SYK inhibition thwarts the BAFF - B-cell receptor crosstalk and thereby antagonizes Mcl-1 in chronic lymphocytic leukemia

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Supplemental Methods.

Immunoblotting, immunoprecipitation and the NFκB Activity Assay

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM NaF, 1 mM sodium phosphate, 1 mM NaVO3, 1 mM EDTA, 1 mM EGTA), supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN), phosphatase inhibitor cocktail 2 and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich). Proteins were analyzed by immunoblotting as previously described [2].

The following antibodies were used: pAkt^{S473}, BAFF, Bcl-2, Bcl-xL, Bim, pERK1/2$^{T202/Y204}$, GAPDH, pIκBα (S32/36), IKKα, pLYN^{Y507}, NIK, Mcl-1, NFκB2(p100/52), PARP, RelA(p65), pSTAT3^{Y705}, STAT3, pSYK^{Y352}, SYK (#2712), SYK (D3Z1E, for IP), TRAF2, TRAF3 (Cell Signaling Technology, Danvers, MA); anti-HA, β-actin (Sigma-Aldrich).

Protein lysates were also analyzed for NFκB activity using the TransAM NFκB Family ELISA Kit, as per the manufacturer instructions (ActiveMotif, Carlsbad, CA).

For immunoprecipitation experiments, cell protein lysates were pre-cleared and incubated at 4ºC overnight with 2 μg of the indicated primary antibody or with rabbit IgG as isotype-specific control (Santa Cruz Biotechnology, Santa Cruz, CA). Lysates were incubated with 20 μL of 50% protein A agarose beads slurry (Cell Signaling) for 3 hours at 4ºC. After washes, samples were heated to 95ºC for up to 5 min and analyzed by immunoblotting. 10% of source protein was used as input control.

For immunocytochemistry, 3x10^5 CLL cells adhered onto polylysine-coated coverslips were processed as previously described [2] and probed against p65/RelA or p100/NFκB2 followed by AlexaFluor-594 goat anti-mouse antibodies (Life Technologies). Fluorescent images were

captured Zeiss AxioCam MRm camera mounted on a Zeiss Observer.Z1 microscope (Zeiss, Jena, Germany).

Real-time PCR

1 x 10^7 CLL cells were collected, and total RNA was isolated using the RNEasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 500 ng total RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Real-time PCR (RT-PCR) was performed in a QuantStudio 7 Flex thermal cycler (Life Technologies), using PerfeCTa Universal Master Mix (Quanta Bioscience, Gaithersburg, MD) according to the manufacturer's instructions. The following probes were used: MCL1: Hs01050896_m1; BCL2A1: Hs00187845_m1; BCL2L1: Hs00236329_m1; CFLAR: Hs00153439_m1; STAT5A: Hs00234181_m1 (Life Technologies). Amplification of the sequences were compared to the reference probe *GAPDH* (#4332649, Applied Biosystems). Samples were analyzed in duplicate, and the comparative C_t method was used for relative quantification.

Cell electroporation

Raji cells (ATCC) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Electroporation of Raji cells was performed using the Amaxa Human B-cell Nucleofection Kit (Lonza, Walkersville, MD). 5x10^6 cells were mixed with 100 µL of Amaxa Solution V, and 2 µg of siRNA/DNA was nucleofected using program O-017. Transfection efficiency, assessed by transfection with 2 µg pMaxGFP plasmid, was 70-90% with cell viability of >90% at 24 hours. pCMV4-NIK-HA was a gift from Shao-Cong Sun (Addgene plasmid 27554) [3].

