Supplemental Material

Detailed methods

Primary culture of neonatal rat ventricular myocytes and RAW264.7 macrophages

Primary cultures of neonatal rat ventricular myocytes (NRVMs) were prepared from 1-day-old Sprague-Dawley rats (Kyudo Inc, Japan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described previously[27]. RAW264.7 macrophages were purchased from RIKEN BRC.

Plasmids and viral vector construction

The adenoviral vectors used to overexpress ZBP1-mcherry-flag and ZBP1 in our study were constructed and packaged by VectorBuilder. The vector ID is VB210915-1271fwa and VB191211-3603xtk, respectively, which can be used to retrieve detailed information about the vector on vectorbuilder.com.

mtDNA and CpG-ODN transfection

mtDNA was isolated from Sprague-Dawley rat liver by using mtDNA extractor CT kit (WAKO, 291-55301). To assess the purity of mtDNA, the copy number of DNA encoding Cox1 and At3 were measured by quantitative real-time PCR. CpG-ODN (tlrl-dsl01), FITC-labeled CpG-ODN (tlrl-1668f) and negative control for CpG-ODN (tlrl-1668c) were purchased from Invivogen. We used Lipofectamine 2000 (Thermo Fisher Scientific, 11668027) as a transfection reagent. The forward (F) and reverse (R) primer sequences were as follows: Cox1 (rat), F 5’-CTGAGCGGGAATAGTGGGTA-, 3’, R 5’-GTCAGTTTCCAAAGCCTCCA-3′; At3 (rat), F 5’-GCCGGAATCCTACACTTTCA-, R 5’-TCCGTGACAGATAGCACAGC-3’. The same procedures were conducted with Sprague-Dawley rat hearts to investigate whether the same response was induced regardless of tissues.

siRNA and transfection

Silencing of ZBP1, RIPK3, NF-κB, TLR9, RIPK1, and STING gene expressions in primary neonatal rat ventricular myocytes (NRVMs) were achieved by the small interfering RNA (siRNA) technique. The sequences of the siRNA duplexes were selected from the coding regions of the target mRNAs. Silencer select siRNA specific to decrease the expression of each gene mRNA and negative control (#4390843) were purchased from Thermo Fisher Scientific. The sense strand of siRNA used were as follows: ZBP1, GCAACGAGAUGAACCUCGAtt; RIPK3, GGAACGCACCAAACCCAAUtt; NF-κB, ACCCAGGAGUGUUCACAGAtt; TLR9, GGACCUGUACCAUUCGAAAtt; RIPK1, GCACUACUUACAUGACGAATT; STING, CCAAGAAUCCGAAGAGGGATT. Transfection of cultured cardiomyocytes was carried out by Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the proposed protocol.

Immunoblot analyses

Cardiomyocyte lysates and heart homogenates were prepared in RIPA lysis buffer containing Tris-HCl, NaCl, NP-40, sodium deoxycholate, and SDS. For immunoblot analyses, we used monoclonal antibodies against ZBP1 (#AG-20B-0010-C100, Adipogen Life Science), RIPK3 (#15828, Cell Signaling Technology), RIPK1 (#3493, Cell Signaling Technology), phosphorylated-RIPK1 (#53286, Cell Signaling Technology), STING (#50494, Cell Signaling Technology), NF-κB (#8242, Cell Signaling Technology), phosphorylated NF-κB (Ser536) (#3033, Cell Signaling Technology), NLRP3 (#15101, Cell Signaling Technology), Bcl-2 (#3498, Cell Signaling Technology), TBK1 (#3013, Cell Signaling Technology), phosphorylated

TBK1 (Ser172) (#5483, Cell Signaling Technology), and GAPDH (sc32233, Santa Cruz Biotechnology), and polyclonal antibodies against LC3 (#4108, Cell Signaling Technology), COX4 (#4844, Cell Signaling Technology), and BAX (#2772, Cell Signaling Technology).

Quantitative real-time PCR

Total RNA extraction and quantitative PCR (qPCR) were performed as described previously, with some modifications. Briefly, total RNA was extracted using an RNeasy Mini Kit (Qiagen), RNA was converted to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO), and the reactions were run in an Applied Biosystems QuantStudio3 for the THUNDERBIRD SYBR qPCR Mix. For measurement of mtDNA in cytosol, heart homogenates were prepared in 100 mM Tricine-NaOH solution, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA and protease inhibitor, then were centrifuged at 700g for 10 min at 4 °C[28]. Protein concentration and volume of the supernatant were normalized, followed by centrifugation at 100,000g for 60 min at 4 °C for the production of a supernatant corresponding to the cytosolic fraction. DNA was isolated from 200 μl of the cytosolic fraction as described above by using DNeasy Blood & Tissue Kit (Qiagen). The copy number of DNA encoding *Dloop*, *Tert*, and *B2m* were measured by quantitative real-time PCR with same volume of the DNA solution. Cytosolic fraction of NRVMs was prepared as the same procedure described above.

The forward (F) and reverse (R) primer sequences were as follows: *Rps18* (mouse), F 5’-TTCTGGCCAACGGTCTAGACAAC-, 3’, R 5’-CCAGTGGTCTTGGTGTGCTGA-3′; *Rps18* (rat), F 5’-AAGTTTCAGCACATCCTGCGAGTA -, R 5’-TTGGTGAGGTCAATGTCTGCTTTC-3’; *Zbp1* (mouse), F 5’-AAGAGTCCCCTGCGATTATTTG-3’, R 5’-TCTGGATGGCGTTTGAATTGG-3’; *Zbp1* (rat), F 5’-AGATCCTGCAGGTGTTGAGTGATG-3’; R 5’ GCCCTCTTTCTTCAGGCGGTA-3’; *Il1b* (mouse), F 5’-TCCAGGATGAGGACATGAGCAC3’, R 5’-GAACGTCACACACCAGCAGGTTA-3’; *Il1b* (rat), F 5’ -CTACCTATGTCTTGCCCGTGGAG-, R 5’-GCGAACATCACACACTAGCAGGTC-3’; *Il6* (mouse), F 5’-CCACTTCACAAGTCGGAGGCTTA-3’, R 5’-GCAAGTGCATCATCGTTGTTCATAC-3’; *Il6* (rat), F 5’ -ATTGTATGAACAGCGATGATGCAC-, R 5’ -CCAGGTAGAAACGGAACTCCAGA-; *Tlr9* (rat), F 5’-TGCTGCCCAGTTTGTCAGAG3’, R 5’-ATGAGGCTTCAGTTCACAGGGTAG-3’; *Dloop* (mouse), F 5’-TCCTCCGTGAAACCAACAA-, R 5’-AGCGAGAAGAGGGGCATT-3’; *Tert* (mouse), F 5’-CTAGCTCATGTGTCAAGACCCTCTT-3’, R 5’-GCCAGCACGTTTCTCTCGTT-3’; *B2m* (mouse), F 5’-ATGGGAAGCCGAACATACTG-3’, R 5’-CAGTCTCAGTGGG GGTGAAT-3’.

Measurement of cytokine secretion

Using post-MI heart, the cytokine secretion of IL-1β and IL-6 were measured with Quantikine ELISA kit in accordance with the supplier’s protocol (R & D systems).

Animal models

ZBP1 knockout mice were created by professor Shizuo Akira from Osaka University[29] and kindly gifted by National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN, Japan). This strain was backcrossed with C57BL/6J mice in our laboratory. Homozygous KO mice and WT mice were used. We created MI in 12- to 13-week-old, male KO mice (KO + MI) and WT littermates (WT + MI) by ligating the left coronary artery. Sham operation without coronary artery ligation was also performed in KO (KO + sham) and WT (WT + sham) mice.

Recent studies showed that there were gender differences in inflammatory responses[30]. We used male mice and excluded female mice to exclude potential effects of gender differences on the results. All animals were randomly assigned to different experimental groups. We used randomisation within blocks based on date of birth. To minimise potential confounders such as cage location, mice were housed in the same rack. AI was aware of the group allocation during allocation, at the conduct of the experiment, the outcome assessment, and the data analysis. Data acquisition and analysis were performed by investigators who were blinded to the group assignment. Immunoblot analysis was conducted by using non-infarct area of the hearts at 3 days after coronary artery ligation. After 3 and 28 days, echocardiography was performed and then the heart was extracted for histological analysis. Heart weight and left ventricular weight were assessed at 28 days after surgery. All of the animals were included except for those died after the surgery. All procedures involving animals and animal care protocols were approved by the Committee on Ethics of Animal Experiments of the Kyushu University Graduate School of Medicine and Pharmaceutical Sciences (A29-390), and were performed in accordance with the Guideline for Animal Experiments of Kyushu University and the Guideline for the Care and Use of Laboratory Animals published by the US National Institutes of Health (revised in 2011) and comply with the ARRIVE guideline. The ARRIVE checklist is provided as supplemental data.

Pressure overload-induced heart failure model

To assess the expression of ZBP1 and its downstream signaling, pressure overload-induced heart failure model was used. The 10- to 12-week-old male C57BL/6J mice were subjected to TAC surgery. Sham-operated animals underwent the same operation without aortic constriction. The TAC or sham-operated mice were sacrificed at 4 weeks.

Echocardiography

Under light anesthesia with 1-2% isoflurane, two-dimensional targeted M-mode images were obtained from the short axis view at the papillary muscle level using a Vevo 2100 ultrasonography system (Visual Sonics, Toronto, Canada) as previously described[31]. We assessed left ventricular diastolic/systolic diameter, left ventricular ejection fraction, and fractional shortening. Fractional shortening is calculated from: %FS= [(diastolic LV diameter-systolic LV diameter)/diastolic LV diameter) × 100].

TUNEL staining

TUNEL staining was conducted as described[32]. Deparaffinized sections were incubated with proteinase K and DNA fragments were labeled with fluorescein-conjugated dUTP using in situ Apoptosis Detection kit (MK500, Takara). Nuclear density was determined by manual counting of DAPI-stained nuclei in 10 fields for each animal using a 40x objective.

Cell viability assay

Cell viability was assessed using Cell Titer Blue assays (Promega, Madison, WI) in accordance with the supplier's protocol.

Histological analyses

The LV accompanied by the septum was cut into base and apex portions, fixed with 10% formalin and submitted for Masson's trichrome staining. Collagen volume was determined by quantitative morphometry of tissue sections from the mid-LV stained with Masson's trichrome as described previously[33]. To measure infarct size 28 days after MI, the heart was excised and five-

micron sections were cut and stained with Picro-Sirius Red. Infarct size (as a percentage) was calculated as total infarct circumference divided by total cardiac circumference.

Immunohistochemistry

Heart sections fixed with 10% formalin were embedded in paraffin. After deparaffinization, endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 5 minutes. For antigen retrieval, sections were boiled for 20 minutes in citrate buffer (pH=6.0). After blocking with 3% skim milk, sections were incubated overnight at 4°C with the following antibodies: anti-mouse macrophage antibody (Mac3; BD Pharmingen, #550292), anti-mouse ZBP1 antibody (#AG-20B-0010-C100, Adipogen Life Science), or anti-rabbit RIPK3 antibody (#15828, Cell Signaling Technology), followed by incubation with biotin-conjugated secondary antibodies. The mouse isotype control (#5415, Cell Signaling Technology) or rabbit isotype control (#3900, Cell Signaling Technology) were used as a negative control. Then, the sections were washed and treated with avidin-peroxidase. The sections were stained using the DAB substrate kit (Wako Pure Chemical Industries, Osaka, Japan), and nuclei were counterstained with hematoxylin. Multiple observers who were blinded to the experiment protocol performed the quantitative analysis. All images were captured with a microscope (BZ-X800, Keyence).

