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Supplementary Information for

TAK1 inhibition elicits mitochondrial ROS to block intracellular bacterial colonization

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Figures S1 to S5

López-Pérez et al. Supplementary Fig. S1

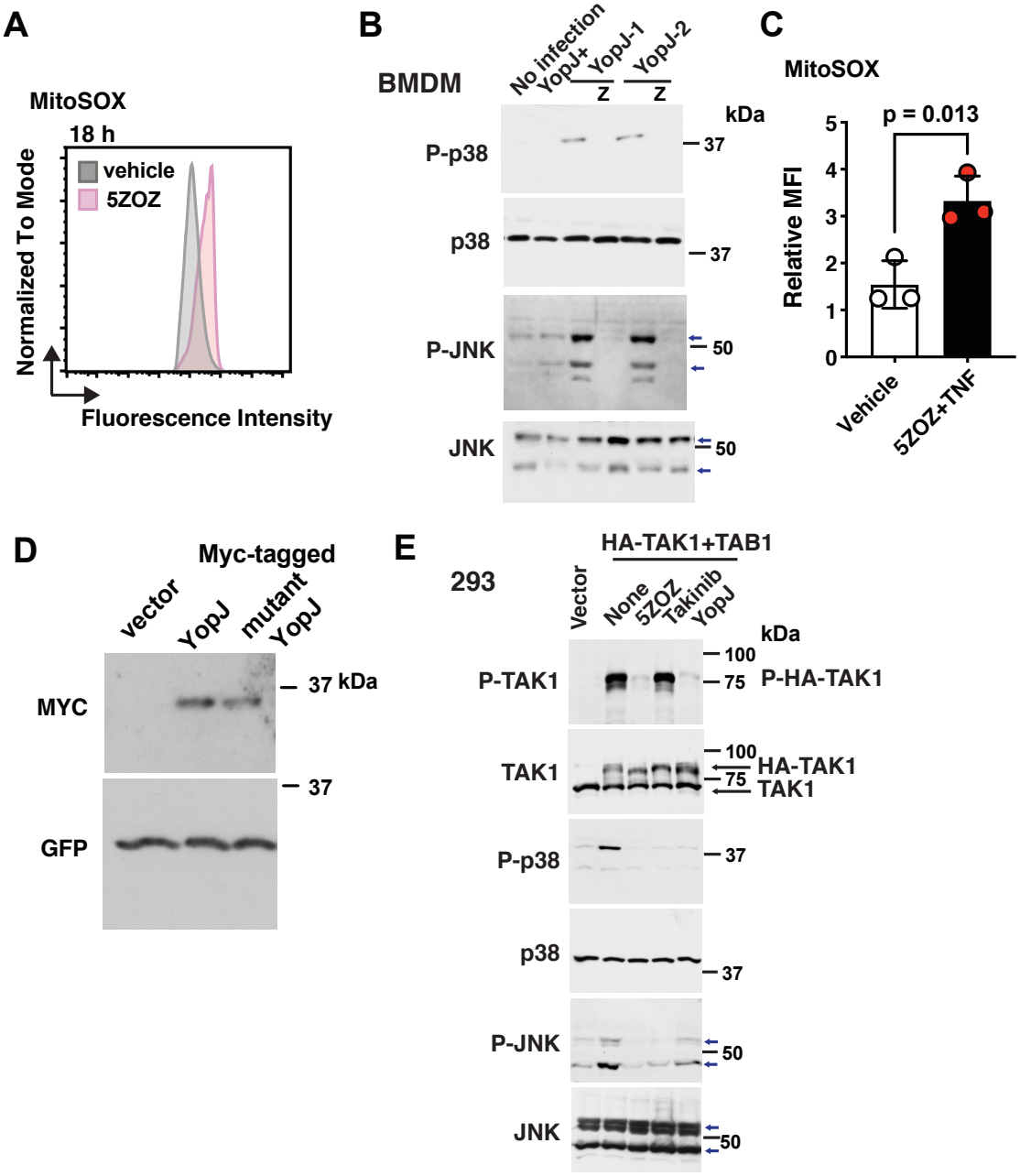


Figure S1. TAK1 inhibition elicits ROS, related to Fig. 1

(A) Representative flow cytometric data with 300 nM 5ZOZ treatment at 18 h shown in Fig, 1A.

