Constitutive and B-cell receptor-induced activation of STAT3 are important signaling pathways targeted by bortezomib in leukemic mantle cell lymphoma

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Online Supplementary Design and Methods

Cell lines, cell culture and reagents

Patients' cells were used either freshly isolated or cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide and 20% heat-inactivated fetal calf serum. Results obtained with freshly or frozen cells were identical and reproducible. MCL leukemic cells (3×10^6 cells/mL) were cultured for 24 h in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. For BCR stimulation, cells were cultured in the same medium on plates coated with rabbit anti-human IgM antibody (10 μg/mL; Jackson ImmunoResearch, Baltimore, MD, USA). Jeko-1 and Rec-1 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunshwieig, Germany) and the HBL-2 cell line was a generous gift from Pr B. Sola (Caen, France). Both Jeko-1 and Rec-1 cell lines exhibit unmutated *IGHV* gene (*IGHV2-70*; 99.6% homology and *IGHV1-2*; 98.5% homology, respectively) and the HBL-2 cell line displays mutated *IGHV* gene (*IGHV3-11*, 97.4% homology). MCL cell lines were maintained in culture in the same media as MCL patients' cells. Normal B-lymphocytes were purified from a pool of PBMC from healthy individuals. Anti-cytokine and anti-cytokine receptor antibodies were purchased from R&D systems (Minneapolis USA). Fludarabine was from Bayer Schering Pharma (Loos, France), bortezomib from Janssen-Cilag (Beerse, Belgium), Stattic and AG490 from Merck KGaA, (Darmstadt, Germany).

Apoptosis and cell viability assays

Cell apoptosis was analyzed on a Coulter EPICS XL (Beckman Coulter, Villepinte, France) by annexin V-FITC and propidium iodide (PI) staining (BD Biosciences, San Jose, CA). The percentage of apoptotic cells corresponded to the percentage of annexin V-positive cells, including PI-negative and PI-positive cells. All measurements were done in duplicate. Cell viability was evaluated by the methyltetrazolium salt (MTS) assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA) according to the manufacturer's instructions. All points were done in triplicate.

Quantification of IL6 mRNA by quantitative reverse transcriptase polymerase chain reaction analysis

The expression of *IL6* mRNA was analyzed in unstimulated and anti-IgM stimulated cells for 3 and 6 hours by qRT-PCR using RT^2 profiler PCR arrays according to the manufacturer's instructions (SA Biosciences, Frederick, MD, USA). *IL6* is one of the genes included in the "p53 signaling pathway" array (SA Biosciences). Target gene expression (*IL6*) was normalized to the mean Ct values from four housekeeping genes (β_2-microglobulin, β-actin ribosomal protein L13A, hypoxanthine phosphorybosyl transferase-1 and β-actin), then normalized to unstimulated control cells to determine the fold-change. Relative fold change of expression was calculated by the ΔΔCt method and the values are expressed as $2^{-\Delta\Delta Ct}$. All points were done in duplicate.

Quantification of cytokines in cell culture supernatants by enzyme-linked immunosorbent assay

MCL cells (3×10^6) were grown in 1 mL of culture medium for 24 h. Secreted IL6 or IL10 was measured by ELISA (Promokine PromoCell GmbH, Heidelberg, Germany) from 50 μL of supernatant. The optical density was determined by a spectrophotometer (Multiskan MS, Thermo, Cergy-Pontoise, France) set at 450 nm. Duplicate measurements were averaged.

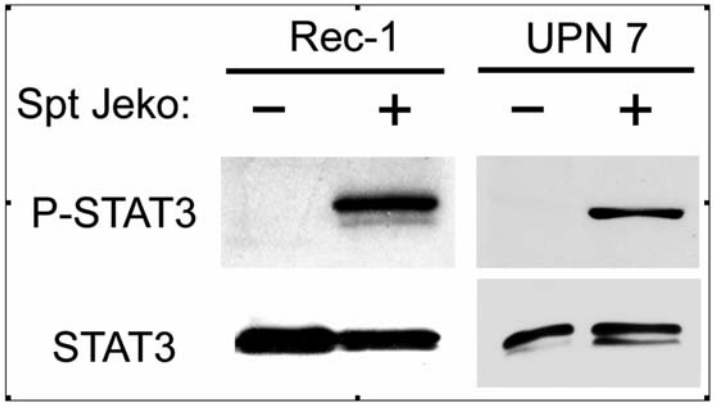
Immunofluorescence

Cytospin cells were fixed in formaldehyde 3.7%, permeabilized in Triton X-100 0.1% and blocked with fetal calf serum 5%, Tween-20 0.1% in phosphate-buffered saline. Cells were incubated with primary anti-tyr705 phospho STAT3 antibody for 1 h (Cell Signaling) followed by a Alexa Fluor 488-labeled secondary goat anti-rabbit antibody (Invitrogen-Molecular Probes). Fluorescent images were digitally acquired using a Zeiss Axioplan2 Deconvolution microscope (magnification x60) (CarlZeiss, Le Pecq, France) and analyzed with Metafer4 (Metasystems, Altlussheim, Germany).

