Materials and Methods

Animals

We generated transgenic mice with myeloid cell-specific loss-of-function (LysM-Cre *Ncoa4*$^{F/F}$, KO) and gain-of-function (LysM-Cre *Ncoa4*$^{Loxp\text{-}Stop\text{-}Loxp\ (LSL)/LSL}$, OE) of NCOA4 by crossing myeloid cell-specific LysM-Cre mice with *Ncoa4*$^{F/F}$ and *Ncoa4*$^{LSL/LSL}$ mice, as previously described (Santana-Codina et al., 2022). Animal studies were performed in accordance with the Institute of Laboratory Animal Resources guidelines, approved by the Institutional Animal Care and Use Committee (IACUC) at the University of New Mexico Health Sciences Center (Protocol# 20-201060-HSC and 23-201434-HSC) and followed the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). All mice were maintained in a standard cage on a 12-h light-dark cycle, in a temperature-controlled room and were allowed *ad libitum* access to a standard chow diet and water. Both male and female animals were used in this study.

Salmonella colitis study

At 6- to 8- week-old age, mice with a body weight of 18-22g were orally gavaged with 0.1 mL of streptomycin (200mg/mL solution in 1 x PBS) and were inoculated intragastrically 24h later with either 0.1mL of sterile LB broth or bacterial culture containing 1 x 10^9 cfu of *Salmonella* Typhimurium for a 20g mouse. The body weights of mice were monitored daily, and colon lengths of mice were recorded after euthanization. For the drug treatments, mice were treated with 0.064% Tempol given in the drinking water or Oltipraz (120mg/kg i.p. in corn oil daily) or 10mg/kg free curcumin (1.25mg/mL i.p in 1x PBS once, 200µLvolume) or 30mg/kg curcumin-nanoparticles (~ 35% curcumin loading, i.p. once, 10 mg/kg loaded curcumin) or Ferrostatin-1 (1mg/kg by i.p. in 1% DMSO daily) one day after *Salmonella* treatment. For the low iron treatment, we changed the standard chow diet to either control (40mg/kg ferric citrate iron,

40Fe) or low iron diet (3.5 mg/kg ferric citrate iron, 3.5 Fe) from Research Diets Inc. (New Brunswick, NJ).

Histology and immunofluorescence staining

Spleen and colon tissues were removed and washed with 1x PBS and fixed with 10% formalin at room temperature overnight and embedded in paraffin. Tissue sections (5 μm) were deparaffinized in xylene and rehydrated in ethanol gradient. Histologic analysis was done on hematoxylin and eosin (H&E)-stained paraffin sections and microscopically analyzed by a gastrointestinal pathologist (Dr. Martin) in a blind manner. The images were taken and analyzed under a fluorescence microscope (EVOS, Thermo Fisher Scientific).

Perl's Iron Staining

Sections were incubated in the mixture of 2% hydrochloric acid and 1% ferrocyanide solution (1:1) for 30 minutes. After incubation, the sections were washed in tap water for 5 minutes. After washing in tap water for 5 minutes, the slides were sealed with coverslip using Permount Mounting Medium (Fisher Scientific, Hampton, NH).

Bone marrow derived macrophage (BMDM) cell culture

First, we euthanized the mouse with CO_2 and then sterilized the mouse abdomen area and skin with 70% ethanol. Opened the abdominal cavity with sterile scissors and removed the surface muscles and proceeded to find the pelvic-hip joint. There we cut the hind leg above the pelvic-hip joint. Once we got the leg, we removed the muscles and residue tissues collecting the femur and tibia from each leg, then placed them in a six well plate filled with 1X sterile PBS. After collection of bones, we cut the bones at both ends and with a needle flush the bone marrow out into a 24 well plate. The bone marrow-derived monocytes are cultured in Dulbecco's modified Eagle

medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin and 10ng/ml macrophage colony-stimulating factor (M-CSF), with fresh media changes every 3 days. By 7th day, the monocytes have differentiated to macrophages and can be collected for the respective analysis.

BMDM staining.

For the preparations of the staining solutions, a general manufacturer's protocol was adhered to. Cells were plated at a concentration of 500 cells/mL in 24-well plates and cultured overnight at 37 °C in 5% CO2. Next day culture medium was discarded, cells were washed with 1x PBS, then treated with 1μM FerroOrange for cellular iron (Dojindo), 1μM Mito-FerroGreen for mitochondrial iron (Dojindo) or, 5μM Bodipy C11 for lipid peroxidation (ThermoFisher), all in 1x PBS. After treatment, the cells were incubated at 37°C for 30 minutes. For the imaging at the single-cell level, 1 million cells were captured using the Imagestream-X MarkII Imaging Flow Cytometer (Amnis), and the collected data were subsequently analyzed using IDEAS 5.0 software (Amnis).

Real-time qPCR analysis

RNA was extracted using an IBI reagent Kit (IB47602, IBI Scientific, Dubuque, IA). qPCR was performed on the Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). The mRNA level was measured by qPCR. The primers are listed in **Table S1**. The gene levels were normalized by 18S. Expression level changes were calculated by comparing to the control.