(B) YopJ expressing *Yersinia* infection blocked TAK1 downstream events. Wild type BMDMs were incubated (MOI 10) with indicated *Yersinia* strains, YopJ expressing (YopJ+) and two *yopJ*-deficient (YopJ-1, YopJ-2), for 2 h. Extracellular *Yersinia* was killed by gentamicin, and BMDMs

were treated with 300 nM 5ZOZ (Z) or left untreated for 30 min. Blue arrows indicate the positions of two forms of JNK1 and JNK2.

(C) HeLa-RIPK3 cells were treated with 25 ng/ml TNF and 750 nM 5ZOZ or with vehicle for 18 h. All data points of the median intensity of MitoSOX are shown. Means±SD and unpaired Students' t-test two-tail p value are shown.

(D) The expression levels of YopJ and mutant YopJ in the experiments shown in Fig. 1 C were analyzed by immunoblotting. HeLa-RIPK3 cells (1×10^5 cells) were transfected with GFP vector (0.1 μg) together with expression vectors for Myc-tagged vector, Myc-YopJ or Myc-mutant YopJ (1.0 μg) (6-well plate), and were harvested at 48 h-post transfection. GFP protein levels are shown as a loading control.

(E) Efficacy of TAK1 inhibitors and YopJ. An active form of TAK1 (co-overexpression of HA-tagged TAK1 and an adapter TAB1) was transfected in 293 cells. Some cells were co-transfected with a YopJ expression vector. Cells were treated with 300 nM 5ZOZ or 10 μM Takinib simultaneously with the transfection and at 30 min prior to the harvest. Cells were harvested at 24 h-post transfection. Blue arrows indicate the positions of two forms of JNK1 and JNK2.