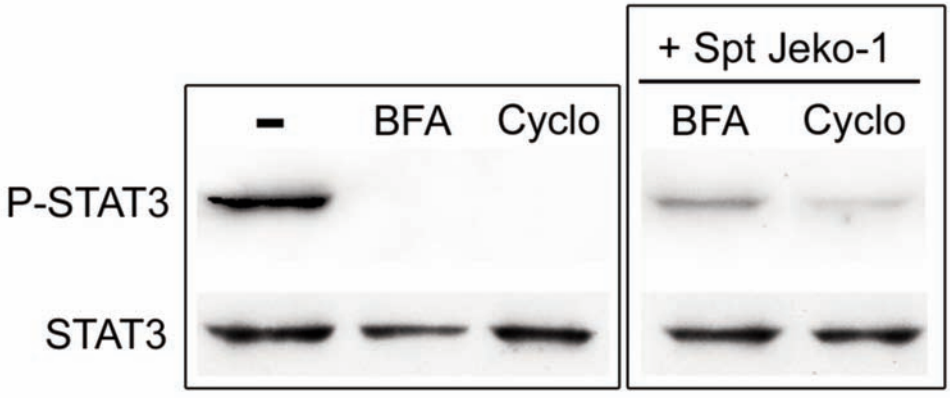
Online Supplementary Table S1. Characteristics of the 20 MCL cases (UPN).

UPN	Sex	Age	VH*	DH*	JH*	IGHV homology*# (%)	IGHV mutational status*†	Lymphocytosis giga/L	% tumor cells§
1	M	59	VH7-4-1	D3	JH5	95	M	15.04	65
2	M	78	VH3-30	D2	JH6	94.3	M	47.34	90
3	M	56	VH4-4	D2	JH6	94	M	50.22	90
4	F	63	VH4-34	D2	JH5	93.8	M	5.5	59
5	M	50	VH4-39	D5	JH3	96.7	M	53.39	88
6	F	69	VH3-39	D3	JH4	100	UM	20.03	80
7	M	82	VH1-2	D1	JH4	100	UM	34.8	91
8	M	68	VH3-23	D2	JH4	99	UM	42.91	86
9	M	66	VH3-11	/	JH6	98.1	UM	4.79	40
10	F	74	VH3-74	D3	JH4	98	UM	34.86	83
11	M	81	VH3-23	D2	JH4	98.9	UM	5.47	80
12	M	71	VH4-31	/	JH6	96.6	M	158.9	96
13	M	71	VH1-2	D3	JH4	95.7	M	35.69	91
14	M	76	VH1-8	D6	JH5	99.6	UM	11.84	66
15	M	80	VH4-34	D2	JH4	99.6	UM	174.4	86
16	F	74	VH4-61	D1	JH5	93.3	M	10.66	77
17	M	69	VH3-9	D1	JH4	95.5	M	8.35	76
18	M	63	VH3-74	D3	JH6	100	UM	13.71	88
19	M	69	VH3-11	D1	JH4	100	UM	4.6	24
20	M	71	VH3-7	D6	JH4	99.6	UM	342.7	83

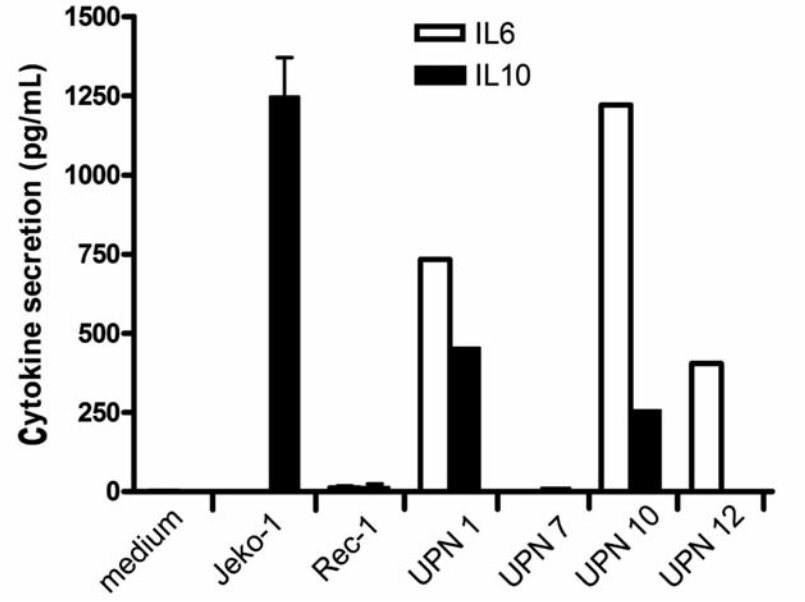
*IGHV *rearrangement and mutational status were analyzed by sequencing the FR1c-JH PCR product and alignment in the IMGT database* #IGHV *homology was calculated as the percentage of homology to the closest germ line* VH *gene.* †*Unmutated (UM):* IGHV *homology ≥ 98%; Mutated (M):* IGVH *homology < 98%.* §*Percentage of tumor B-lymphocytes in the PBMC sample.*