Antibodies and plasmids

Primary antibody against ferroportin (FPN or SLC40A1, clone:8G10NB) was from Novus Biologicals (Centennial, CO). Primary antibody against ferritin heavy chain 1(FTH1 or D1D4

#4393S) and secondary antibodies against rabbit (7074S) or mouse IgG (7076S) were from Cell Signaling Technology (Danvers, MA). Primary antibodies against kelch like ECH associated protein 1(KEAP1, sc-514914, 39-65aa), heme oxygenase 1 antibody (HO-1, encoded by Hmox1 gene, F-4, sc390991), NAD(P)H quinone dehydrogenase 1 (NQO1, sc-32793) and beta-actin (β-actin, C4, sc-47778) were from Santa Cruz Biotechnology (Dallas, TX). Primary antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2, A1244) and nuclear receptor coactivator 4 (NCOA4, A5695) were from ABclonal (Woburn, MA). MYC-tag Rabbit Polyclonal antibody (16286-1-AP), HA tag Rabbit Polyclonal antibody (51064-2-AP), KEAP1 Polyclonal antibody (10503-2-AP, 325-624aa) and GAPDH Monoclonal antibody (60004-1-Ig) were from Proteintech (Rosemont, IL). T9 NCOA4 LONG (L) NO STOP_1 pHAGE CTAP Puro with HA/Flag tag was from Dr. Joseph Mancias. Nrf2-FL in pCI, 5'-Myc-Keap1 in pcDNA3 and truncation plasmids were from Dr. Donna Zhang.

Cell line culture

RAW264.7 murine macrophage cell line, SW480 colorectal cancer cell line and HEK293T cell line were obtained from ATCC and maintained at 37°C in 5% CO2 and 21% O2.
Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic.

Co-immunoprecipitation assay

HEK293T cells were seeded into a 10cm plate and transfected with DNA plasmid using either Lipofectamine 2000 (Invitrogen) or polyethylenimine (PEI; Polysciences Inc., Warrington, PA), according to the manufacturer's instructions. Cell lysates were collected at 48 h post transfection in NP40 buffer containing 50mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40. A protease inhibitor cocktail made from Pierce™ Protease Inhibitor Tablets (EDTA-free, PIA32955, VWR) was also added to the NP40 buffer freshly. Cell lysates were incubated with 2 μg of antibody and 20 μl of Protein G magnetic beads on a rotator at 4°C overnight. To detect

protein expression in the total cell lysates, 10 µl of the cell lysates in NP40 buffer was mixed with 10 µl of 2× sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 100 mM DTT, 0.1% bromophenol blue) and boiled for 5 min. The immunoprecipitated complexes were washed with NP40 buffer three times and eluted in sample buffer by boiling for 5 min. Samples were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes for immunoblot analysis.

Western blot analysis

Animal tissues were lysed and homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer and incubated on ice for 15 min. After incubation, tissue extracts were centrifuged for 15 min at 4 °C. The supernatant was collected for protein concentration quantification using the Bradford assay. Equal amounts (30–50 µg) of protein were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), along with molecular weight markers. The gels were run for 1 h 30 min at 110 V. The proteins inside the gels were transferred onto nitrocellulose membranes for 1 h at 100 V using the wet transfer method as previously described (Xue et al, 2016). The membranes were blocked with 3% milk for 1 h, incubated in primary antibodies overnight. Next day, they were rinsed with Tris Buffered Saline containing 0.1% Tween20 (TBST) for 3 times (5 min each), incubated in secondary antibodies for 1 h, and rinsed with TBST for 3 times (5 min each). The membranes were visualized using chemiluminescent substrate by capturing the chemiluminescent signals (BioRad). The band intensities of targeted proteins were read by using Image Lab software and scored with ImageJ software.

Statistical Analysis

Experiments were replicated three times. Data were expressed as mean ± SD. Statistical analyses were conducted using Student's t-test, one-way, or two-way analysis of variance (ANOVA) using Graphpad Prism. A p-value less than 0.05 was deemed statistically significant.