López-Pérez et al. Supplementary Fig. S2

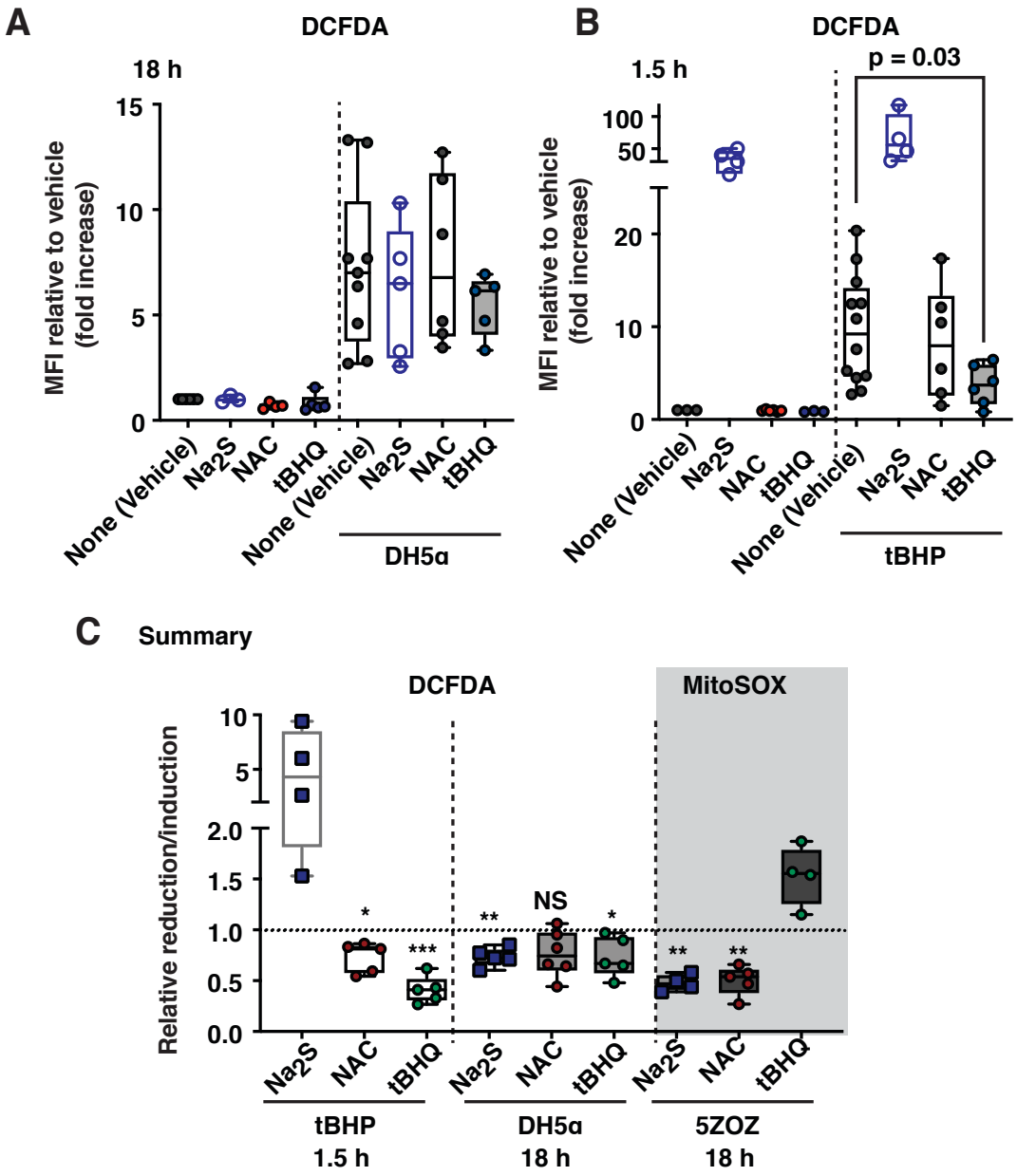


Figure S2. Characterization of antioxidants, related to Fig. 2

(A) BMDMs were incubated with E. coli DH5α at 100-fold number of BMDMs for 1 h. The culture medium was changed to one with antibiotics 50 I.U./ml penicillin-streptomycin and 10 μg/ml

gentamicin, and sodium sulfide (0.4 mM), cysteine (3 mM), NAC (3 mM) or tBHQ (20 μM) were added to the medium. ROS levels were analyzed at 18 h-post treatment.

(B) BMDMs were incubated with an oxidative agent, tBHP (1 mM), together with sodium sulfide (0.4 mM), cysteine (3 mM), NAC (3 mM) or tBHQ (20 μM) for 1.5 h.

(C) Summary of ROS modulating chemicals' effect. Data in each experiment shown in Fig. 2, and this supplementary Fig. S2 were recalculated to the proportion of induction or reduction relative to the MFIs of samples with tBHP, DH5α or 5ZOZ in the absence of antioxidants.

All data points are shown. One-way ANOVA, multiple comparisons, Tukey test (A,B). P values are indicated. One sample and Wilcoxon test, *, $p < 0.05$: **, $p < 0.01$; ***, $p < 0.001$ (C).