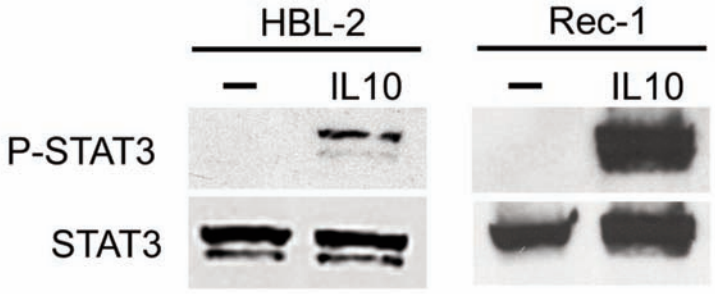
Online Supplementary Figure S1. Addition of cell culture supernatant from Jeko-1 cells induced STAT3 phosphorylation in Rec-1 and primary (UPN7) MCL cells, both lacking basal STAT3 phosphorylation. Lane 1 (-), no supernatant added; lane 2 (+), cells resuspended for 60 min in the supernatant from Jeko-1 cells (Spt Jeko).



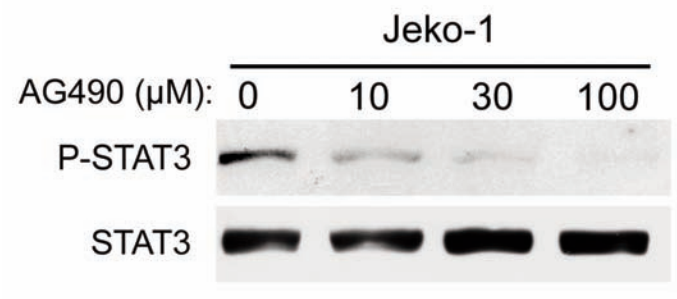
Online Supplementary Figure S2. Treatment of Jeko-1 cells with inhibitors of protein synthesis and secretion leads to inhibition of STAT3 phosphorylation. Jeko-1 cells were treated for 2 h with no inhibitor (Ctrl) or with 20 μM brefeldin A (BFA) or 20 μM cycloheximide (cyclo) (left panel). BFA- or cycloheximide-treated Jeko-1 cells were washed and incubated for 60 min in the supernatant of untreated Jeko-1 cells (spt Jeko-1) (right panel).



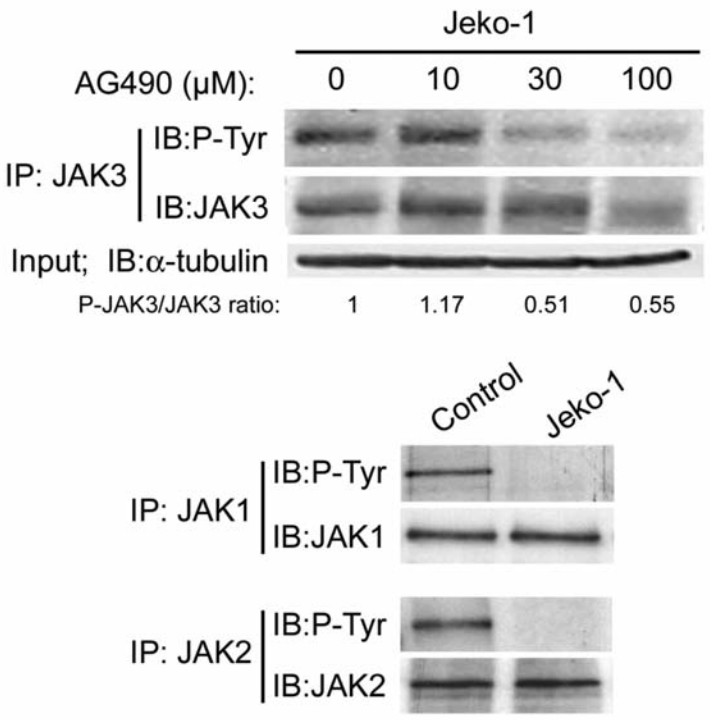
Online Supplementary Figure S3. Levels of IL6 and IL10 secreted in supernatants of Jeko-1 (n=3), Rec-1 (n=3) and primary MCL cells. Protein contents were measured by ELISA in duplicate samples of supernatant after 24 h of culture.



