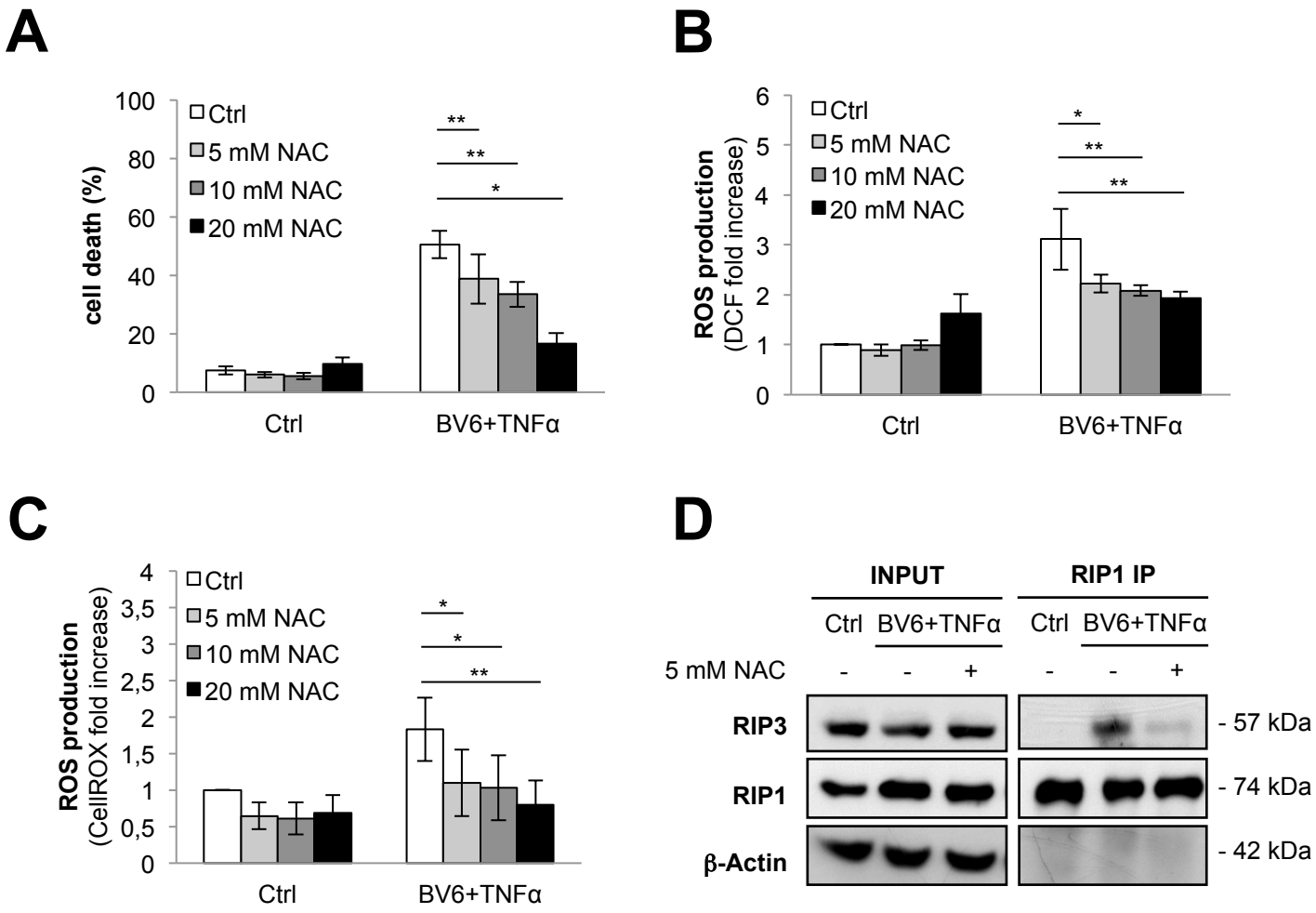
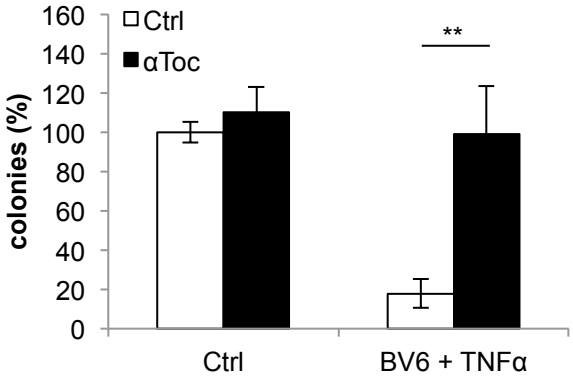
Supplemental Figure 1