López-Pérez et al. Supplementary Fig. S3

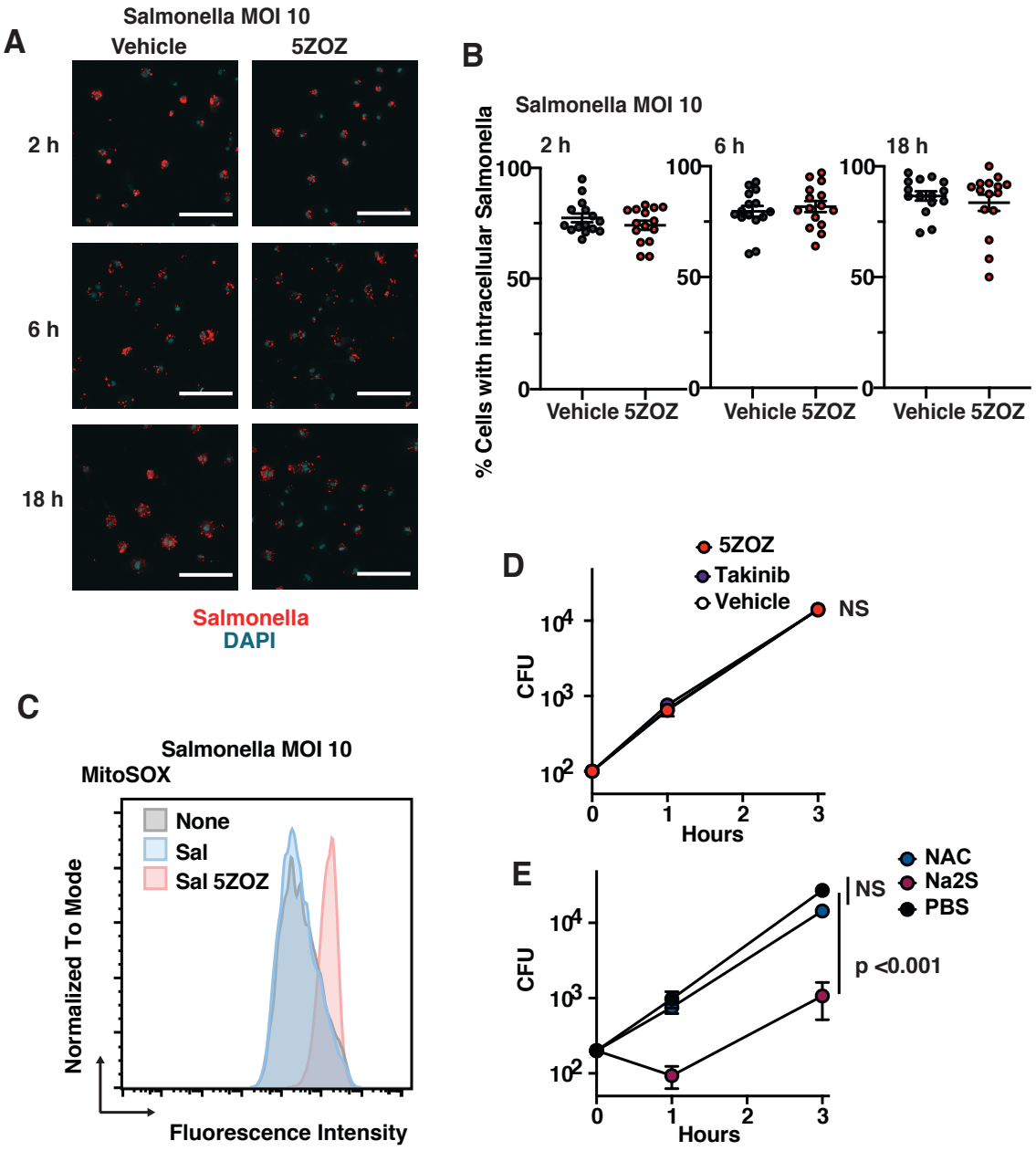


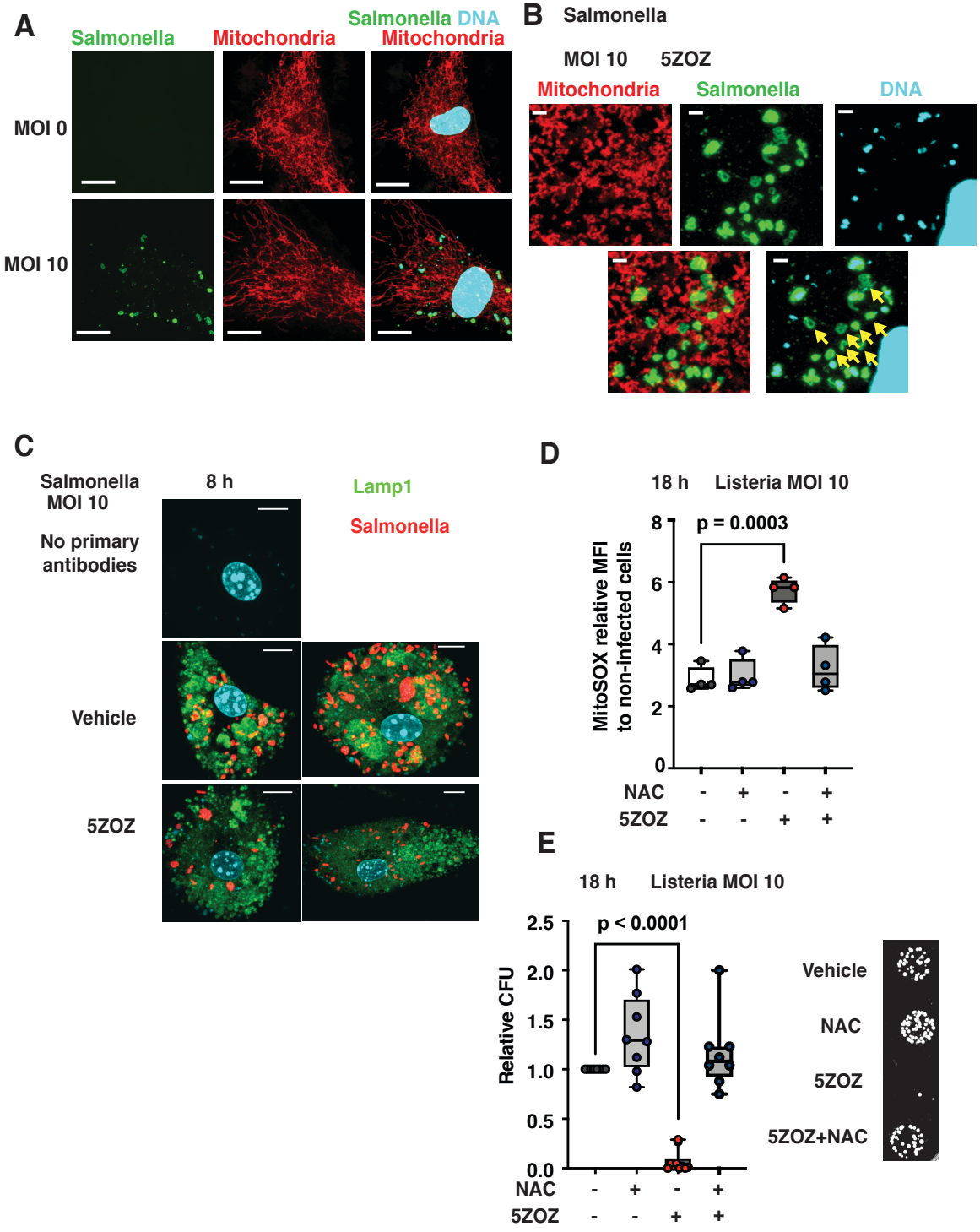
Figure S3. Intracellular *Salmonella*, related to Fig. 3

(A, B) Intracellular *Salmonella* was visualized by immunofluorescence staining at 2, 6 and 18 h-post infection (MOI 10). BMDMs having >1 intracellular *Salmonella* were counted (B). 4 randomly selected areas (> 50 cells per image) were photographed in 3 independent BMDMs of 3 different animals. Each data point represents one photographed image. Scale bars, 100 μm.

(C) Representative flow cytometirc data of Fig. 3A. MitoSOX in SytoxGreen-negative BMDMs with or without *Salmonella* (MOI 10) and 300 nM 5ZOZ is shown.

(D, E) Direct effects of the chemicals on *Salmonella* growth were determined. *Salmonella* was incubated with vehicle (DMSO), 300 nM 5ZOZ or 10 μM Takinib (D), or vehicle (PBS), 0.4 mM

sodium sulfide, or 3 mM NAC (E) in LB broth at 30 °C. Culture broth with serial dilutions was spotted on LB agar. Means ±SD (N = 3) are shown. Simple linear regression test was performed. NS, not significant.



