

Noncanonical cell death program independent of caspase activation cascade and necroptotic modules is elicited by loss of TGF β -activated kinase 1

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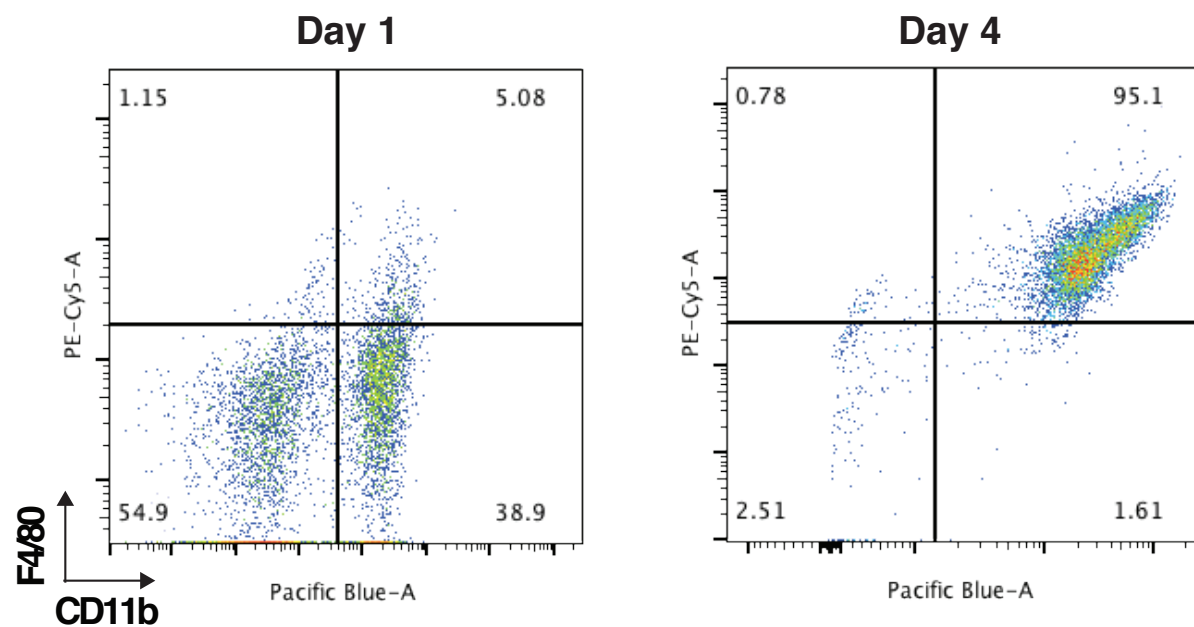