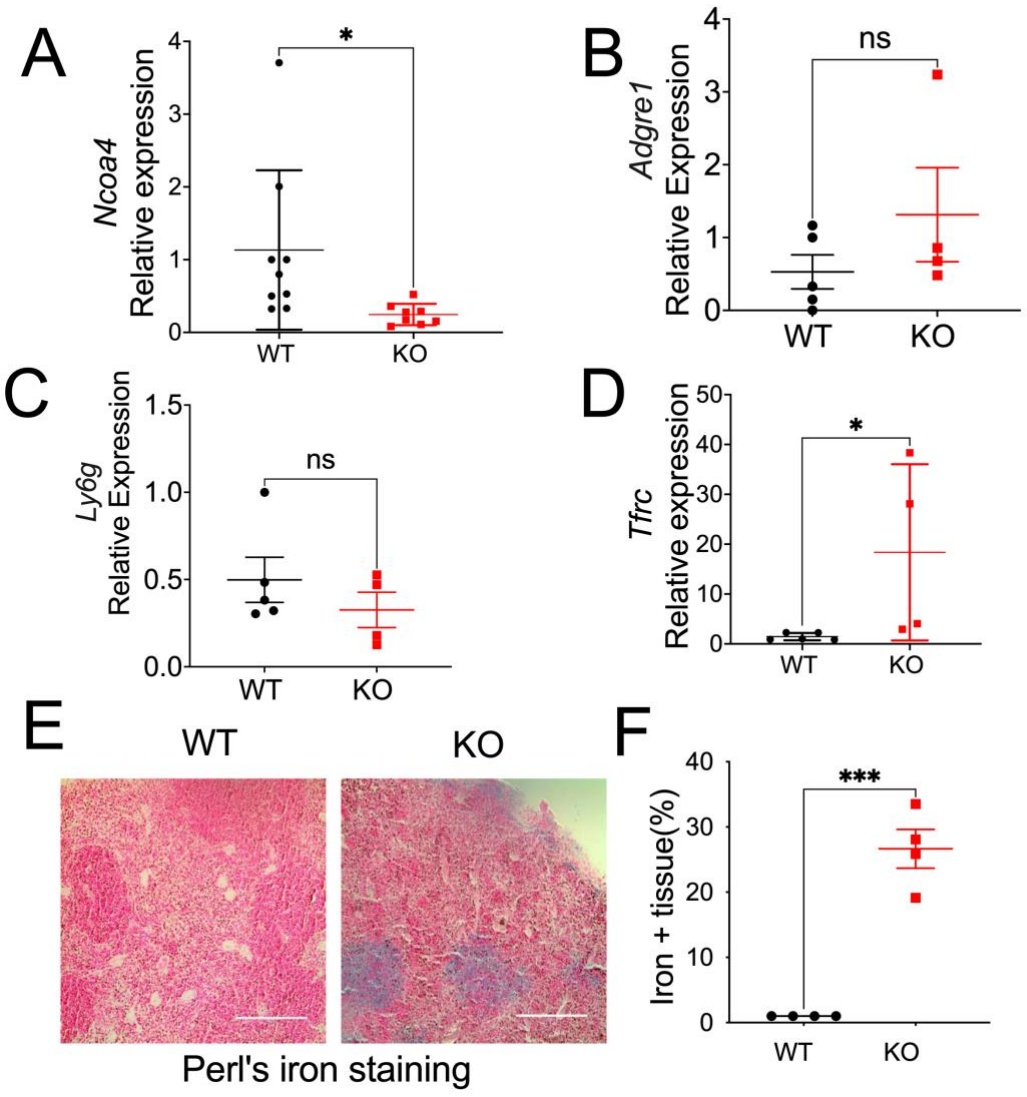

Fig. S1: Myeloid NCOA4 KO leads to an increase in spleen iron levels compared to the WT mice. Using LysM-Cre *Ncoa4*$^{F/F}$ mice (KO), the study conducted qPCR analysis for gene expression of *Ncoa4* (**A**), macrophage marker *Adgre1* (**B**), *Ly6g* (**C**) and *Tfrc* (**D**). Perl's iron staining for visualizing iron deposits (**E**), and quantification of iron levels (**F**). The results, analyzed by Student's t-test, reveal a significant increase in spleen iron levels in the KO group (n=4-8) compared to the wild-type (WT, n=4-9) group, as denoted by *p<0.05 and ***p<0.001. ns, not significant.