Immunofluorescence

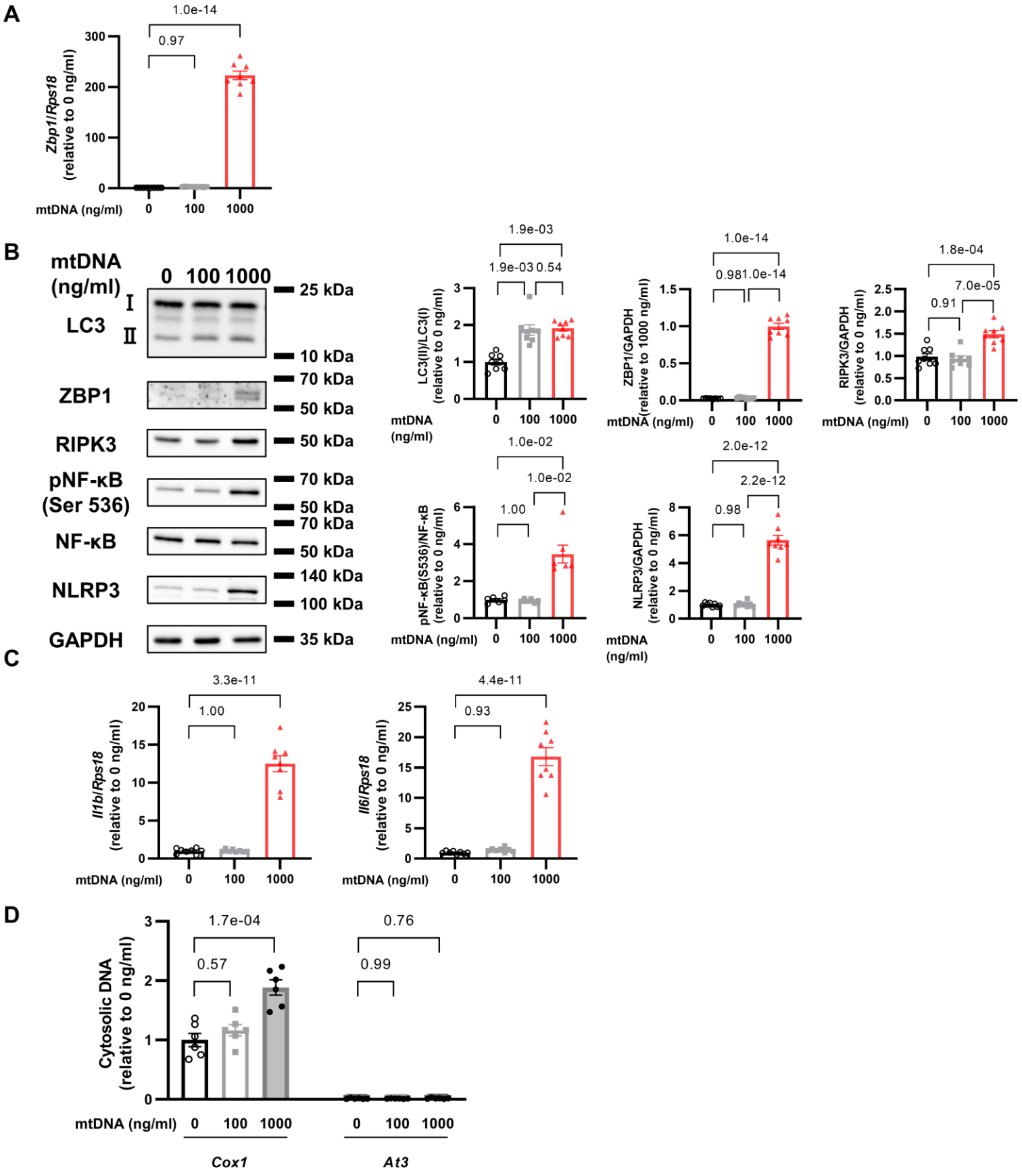
Cardiomyocytes were infected with adenovirus harboring mcherry-tagged ZBP1. FITC-labeled CpG-ODN were transfected 24 hours after infection. After 24 hours, nuclei were stained with Hoechst 33342 (Invitrogen), and cells were observed with a confocal microscope (Nikon). To assess the effects of ZBP1 on the interaction between TLR9 and CpG-ODN, we transfected FITC-labeled CpG-ODN after transfection of ZBP1 siRNA (See “Detailed methods” in Online material). Cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature followed by permeabilization in 0.1% Triton X-100 for 15 min. After blocking in 1% BSA (A8806, Sigma), cells were incubated with anti-TLR9 (# PA520203, Invitrogen) primary antibody for 1 hour at room temperature, followed by incubation for 60 min at room temperature in secondary antibodies (Donkey Anti-Rabbit IgG H&L Alexa Fluor® 647 preadsorbed, # ab150063, Abcam). The rabbit isotype control (#3900, Cell Signaling Technology) and FITC not labeled CpG-ODN were used as a negative control for TLR9 and FITC-labeled CpG-ODN, respectively. Nuclei were stained with Hoechst 33342 (Invitrogen), and cells were observed with a confocal microscope (Nikon).

Statistical analysis

All values are expressed as mean ± SEM. Normal distribution was confirmed by Shapiro-Wilk test. Statistical analyses were described in each Figure Legend. A two-way ANOVA followed by Tukey post hoc multiple comparisons test was used when there were 2 levels of grouping, and the normality was not violated. If violated, Wilcoxon rank sum test with or without Bonferroni correction was used. $P < 0.05$ was considered to be statistically significant. All analyses were performed with the SAS statistical package (version 9.4, SAS Institute, Cary, North Carolina). The authors had full access to and take full responsibility for the integrity of the data.

Supplemental Figures

1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mtDNA (ng/ml) 0 100 1000
B
mtDNA (ng/ml)
0 100 1000
LC3
I
II
25 kDa
10 kDa
ZBP1
70 kDa
50 kDa
RIPK3
50 kDa
pNF-κB (Ser 536)
70 kDa
50 kDa
NF-κB
70 kDa
50 kDa
NLRP3
140 kDa
100 kDa
GAPDH
35 kDa
1.9e-03
1.9e-03 0.54
LC3(II)/LC3(I) (relative to 0 ng/ml)
1.0e-14
0.98 1.0e-14
ZBP1/GAPDH (relative to 1000 ng/ml)
1.8e-04
7.0e-05
0.91
RIPK3/GAPDH (relative to 0 ng/ml)
1.0e-02
1.0e-02
1.00
pNF-κB(S536)/NF-κB (relative to 0 ng/ml)
2.0e-12
2.2e-12
0.98
NLRP3/GAPDH (relative to 0 ng/ml)
C
3.3e-11
1.00
Il1b/Rps18 (relative to 0 ng/ml)
4.4e-11
0.93
Il6/Rps18 (relative to 0 ng/ml)
D
1.7e-04
0.57
0.76
0.99
Cytosolic DNA (relative to 0 ng/ml)
mtDNA (ng/ml) 0 100 1000
Cox1
At3

3

Figure S1. mtDNA from rat hearts increases ZBP1 and activates inflammatory signaling in cardiomyocytes. (A) *Zbp1* levels in NRVMs treated with 100 or 1000 ng/ml of mtDNA extracted from rat hearts (n = 8). The experiment was conducted 2 times. (B) Representative immunoblots in cultured neonatal rat ventricular myocytes (NRVMs) treated with 100 or 1000 ng/ml of mtDNA extracted from rat hearts. n = 8 except for NF-κB (n = 6). The experiment was conducted 3 times except for NF-κB (2 times). (C) *Il1b* and *Il6* mRNA levels in NRVMs treated with 100 or 1000 ng/ml of mtDNA extracted from rat hearts (n = 8). The experiment was conducted 2 times. (D) Cytosolic DNA levels in NRVMs stimulated with 100 or 1000 ng/ml mtDNA was quantified by real-time PCR (n = 6). The experiment was conducted 2 times. Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (B LC3 and NF-κB; adjust = 3), and two-way ANOVA followed by Tukey multiple comparisons test (A, B except for LC3 and NF-κB, C and D).

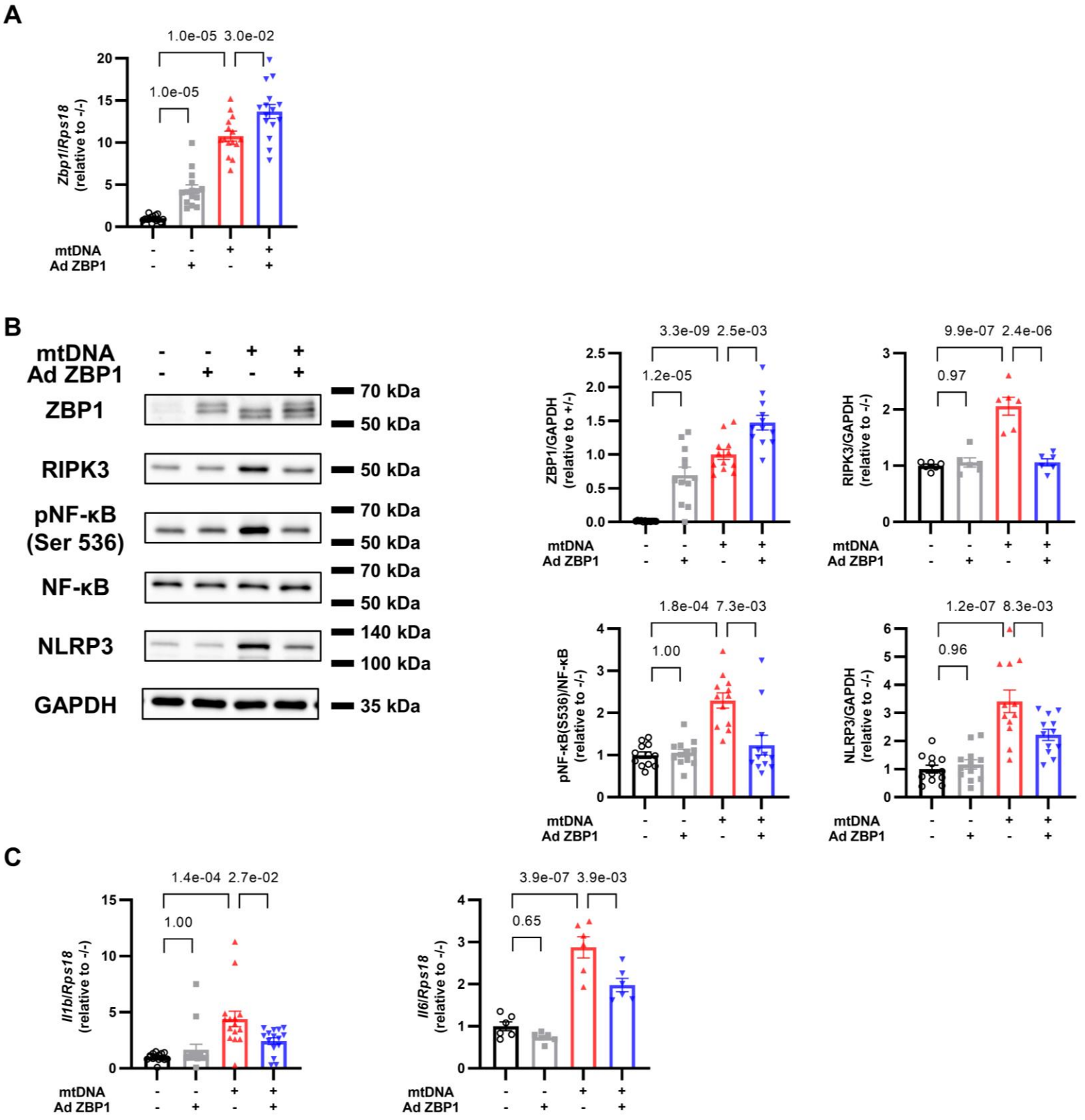
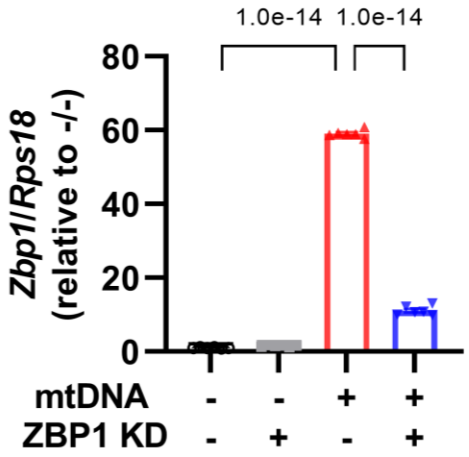