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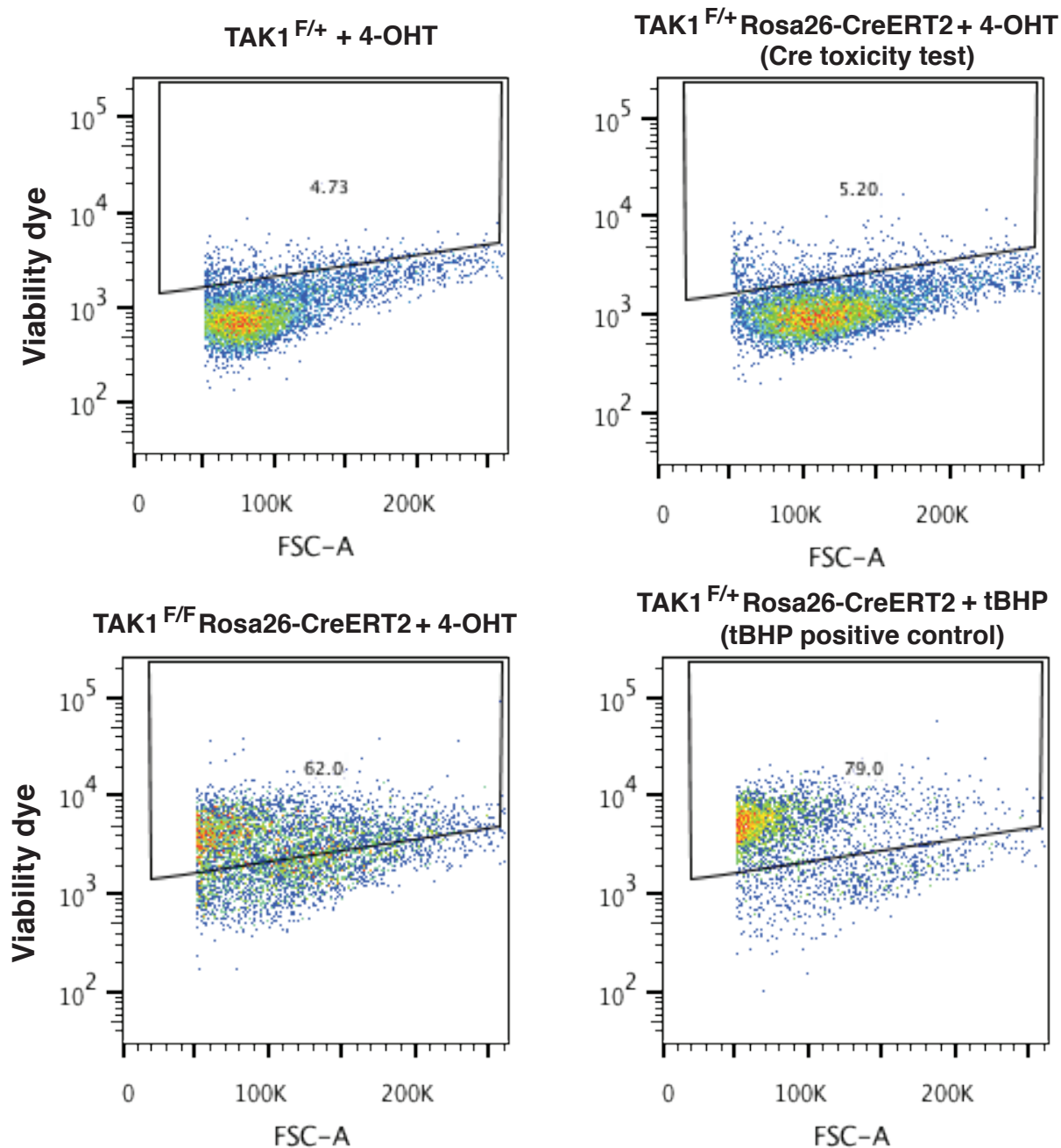
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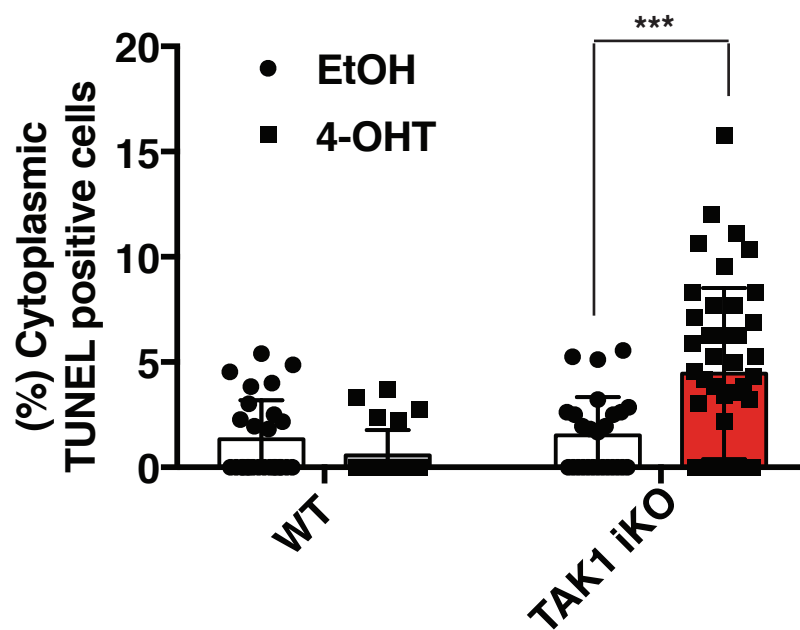
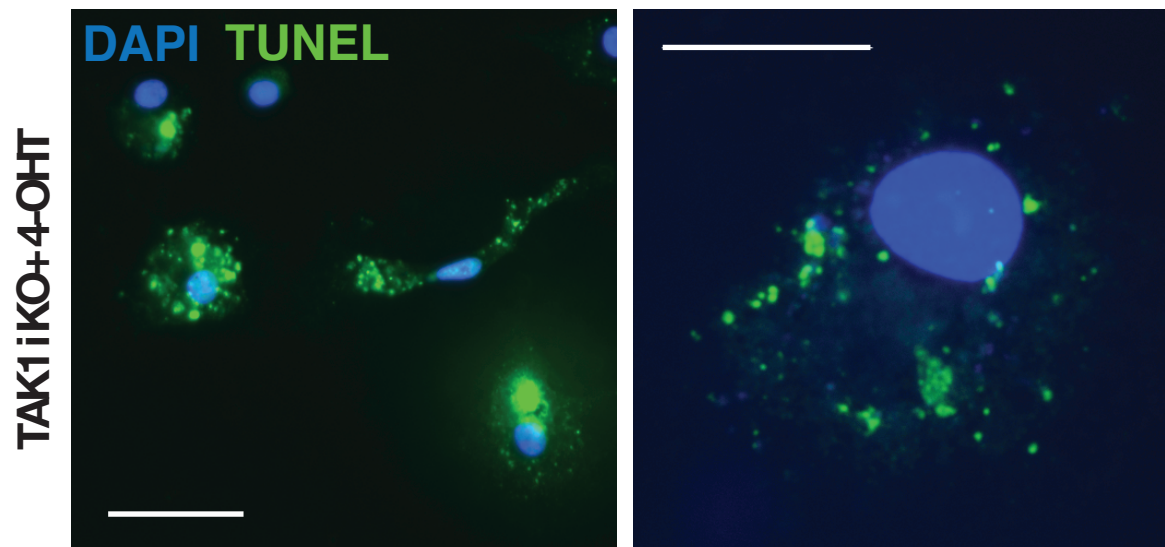
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