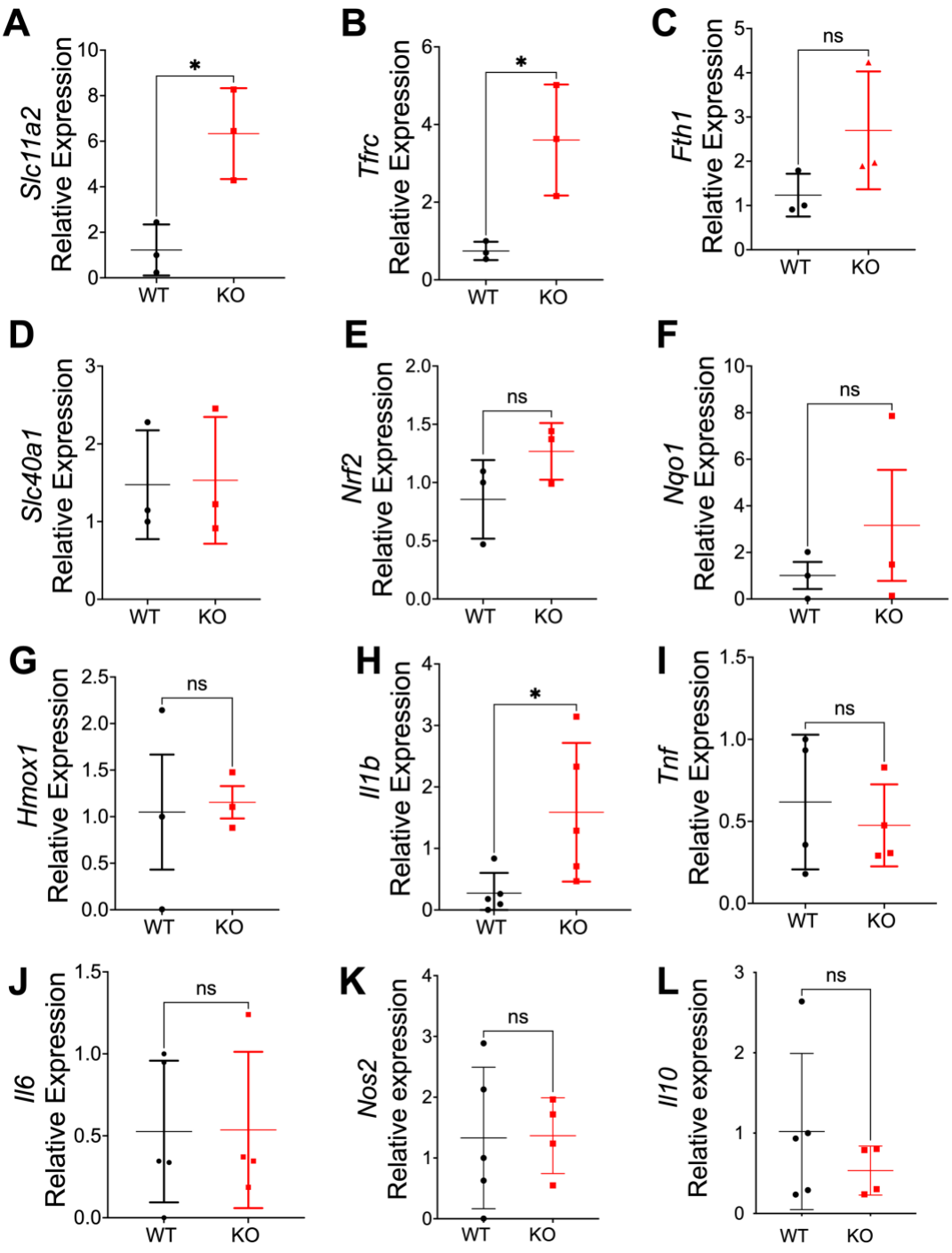

Fig. S2: Myeloid NCOA4 knockout robustly increases iron uptake transporters in BMDM cells. qPCR analysis for (**A**) *Slc11a2*, (**B**) *Tfrc*, (**C**) *Fth1*, (**D**)*Slc40a1*, (**E**) *Nrf2*, (**F**)*Nqo1*, (**G**) Hmox-1, (**H**) *Il1b*, (**I**)*Tnf*, (**J**) *Il6,* (**K**) Nos2, and (**L**) *Il10* in BMDM cells from myeloid NCOA4 KO (n=3-4) and their WT (n=3-5) control mice. ns, not significant. *p<0.05. Student's t- test.