A
1.0e-05 3.0e-02
1.0e-05
Zbp1/Rps18 (relative to -/-)
mtDNA
Ad ZBP1
B
mtDNA
Ad ZBP1
ZBP1
RIPK3
pNF-κB (Ser 536)
NF-κB
NLRP3
GAPDH
70 kDa
50 kDa
70 kDa
50 kDa
70 kDa
50 kDa
140 kDa
100 kDa
35 kDa
3.3e-09 2.5e-03
1.2e-05
ZBP1/GAPDH (relative to +/-)
9.9e-07 2.4e-06
0.97
RIPK3/GAPDH (relative to -/-)
1.8e-04 7.3e-03
1.00
pNF-κB(S536)/NF-κB (relative to -/-)
1.2e-07 8.3e-03
0.96
NLRP3/GAPDH (relative to -/-)
C
1.4e-04 2.7e-02
1.00
Il1b/Rps18 (relative to -/-)
3.9e-07 3.9e-03
0.65
Il6/Rps18 (relative to -/-)

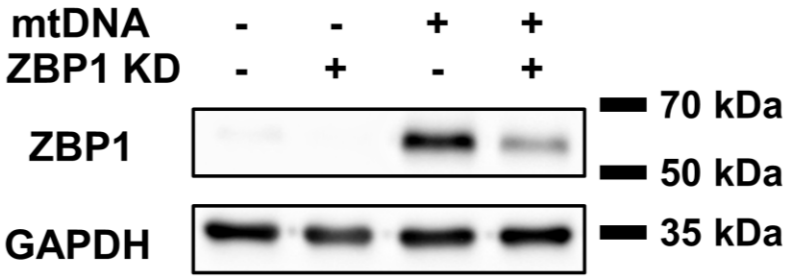
Figure S2. ZBP1 overexpression attenuates inflammatory responses in cardiomyocyte treated with mtDNA.

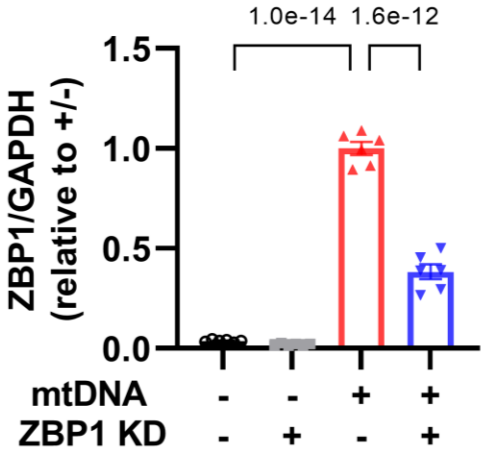
(A) mRNA levels of ZBP1 in NRVMs treated with or without adenovirus harboring ZBP1 (1 moi) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours (n = 15). The experiment was conducted 5 times. (B) Representative immunoblots of ZBP1 (n = 12, number of experiments = 4), RIPK3 (n = 6, number of experiments = 2), phosphorylated NF-κB (Ser 536), NF-κB (n = 12, number of experiments = 4), NLRP3 (n = 12, number of experiments = 4), and GAPDH in NRVMs treated with or without transfection of adenovirus harboring ZBP1 (1 moi) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. (C) mRNA levels of Il1b (n = 15, number of experiments = 5) and Il6 (n = 6, number of experiments = 2) in NRVMs treated with or without adenovirus harboring ZBP1 (1 moi) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (A, B except for NF-κB, and C *Il1b*; adjust = 3), and two-way ANOVA followed by Tukey multiple comparisons test (B except for NF-κB and C *Il6*).

A

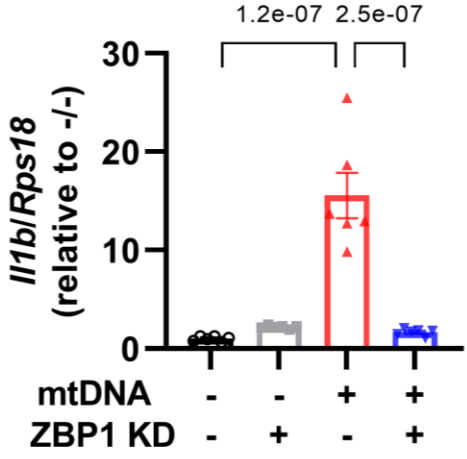

1.0e-14 1.0e-14
Zbp1/Rps18 (relative to -/-)
80
60
40
20
0
mtDNA - - + +
ZBP1 KD - + - +

B


mtDNA - - + +
ZBP1 KD - + - +
ZBP1
GAPDH
70 kDa
50 kDa
35 kDa


1.0e-14 1.6e-12
ZBP1/GAPDH (relative to +/-)
1.5
1.0
0.5
0.0
mtDNA - - + +
ZBP1 KD - + - +

C


1.2e-07 2.5e-07
Il1b/Rps18 (relative to -/-)
30
20
10
0
mtDNA - - + +
ZBP1 KD - + - +

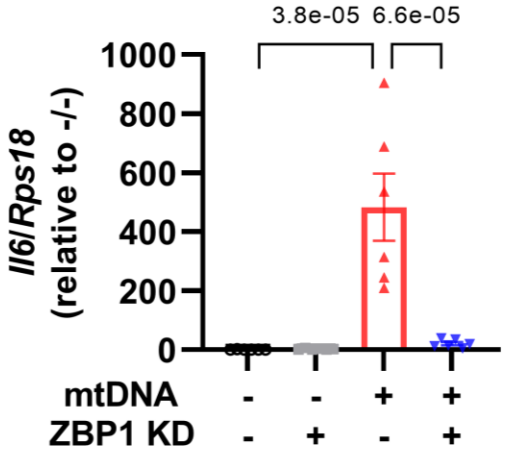

3.8e-05 6.6e-05
Il6/Rps18 (relative to -/-)
1000
800
600
400
200
0
mtDNA - - + +
ZBP1 KD - + - +

Figure S3. ZBP1 knockdown attenuates increases in inflammatory cytokines in macrophages treated with mtDNA. (A) mRNA levels of Zbp1 in indicated groups (n = 6). The experiment was conducted 2 times.(B) Representative immunoblots of ZBP1 and GAPDH in RAW264.7 treated with or without small interfering RNA (siRNA) for ZBP1 (10 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. (C) mRNA levels of Il1b and Il6 in indicated groups (n = 6). The experiment was conducted 2 times. Error bars denote standard errors. Data were analyzed using the two-way ANOVA followed by Tukey multiple comparisons test.

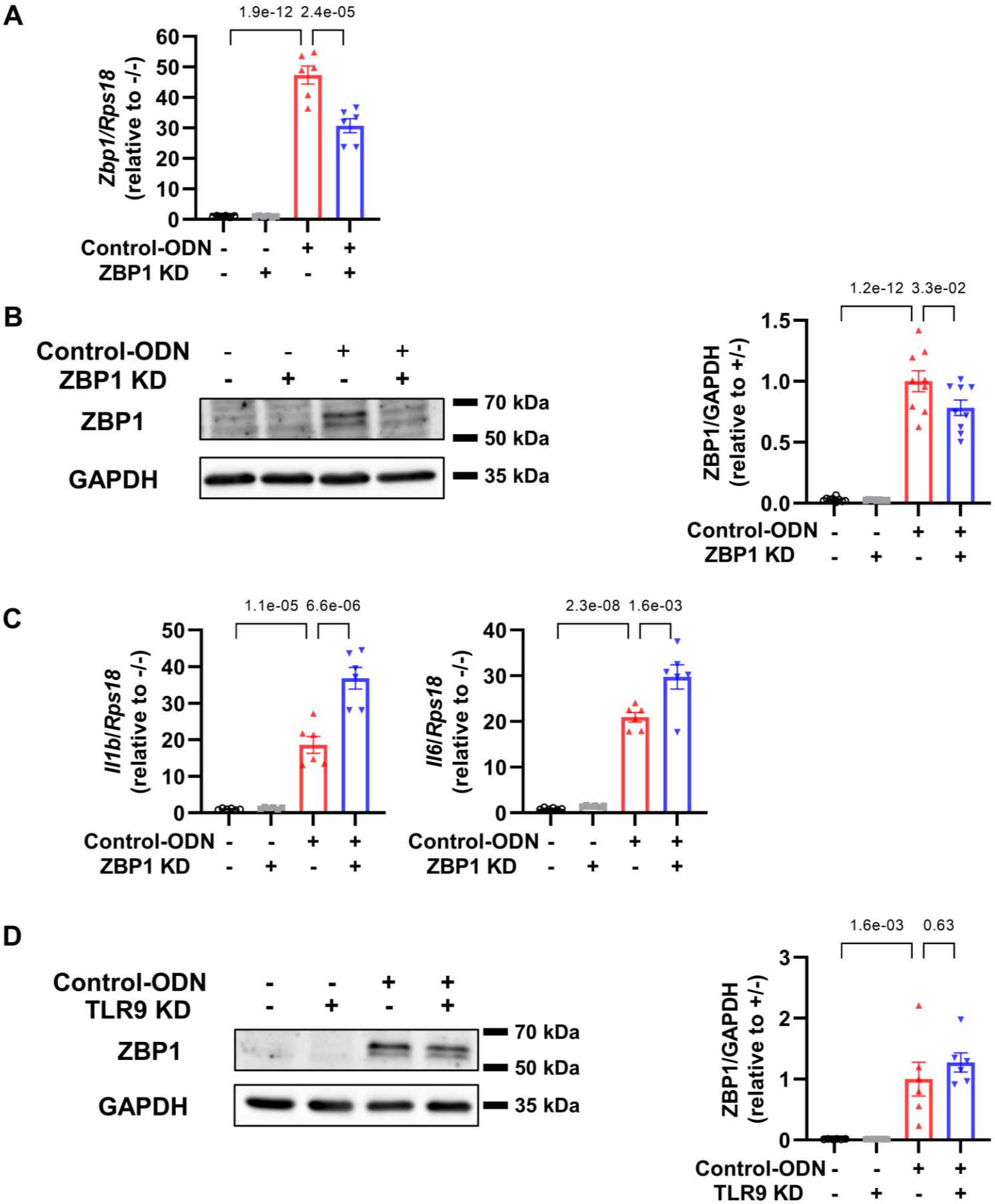

A
1.9e-12 2.4e-05
Zbp1/Rps18 (relative to -/-)
60
50
40
30
20
10
0
Control-ODN - - + +
ZBP1 KD - + - +
B
Control-ODN - - + +
ZBP1 KD - + - +
ZBP1
GAPDH
70 kDa
50 kDa
35 kDa
1.2e-12 3.3e-02
ZBP1/GAPDH (relative to +/-)
1.5
1.0
0.5
0.0
Control-ODN - - + +
ZBP1 KD - + - +
C
1.1e-05 6.6e-06
Il1b/Rps18 (relative to -/-)
50
40
30
20
10
0
Control-ODN - - + +
ZBP1 KD - + - +
2.3e-08 1.6e-03
Il6/Rps18 (relative to -/-)
40
30
20
10
0
Control-ODN - - + +
ZBP1 KD - + - +
D
Control-ODN - - + +
TLR9 KD - + - +
ZBP1
GAPDH
70 kDa
50 kDa
35 kDa
1.6e-03 0.63
ZBP1/GAPDH (relative to +/-)
3
2
1
0
Control-ODN - - + +
TLR9 KD - + - +

Figure S4. ZBP1 knockdown attenuates inflammation but TLR9 knockdown does not affect ZBP1 in cardiomyocytes treated with control-ODN.