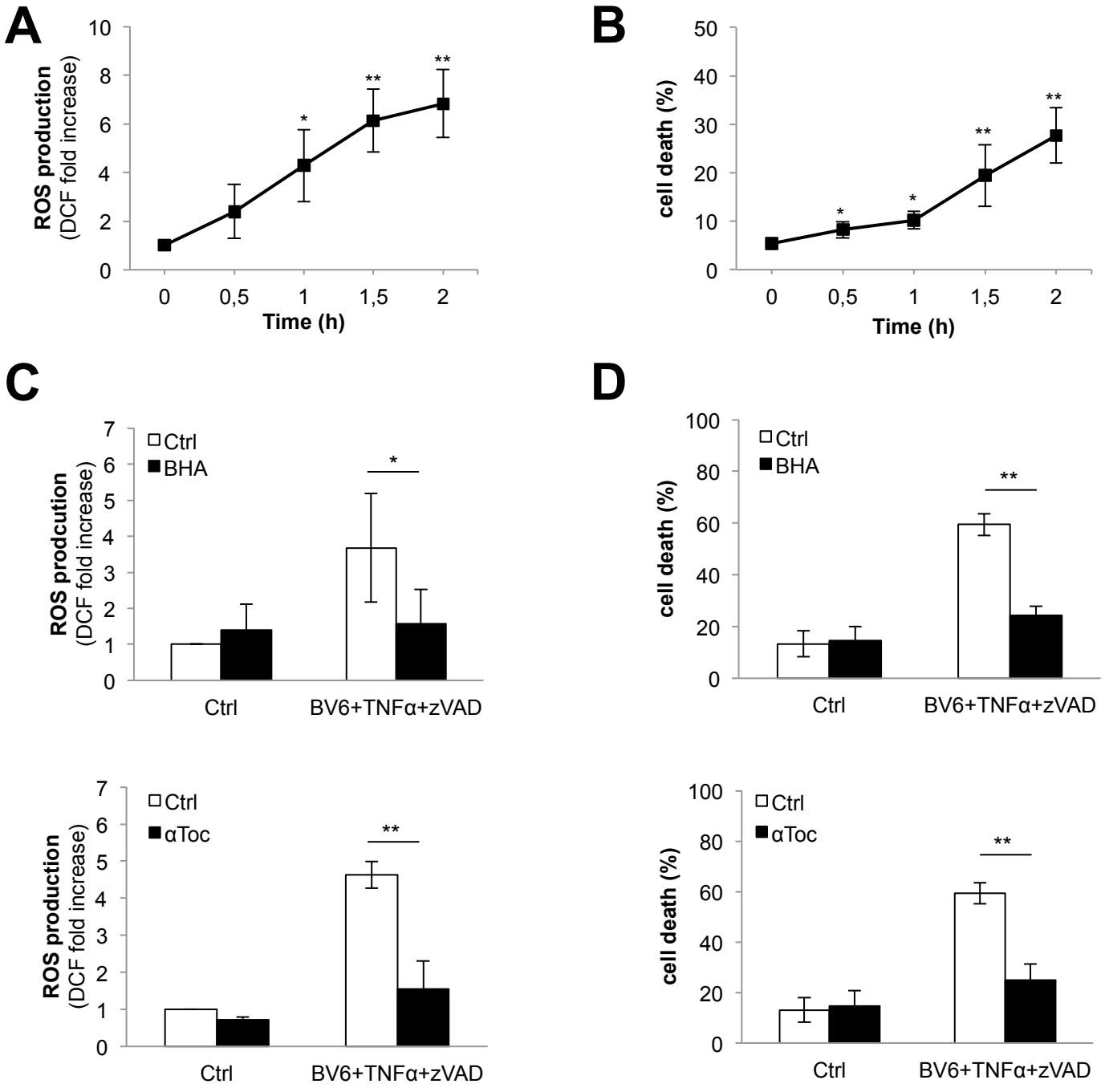
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