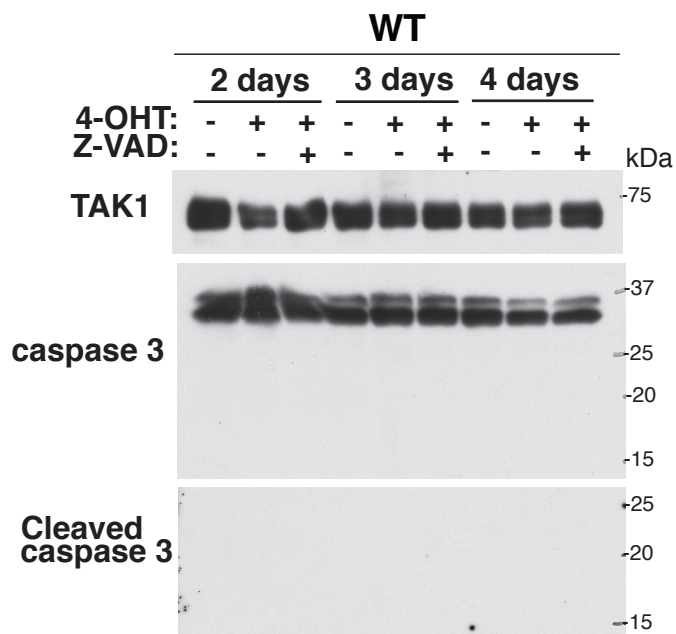
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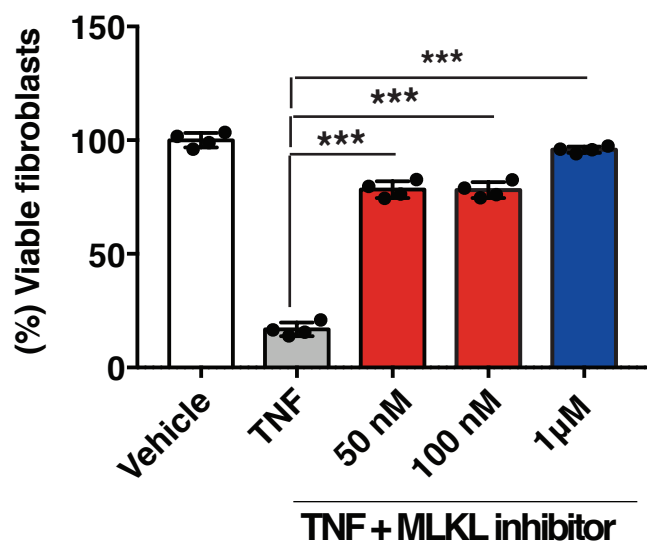
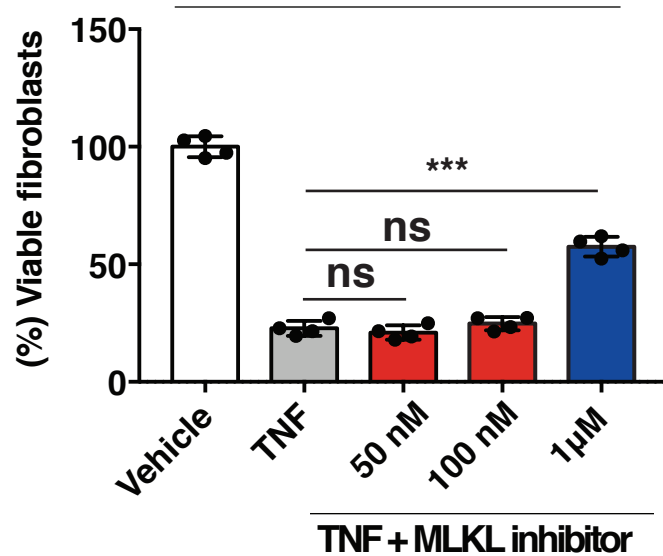
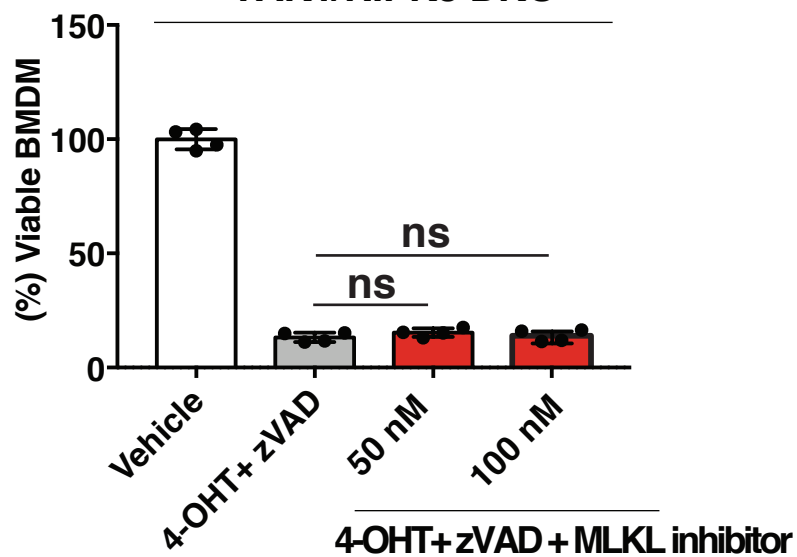
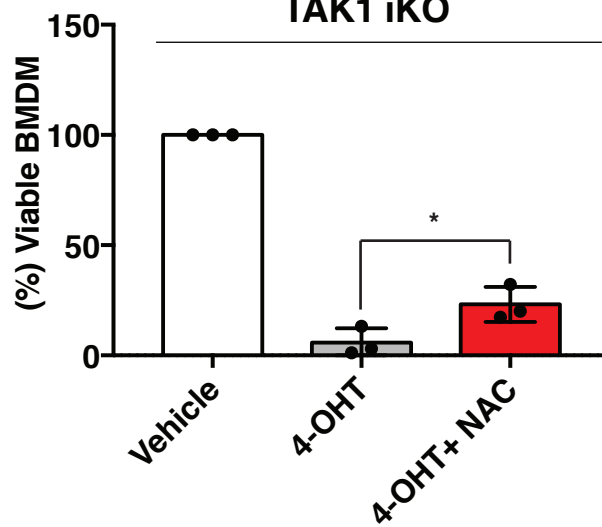