(A) mRNA levels of Zbp1 in NRVMs treated with or without siRNA for ZBP1 (10 nM) in the presence or absence of control-ODN (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. (B) Representative immunoblots of ZBP1 and GAPDH in NRVMs treated with or without siRNA for ZBP1 (10 nM) in the presence or absence of control-ODN (1000 ng/ml) for 24 hours (n = 9). The experiment was conducted 3 times. (C) mRNA levels of Il1b and Il6 in NRVMs treated with or without siRNA for ZBP1 (10 nM) in the presence or absence of control-ODN (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. (D) Representative immunoblots of ZBP1 and GAPDH in NRVMs treated with or without small interfering RNA (siRNA) for TLR9 (10 nM) in the presence or absence of control-ODN (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. Error bars denote standard errors. Data were analyzed using the two-way ANOVA followed by Tukey multiple comparisons test.

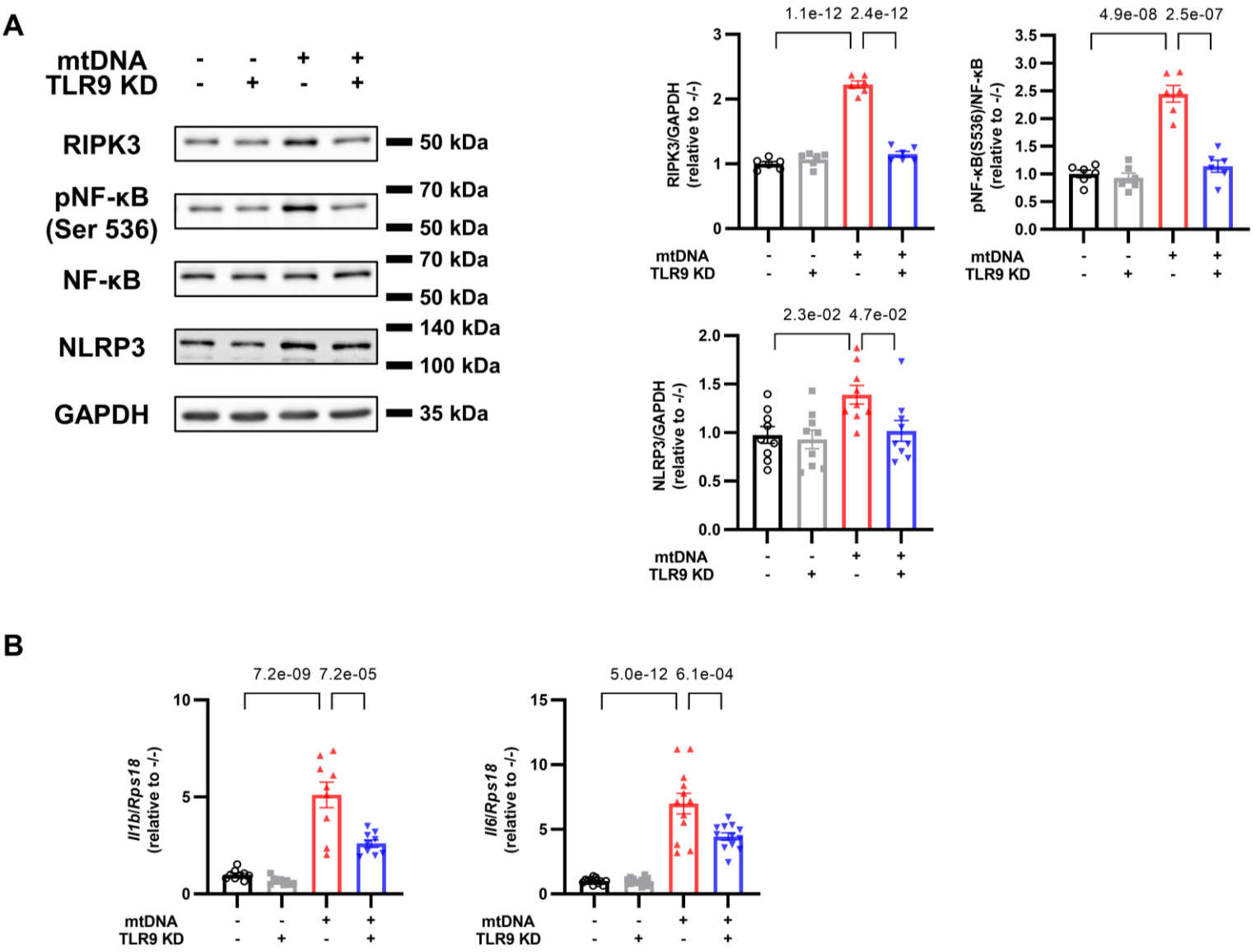
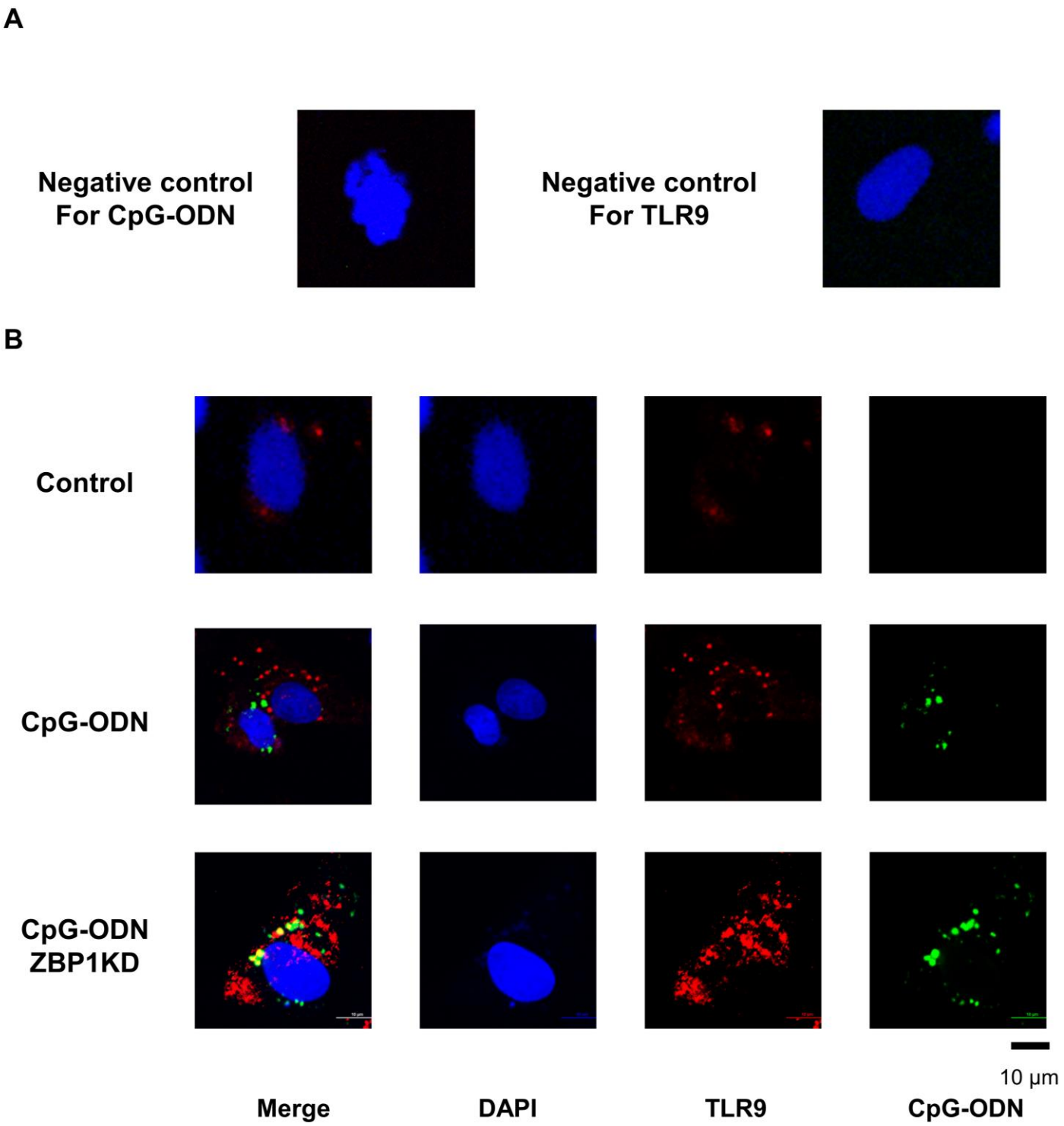


Figure S5. TLR9 knockdown attenuates downstream inflammatory signaling.

(A) Representative immunoblots of RIPK3 (n = 6, number of experiments = 2), phosphorylated NF-κB (Ser 536), NF-κB (n = 6, number of experiments = 2), NLRP3 (n = 9, number of experiments = 3), and GAPDH in NRVMs treated with or without siRNA for TLR9 (10 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. (B) mRNA levels of *Il1b* and *Il6* in NRVMs treated with or without siRNA for TLR9 (10 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours (n = 9). The experiment was conducted 3 times. Error bars denote standard errors. Data were analyzed using the two-way ANOVA followed by Tukey multiple comparisons test.


A
Negative control
For CpG-ODN
Negative control
For TLR9
B
Control
CpG-ODN
CpG-ODN
ZBP1KD
10 μm
Merge
DAPI
TLR9
CpG-ODN

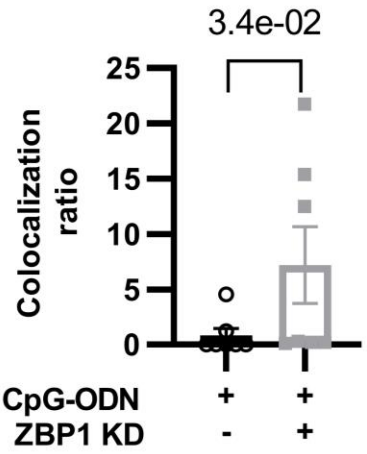

3.4e-02
Colocalization ratio
25
20
15
10
5
0
CpG-ODN
ZBP1 KD
+
-
+
+

Figure S6. ZBP1 knockdown increases interaction between TLR9 and CpG-ODN. (A) Immunofluorescence images of negative control for CpG-ODN (left panel) and TLR9 (right panel). (B) Representative immunofluorescence images in NRVMs treated with or without siRNA for ZBP1 (10 nM) in the presence or absence of FITC labeled-CpG ODN (100 nM) for 24 hours (n = 7). Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test.