Microarray Analysis

$1x10^7$ CLL cells were negatively selected using the B-cell Isolation Kit (Miltenyi Biotec; #130-093-660). RNA was prepared as above. The RNA gene expression microarray experiments were carried out by the Dartmouth Genomics & Microarray Laboratory (Lebanon, NH). Beadarrays with probes for all known human genes (Illumina, San Diego, CA) were used for RNA profiling. Reverse transcription using an oligo(dT) primer bearing a T7 promoter and the high yield ArrayScript™ reverse transcriptase were used to make cDNA. The cDNA was made double-stranded and purified to use as a template for *in vitro* transcription with T7 RNA Polymerase and the included biotin-NTP mix. The labeled cRNA was purified and 1.5 µg used for hybridization to the beadarrays for 16 hours at 55°C. Following hybridization, the beadarrays were washed and stained with streptavidin-Cy3 (GE Healthcare, Piscataway, NJ). Fluorescent images were obtained with an Illumina 500GX scanner and processed with the BeadScan software (Illumina). Full results are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE89428. Fisher exact test was used to identify ontology groups and pathways statistically enriched in the gene set. BCR pathway genes were sourced from Pathways Studio, KEGG Pathways and WikiPathways (157 genes total). NFκB targets were sourced from http://www.bu.edu/nf-kb/gene-resources/target-genes/

Immunohistochemistry

Immunohistochemistry was performed on lymphatic tissues from 10 CLL patients at the department of Pathology at Dartmouth-Hitchcock Medical Center. All staining was performed after antigen retrieval [using intermittent heating for 4 cycles of 5 min each in a 625-W

microwave oven to maintain the temperature of the buffer [(0.01 M citrate buffer, pH 6.0) at 95°C] and run in parallel with known positive and negative controls. After incubation with primary antibodies (1:100) and relevant secondary antibodies, slides were immunostained using the Biotin-Streptavidin amplified system.

Supplemental Figure Legends.

Supplemental Figure 1. **Expression of BAFF ligands and receptors in peripheral blood- and bone marrow-derived CLL cells**. CLL cells from paired peripheral blood-bone marrow samples were subjected to flow cytometry analysis using the indicated antibodies (N=6). A representative samples is shown.

Supplemental Figure 2. **BAFF ligand expression in BAFF-CHO cells**. *(A)* BAFF-CHO and control cells were lysed and BAFF expression was confirmed by immunoblotting (using anti-HA), *(B)* BAFF was detected by flow cytometry (CD257-PE).

Supplemental Figure 3. BAFF stimulation upregulates NFκB target genes and induces Mcl-1. *(A)* CLL cells from 6 individual patients were co-cultured with BAFF-expressing or control stroma for 24 h. Total RNA was reverse-transcribed and subjected to real-time PCR with the indicated probes (in duplicates); * - $p<0.05$ compared to control. *(B)* CLL cells from 4 individual patients were co-cultured with BAFF-expressing stroma for 24 h. Proteins were subjected to immunoblotting.

Supplemental Figure 4. BAFF modulates genes within the BCR pathway. CLL cells from 3 individual samples were co-cultured with BAFF-expressing stroma for 24 h. RNA was isolated from the purified CLL B-cells and microarray analysis was performed as described in the methods. Heatmap shows hierarchical clustering of the 63 differentially expressed genes within the BCR pathway (yellow=upregulation, blue=downregulation).

Supplemental Figure 5. **Direct toxicity of BCR-signaling inhibitors**. CLL cells (n=10) were incubated with the indicated drugs, or vehicle control, for 24 h. Apoptosis within the $CD19^{+}$

subset of cells was determined by Annexin V and 7-AAD staining. Data are the mean ± SE. *, $p<0.05$ compared to vehicle control.

Supplemental Figure 6. **Pevonedistat, an inhibitor of NEDD8-activating enzyme, abrogates NFκB and BAFF-mediated survival in CLL cells**. CLL cells were incubated with BAFF-expressing stroma for 24 h, and then with the indicated drugs for up to 24 h (B-C). Cells were lysed and subjected to *(A)* immunoblotting, *(B)* immunocytochemistry using p100/52 or RelA/p65 antibodies (red) and DAPI (blue) and *(C)* flow cytometry for Annexin V (mean±SE shown; * - $p<0.05$ compared to control stroma).