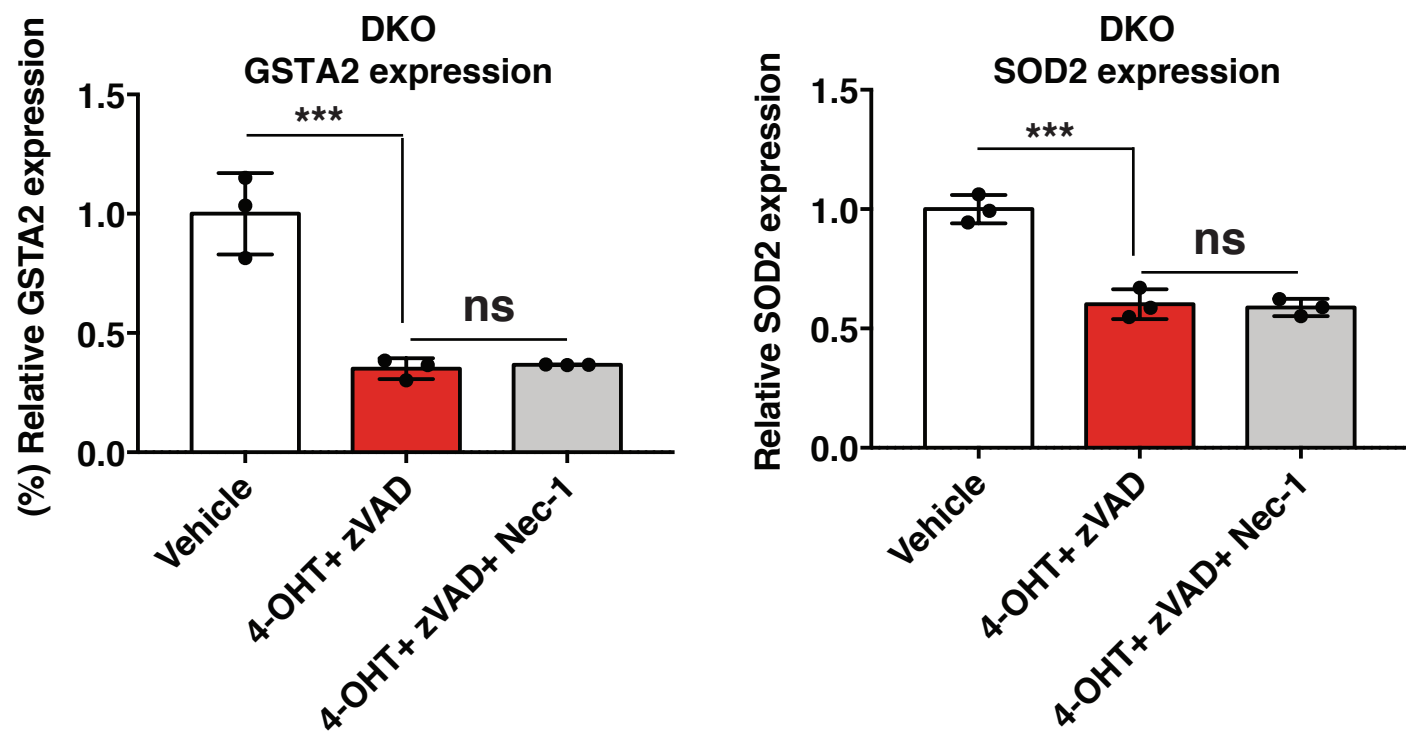
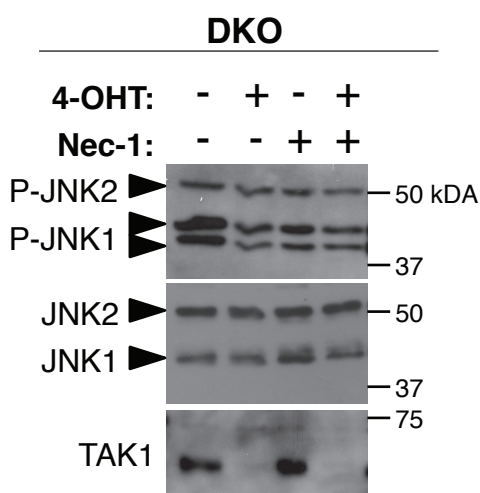
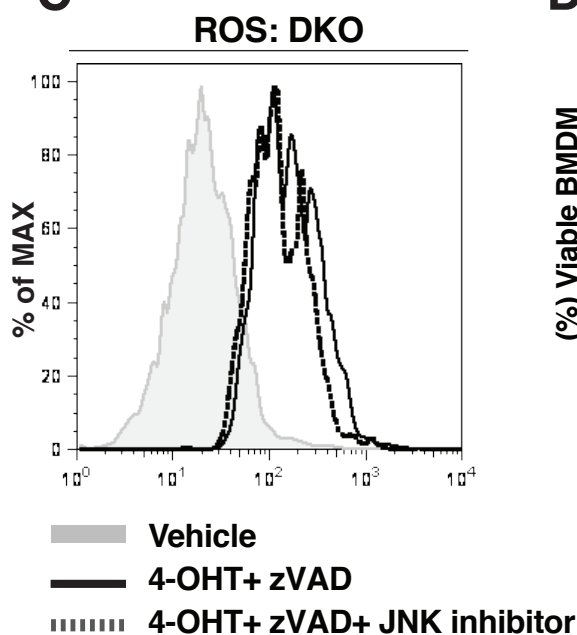