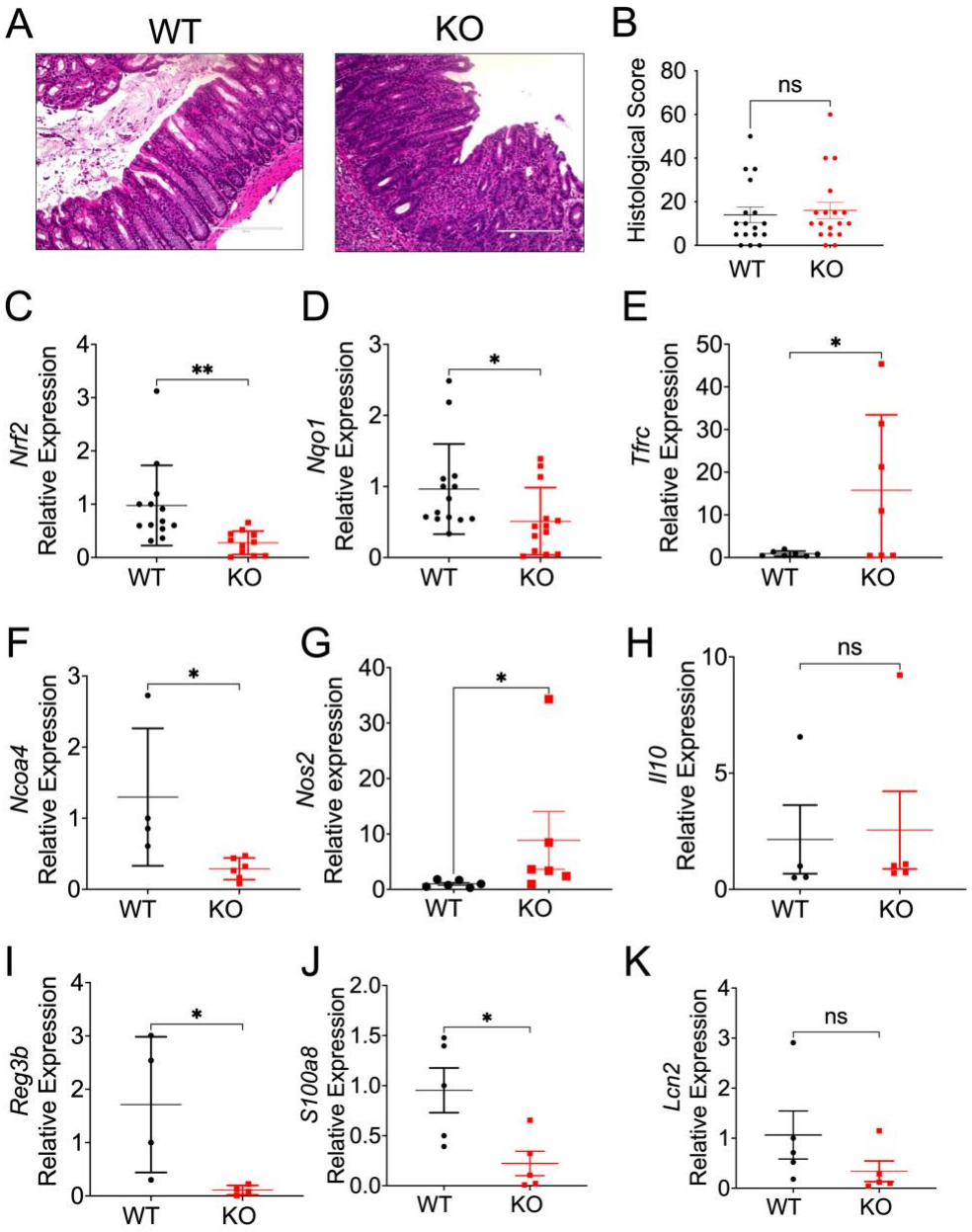

Fig. S3: Colon tissues from *Salmonella*-treated myeloid NCOA4 knockout mice exhibit dysregulation in oxidative stress levels, iron metabolism, inflammatory response, and antimicrobial peptide levels. (**A**) H&E staining (Bar 400μM), (**B**) Histological score and qPCR analysis of (**C**) *Nrf2*, (**D**) *Nqo1*, (**E**) *Tfrc*. (**F**) *Ncoa4*, (**G**) *Nos2*, (**H**) *Il10,* and (**I**) *Reg3b*, (**J**) *S100a8 and* (**K**) *Lcn2* were conducted on colon tissues from control or *Salmonella*-treated KO mice (n=4-18) and WT mice (n=4-17). ns, not significant. *p<0.05, **p<0.01. Student's t-test.