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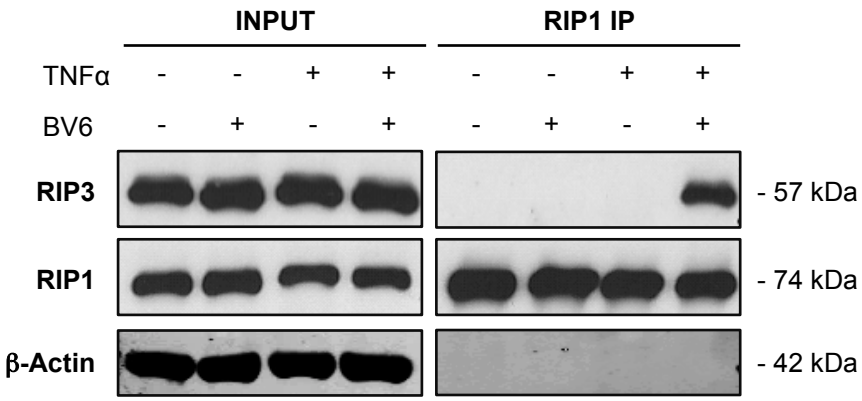
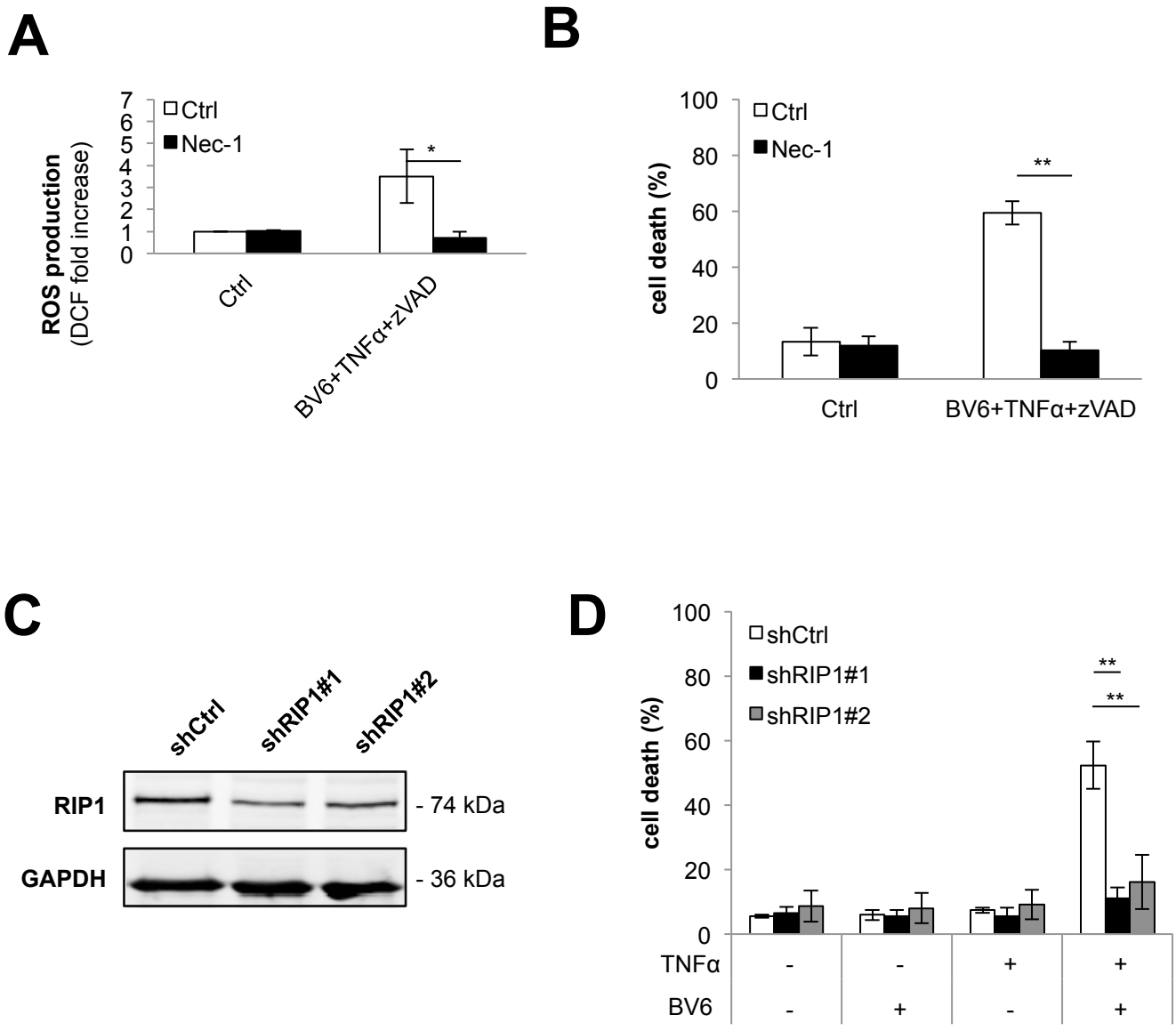
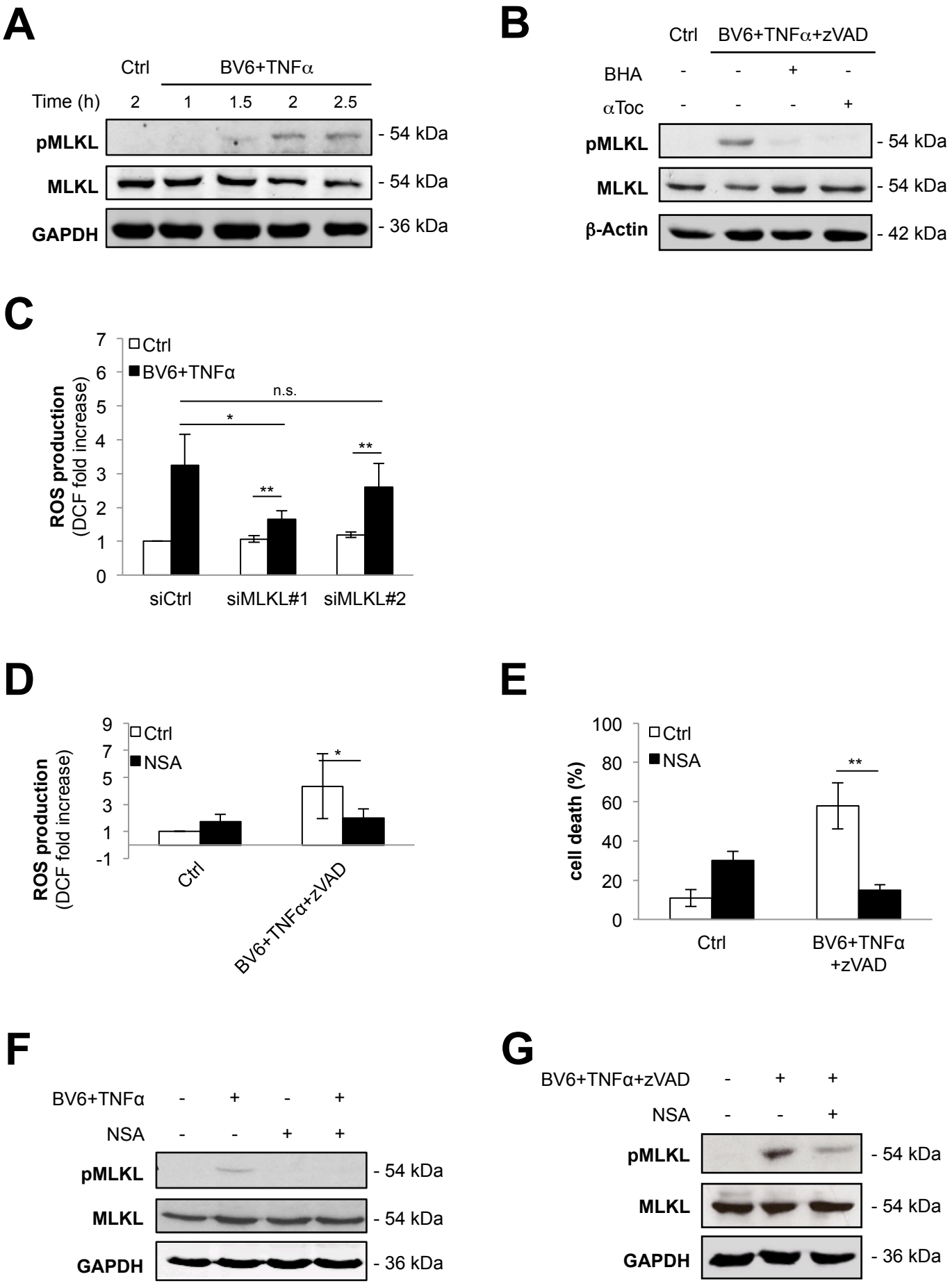