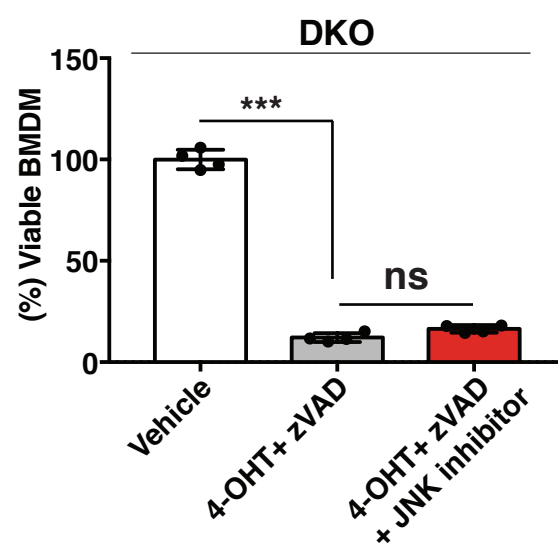
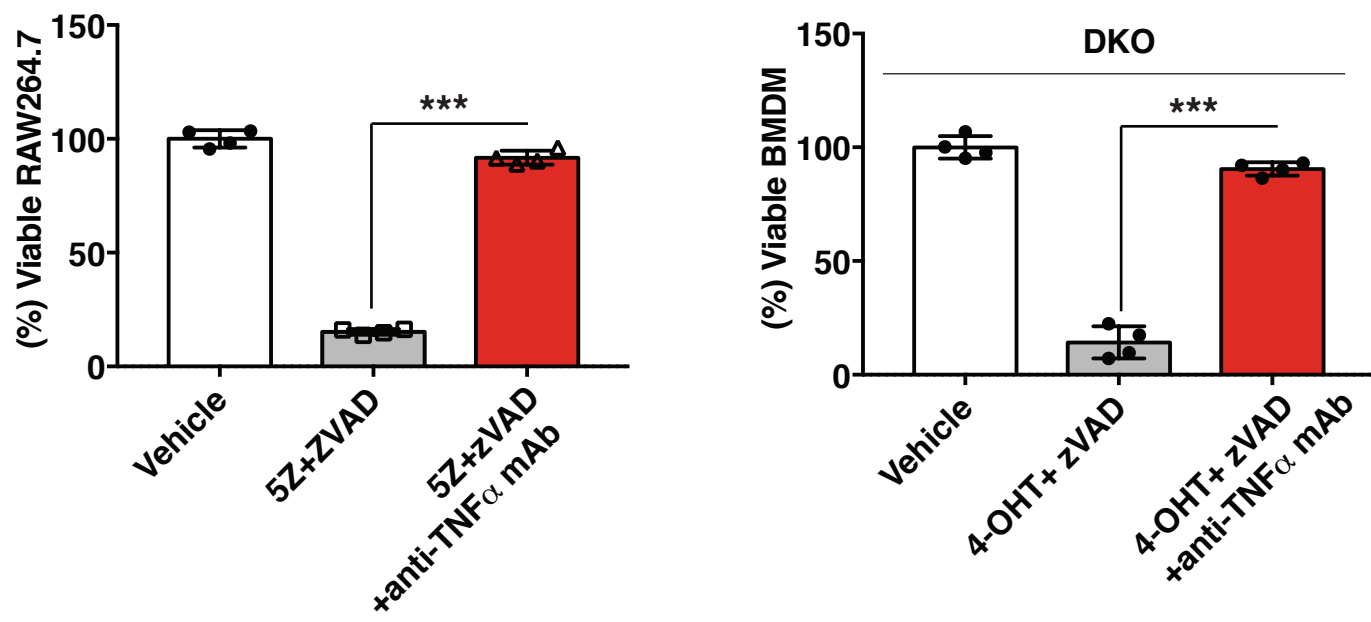
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