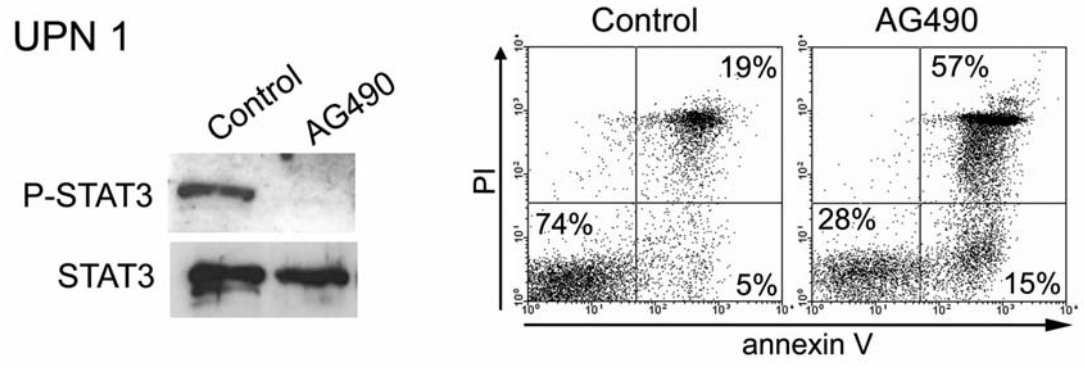
Online Supplementary Figure S4. IL10 treatment induced STAT3 phosphorylation in MCL cell lines lacking basal STAT3 phosphorylation. Rec-1 cells and HBL-2 cells were incubated for 24 h in the absence (-) or in the presence of IL10 (50 ng/mL, R&D Systems). P-STAT3 and total STAT3 expression were analyzed by western blotting as described in the *Design and Methods* section.



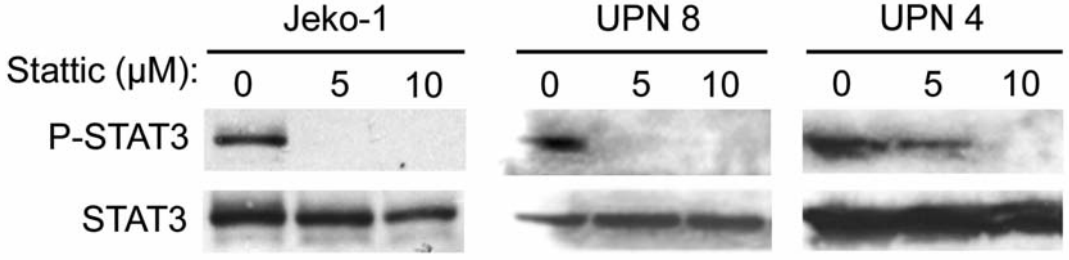
Online Supplementary Figure S5. Treatment of Jeko-1 cells for 24 h with the JAK inhibitor AG490 induced a dose-dependent decrease of STAT3 phosphorylation.