Supplemental Figure 7. **BAFF-mediated regulation of Mcl-1**. *(A)* CLL cells (N=4) were co-cultured with BAFF-expressing stroma for 24 h, treated with SYK inhibitors (entospletinib, R406) or MEK inhibitors (MEK inhibitor VII, PD184352, selumetinib, trametinib) for 24 h in the presence of caspase inhibitor QVD-OPh (1 μM), followed by collection of mRNA and protein. *(B)* CLL cells were cultured off stroma. Whole cell protein lysates were collected at the indicated timepoints and subjected to immunoblotting. 1 of 3 independent experiments is shown.

Supplemental Figure 8. SYK-TRAF2/3 interaction. *(A)* Raji cells were transfected with pCMV-NIK or control plasmid. 48 h later, cells were stimulated or not with 25 ng/mL sol-BAFF for 30 min. Whole-cell lysates were subjected to immunoblotting. *(B)* CLL cells were co-cultured with BAFF-expressing stroma for 24 h, followed by treatment with IKK inhibitors for 6h. Whole cell protein lysates were subjected to immunoblotting. Representative blots of three independent experiments are shown. *(C)* Cells were stimulated with 25 ng/mL sol-BAFF for 30 min. Protein lysates were immunoprecipitated with IKK1 or control antibodies.

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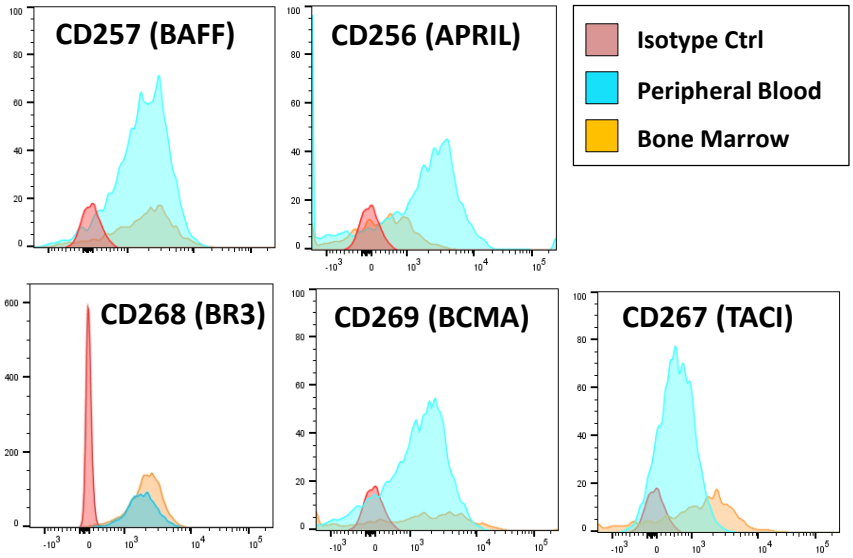
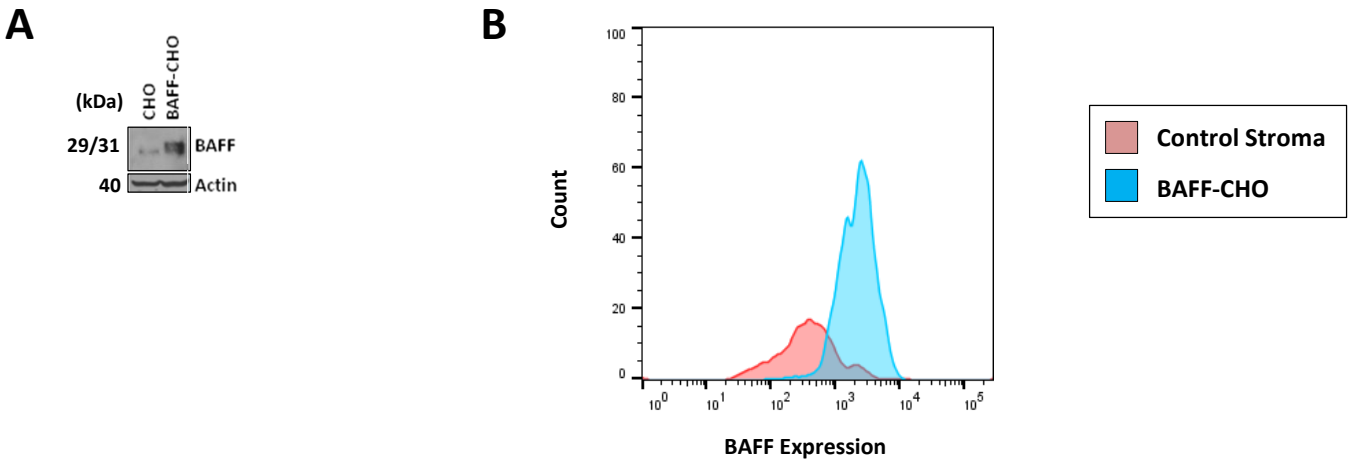
Supplemental Figure 1