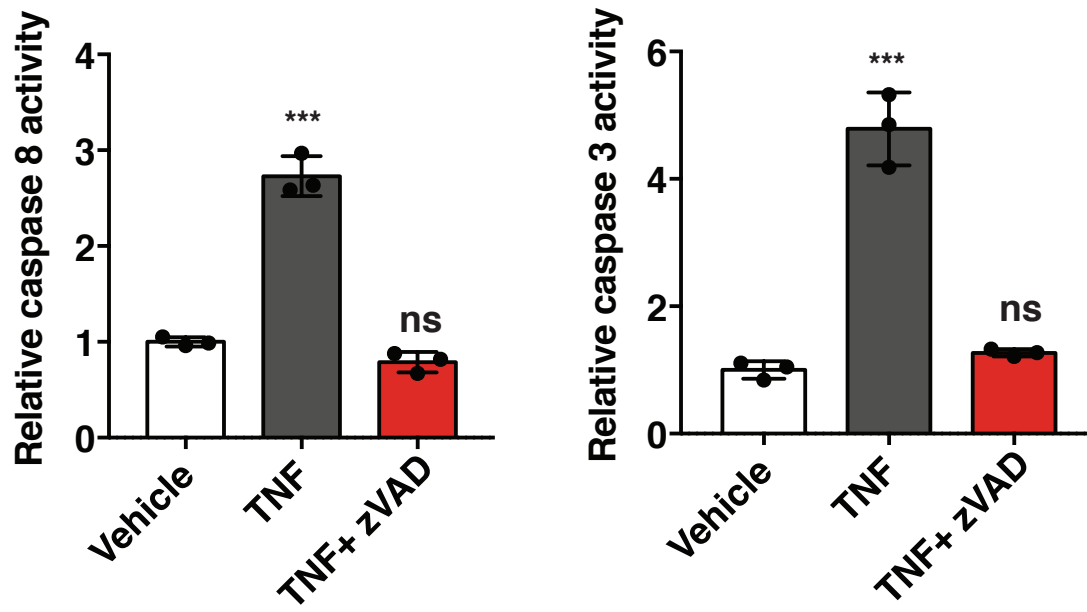
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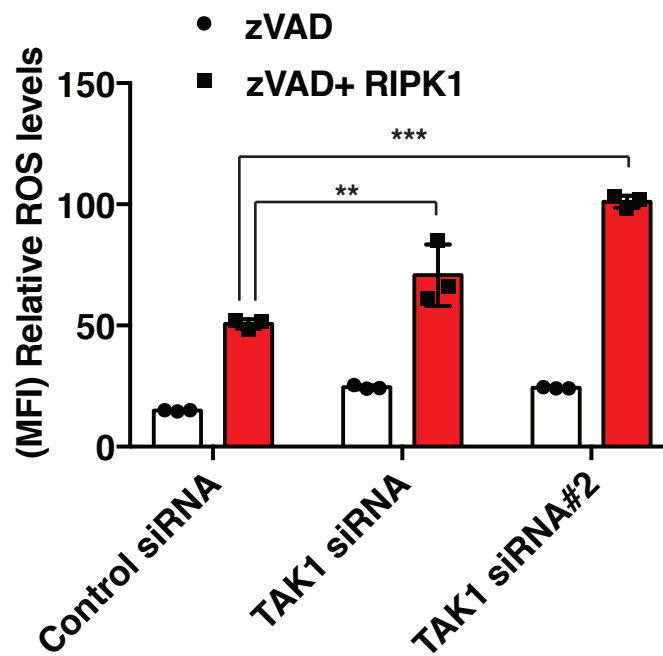