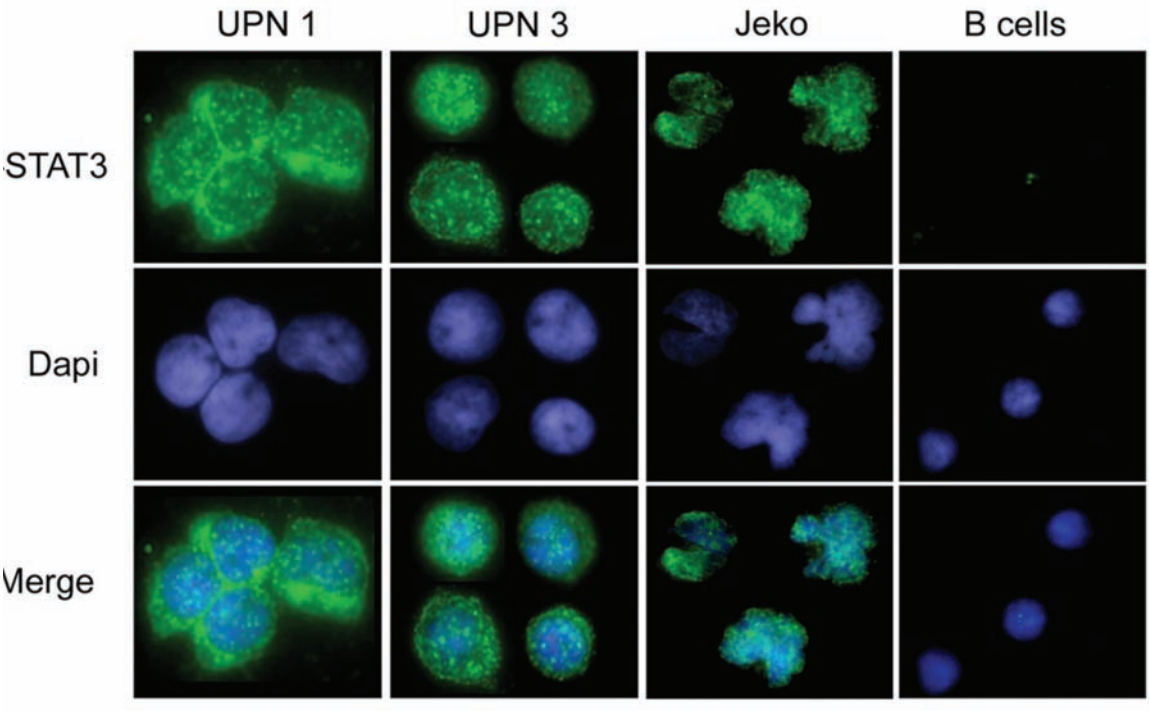
Online Supplementary Figure S6. Constitutive phosphorylation of JAK3 but not JAK1 and JAK2 was detected in Jeko-1 cells and treatment with AG490 reduced phosphorylation of Jak3. Jeko-1 cells were treated for 24 h with increasing concentrations of AG490 or left untreated. Cells were lysed in ice-cold 1% Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, and 1 mM EDTA) containing phosphatase and protease inhibitors for 30 min on ice. Insoluble material was removed by centrifugation at 27000g and total cell lysates (500 μg) were immunoprecipitated (IP) with anti-Jak antibodies (anti-Jak1 and anti-Jak2 from Millipore; anti-Jak3 from Santa Cruz Biotechnology) before immunoblotting (IB) with anti-pTyr 4G10 antibody (Millipore). The blot was then stripped and reblotted with the corresponding anti-Jak antibodies. For IP Jak3, input extracts were also immunoblotted with α-tubulin as a control of equivalent protein amounts for immunoprecipitation. Control IP Jak1: IL4-stimulated Ramos human lymphoblastoid cell line. Control IP Jak2: Erythropoietin-stimulated UT7 human megakaryocytic cell line.



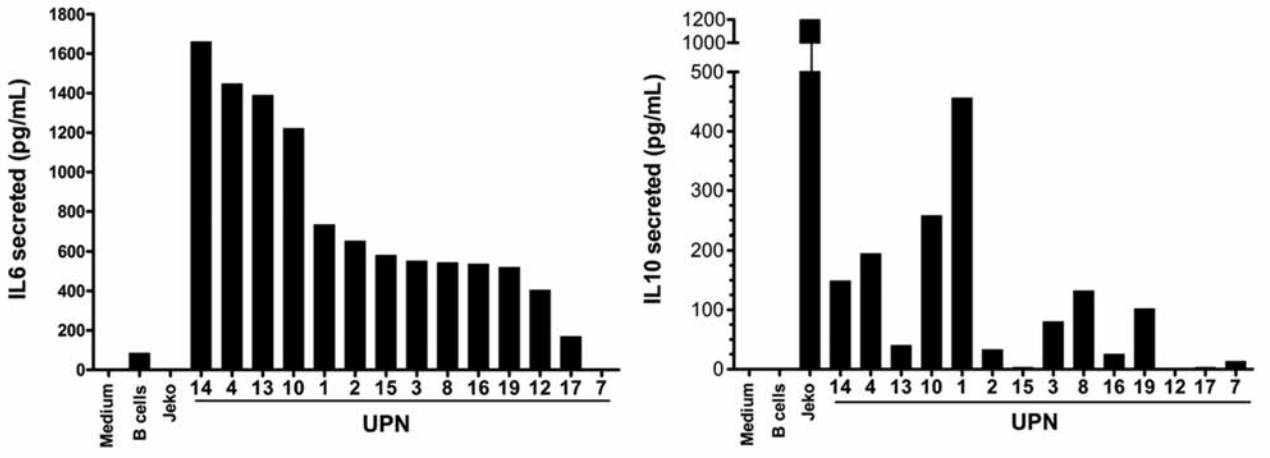
Online Supplementary Figure S7. Inhibition of STAT3 phosphorylation by AG490 (100 μM) (left panel) is associated with an increase of apoptosis (right panel) in primary MCL cells (UPN1). Apoptosis was measured by flow cytometry (annexin V/PI).