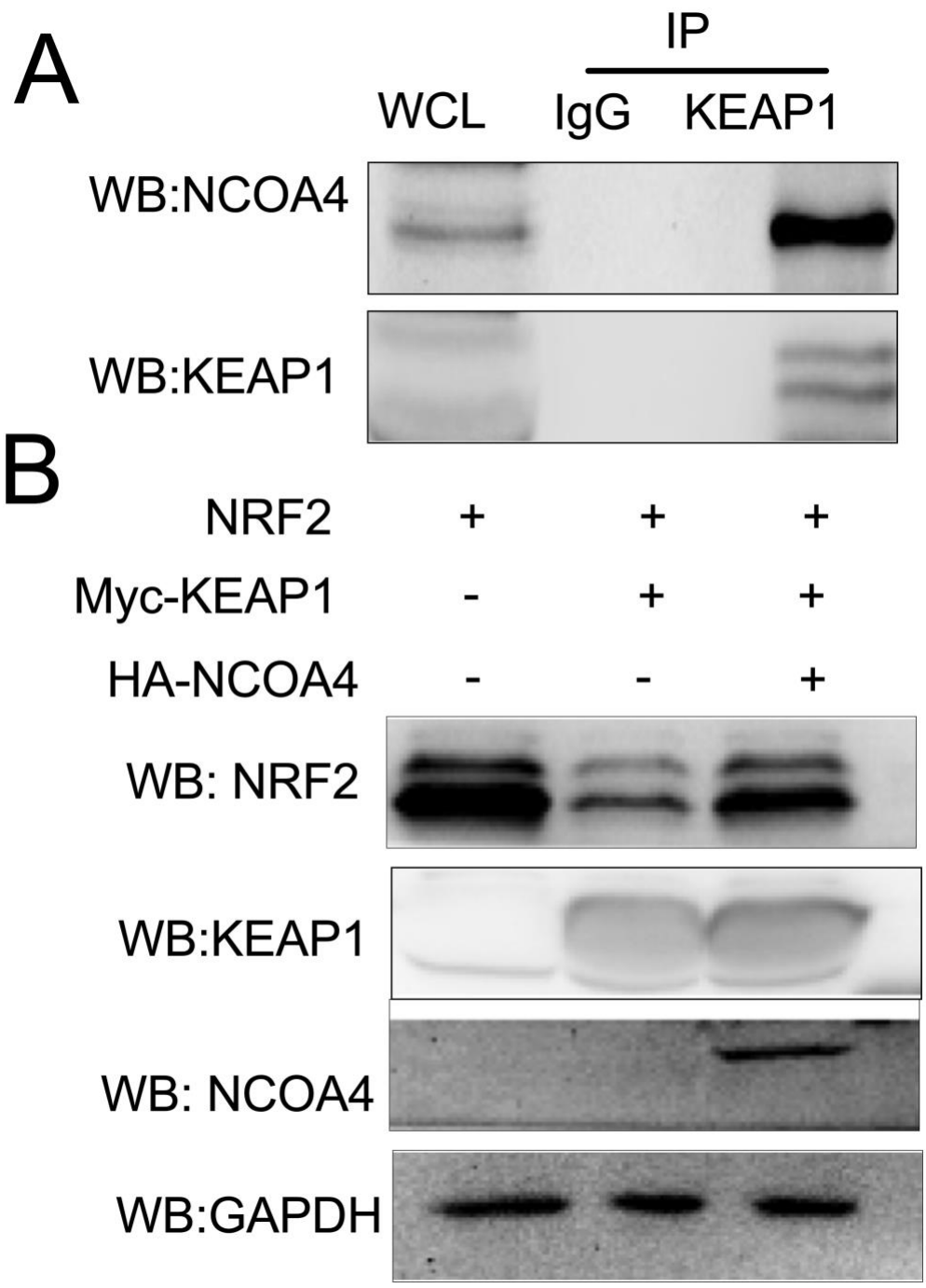

Fig. S4: NCOA4 endogenously interacts with KEAP1 and competes with NRF2 for KEAP1 binding. (**A**) Western blot (WB) analysis of whole cell lysate (WCL) and WCL immunoprecipitation (IP) with IgG or KEAP1 antibody in human SW480 cells. (**B**) WB analysis in HEK293T cells co-transfected with NRF2, Myc-KEAP1 and HA-NCOA4.

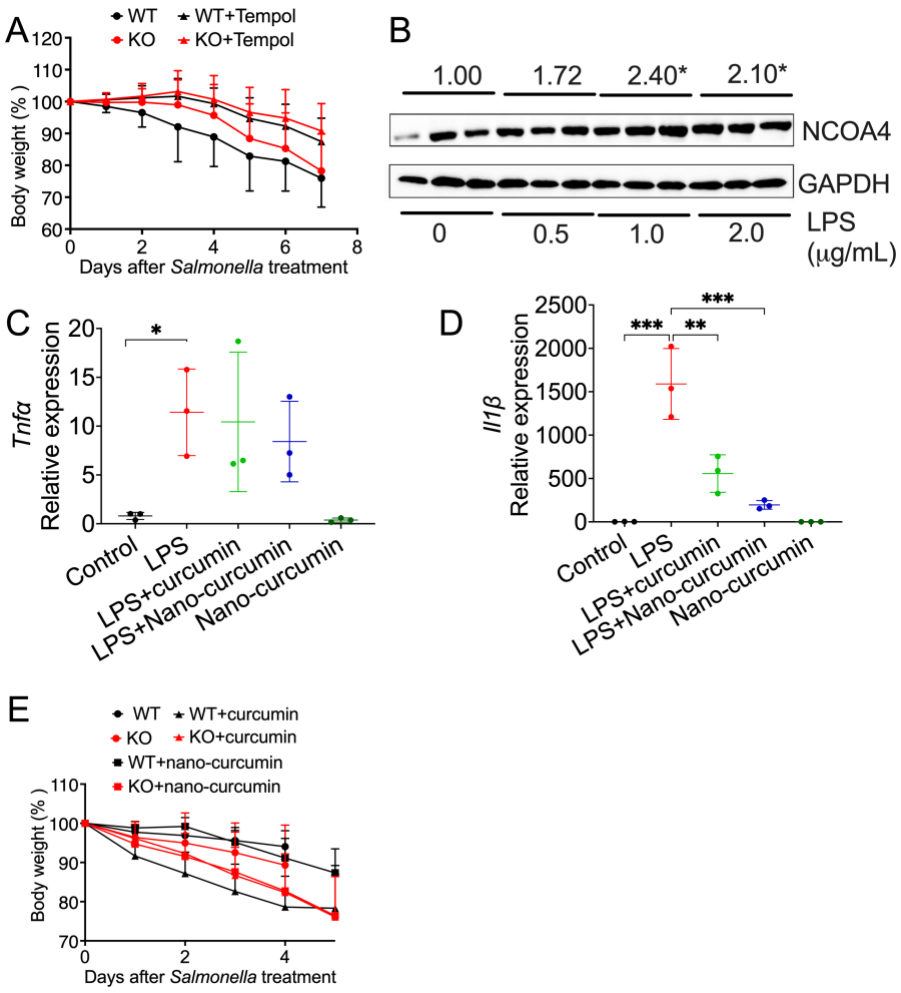

Fig. S5: Myeloid Cell Targeting Nanoparticles Conjugated Curcumin Reduces LPS-Induced proinflammatory cytokines in RAW264.7 cells and do not affect body weights in *Salmonella* challenged mice. (**A**) Body weight change from *Salmonella*-treated KO mice (n=3-4) and WT mice (n=3-4) that received vehicle or 0.064% Tempol in drinking water. (**B**) WB analysis in RAW264.7 cells treated with LPS. qPCR analysis of the proinflammatory cytokines (**C**) *Tnfα*, and (**D**) *Il1β* in RAW264.7 cells treated with LPS, curcumin and nano-curcumin. (**E**) Body weight change from *Salmonella*-treated KO mice (n=3-7) and WT mice (n=3-6) that received vehicle curcumin or nano-curcumin. Values above blots represent mean. Statistical significance was denoted as *p<0.05, **p<0.01, ***p<0.001. One-way ANOVA followed by Dunnett's multiple comparisons test was applied.