Supplemental Figure 2



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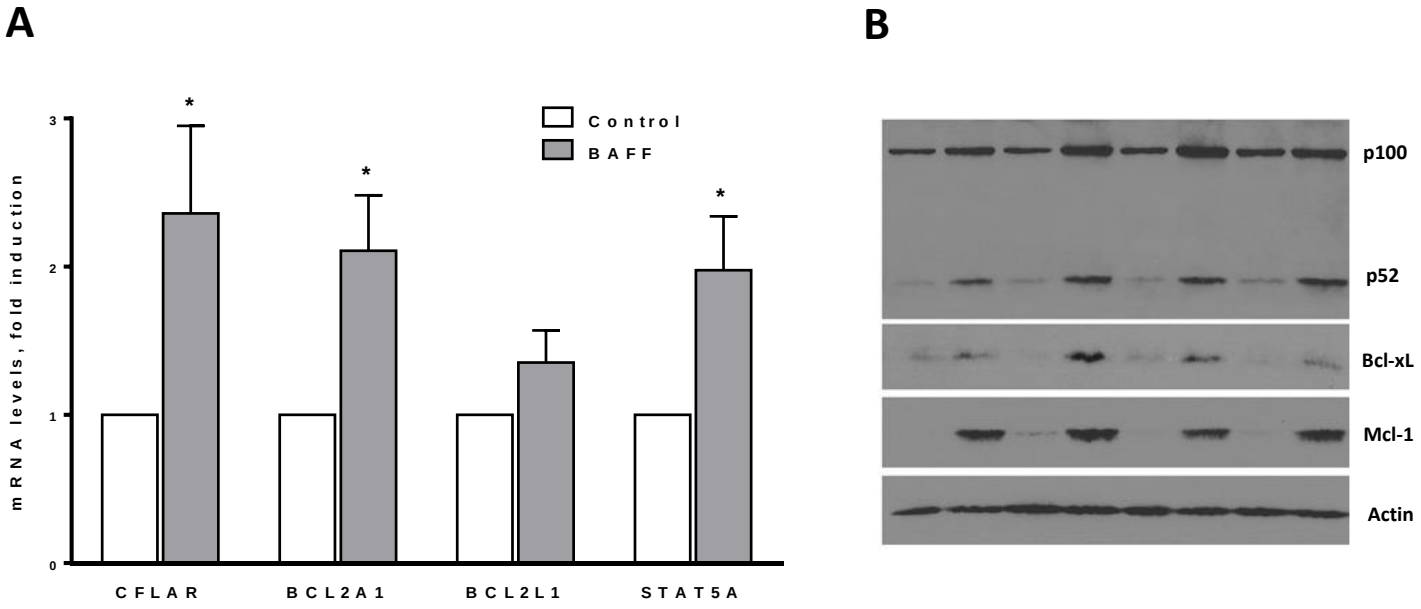