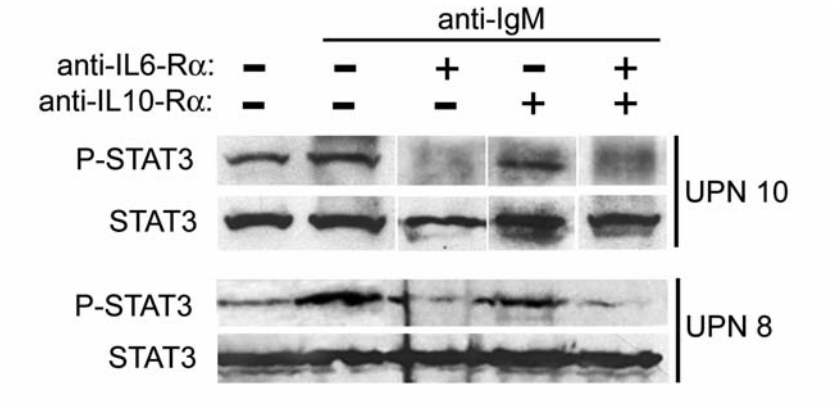
Online Supplementary Figure S8. Treatment (24 h) of Jeko-1 cells, and primary MCL cells (UPN8, UPN4) with Stattic, a small-molecule inhibitor of STAT3 dimerization and activation, induced a dose-dependent decrease of STAT3 phosphorylation.



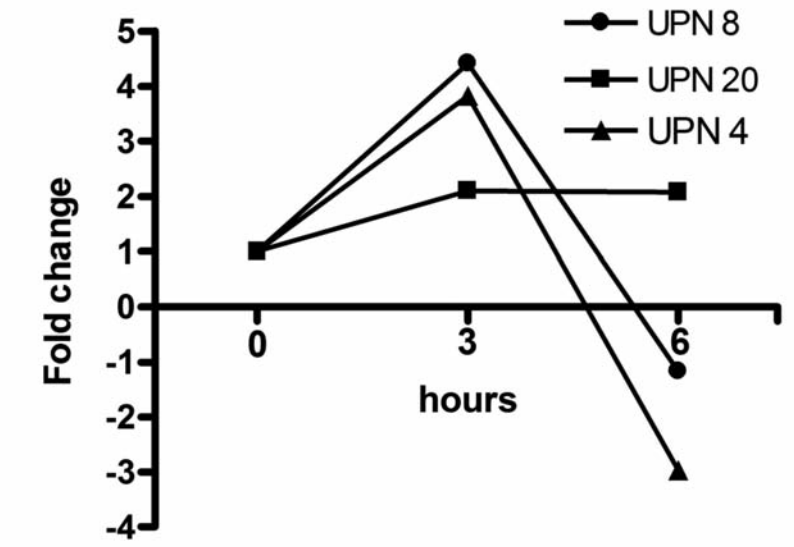
Online Supplementary Figure S9. Immunofluorescent staining of P-STAT3 (Alexa 488, green) and nuclear staining (DAPI, blue) in cells from two primary MCL cases (UPN1, UPN3), the Jeko-1 cell line (Jeko) and purified normal B cells (B cells). Magnification x 60.



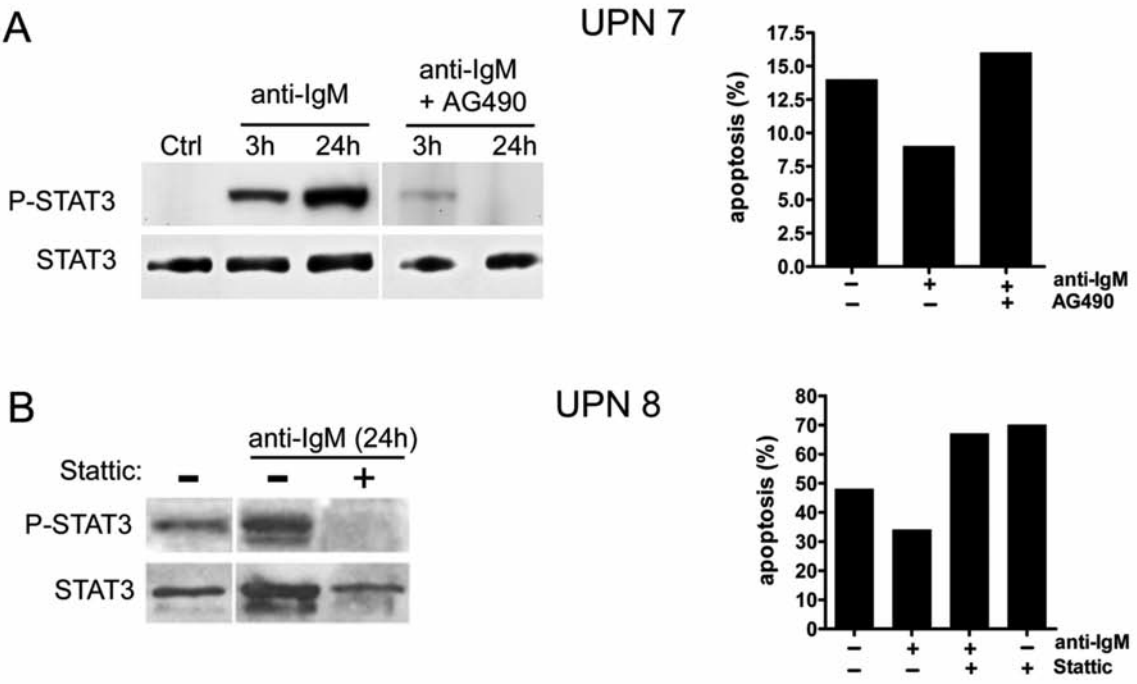
Online Supplementary Figure S10. Levels of IL6 and IL10 secreted in supernatants of 14 MCL cases (UPN), normal B cells and Jeko-1 cells were measured by ELISA after 24 h of culture.