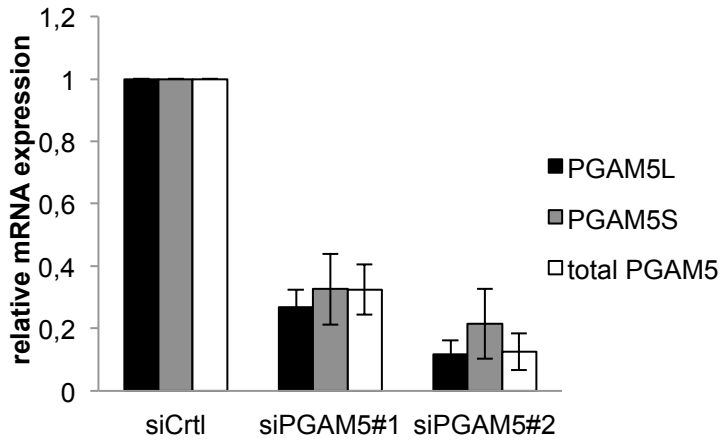
Supplemental Figure 6



Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure Legends

Supplemental Figure 1. Analysis of BV6/TNFα-induced cell death in FADD-deficient Jurkat cells.

A, Wildtype (WT) and FADD-deficient (FADD def) Jurkat cells were analyzed for FADD protein expression levels by Western blotting.

B, FADD-deficient Jurkat cells were treated with BV6 (1 μM) and/or TNFα (1 ng/ml) for four hours. Cell death was analyzed by FSC/SSC analysis or PI staining using flow cytometry. Data represent mean ± SD of at least three independent experiments performed in triplicate.

Supplemental Figure 2. NAC reduces BV6/TNFα-induced cell death, ROS production and RIP1/RIP3 necrosome formation.

A-D, FADD-deficient Jurkat cells were preincubated with NAC (5 mM, 10 mM and 20 mM) for one hour prior to stimulation with BV6 (1 μM) and/or TNFα (1 ng/ml) for four hours (A-C) or one and a half hours (D). Cell death was determined by FSC/SSC analysis and flow cytometry (A). ROS production in PI-negative cells was determined by flow cytometry using fluorescent dyes CM-H_2DCFDA (B) or CellROX (C) and is depicted as fold increase. Data represent mean ± SD of at least three independent experiments performed in triplicate. *P<0.05, **P<0.01. RIP1 was immunoprecipitated using anti-RIP1 antibody and detection of indicated proteins was done by Western blotting (D).

Supplemental Figure 3. α-Tocopherol protects against BV6/TNFα-induced reduction of long-term clonogenic survival.