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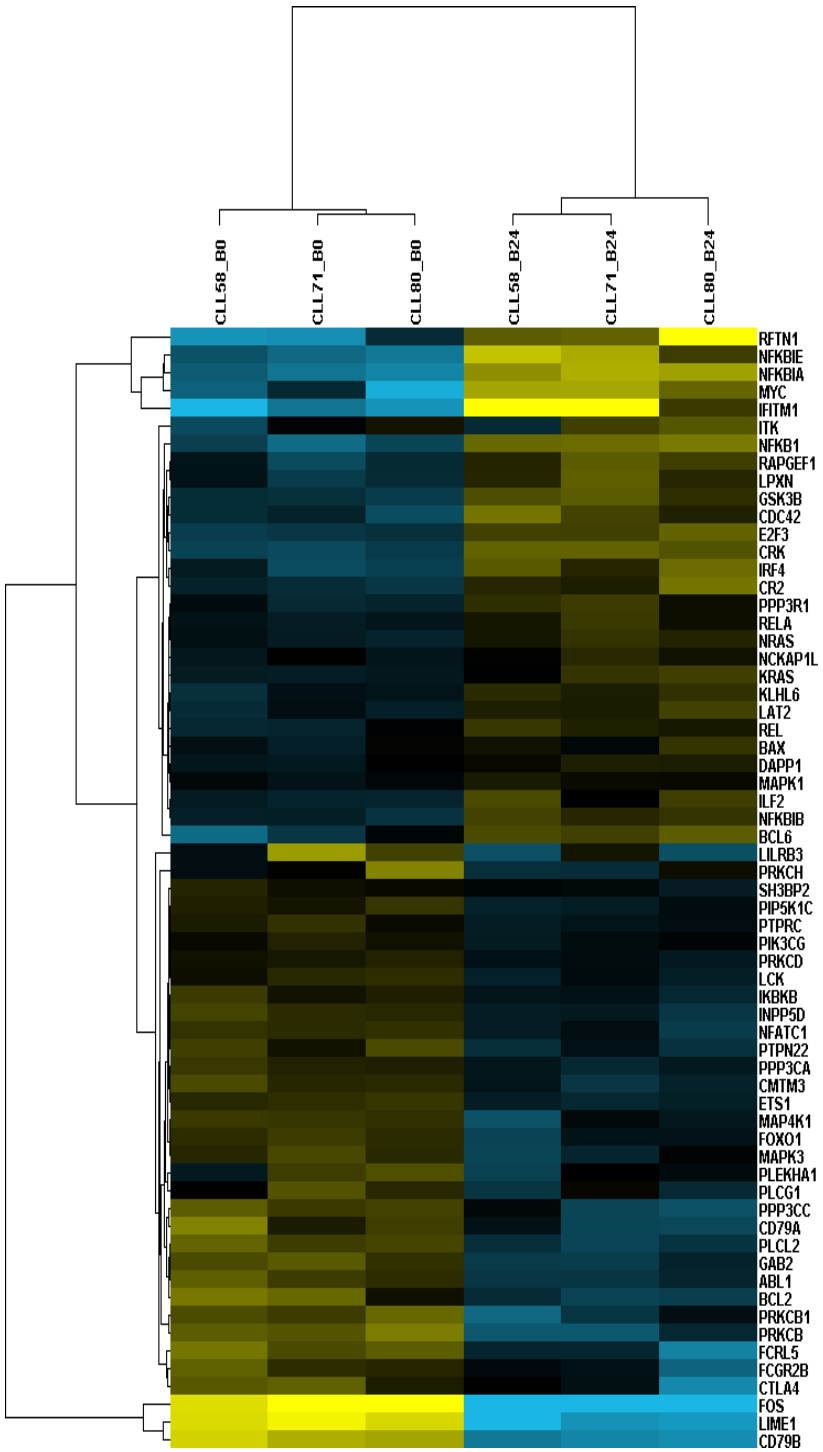