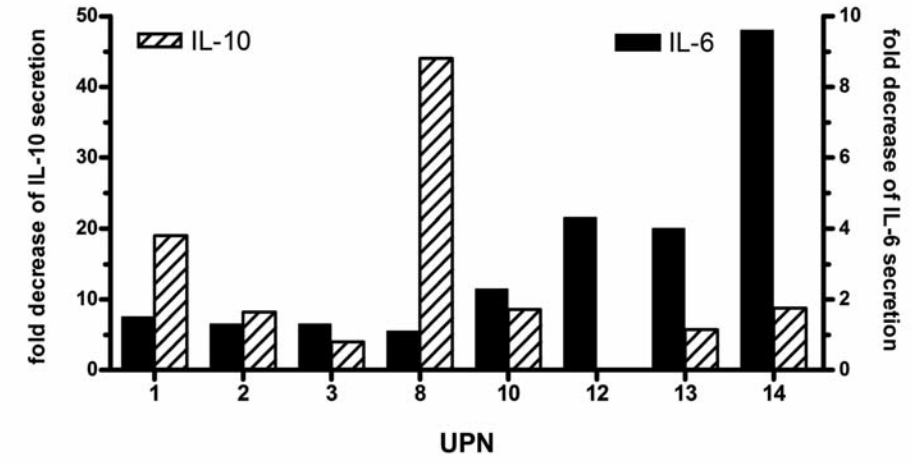
Online Supplementary Figure S11. Blocking the IL6 pathway with an anti-IL6 receptor inhibited both constitutive and BCR-induced STAT3 phosphorylation in primary MCL cells (UPN8, UPN10). STAT3 phosphorylation was analyzed following 24 h of BCR stimulation (anti-IgM) in the absence (-) or in the presence (+) of anti-IL6 receptor-α (anti-IL6-Rα) and/or anti-IL10 receptor-α (anti-IL10-Rα) blocking antibodies (40 μg/mL).



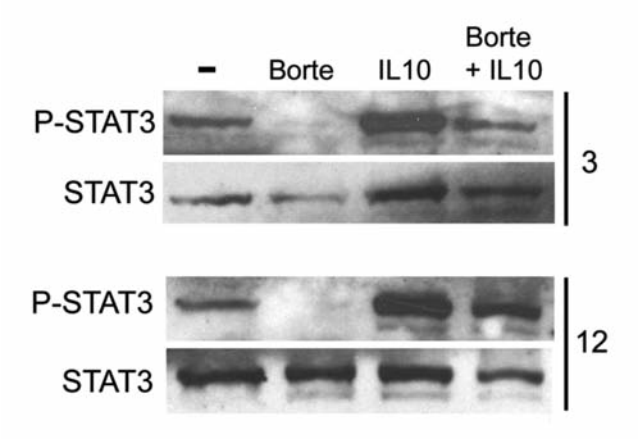
Online Supplementary Figure S12. BCR engagement induced an increase in *IL6* mRNA level in primary MCL cells. The expression of *IL6* mRNA was analyzed in duplicate by qRT-PCR either in unstimulated or anti-IgM stimulated cells for 3 and 6 h. *IL6* expression was normalized to the mean Ct values from four housekeeping genes, then normalized to unstimulated control cells to determine the fold-change. Relative fold change of expression was calculated by the ΔΔCt method and the values are expressed as $2^{-\Delta\Delta Ct}$.