FADD-deficient Jurkat cells were preincubated with α-Toc (300 μM) for one hour prior to stimulation with BV6 (1 μM) and TNFα (1 ng/ml) for one hour. Long-term clonogenic survival was assessed after 8-10 days by colony assay as described in Material and Methods and the percentage of colony formation relative to untreated controls is shown. Data represent mean ± SD of three independent experiments performed in triplicate; *P<0.05; **P<0.01.

Supplemental Figure 4. ROS are required for BV6/TNFα/zVAD-induced necroptotic cell death in MV4-11 cells.

A and B, MV4-11 cells were treated with zVAD (20 μM), BV6 (0.5 μM) and TNFα (1 ng/ml) for indicated time points. ROS production in PI-negative cells was determined by flow cytometry using fluorescent dyes CM-H_2DCFDA (A) and is depicted as fold increase. Cell death was determined by FSC/SSC analysis and flow cytometry (B).

C-E, MV4-11 cells were preincubated with zVAD (20 μM) in combination with BHA (100 μM) or αToc (200 μM) for one hour prior to treatment with BV6 (0.5 μM) and/or TNFα (1 ng/ml) for two hours (C), five hours (D) or one hour (E). ROS production in PI-negative cells was determined by flow cytometry using fluorescent dyes CM-H_2DCFDA (C) and is depicted as fold increase. Cell death was determined by FSC/SSC analysis and flow cytometry (D). RIP1 was immunoprecipitated using anti-RIP1 antibody and detection of indicated proteins was done by Western blotting (E); asterisk indicates heavy chain (IgG_H).

Data represent mean ± SD of at least three independent experiments performed in triplicate; *P<0.05, **P<0.01 comparing BV6/TNFα/zVAD-treated cells to untreated

cells (A, B) or to BV6/TNFα/zVAD-treated cells in the presence of ROS scavengers (C, D).

Supplemental Figure 5. BV6 and TNFα cooperate to induce RIP1/RIP3 necrosome formation.

FADD-deficient Jurkat cells were treated with BV6 (1 μM) and/or TNFα (1 ng/ml) for one and a half hours or left untreated. RIP1 was immunoprecipitated using anti-RIP1 antibody and indicated proteins were detected by Western blotting.

Supplemental Figure 6. RIP1 is required for BV6/TNFα-induced necroptosis.

A and B, MV4-11 cells were pretreated with zVAD (20 μM) and Nec-1 (30 μM) one hour prior to treatment with BV6 (0.5 μM) and/or TNFα (1 ng/ml) for two hours (A) or five hours (B). ROS production in PI-negative cells was determined by flow cytometry using fluorescent dyes CM-H_2DCFDA (A) and is depicted as fold increase. Cell death was determined by FSC/SSC analysis and flow cytometry (B). Data represent mean ± SD of at least three independent experiments performed in triplicate; *P<0.05, **P<0.01.

C and D, FADD-deficient Jurkat cells were transduced with control vector (shCtrl) or vectors containing shRNAs against RIP1 (shRIP1#1, shRIP1#2). Expression of RIP1 was analyzed by Western blotting (C). Cells were treated with BV6 (1 μM) and/or TNFα (1 ng/ml) for four hours and cell death was determined by FSC/SSC analysis and flow cytometry (D).

Supplemental Figure 7. ROS act upstream and downstream of MLKL activation upon BV6/TNFα-treatment.

A and B, FADD-deficient Jurkat cells were treated with BV6 (1 μM) and/or TNFα (1 ng/ml) for the indicated time points (A). MV4-11 cells were pretreated with zVAD (20 μM) in combination with BHA (100 μM) or αToc (200 μM) one hour prior to treatment with BV6 (0.5 μM) and/or TNFα (1 ng/ml) for one and a half hours (B). Expression of phospho-MLKL (pMLKL) and MLKL was detected by Western blotting.

C, FADD-deficient Jurkat cells were transfected with non-silencing control siRNA (siCtrl) or siRNAs against MLKL (siMLKL#1, siMLKL#2) and were treated with BV6 (1 μM) and/or TNFα (1 ng/ml) for two hours. ROS production in PI-negative cells was determined by flow cytometry using fluorescent dye CM-H_2DCFDA and is depicted as fold increase. Data represent mean ± SD of at least three independent experiments performed in triplicate; *P<0.05, **P<0.01, n.s. not significant.