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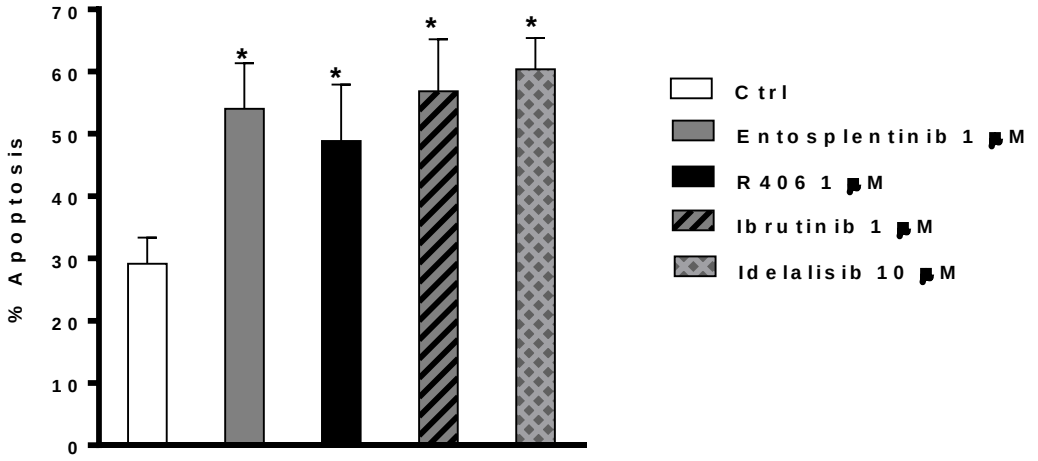