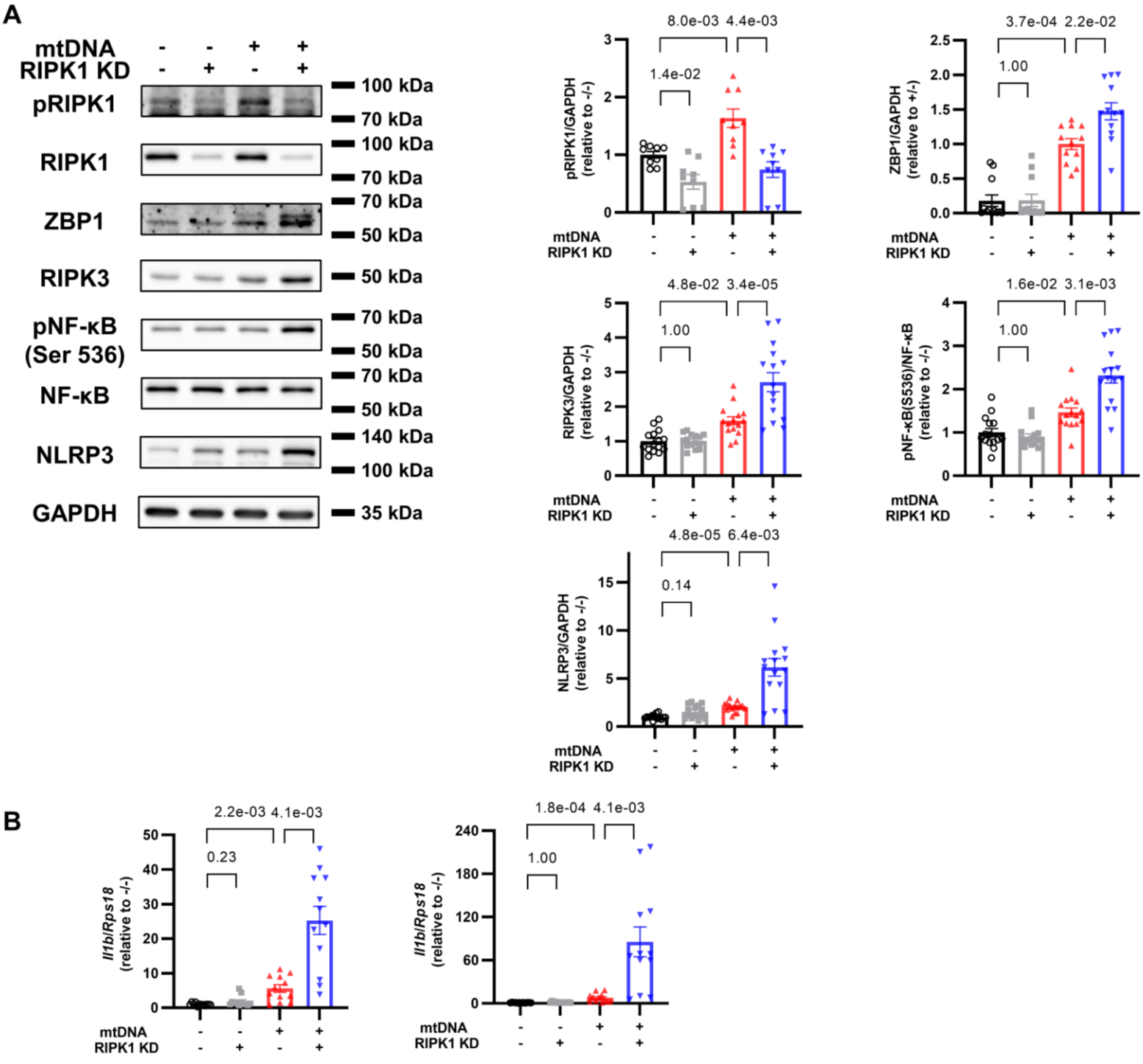

A
mtDNA - - + +
RIPK1 KD - + - +
pRIPK1
RIPK1
ZBP1
RIPK3
pNF-κB (Ser 536)
NF-κB
NLRP3
GAPDH
100 kDa
70 kDa
100 kDa
70 kDa
70 kDa
50 kDa
50 kDa
70 kDa
50 kDa
70 kDa
50 kDa
140 kDa
100 kDa
35 kDa
pRIPK1/GAPDH (relative to -/-)
8.0e-03 4.4e-03
1.4e-02
ZBP1/GAPDH (relative to +/-)
3.7e-04 2.2e-02
1.00
RIPK3/GAPDH (relative to -/-)
4.8e-02 3.4e-05
1.00
pNF-κB(S536)/NF-κB (relative to -/-)
1.6e-02 3.1e-03
1.00
NLRP3/GAPDH (relative to -/-)
4.8e-05 6.4e-03
0.14
mtDNA
RIPK1 KD
B
Il1b/Rps18 (relative to -/-)
2.2e-03 4.1e-03
0.23
1.8e-04 4.1e-03
1.00

Figure S7. RIPK1 knockdown suppresses ZBP1 expression and exacerbates downstream inflammatory signaling.

(A) Representative immunoblots of phosphorylated RIPK1, RIPK1 (n = 9, number of experiments = 3), ZBP1 (n = 12, number of experiments = 4), RIPK3 (n = 15, number of experiments = 5), phosphorylated NF-κB (Ser 536), NF-κB (n = 15, number of experiments = 5), NLRP3 (n = 15, number of experiments = 5), and GAPDH in NRVMs treated with or without siRNA for RIPK1 (5 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. (B) mRNA levels of *Il1b* and *Il6* in NRVMs treated with or without siRNA for RIPK1 (5 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours (n = 12). The experiment was conducted 4 times. Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (A except for RIPK3, and B; adjust = 3), and two-way ANOVA followed by Tukey multiple comparisons test (A RIPK3).

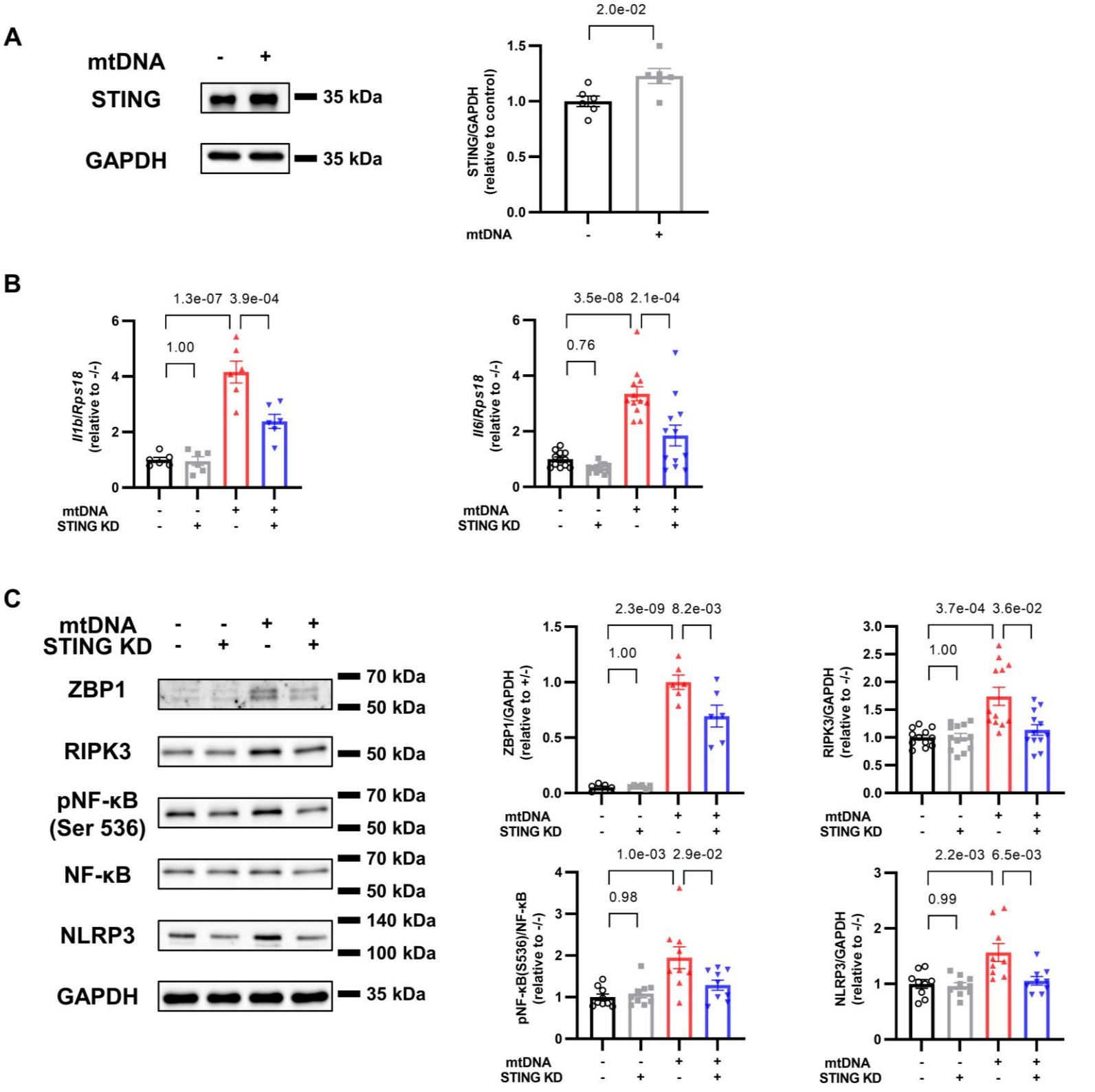
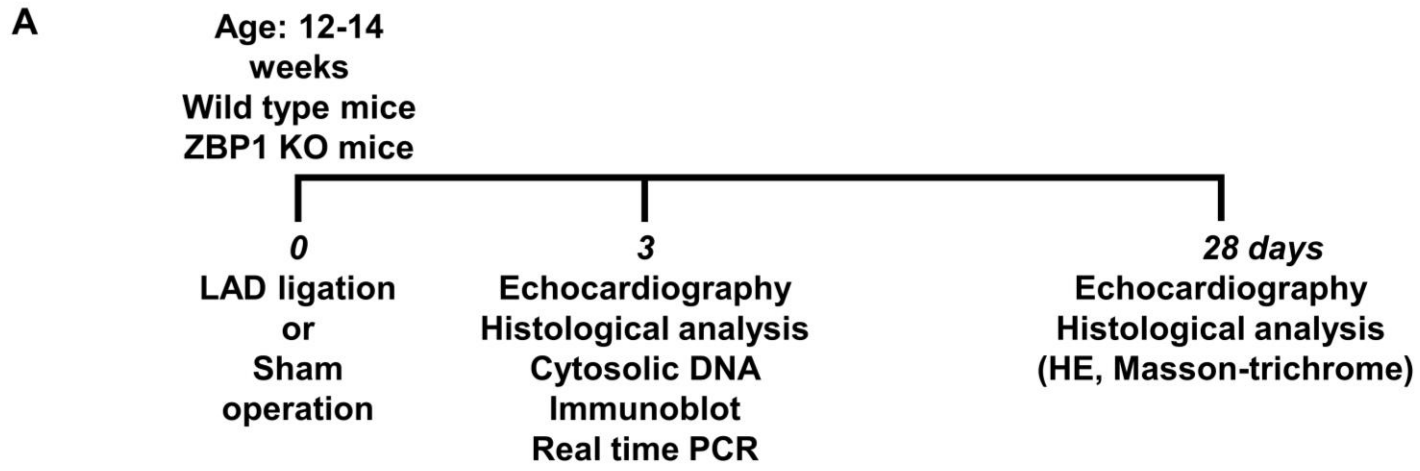
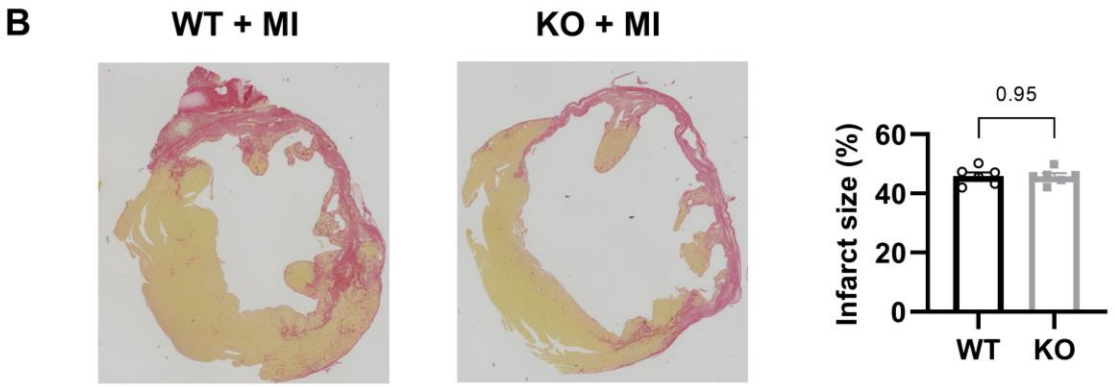