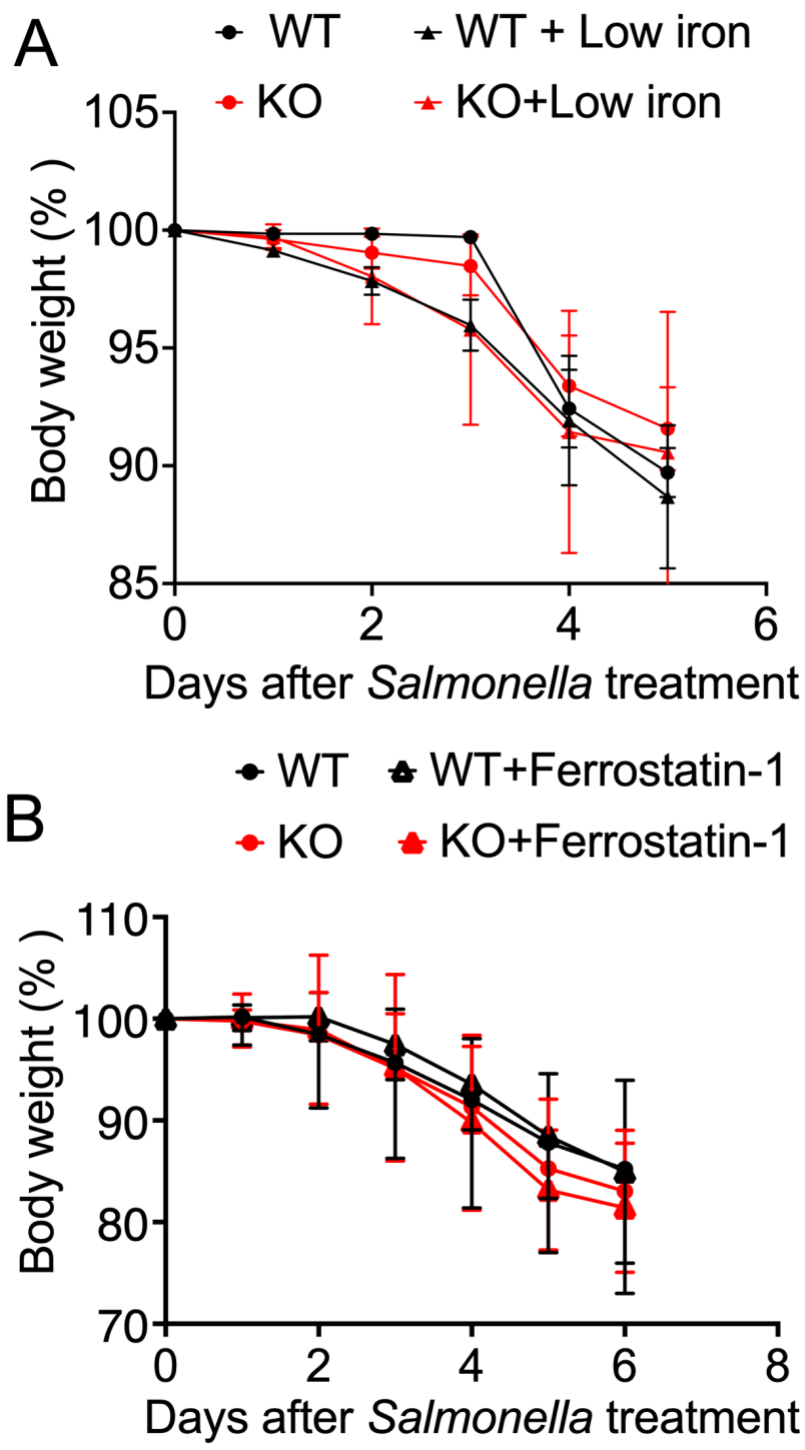

Fig. S6: Low iron diet and ferroptosis inhibition do not affect body weights in *Salmonella* challenged mice. Body weight change from *Salmonella*-treated KO mice (n=3-4) and WT mice (n=4-5) that received (**A**) a control iron diet or a low iron diet, (**B**) vehicle or Ferrostatin-1.

