on CLL cells (Fig. 1*b*). This effect was independent of the IGHV status: patients with mutated or non-mutated IGHV status did not show a statistically different response to any ligand combination (data not shown).

### IL4 and sCD40L response curves in CLL cells reveal mechanistic differences to non-malignant B cells

As sCD40L and IL4 were the most efficient ligands in protecting CLL cells from apoptosis, the survival response of CLL cells and non-malignant B cells was compared after incubation with these ligands individually. To this end, cells from CLL patients ($n = 3$) and non-malignant B cells from healthy donors ($n = 3$) were treated with increasing concentrations of either IL4 or sCD40L. CLL cells survived much better than non-malignant B cells in the presence of both ligands tested. The plateau value at saturating ligand concentration was $54 \pm 2\%$ (IL4) and $53 \pm 2\%$ (sCD40) surviving cells after 4 days as opposed to about $30 \pm 4\%$ for the non-malignant B-lymphocytes (Fig. 2). This suggests distinct differences in ligand–receptor interactions and/or intracellular signaling for CLL cells. For IL4, the apparent dissociation

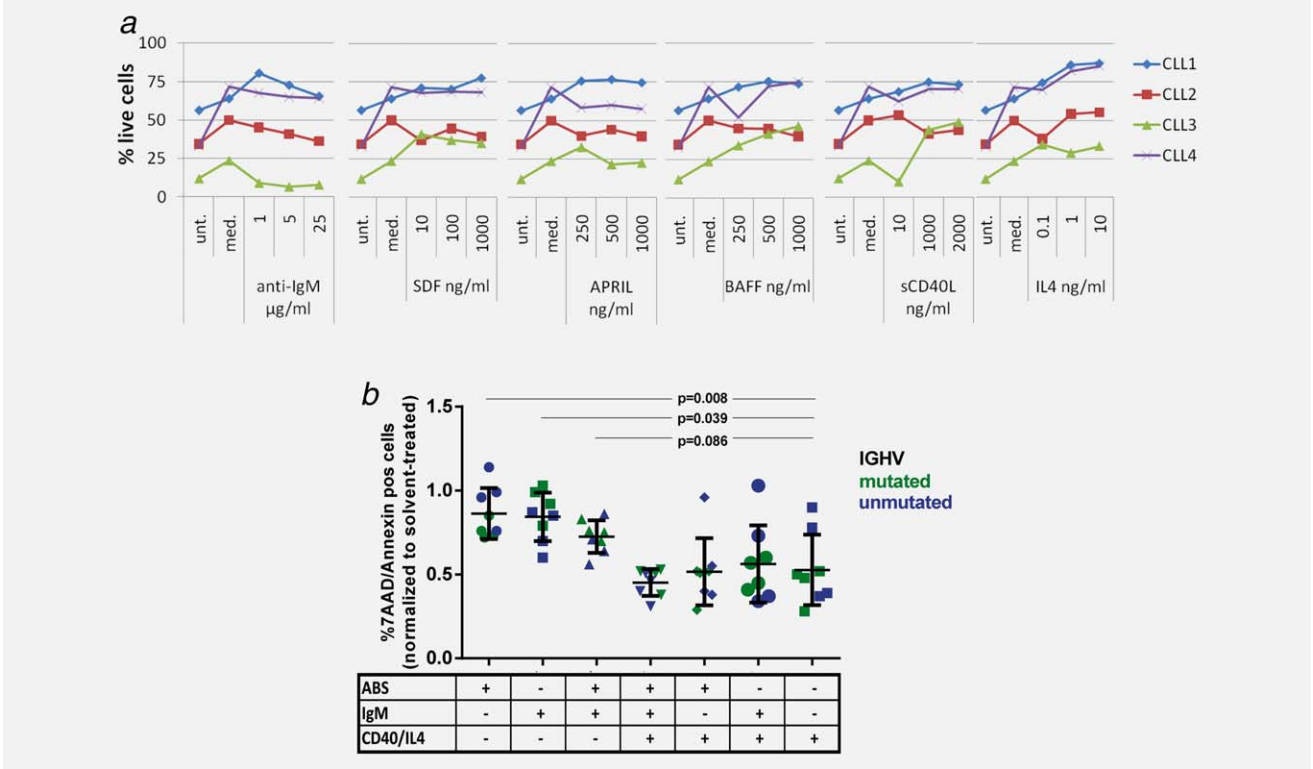


**Figure 1.** Identification of optimal ligand concentrations for CLL cell survival. (*a*) CLL cells from four patients were treated with increasing concentrations of different ligands for 4 days or left untreated. The percentage of dead cells (7-AAD-positive cells) was measured 4 days after treatment by quantifying 7-AAD staining by flow cytometry. The optimal ligand concentrations were determined by saturation in the survival response. Optimal concentrations of ligands were determined to be: CD40L 1,000 ng/ml, IL4 1 ng/ml, SDF 100 ng/ml, APRIL 250 ng/ml, BAFF 250 ng/ml and anti-IgM F(ab′) 5 µg/ml. (*b*) CLL cells from eight patients were treated with combinations of ligands and apoptosis quantified as 7-AAD-positive cells after 48 hr. IL4 and CD40L were most effective in rescuing CLL cells from apoptosis (*p*-values for difference between CD40/IL4 *vs*. other ligand combinations determined by two-tailed Mann–Whitney *U* test). Concentrations of ligands used: CD40L 1,000 ng/ml, IL4 1 ng/ml, SDF 100 ng/ml, APRIL 250 ng/ml, BAFF 250 ng/ml and anti-IgM $F(ab')_2$ 5 µg/ml. Ligand concentrations were chosen according to previous results in (*a*). ABS = APRIL, BAFF, SDF-1. Data from patients with mutated IGHV status are depicted in green and with unmutated IGHV in blue. See also Supporting Information Table S1 for patient characteristics.

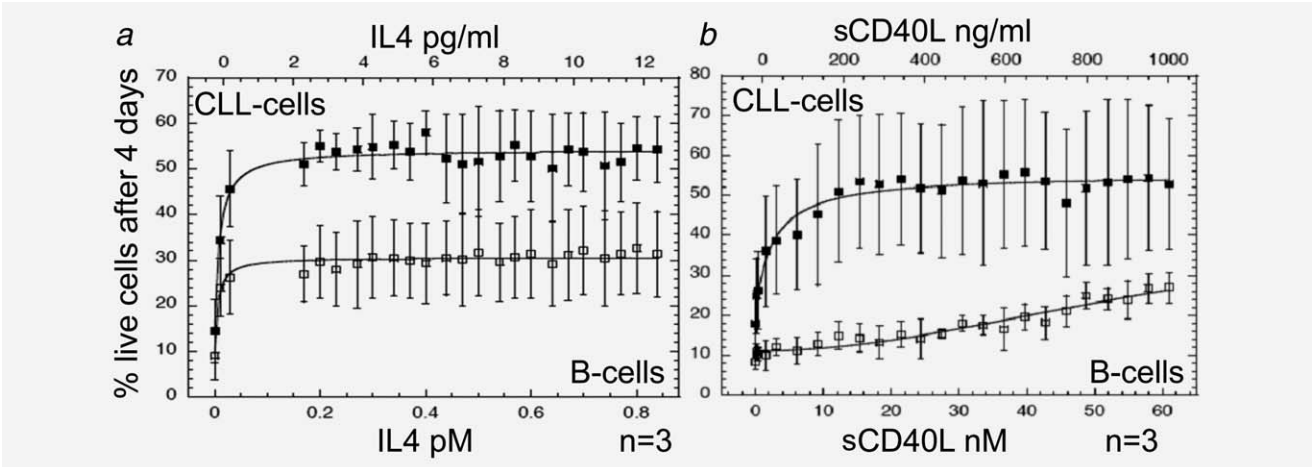