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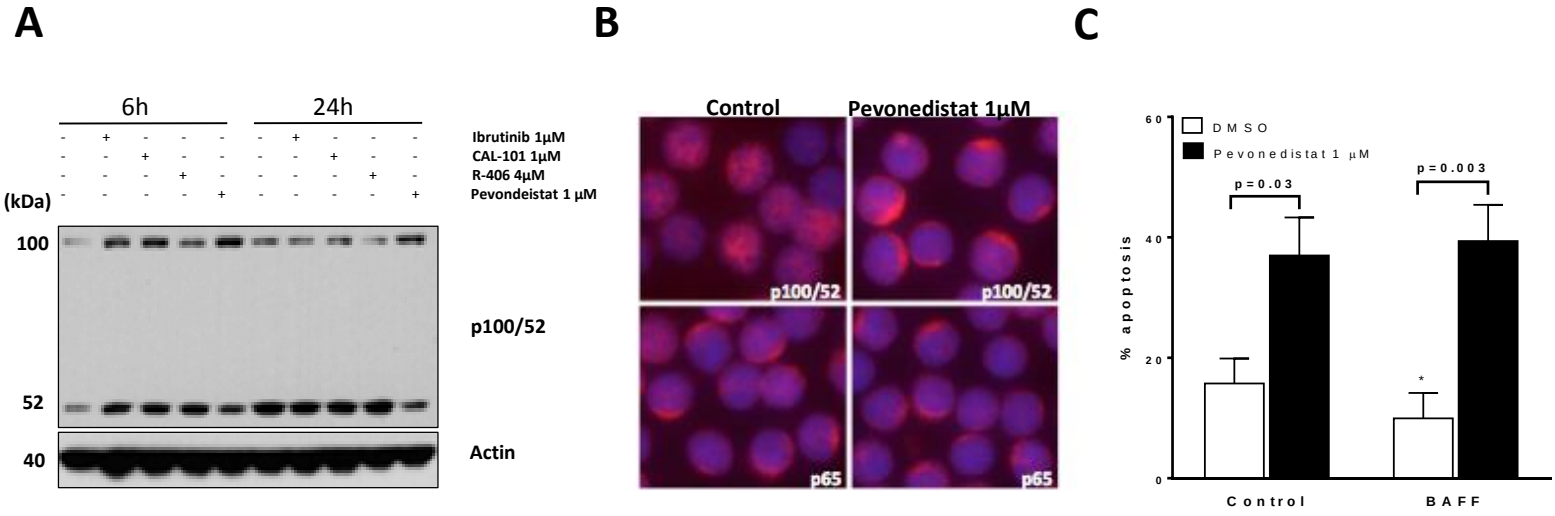
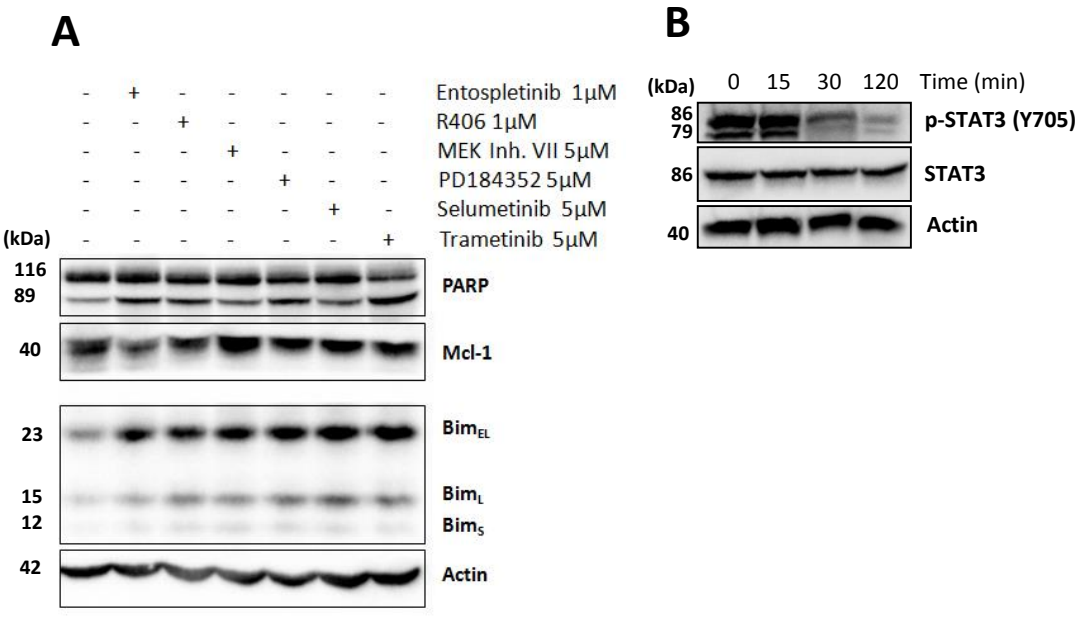
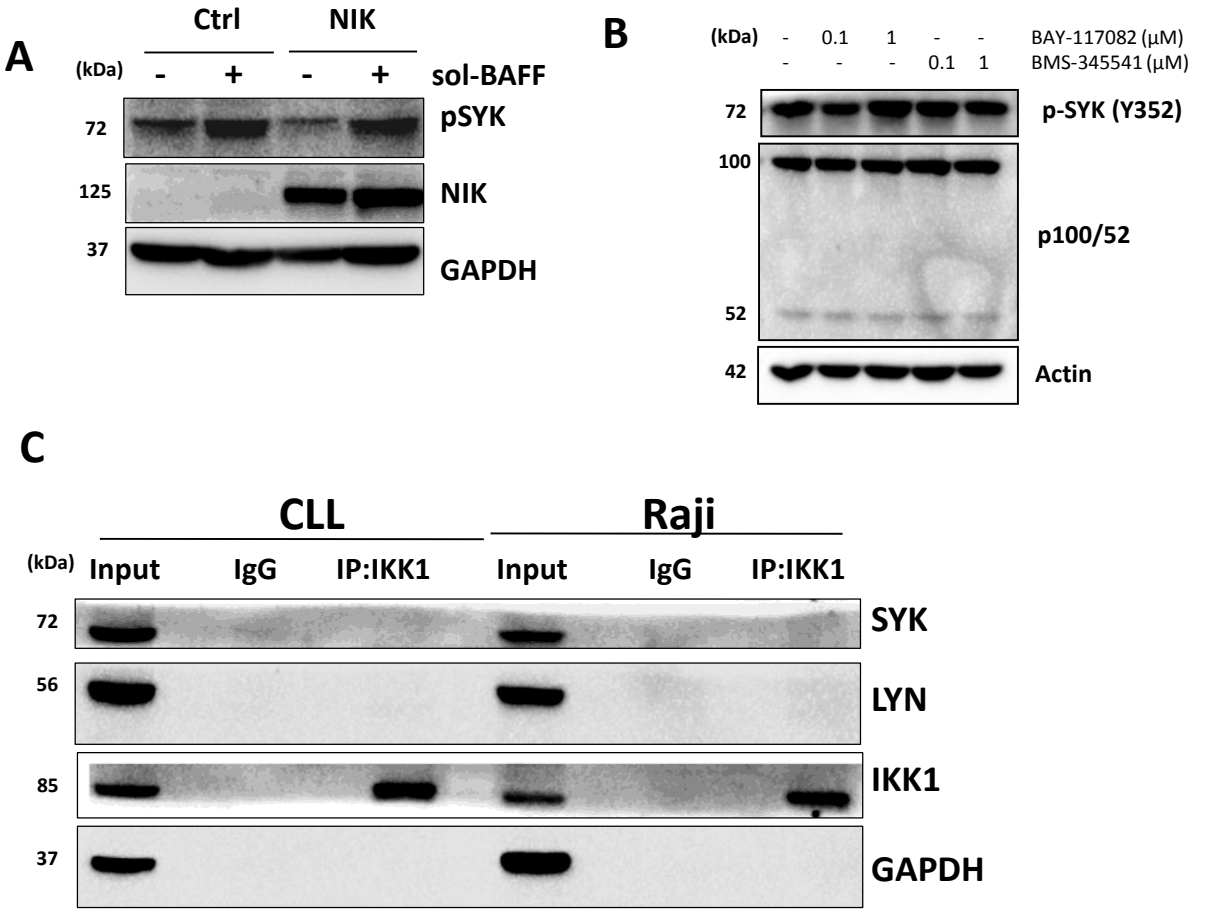