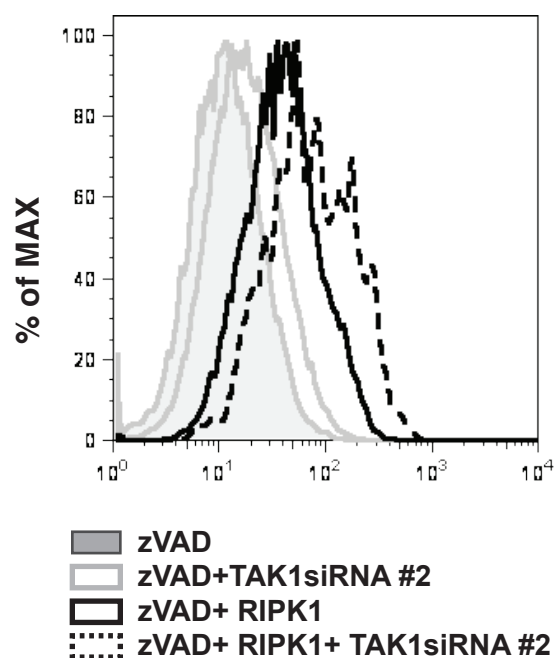
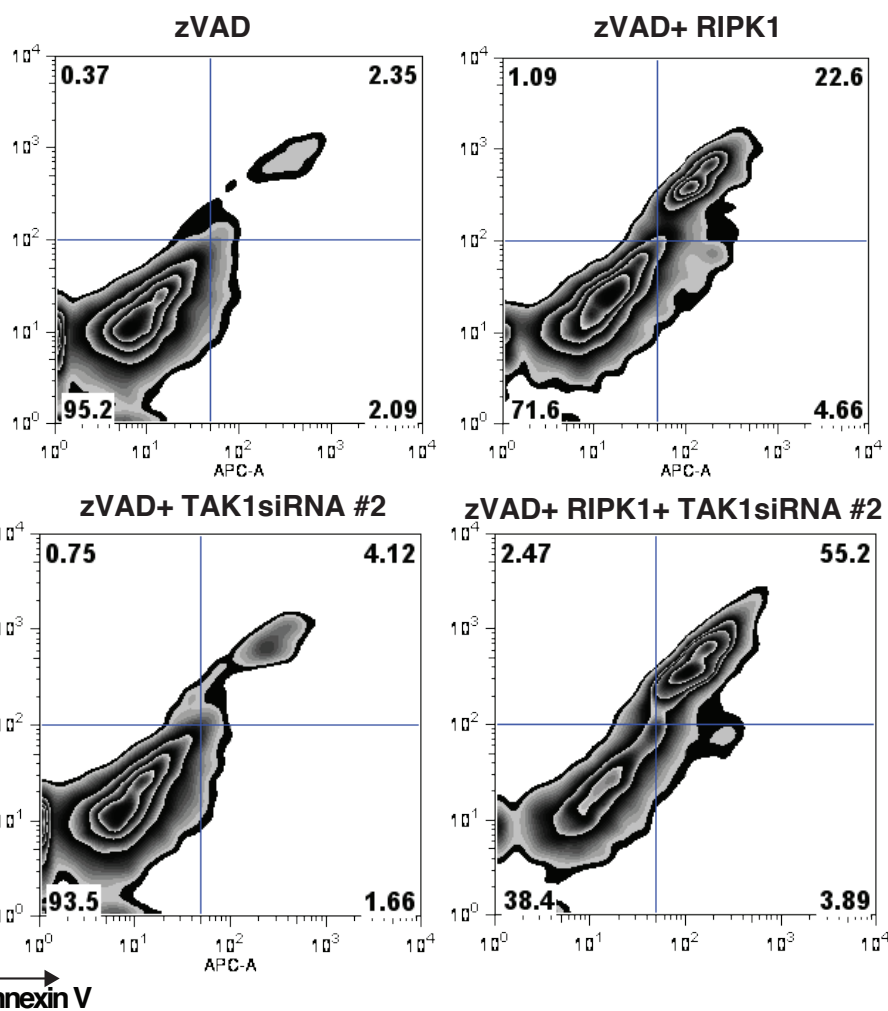
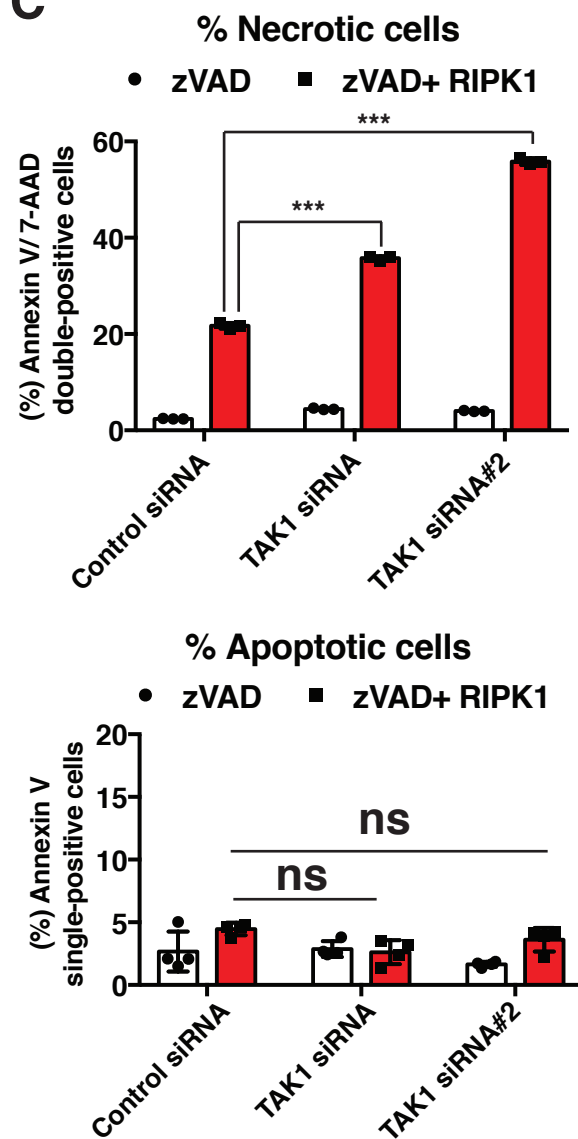