**Figure 2.** CLL cells are more responsive to IL4 and sCD40L compared to non-malignant B cells. CLL cells (CD19+ sorted) ($n = 3$) and non-malignant B cells ($n = 3$) were treated with systematically varied concentrations of (*a*) IL4 and (*b*) sCD40L. Proportion of dead cells (7-AAD-positive cells) was measured after 4 days of treatment using flow cytometry. Shaded boxes: CLL cells; unshaded boxes: non-malignant B cells.

constant $K_d$ of 0.01 pM in the absence of cooperativity (alpha = 1) was the same for both malignant and non-malignant cells. Thus, the increased stimulation of CLL cells by IL4 occurred in the downstream signaling cascade without changes in the affinity between ligand and receptor (Fig. 2*a*). In contrast, the response curve of sCD40L was sigmoidal in the non-malignant cells, while this feature was absent in the CLL cells, displaying a qualitative difference for the two different cell types. For non-malignant cells the binding occurred with relatively low affinity with $K_d = 70$ pM and some cooperative binding (alpha = 2). In contrast, the response of the CLL cells to sCD40L addition lost cooperativity and rather followed a simple binding model with a largely increased binding affinity of the ligand as reflected by the much smaller value of $K_d = 2.6$ pM for half-saturating ligand concentration with respect to the plateau of the surviving cell fraction (Fig. 2*b*). Thus, CLL cells appear to be deregulated in terms of an almost 30-fold higher binding affinity for the interaction of sCD40L with its cellular receptors.

### CD40 does not display different receptor distribution on CLL cells compared to non-malignant B cells

For the IL4 receptor it has been shown that CLL cells carry threefold more molecules on their cell membranes compared to non-malignant B cells.[21] This can readily explain the stronger response and different saturation points in CLL *versus* non-malignant cells.