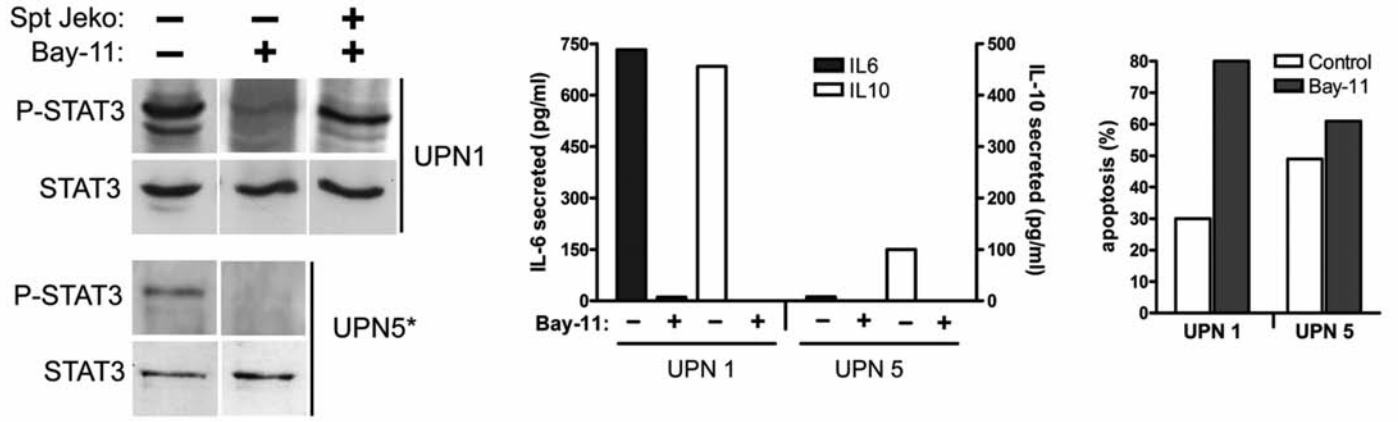
Online Supplementary Figure S13. Treatment of primary MCL cells with 100 μM of AG490 (A) or 10 μM of Stattic (B) abolished both BCR-induced STAT3 phosphorylation (left panels) and BCR-induced cell survival (24 h) (right panels).



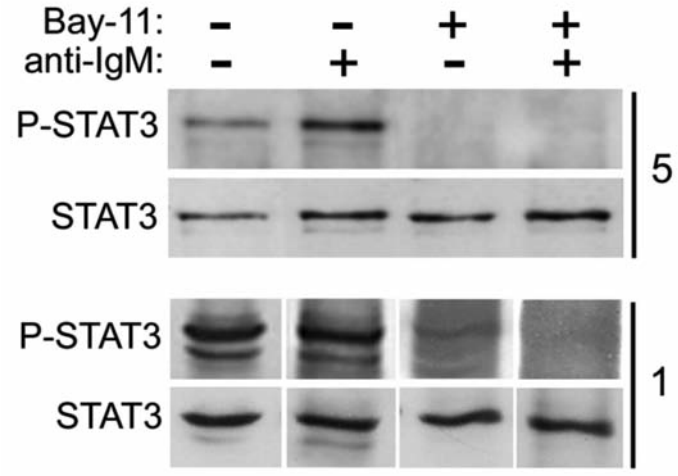
Online Supplementary Figure S14. Treatment of MCL cells with bortezomib (10 nM) induced a decrease of IL10 and IL6 secretion. Fold decrease of IL6 and IL10 secretion was measured in duplicate by ELISA in culture supernatants after 20 h of bortezomib treatment.



Online Supplementary Figure S15. Exogenous addition of IL10 overcomes bortezomib-mediated blockade of STAT3 phosphorylation. Primary MCL cells (UPN 3, UPN 12) were treated for 20 h in the absence (-) or in the presence of either bortezomib (Borte, 10 nM) or IL10 (50 ng/mL, R&D systems) or bortezomib and IL10 (Borte + IL10). P-STAT3 and total STAT3 expression were analyzed by western blotting as described in the *Design and Methods* section.



Online Supplementary Figure S16. Treatment of primary MCL cells with BAY 11-7082 (4 μM) for 24h induced inhibition of constitutive STAT3 phosphorylation (left panel), inhibition of IL6 and IL10 secretion (middle panel) and enhanced cell apoptosis (right panel). Secretion of IL6 and IL10 was measured by ELISA and cell apoptosis evaluated by flow cytometry; Spt Jeko: addition of supernatant from Jeko-1 cells during Bay 11 treatment (24 h).*Due to limited cell number availability, incubation with supernatant Jeko was not possible for UPN5.



Online Supplementary Figure S17. The inhibition of NF-κB suppresses both constitutive and BCR-induced STAT3 phosphorylation. Primary MCL cells (UPN1, UPN5) were treated (+) or not (-) for 24 h with Bay 11-7082 (4 μM, Sigma) an inhibitor of NF-κB. Cells were simultaneously activated (+) or not (-) with immobilized anti-IgM. P-STAT3 and total STAT3 expression were analyzed by western blotting as described in the *Design and Methods* section.