Supplemental Figure 7



Supplemental Figure 8



Supplemental Table 1

Drug Name	Class	Source
A-1210477	Mcl-1 Inhibitor	Abbvie, Inc. (Chicago, IL)
BAY-11-7082	IKK Inhibitor	Sigma-Aldrich (St. Louis, MO)
Bendamustine	Alkylating Agent	Sigma-Aldrich (St. Louis, MO)
BMS-345541	IKK Inhibitor	Selleck Chemicals (Houston, TX)
Entospletinib (GS-9973)	SYK Inhibitor	Selleck Chemicals (Houston, TX)
Fludarabine	Purine Analog	Sigma-Aldrich (St. Louis, MO)
Ibrutinib (PCI-32765)	BTK Inhibitor	Selleck Chemicals (Houston, TX)
Idelalisib (CAL-101, GS-1101)	PI3Kδ Inhibitor	Selleck Chemicals (Houston, TX)
MEK Inhibitor VII	MEK1/2 Inhibitor	Calbiochem (Billerica, MA)
PD184352 (CI-1040)	MEK1/2 Inhibitor	Selleck Chemicals (Houston, TX)
Pevonedistat (MLN4924)	Nedd8-activating enzyme inhibitor	Millennium Pharmaceuticals, Inc. (Cambridge, MA)
QVD-OPh	Caspase inhibitor	Sigma-Aldrich (St. Louis, MO)
R406	SYK Inhibitor	Selleck Chemicals (Houston, TX)
Ruxolitinib	JAK1/2 Inhibitor	Selleck Chemicals (Houston, TX)
Selumetinib (AZD6244)	MEK1/2 Inhibitor	Selleck Chemicals (Houston, TX)
Trametinib (GSK1120212)	MEK1/2 Inhibitor	Selleck Chemicals (Houston, TX)
Venetoclax (ABT-199)	Bcl-2 Inhibitor	Activ Biochem (Maplewood, NJ)