For sCD40L, we observed a stronger response in CLL cells. However, in addition a loss of cooperativity was observed in the dose–response of CLL cells. One explanation could be changes in the spatial distribution of CD40 receptor similar to what has been described for other members of the TNF superfamily of ligands that self-assemble.[22] For recombinant sCD40L, cooperative binding has been reported previously,[23] even though for synthetic sCD40L the hill constant of was $h = 2.4 \pm 0.2$ [24] and somewhat higher than what we observed for the dose–response of CLL cells. As expected, blocking the trimerization of sCD40L also resulted in loss of cooperative binding to CD40 receptor ($h = 1 \pm 0.04$).[24] Trimerization of sCD40L/cd40 is required for signaling and could explain differences in dose–response curves. Accordingly, we stained PBMC of CLL patients and CD19 sorted B cells from PBMC of healthy donors for CD40 and IL4 to assess whether differences in receptor clustering could be detected that could explain qualitative changes in dose response in CLL cells. To quantitate the different cellular subpopulations in large samples, we used an imaging-based flow cytometer.[19] After stimulation of CLL cells and non-malignant B cells with sCD40L, we assessed receptor clustering at the cellular membrane that could explain aberrant loss of cooperativity observed in CLL cells. Interestingly, CLL cells and non-malignant B cells showed a heterogeneous distribution of IL4 and CD40L receptors (Fig. 3*a* and 3*b* and Supporting Information Fig. S2). No difference in receptor clustering was observed between malignant and non-malignant cells (Fig. 3*c*). Stimulation with ligands resulted in a slightly reduced CD40 clustering to a similar extent in both CLL cells and non-malignant B cells. In addition, no difference in receptor internalization was detected between CLL and control (Fig. 3*d*). Therefore, the loss of cooperativity of the sCD40L dose–response in CLL cells could not be correlated with different receptor clustering of CD40 or internalization events, making a causative connection between these two processes unlikely.

As a next step we therefore tested whether downstream intracellular differences can be observed in CLL cells in sCD40L and also in IL4 signaling.

### IL4 stimulation leads to increased intracellular STAT6 levels and phosphorylation

IL4 has been reported to induce phosphorylation of STAT6, which homodimerizes and translocates to the nucleus.[25,26] In the nucleus it acts as a transcription factor and induces target genes in concert with c-Jun, SP-1, C/EBP and NF-kB that are required for the development of Th2 helper cells.[27] Among these target genes is CD23, which is secreted from CLL cells and has prognostic relevance in CLL, underlining the importance of the IL4/STAT6 signaling cascade in CLL.[28] We therefore quantified the activation of STAT6 in CLL cells compared to non-malignant lymphocytes after stimulation with IL4 and found an enhanced phosphorylation of STAT6 in CLL cells both by Western-blot (Fig. 4*a* and Supporting Information Fig. S3) and flow cytometry (Fig. 4*b*). This increased activation can explain the aberrant dose–response curves compared to non-malignant B cells. Intriguingly, weak phosphorylation could be observed in CLL cells already in the non-stimulated situation. This is in-line with a response of CLL cells to the JAK3-selective inhibitor PF-956980 inhibitor that blocks IL4-mediated signaling.[29]

### sCD40L stimulation induces stronger NF-kB signaling in CLL cells compared to non-malignant B cells

In the light of the loss of cooperativity that we observed in CLL cells for stimulation with sCD40L it is of interest that sCD40L has been shown to induce both survival and proliferation in non-malignant B cells.[30,31] In the latter, survival is induced *via* NF-kB signaling while induction of proliferation cannot be prevented by knockout of p50.[32] In contrast, in CLL cells sCD40L by itself does not induce proliferation.[33] CLL cells can only be stimulated to undergo proliferation when in addition to incubation with sCD40L (*i*) if IL4R is stimulated in addition (see Supporting Information Fig. S1*a* produced with data from Jacob *et al.*[33]) or if (*ii*) the CD40 receptor is crosslinked using monoclonal antibodies that do not compete with sCD40L binding (see Supporting Information Fig. S1*c*). For the sCD40L/CD40 interaction a targeted loss of trimerization by exchanging amino acid G144P leads to loss of cooperativity.[24] Intriguingly, at the same time monomerization of sCD40L has been shown to reduce downstream NF-kB signaling.[24] We therefore speculated whether