A
mtDNA - +
STING
35 kDa
GAPDH
35 kDa
2.0e-02
STING/GAPDH (relative to control)
mtDNA
B
1.3e-07 3.9e-04
1.00
Il1b/Rps18 (relative to -/-)
mtDNA
STING KD
3.5e-08 2.1e-04
0.76
Il6/Rps18 (relative to -/-)
C
mtDNA - - + +
STING KD - + - +
ZBP1
70 kDa
50 kDa
RIPK3
50 kDa
pNF-κB (Ser 536)
70 kDa
50 kDa
NF-κB
70 kDa
50 kDa
NLRP3
140 kDa
100 kDa
GAPDH
35 kDa
2.3e-09 8.2e-03
1.00
ZBP1/GAPDH (relative to +/-)
3.7e-04 3.6e-02
RIPK3/GAPDH (relative to -/-)
1.0e-03 2.9e-02
0.98
pNF-κB(S536)/NF-κB (relative to -/-)
2.2e-03 6.5e-03
0.99
NLRP3/GAPDH (relative to -/-)

Figure S8. STING knockdown suppresses ZBP1 expression and attenuates inflammatory signaling.

(A) Representative immunoblots in NRVMs treated with or without mtDNA (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. (B) mRNA levels of *Il1b* (n = 6, number of experiments = 2) and *Il6* (n = 12, number of experiments = 4) in NRVMs treated with or without siRNA for STING (1 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. (C) Representative immunoblots of ZBP1 (n = 6, number of experiments = 2), RIPK3 (n = 12, number of experiments = 4), phosphorylated NF-κB (Ser 536), NF-κB (n = 9, number of experiments = 3), NLRP3 (n = 9, number of experiments = 3), and GAPDH in NRVMs treated with or without siRNA for STING (1 nM) in the presence or absence of mtDNA (1000 ng/ml) for 24 hours. Error bars denote standard errors. Data were analyzed using the Student's t-test (A), Wilcoxon rank sum test (C RIPK3; adjust = 3), and two-way ANOVA followed by Tukey multiple comparisons test (B and C except for RIPK3).


A
Age: 12-14 weeks
Wild type mice
ZBP1 KO mice
0
LAD ligation or Sham operation
3
Echocardiography
Histological analysis
Cytosolic DNA
Immunoblot
Real time PCR
28 days
Echocardiography
Histological analysis
(HE, Masson-trichrome)


B
WT + MI
KO + MI
0.95
Infarct size (%)
60
40
20
0
WT
KO

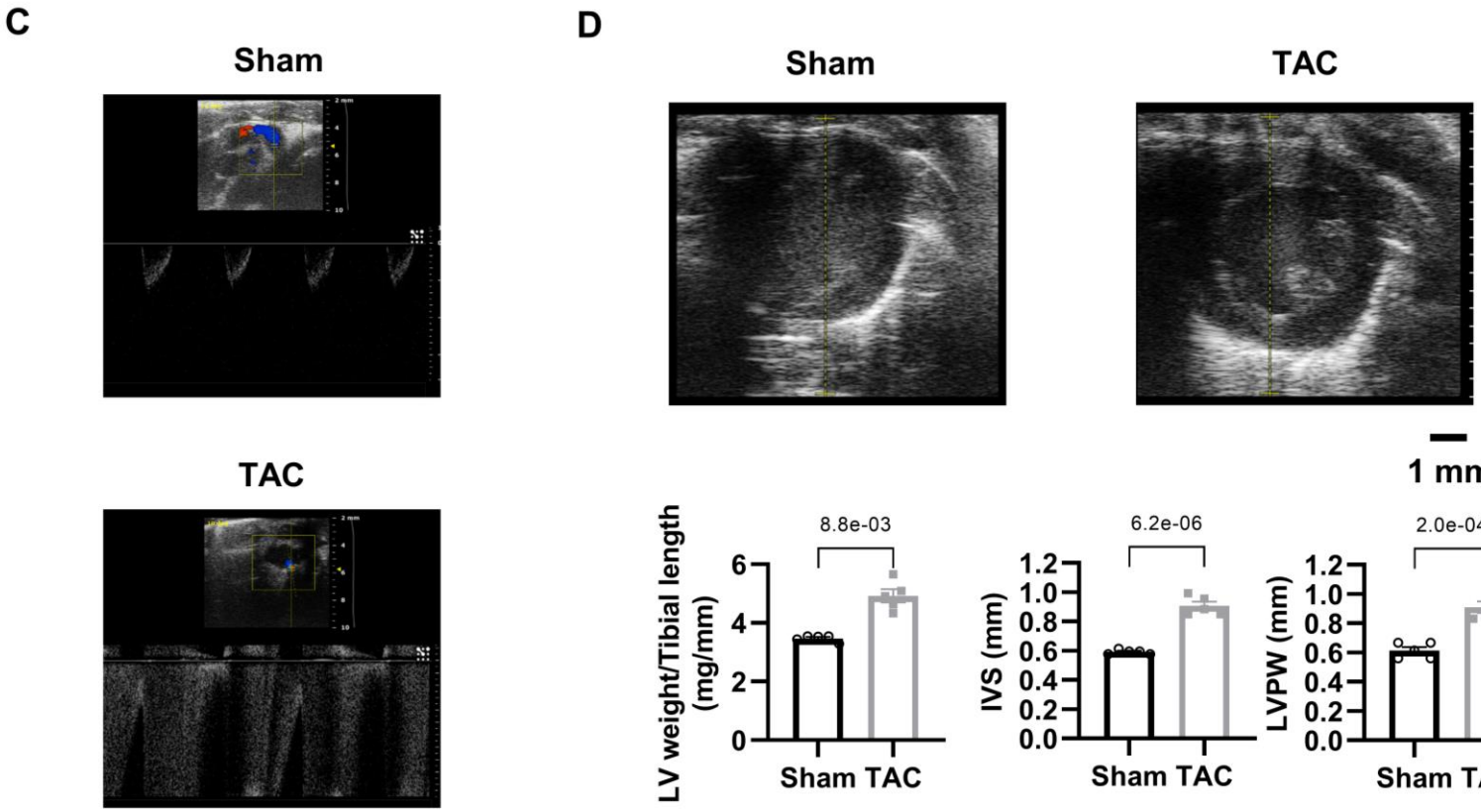

C
Sham
TAC
D
Sham
TAC
1 mm
8.8e-03
6.2e-06
2.0e-04
LV weight/Tibial length (mg/mm)
IVS (mm)
LVPW (mm)
Sham TAC
Sham TAC
Sham TAC

Figure S9. Animal experiment protocol and pressure overload model.

(A)Twelve to fourteen-weeks-old wild type or ZBP1 homozygous knockout (KO) mice were subjected to left anterior descending artery ligation or sham operation. Echocardiography, histological analysis, cytosolic DNA measurement, immunoblot, and real time PCR was conducted by using hearts at 3 days after operation. After 28 days, echocardiography and histological analysis was performed. (B) Picro-Sirius Red-stained heart section in each group. Infarct size was assessed with infarct circumference/cardiac circumference in each group 28 days after MI (n = 6). (C and D) The representative echocardiographic color Doppler (C) or 2D images (D) of mice hearts 7 days after transverse aortic constriction or sham operation (n = 5). Error bars denote standard errors. Data were analyzed using the Student's t-test (B and D interventricular septum thickness, left ventricular posterior wall thickness), Wilcoxon rank sum test (D left ventricular weight).