Supplementary Figure S4. Intracellular bacteria, related to Fig. 4

(A) No non-specific staining was observed in immunofluorescence staining with anti-*Salmonella*. BMDMs were infected with *Salmonella* (MOI of 10) or left uninfected (MOI of 0), and

immunofluorescence staining was performed at 18 h-post infection. All images were captured with a confocal microscopy using the same photomultiplier voltage setting. Scale bars, 10 μm

(B) Additional examples of destroyed intracellular *Salmonella* in the experiment shown in Fig. 4A. BMDMs were infected at MOI of 10, and were treated with 300 nM 5ZOZ. Projected images covering the entire thickness of the cells are shown. (Upper panels) individual staining; (lower panels) merged image of MitoTracker Red and *Salmonella* left, and merged image of *Salmonella* and DAPI. Examples of damaged *Salmonella* are indicated in yellow arrows. Scale bars, 2 μm

(C) Intracellular *Salmonella* was observed in both cytoplasm and lamp1-positive vacuoles (*Salmonella* containing vacuoles). BMDMs were infected with *Salmonella* (MOI of 10), and immunofluorescence staining was performed at 8 h-post infection. All images were captured with a confocal microscopy using the same photomultiplier voltage setting. Scale bars, 10 μm

(D, E) Wild type BMDMs were infected with *Listeria* with MOI of 10. ROS (D) and intracellular *Listeria* number (E) were determined as described in *Salmonella* infection. Representative *Listeria* colonies from BMDM cell lysate at 18 h-post infection are shown (E, right panel).

One-way ANOVA, multiple comparisons, Tukey test.

López-Pérez et al. supplementary Fig. S5

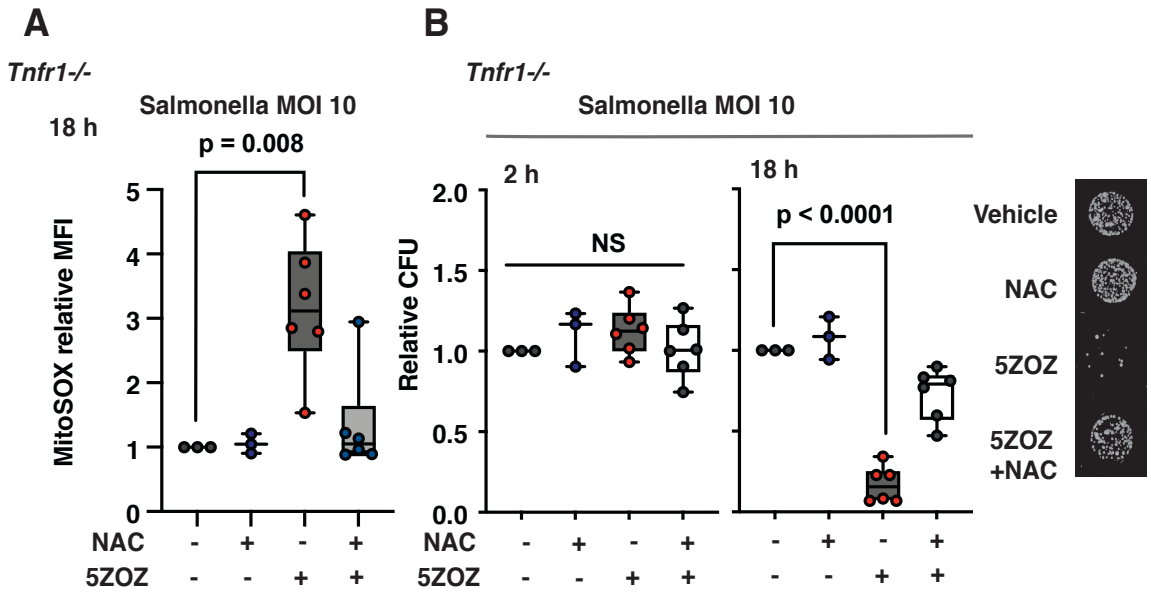


Figure S5 TAK1 inhibition limits intracellular bacteria growth independently of TNF signaling, related to Fig. 5

(A, B) *Tnfr1-/-* BMDMs were infected with *Salmonella* with MOI of 10, and extracellular *Salmonella* was killed by gentamicin. 300 nM 5ZOZ and 3 mM NAC were added to medium when starting gentamicin treatment. Mitochondrial ROS (A) and intracellular *Salmonella* numbers at 2h and 18 h post infection (B) were determined. One-way ANOVA, multiple comparisons, Tukey test.