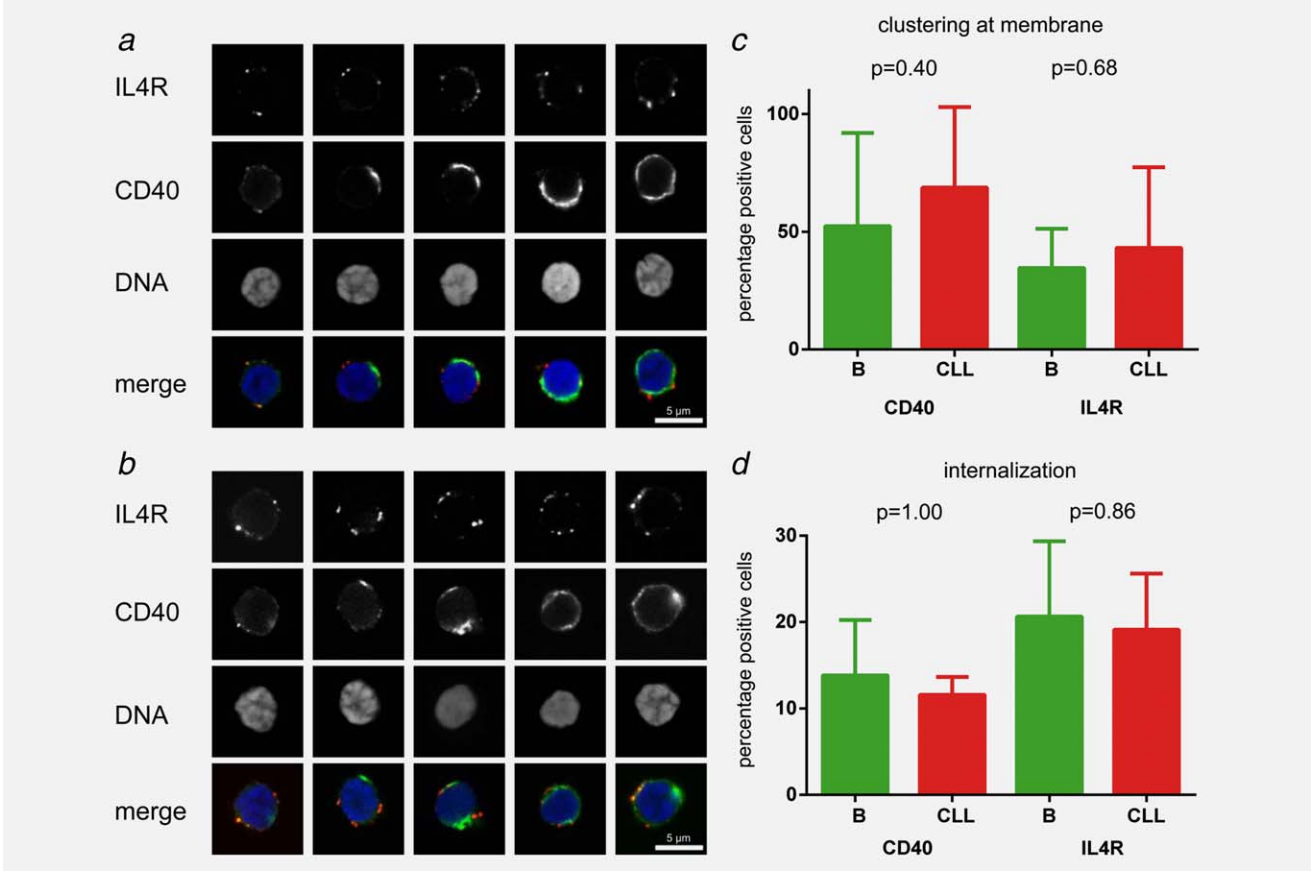


**Figure 3.** Higher responsiveness of CLL cells to IL4 and CD40L does not correlate with receptor membrane clustering or intracellular density. (*a*) IL4 receptor and CD40 were detected on the surface of CLL cells and (*b*) non-malignant CD19-positive B cells using immunofluorescence. DNA was stained with DAPI. Signal intensity and distribution varied in cells. Shown are representative examples of weaker staining (left) and stronger and more uniformly distributed staining (right). Cells were stimulated with 0.4 pg/ml IL4 and 150 ng/ml CD40L for 5 hr and receptors stained with directly labeled antibodies. (*c*) Using image-based flow cytometry, surface IL4 receptor and CD40 quantity and distribution were characterized in populations of CLL cells and non-malignant CD19-positive B cells from two (CD40) or three (IL4R) healthy donors and four (CD40) or five (IL4R) CLL samples. Stimulation did not change spatial distribution of CD40 and IL4R between CLL cells compared to non-malignant B cells. (*d*) For internalization, no consistent changes could be observed on CLL cells compared to non-malignant B cells after stimulation with IL4 or CD40L. Error bars reflect standard deviation. *p*-Values were calculated using two-tailed Mann–Whitney *U* test. Cells were stimulated with 0.4 pg/ml IL4 and 150 ng/ml CD40L for 5 hr and receptors stained with directly labeled antibodies as in panels *a* and *b*. More than 2,000 cells were analyzed per condition. For details and examples, see Supporting Information Figure S2.

the loss of cooperativity that coincides with a shift from induction of proliferation towards the induction of survival could be linked by enhanced stimulation of NF-kB by sCD40L in CLL cells. To this end we quantified NF-kB activity using a chemiluminescent oligonucleotide coupled ELISA that we established previously (co-ELISA[4]) and that allows to robustly measure activity of NF-kB in cell lysates directly. Similar to the effects we observed with IL4, signaling downstream of sCD40L was enhanced in CLL cells both before and after stimulation when compared to non-malignant B cells (Fig. 4*c*). Although activity of NF-kB was heterogeneous both in non-malignant and malignant B cells, the median activity in samples derived from different donors was higher in CLL cells compared to stimulated non-malignant B cells not only in the stimulated but also in the non-stimulated situation.