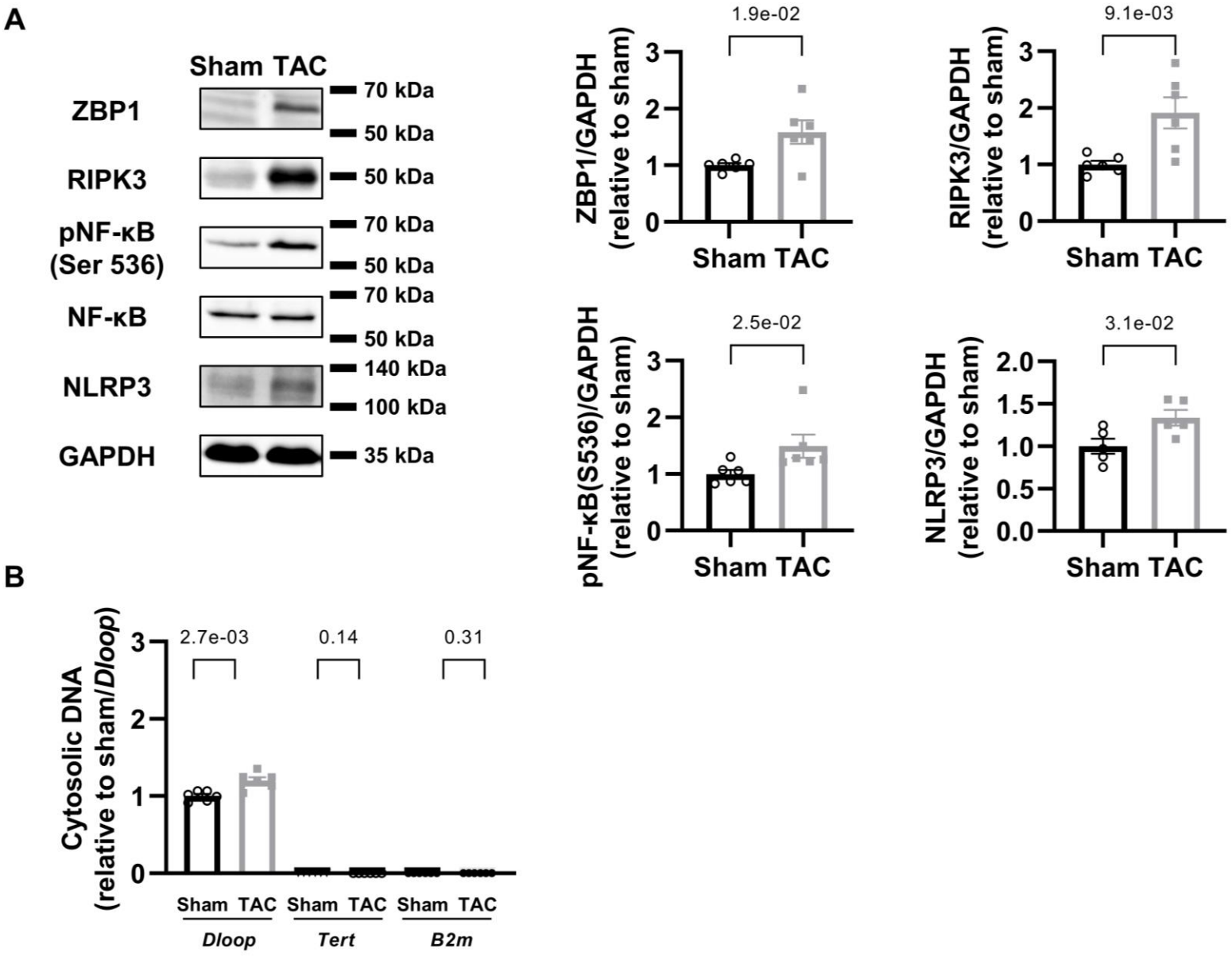


Figure S10. Myocardial ZBP1 and cytosolic mtDNA are increased in pressure overload model.

(A) Representative immunoblots of ZBP1, RIPK3, phosphorylated NF-κB (Ser 536), NF-κB, NLRP3, and GAPDH of mice hearts 28 days after transverse aortic constriction (TAC) or sham operation. n = 6 except for NLRP3 (n = 5). The experiment was conducted 2 times. (B) DNA levels in this cytosolic fraction was quantified by real-time PCR (n = 6). The experiment was conducted 2 times. Data were analyzed using the Student's t test except for A NF-κB (Wilcoxon rank sum test).

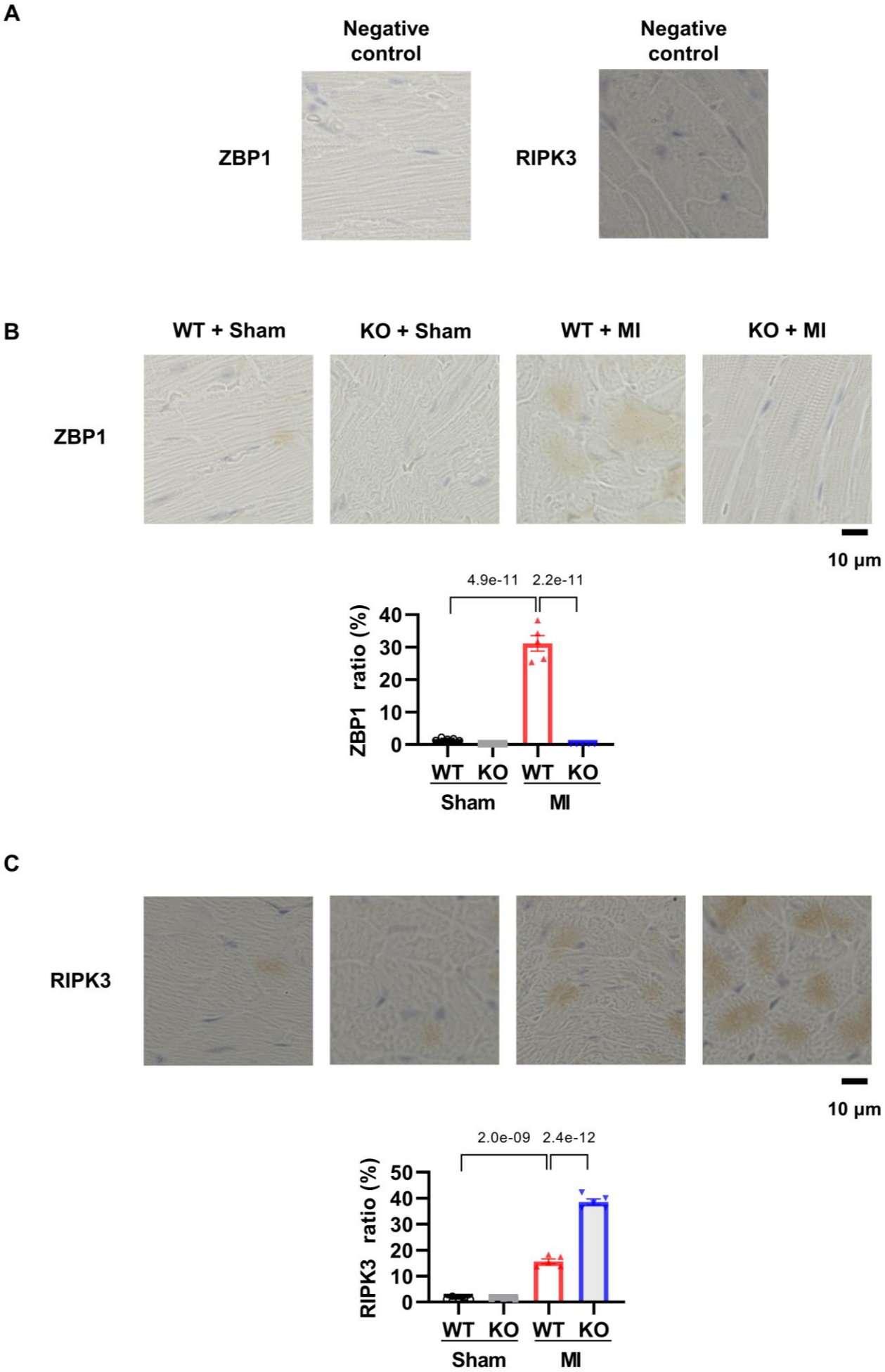
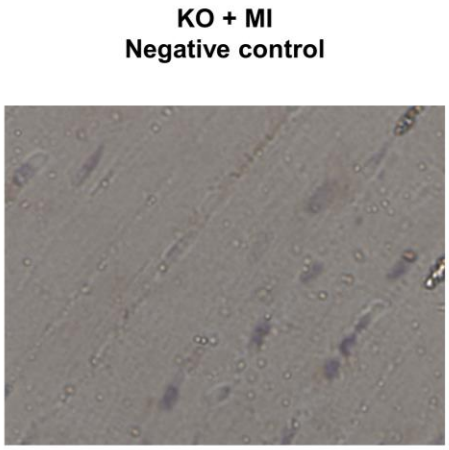
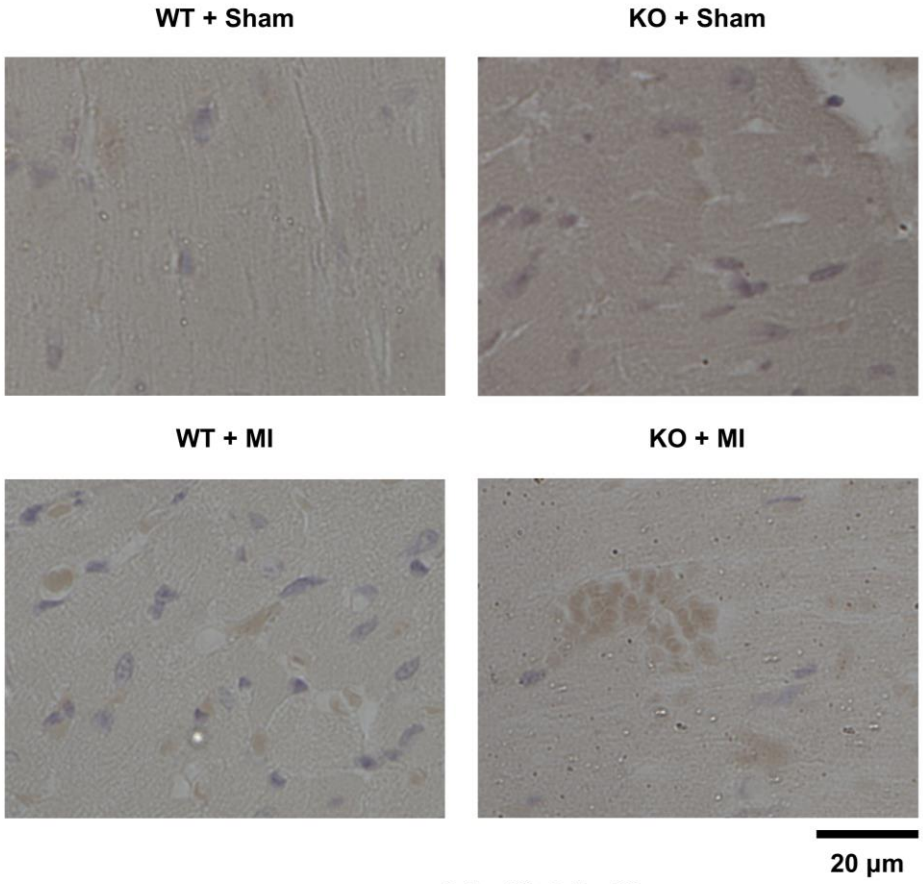

A
Negative control
Negative control
ZBP1
RIPK3
B
WT + Sham
KO + Sham
WT + MI
KO + MI
ZBP1
10 μm
4.9e-11
2.2e-11
ZBP1 ratio (%)
40
30
20
10
0
WT
KO
WT
KO
Sham
MI
C
RIPK3
10 μm
2.0e-09
2.4e-12
RIPK3 ratio (%)
50
40
30
20
10
0
WT
KO
WT
KO
Sham
MI

Figure S11. Assessment of ZBP1 and RIPK3 expression in cardiomyocyte of heart tissues. (A) Immunohistochemistry images of negative control for ZBP1 (left panel) and RIPK3 (right panel). (B) The representative immunohistochemistry images of ZBP1 of mice hearts 3 days after MI operation (n = 5). (C) The representative immunohistochemistry images of RIPK3 of mice hearts 3 days after MI operation (n = 5). Error bars denote standard errors. Data were analyzed using the two-way ANOVA followed by Tukey multiple comparisons test.

A


KO + MI
Negative control

B


WT + Sham
KO + Sham
WT + MI
KO + MI
20 µm

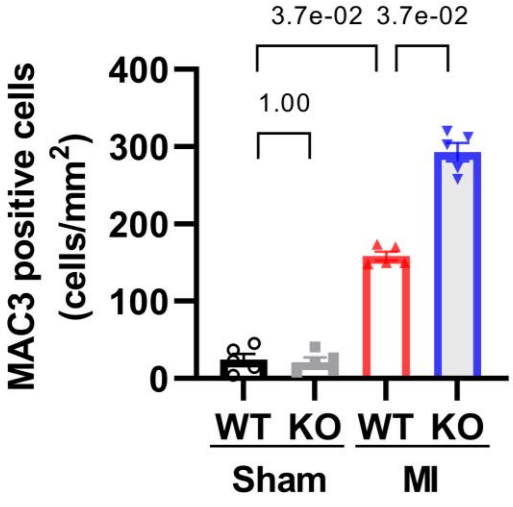

3.7e-02
3.7e-02
1.00
MAC3 positive cells
(cells/mm2)
400
300
200
100
0
WT
KO
WT
KO
Sham
MI

Figure S12. ZBP1 knockout increases macrophage infiltration into post-MI hearts.