D and E, MV4-11 cells were pretreated with zVAD (20 μM) and NSA (1 μM) for one hour prior to treatment with BV6 (0.5 μM) and/or TNFα (1 ng/ml) for two hours (D) or five hours (E). ROS production in PI-negative cells was determined by flow cytometry using fluorescent dye CM-H_2DCFDA (D) and is depicted as fold increase. Cell death was determined by FSC/SSC analysis and flow cytometry (E). Data represent mean ± SD of at least three independent experiments performed in triplicate; *P<0.05, **P<0.01.

F and G, FADD-deficient Jurkat cells were preincubated with NSA (1.5 μM) for one hour prior to treatment with BV6 (1 μM) and TNFα (1 ng/ml) for two hours (F). MV4-11 cells were pretreated with zVAD (20 μM) and NSA (1 μM) for one hour prior to treatment with BV6 (0.5 μM) and/or TNFα (1 ng/ml) for one and a half hours (G). Expression of phospho-MLKL (pMLKL) and MLKL was detected by Western blotting.

Supplemental Figure 8. Effect of PGAM5 knockdown on mRNA levels of PGAM5S and PGAM5L.

FADD-deficient Jurkat cells were transfected with non-silencing control siRNA (siCtrl) or two distinct siRNA sequences against PGAM5 (siPGAM5#1, siPGAM5#2). mRNA expression levels of total PGAM5, PGAM5S and PGAM5L were analyzed by qRT-PCR and are shown as relative mRNA expression normalized to the corresponding siCtrl sample for each PGAM5 isoform. Data represent mean ± SD of two independent experiments performed in triplicate.

Supplemental Materials and Methods

Cell culture and chemicals

AML cell line MV4-11 was obtained from DSMZ (Braunschweig, Germany) and were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, 25 mM HEPES buffer, 50 U/ml penicillin, and 50 µg/ml streptomycin. caspase inhibitor zVAD.fmk was obtained from Bachem (Heidelberg, Germany).

Colony assay

For colony assay, cells were pretreated with αToc for one hour prior to stimulation with BV6 and TNFα for another hour. 2000 cells were then seeded in semisolid culture media (H4100, StemCell Technologies, Vancouver, BC, Canada) as previously described [1].

Western blot analysis

Rabbit-anti-phospho MLKL antibody from Abcam (Cambridge, UK) was used for Western blot analysis.

Quantitative real-time PCR

Total RNA was extracted using peqGOLD Total RNA kit from Peqlab Biotechnology GmbH (Erlangen, Germany) according to the manufacturer's instructions. Total RNA (300 ng) was used to synthetize the corresponding cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas GmbH, St. Leon-Rot, Germany). To quantify gene expression levels, SYBR-Green-based quantitative qRT-PCR was performed using the 7900HT fast real-time PCR system from Applied Biosystems (Darmstadt, Germany). Data were normalized on 28S-rRNA expression as reference gene. The following primers were used: 28S_forward TTGAAAATCCGGGGGAGAG,

28S_reverse ACATTGTTCCAACATGCCAG; total PGAM5_forward: AACGTCATCCGCTACATCGT, total PGAM5_reverse: CCCCAGAACCACAAAATCC; PGAM5S_forward: TCTTCATCTGTCACGCCAAC, PGAM5S_reverse: TCAGCAAGTGAAAGAGGTCAG; PGAM5L_forward CCGACAAGATCACTCGATCC, PGAM5L_reverse: TTAAGTGTGTGCGGCCTGT. Melting curves were plotted to verify the specificity of the amplified products. All determinations were performed in triplicate. The relative expression of the target gene transcript and reference gene transcript was calculated as $\Delta\Delta C_t$. At least two independent experiments were performed for each gene.

Reference:

1. Belz K, Schoeneberger H, Wehner S, Weigert A, Bonig H, Klingebiel T, et al. Smac mimetic and glucocorticoids synergize to induce apoptosis in childhood ALL by promoting ripoptosome assembly. Blood 2014; 124: 240-250.