These changes in intracellular signaling pathways underline the observed differences in the dose response curves of CLL cells *versus* non-malignant B cells both for IL4 and sCD40L. In the case of sCD40L, our finding of enhanced NF-kB activity underscores the switch in CLL cells from enhanced proliferation and survival that is observed in non-malignant B cells towards a prosurvival phenotype only in CLL cells that has been reported to be induced specifically by NF-kB signaling.

## Discussion

Microenvironmental support is emerging as a major factor in tumorigenesis. For several reasons, CLL is an ideal model to study this relation: primary CLL cells are easily accessible and undergo rapid apoptosis *in vitro* that can be rescued by culture on feeder cells. This support consists of signals that

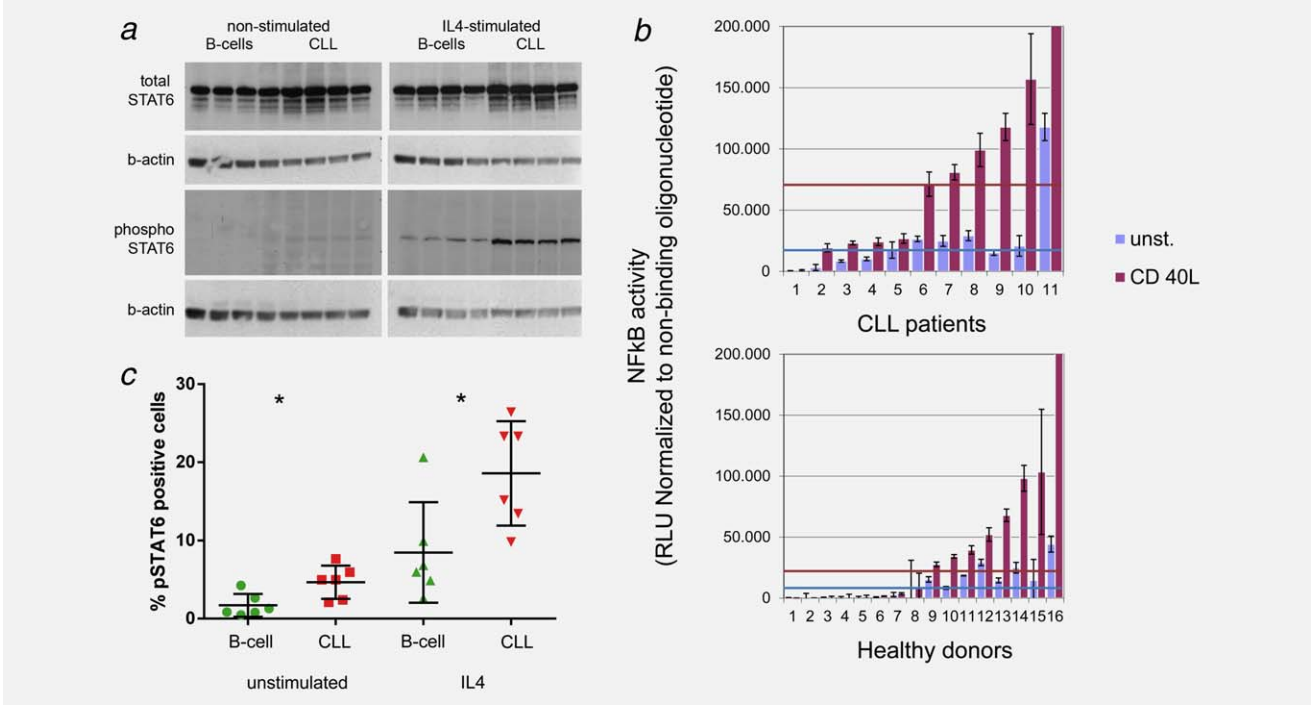


**Figure 4.** Activation of downstream signaling by IL4 and sCD40L on CLL cells. (*a*) B cells from four healthy probands and from four CLL patients were stimulated with 0.4 pg/ml IL4 for 1 hr or left unstimulated and characterized by Western blot for levels of STAT6 and phospho-STAT6. Although total levels of STAT6 may only be slightly increased in CLL cells, a significant higher level of phosphorylated STAT6 was observed in CLL cells after stimulation (right panels) and possibly already before stimulation (left panels). Densitometric analysis showed a statistically significant difference after stimulation (two-tailed Mann–Whitney *U* test, Supporting Information Fig. S3). (*b*) Cells from six healthy probands and six CLL patients were stimulated with IL4 or left unstimulated and characterized by intracellular flow cytometry for levels of phospho-STAT6. Shown are the numbers of cells with signal above background as detected by non-specific IgG. Both in the non-stimulated and in the stimulated cell populations, more cells were positive in samples derived from CLL patients than from healthy donors. $*p < 0.05$ with two-tailed Mann–Whitney *U* test. Cells were stimulated for 1 hr with 0.4 pg/ml recombinant IL4. (C) CD19+ cells from CLL patients (top panel) and healthy probands (bottom panel) were stimulated with 150 ng/ml CD40L for 1 hr (red columns) or left unstimulated (blue columns). Activity of NF-kB (p65) was characterized by co-ELISA. Although both malignant and non-malignant cells show a wide range of NF-kB activity, the median of activity and activation (bold horizontal lines) is higher in CLL cells than in non-malignant cells.