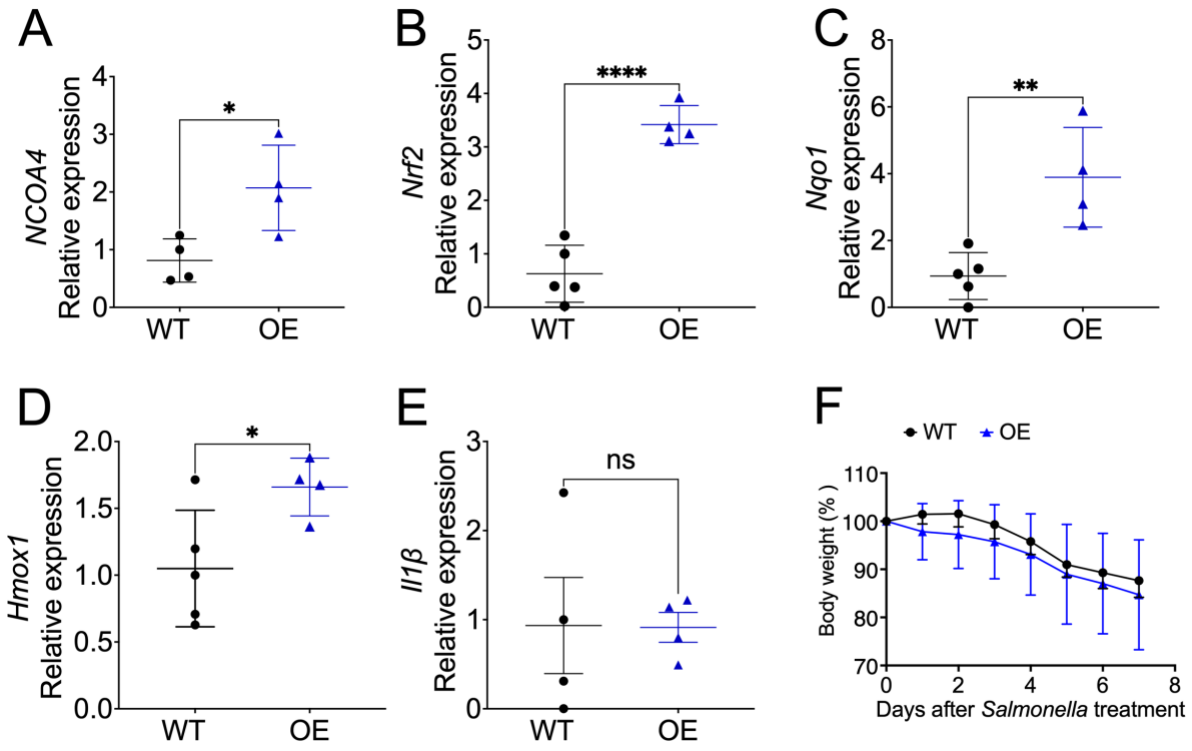

Fig. S7: Myeloid cell specific NCOA4 overexpression activates the transcription of NRF2 signaling. (**A**) qPCR analysis of *NCOA4* in spleens, (**B**) *Nrf2,* (**C**) *Nqo1,* (**D**) *Hmox1,* and (**E**) *Il1β* in BMDM cells from myeloid cell-specific NCOA4 overexpression (OE, n=4) and WT mice (n=4-5). (**F**) Body weight change for WT (n=5) and OE (n=4) mice treated with *Salmonella*. ns, not significant. *p<0.05, **p<0.01, ****p<0.0001, Student's t-test.

Table S1 qPCR primers

18S_forward	GTAACCCGTTGAACCCCATT
18S_ reverse	CCATCCAATCGGTAGTAGCG
hNCOA4 forward	*GCAAATATTGGGCCCTTCCT*
hNCOA4 reverse	*CAATACCGGATGCTGACTTCTG*
mNcoa4 forward	TGCCATTGGTCTTCAGGCTCCT
mNcoa4 reverse	CAGGCATCGCTGAAGAAACTGC
Il1β forward	AAGAGCTTCAGGCAGGCAGTATCA
Il1β reverse	TGCAGCTGTCTAGGAACGTCA
Il6 forward	ACCAGAGGAAATTTTCAATAGGC
Il6 reverse	TGATGCACTTGCAGAAAACA
Tnfa forward	AGGGTCTGGGCCATAGAACT
Tnfa reverse	CCACCACGCTCTTCTGTCTAC
Cxcl1 forward	TCTCCGTTACTTGGGGACAC
Cxcl1 reverse	CCACACTCAAGAATGGTCGC
Tfrc forward	GTTTCTGCCAGCCCCTTATTAT
Tfrc reverse	GCAAGGAAAGGATATGCAGCA
Slc11a2 forward	TTGGCAATCATTGGTTCTGA
Slc11a2 reverse	CTTCCGCAAGCCATATTTGT
Fth1 forward	GGCAAAGTTCTTCAGAGCCA
Fth1 reverse	CATCAACCGCCAGATCAAC
Slc40a1 forward	ATGGGAACTGTGGCCTTCAC
Slc40a1 reverse	TCCAGGCATGAATACGGAGA
Nrf2 forward	TCTATGTCTTGCCTCCAAAGG

Nrf2 reverse	CTCAGCATGATGGACTTGGA
Nqo1 forward	CCAATCAGCGTTCGGTATTA
Nqo1 reverse	GTCTTCTCTGAATGGGCCAG
Hmox1 forward	AAGCCGAGAATGCTGAGTTCA
Hmox1 reverse	GCCGTGTAGATATGGTACAAGGA
Nos2 forward	ACCCTAAGAGTCACCAAAATGGC
Nos2 reverse	TTGATCCTCACATACTGTGGACG
m*Il10* forward	GCTCTTACTGACTGGCATGAG
m*Il10* reverse	CGCAGCTCTAGGAGCATGTG
Ly6g forward	TGGACTCTCACAGAAGCAAAG
Ly6g reverse	GCAGAGGTCTTCCTTCCAACA
Adgre1 forward	CCCCAGTGTCCTTACAGAGTG
Adgre1 reverse	GTGCCCAGAGTGGATGTCT
Reg3b forward	ACTCCCTGAAGAATATACCCTCC
Reg3b reverse	CGCTATTGAGCACAGATACGAG
Lcn2 forward	TGGCCCTGAGTGTCATGTG
Lcn2 reverse	CTCTTGTAGCTCATAGATGGTGC
S100a8 forward	CCAATTCTCTGAACAAGTTTTCG
S100a8 reverse	TCACCATGCCCTCTACAAGA