A**TAB2 KO****TAK1 KO****TAK1/RIPK3 DKO****B****TAK1 iKO**

A**B****C****D****E**

DKO



A**ROS****B****C**

Supplemental Figure Legends

Figure S1. *In vitro* characterization of BMDMs

(A) Freshly isolated bone marrow cells and BMDMs (L929-conditioned culture for 4 days) were incubated with F4/80 and CD11b antibodies and analyzed by flow cytometry. Figure shown is representative of three different experiments.

(B) Cells from mice with the indicated genotypes were incubated with fixable viability dye (Live/Dead) and quantified on a flow cytometer. Percent positive for Live/Dead is shown. Cells were treated with 1 mM tBHP for 1 h as a positive control (bottom right).

Figure S2. Characterization of *Tak1*-deficient BMDM death

(A) TUNEL shows unusual pattern of cytoplasmic puncta staining. TAK1^{iKO} and control (WT) BMDMs were cultured 4 day with 0.3 μ M 4-OHT. Cells were fixed, permeabilized, and labeled with fluorescein-12-dUTP and counterstained with DAPI. Scale bars, 20 μ m (left panel) and 10 μ M (right panel). Representative images of TUNEL staining are shown. Graph shows the percent of TUNEL positive cells per 1000 cells counted. Each dot shows a percentage of TUNEL positive cells per area (approximately 40 cells per area).

(B) Western blotting for TAK1, caspase 3 and cleaved caspase 3 in WT BMDMs treated with 0.3 μ M 4-OHT and 20 μ M zVAD for 2, 3 or 4 days.

Figure S3. Oxidative stress but not RIPK3 or MLKL is responsible for *Tak1*-deficient BMDM death

(A) *Tab2*-deficient (TAB2 KO) and *Tak1*-deficient (TAK1 KO) fibroblasts were pre-treated with vehicle (DMSO) or indicated concentration of GW806742X for 1 h and stimulated with 20 ng/ml TNF for 24 h. DKO BMDMs were treated with 0.3 μ M 4-OHT for 2 days, then subsequently treated with 20 μ M zVAD and GW806742X for additional 2 days. Cell viability was determined by crystal violet assay. Data are the result of 3 independent experiments and show mean percentages $\pm$ SD. ns, not significant; ***p-value < 0.001; one-way ANOVA.

(B) TAK1^{iKO} BMDMs were treated 0.3 μ M 4-OHT for 2 days and subsequently treated with 500 μ M NAC for additional 2 days. Viability was assessed by crystal violet assay. Figure shown are mean percent viable $\pm$ SD. * p-value < 0.05; one-way ANOVA.

Figure S4. Investigation of the mechanism of *Tak1*-deficient BMDM death

(A) DKO BMDMs were treated with 0.3 μ M 4-OHT $\pm$ 50 μ M Nec-1 for 4 days and RNA was extracted. Relative expressions of GSTA2 and SOD2 are shown. Figure shown are mean $\pm$ SD. ns, not significant; *** p-value < 0.001; one-way ANOVA.

(B) Western blotting for TAK1, JNK and P-JNK in DKO BMDMs treated with 0.3 μ M 4-OHT and 20 μ M zVAD for 4 days.

(C and D) DKO BMDMs were treated with 0.3 μ M 4-OHT for 2 days, then subsequently treated with 20 μ M zVAD and 20 μ M SP600125 for additional 2 days. ROS was detected by CellROX dye and cell viability was determined by crystal violet assay. Figure shown are mean percentages $\pm$ SD. ns, not significant; ***p-value < 0.001; one-way ANOVA.

(E) RAW264.7 cells were incubated with 200 nM 5Z-7-oxozeaenol (5Z) with or without 10 μ g/ml of anti-TNF α neutralizing antibody and 20 μ M zVAD for 24h. DKO BMDMs were treated with 0.3 μ M 4-OHT for 2 days, then subsequently treated with 20 μ M zVAD and 10 μ g/ml of anti-TNF α neutralizing antibody for additional 2 days. Cell viability was measured by crystal violet assay. Data are the result of 3 independent experiments and show mean percentages $\pm$ SD. *** p-value < 0.001; one-way ANOVA.

Figure S5. The effect of zVAD on caspase 3 and 8 activities in DKO fibroblasts treated with TNF

DKO fibroblasts were pre-treated with (DMSO) or 20 μ M zVAD for 1 h and stimulated with 20 ng/ml TNF for 6 h. Caspase activities relative to vehicle samples in cellular extracts are shown. Data are the result of 3 independent

experiments and show mean percentages $\pm$ SD. ns, not significant; *** p-value < 0.001 ; one-way ANOVA.

Figure S6. The effect of TAK1 inhibition on RIPK1-induced ROS accumulation and necrosis induction

(A and B) HeLa cells were transfected with another siRNA targeted against TAK1 gene, incubated for 24 h and then transfected with an expression vector for GFP-tagged RIPK1 or GFP together with 20 μ M zVAD, incubated for another 24 h, and stained with CellROX dye or Annexin V-APC and 7-AAD, then analyzed on a flow cytometer.

(C) Percentage of necrotic cells (Annexin V/ 7-AAD double-positive cells and percentage of apoptotic cells (Annexin V single-positive cells are shown. The results shown are representative of three different experiments and show mean percentages $\pm$ SD. ns, not significant; *** p-value < 0.001 ; two-way ANOVA.