are delivered to CLL cells by direct cell contact or through soluble factors. CLL cells themselves produce and express both ligands and receptors for prosurvival cytokines such as IL-2, IL4, IL-8, TNFa as well as VEGF, which modulate survival.[34] However, it is unclear if an enriched microenvironment is the sole cause for prolonged CLL cell survival or whether the CLL cells are also intrinsically more responsive to microenvironmental cues. We therefore tested different combinations of ligands for their effect on the survival response of CLL cells. The T-cell-derived ligands IL4 and sCD40L proved to be most effective in rescuing CLL cells from apoptosis. Moreover, CLL cells were more responsive than non-malignant B cells to systematically varied dosages of IL4 and sCD40L. This indicates that not only increased microenvironmental stimuli but also receptivity to the stimuli are important in the prolonged survival of CLL cells. Why are CLL cells more receptive to IL4 and sCD40L stimulation, and are these differences caused by aberrant receptor ligand interaction or downstream effector molecules?

CLL blood $CD8^+$ T cells and CLL cells contain cytoplasmic IL4. Both of these cell populations are able to secrete IL4 into the culture supernatant.[27] IL4 protects CLL cells from apoptosis by increasing the levels of members of the BCL family of proteins,[35] and the enhanced response of IL4 on CLL cells we observed is probably caused by a threefold increase of the IL4R on CLL cells.[21] In B cells, IL4 stimulates several intracellular signaling pathways including STAT6,[36] which becomes recruited to the IL4R where it is phosphorylated by JAK kinases.[37] The phosphorylated STAT6 forms dimers and translocates to the nucleus to bind DNA and activate transcription of target genes inhibiting apoptosis in B cells.[36,37]

CD40 stimulation of CLL cells by membrane-bound CD40L has been shown *in vitro* to prevent apoptosis and induce proliferation,[16,38] which likely occurs in large part *via* the NF-kB signaling cascade.[39] Secreted CD40L (sCD40L) can also be produced by CLL cells[18] and in contrast to membrane-bound CD40L, sCD40L alone can induce proliferation only in non-malignant B cells but not in CLL cells.[18] In

CLL cells, additional signals and/or crosslinking of the CD40 receptor is required.[18,33] In addition, a subset of CLL cells expresses both sCD40L and its receptor, enabling an autocrine loop by which CLL cells can promote their own survival, while non-malignant B cells express only the CD40 receptor.[18,40]

In the dose–response of non-malignant B cells to sCD40L, we observed cooperativity which is frequently caused by oligomerization of ligand and/or receptors. In line with this concept, recombinant sCD40L has been shown to trimerize[23,41] and interact stoichiometrically with CD40 receptor with a cooperativity factor $h$ of 2.9.[24] In non-malignant B cells, recombinant trimeric sCD40L induces proliferation, differentiation and prevents apoptosis.[41] The downstream signaling pathways of CD40 include NF-kB signaling[42] that drives survival *via* induction of the anti-apoptotic BCL2. In contrast, proliferation is induced *via* non-NF-kB signaling in non-malignant B cells.[32] sCD40L can induce proliferation only in non-malignant B cells, not in CLL cells[33] (Supporting Information Fig. S1). In CLL cells, additional crosslinking of the CD40 receptor and/or IL4 stimulation is required for full functionality of sCD40L. Intriguingly, mutating the sCD40L molecule to prevent trimerization and thereby removing cooperativity leads to a change in the downstream NF-kB signaling.[24] We therefore speculated whether the loss of cooperativity of sCD40L signaling that we observed in CLL cells together with the previously reported loss of the dual functionality of sCD40L (survival and proliferation) in these cells could be linked to changes in NF-kB signaling in CLL cells. To this end, we measured the activity of NF-kB as a factor possibly differentiating between proliferation and apoptosis and found it to be higher in CLL cells. We propose that this enhanced NF-kB signaling drives the sCD40L mediated response towards survival of the malignant cells. The proliferation of the malignant clone that has been shown in the microenvironment of CLL patients[43] would then require either membrane-bound CD40L, for example, on the surface of neighboring T cells[6,44,45] or additional pro-proliferative signaling like, for example, IL4.[46] Interestingly, different levels of NF-kB activation have already been shown to correspond to different prognostic CLL subgroups,[47,48] and efforts have already been undertaken to target NF-KB therapeutically in CLL (AT514,[49] BAY 11–0782[50] and Parthenolide[51]).

In conclusion, we expect that quantitative and comprehensive analyses of the aberrant signaling network of CLL cells are warranted to help understand the malignant phenotype of these cells and to open new avenues for treating CLL patients.

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**Supplemental Material:**

Supplemental Table 1 lists the cytogenetic karyotype and immunoglobulin heavy chain (IGHV) locus mutation status of the patient cohort analysed.

Supplementary Figure S1: *sCD40L does not induce proliferation in CLL cells in contrast to non-malignant B-cells.* Data taken from Jacob et al., Leukemia Res 1998: Non-malignant B-cells from healthy donors were stimulated for three days with sCD40L, monoclonal antibodies against CD40 (S2C6, 5c3 and G28) and with IL4 and subsequently proliferation was measured by incorporation of pulsed H3Thymidine into DNA (y-axis). (A) Non-malignant B-cells from three donors A, B and C show strong proliferation upon treatment with sCD40L (increased incorporation of H3-thymidine by DNA-replication, associated with proliferation). This incorporation of H3-thymidine reflecting cellular proliferation is enhanced by co-incubation with IL4. (B) In contrast, in CLL cells (n=18) sCD40l does not induce proliferation as no H3-thymidine is incorporated into the DNA of these CLL cells. (C) In CLL cells, proliferation can only induced by sCD40L either via coincubation with antibodies that crosslink CD40 (S2C6, 5c3 and G28, panel C) or via coincubation with IL4 (panel A).

Supplementary Figure S2: Example for assessing plasma membrane and internalized signal of IL4R and CD40. Columns 1 and 2 show examples for the mask applied to differentiate cell surface from internal fluorescence signal (blue). Columns 3 and 4 show examples for detection of receptor clusters on the cell surface, whereas columns 5 and 6 show examples for the detection of clusters of internalized receptors (blue). Columns 3-6 do not depict identical single cells. For details see materials and methods section.

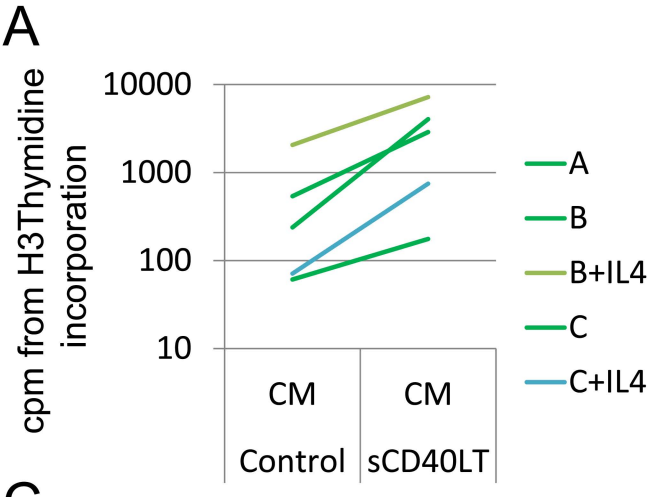
Supplementary Figure S3 is a densitometric analysis of the western signal depicted in figure 4 A, showing that the difference in pSTAT6 levels are statistically stronger in CLL cells as compared to non-malignant B-cells after stimulation with IL4.

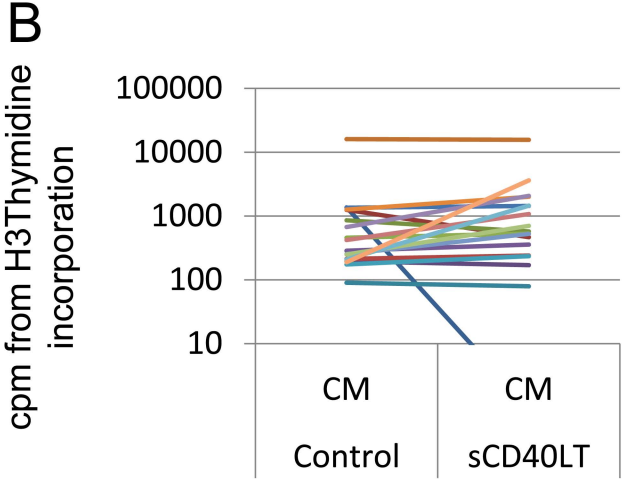
**Supplementary Table 1: Clinical characteristics of CLL patients**

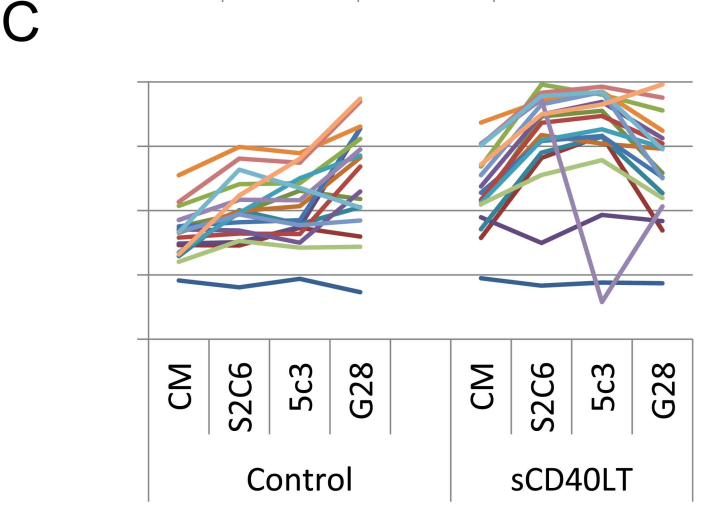
| patient | karyotype | IGHV status * |
|---|---|---|
| 1 | bidel(13q) | mut |
| 2 | del(13q) | non-mut |
| 3 | del(13q) | mut |
| 4 | normal | non-mut |
| 5 | bidel(13q) | mut |
| 6 | del(13q), t(14;19), 18q | mut |
| 7 | normal | mut |
| 8 | del(13q) | mut |
| 9 | del(13q) | non-mut |
| 10 | del(13q) | mut |
| 11 | normal | mut |
| 12 | normal | mut |
| 13 | normal | mur |
| 14 | normal | non-mut |
| 15 | del(17p), TP53mut | non-mut |
| 16 | del(13q) | non-mut |
| 17 | del(13q), del(11q) | non-mut |

* sequences with a germ line homology >=98% were considered unmutated

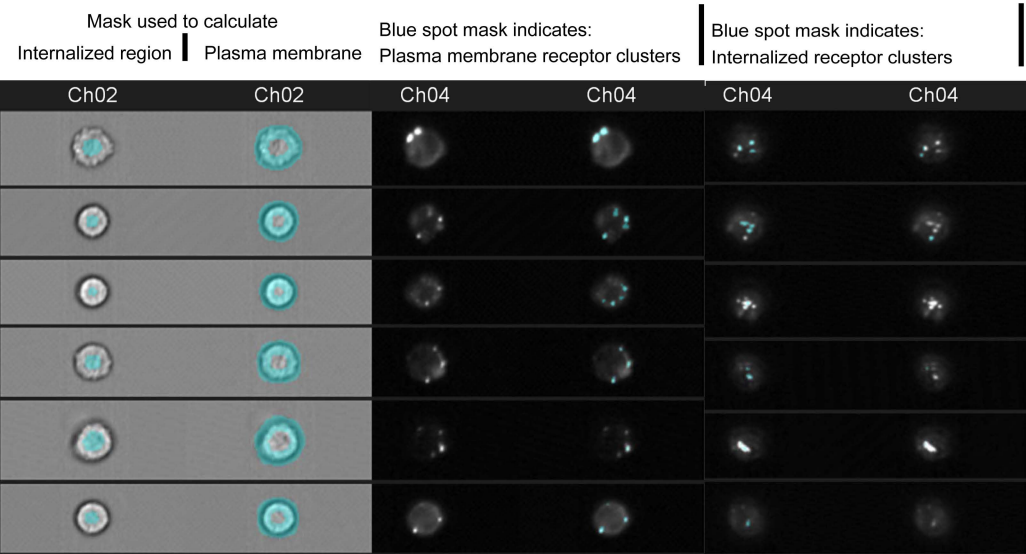
*Suppl. Figure 1: sCD40L does not induce proliferation in CLL cells in contrast to non-malignant B-cells.*



Supplemental Figure 2: Example for assessing plasma membrane and internalized signal of IL4R and CD40. Columns 3-6 do not depict identical single cells.



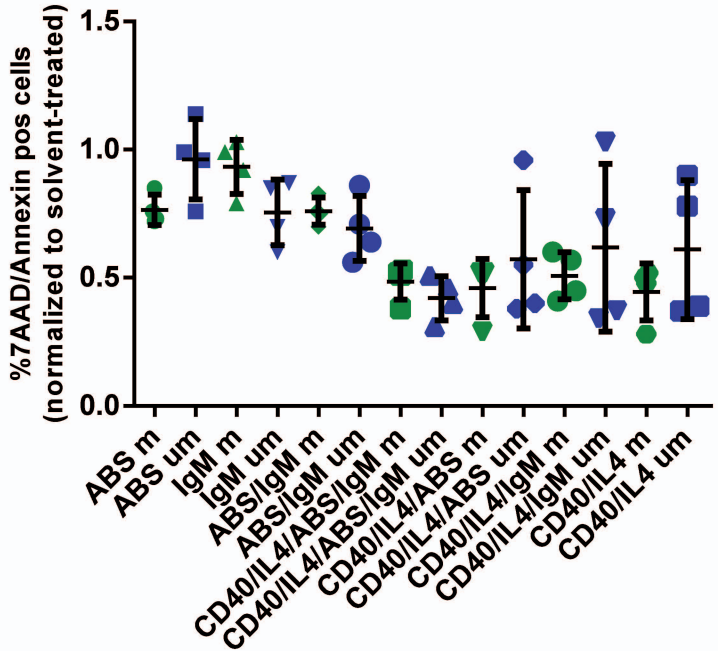



Figure for reviewer #2:
Combinations of ligands lead to reduction of apoptosis. This response was not different between patients with and without IGHV mutation status ("m" vs "um"). ABS: APRIL, BAFF, SDF1

Suppl. Figure 3: densitometric quantification of STAT6 and pSTAT6 signals on Western blot in B- and CLL cells with and without stimulation via IL4. Signal intensities were normalized to all signals on that blotting membrane. Significance was tested with 2-tailed Mann-Whitney. * = p<0.05.