(A) Immunohistochemistry images of negative control for mac 3 in KO + MI mice. (B) The representative immunohistochemistry images of mac3 of mice hearts 3 days after MI operation (n = 5). Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (adjust = 3).

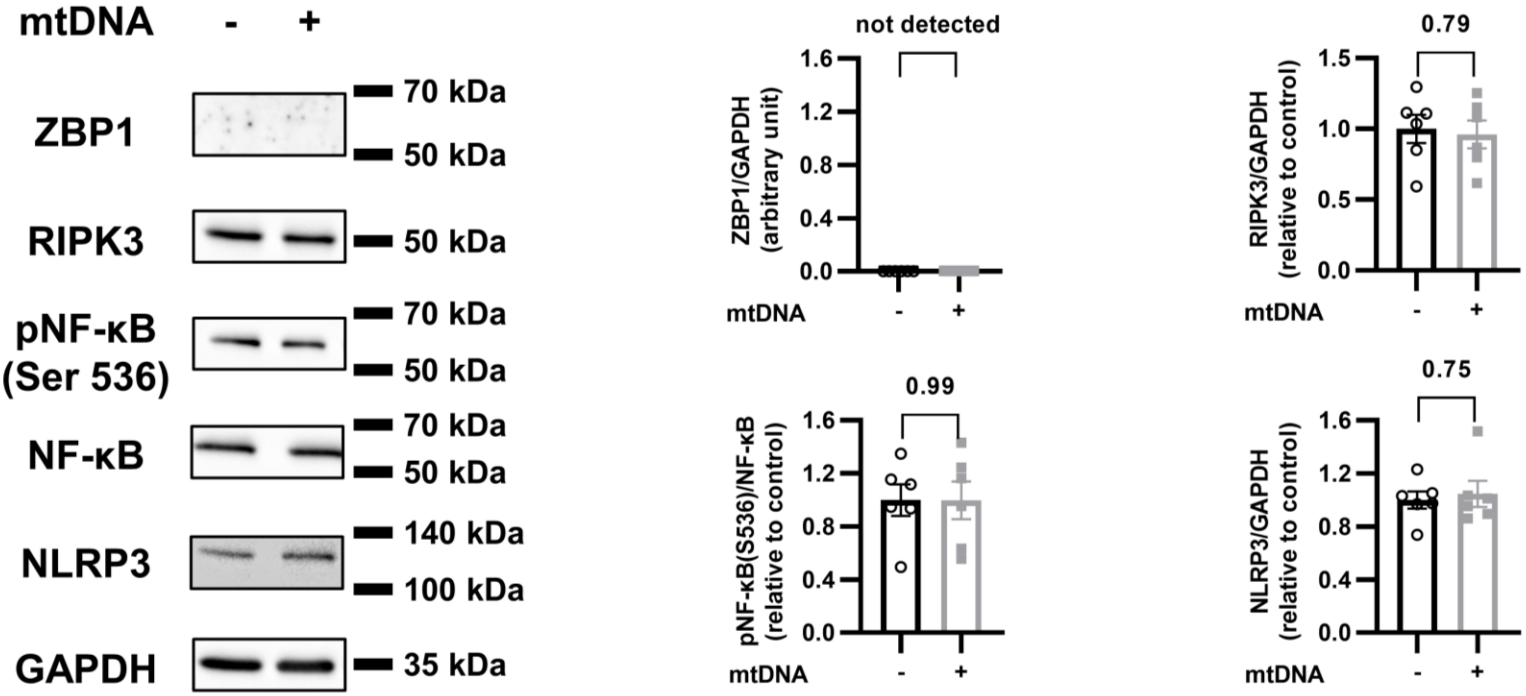
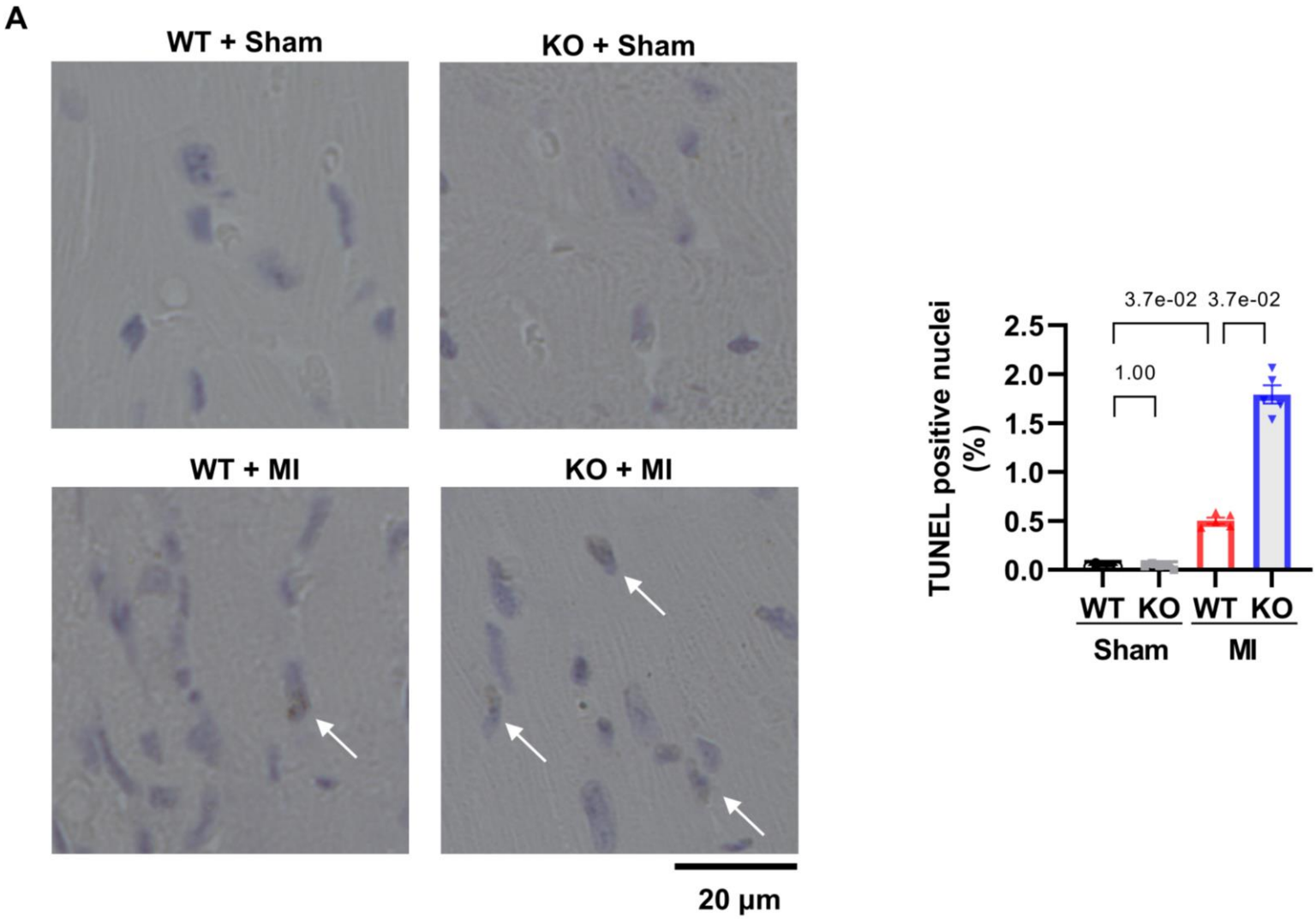


Figure S13. Administration of mtDNA without lipofectamine does not affect ZBP1 pathway.

Representative immunoblots in NRVMs treated by mtDNA without lipofectamine (1000 ng/ml) for 24 hours (n = 6). The experiment was conducted 2 times. Error bars denote standard errors. Data were analyzed using the Student's t-test except for NLRP3 (Wilcoxon rank sum test).


A
WT + Sham
KO + Sham
WT + MI
KO + MI
20 μm
TUNEL positive nuclei (%)
3.7e-02
3.7e-02
1.00
2.5
2.0
1.5
1.0
0.5
0.0
WT KO WT KO
Sham MI

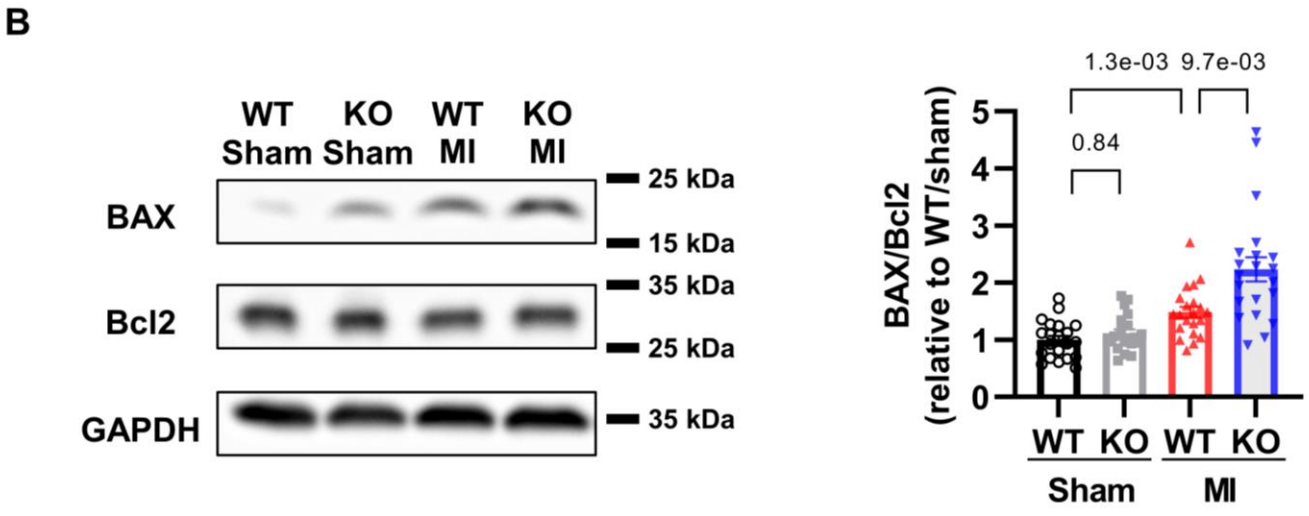

B
WT Sham
KO Sham
WT MI
KO MI
BAX
Bcl2
GAPDH
25 kDa
15 kDa
35 kDa
25 kDa
35 kDa
BAX/Bcl2 (relative to WT/sham)
1.3e-03
9.7e-03
0.84
5
4
3
2
1
0
WT KO WT KO
Sham MI

Figure S14. ZBP1 knockout exacerbates myocardial apoptosis after MI.

(A) TUNEL stained heart section in each group. Arrow heads indicate TUNEL-positive nuclei. The percentage of TUNEL-positive nuclei in the heart was assessed in non-infarcted area (n = 5). (B) Representative immunoblots of BAX, Bcl2, and GAPDH in wild type and ZBP1 knockout mice hearts 3 days after left anterior descending artery ligation or sham operation (n = 21). The experiment was conducted 7 times. Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (adjust = 3).

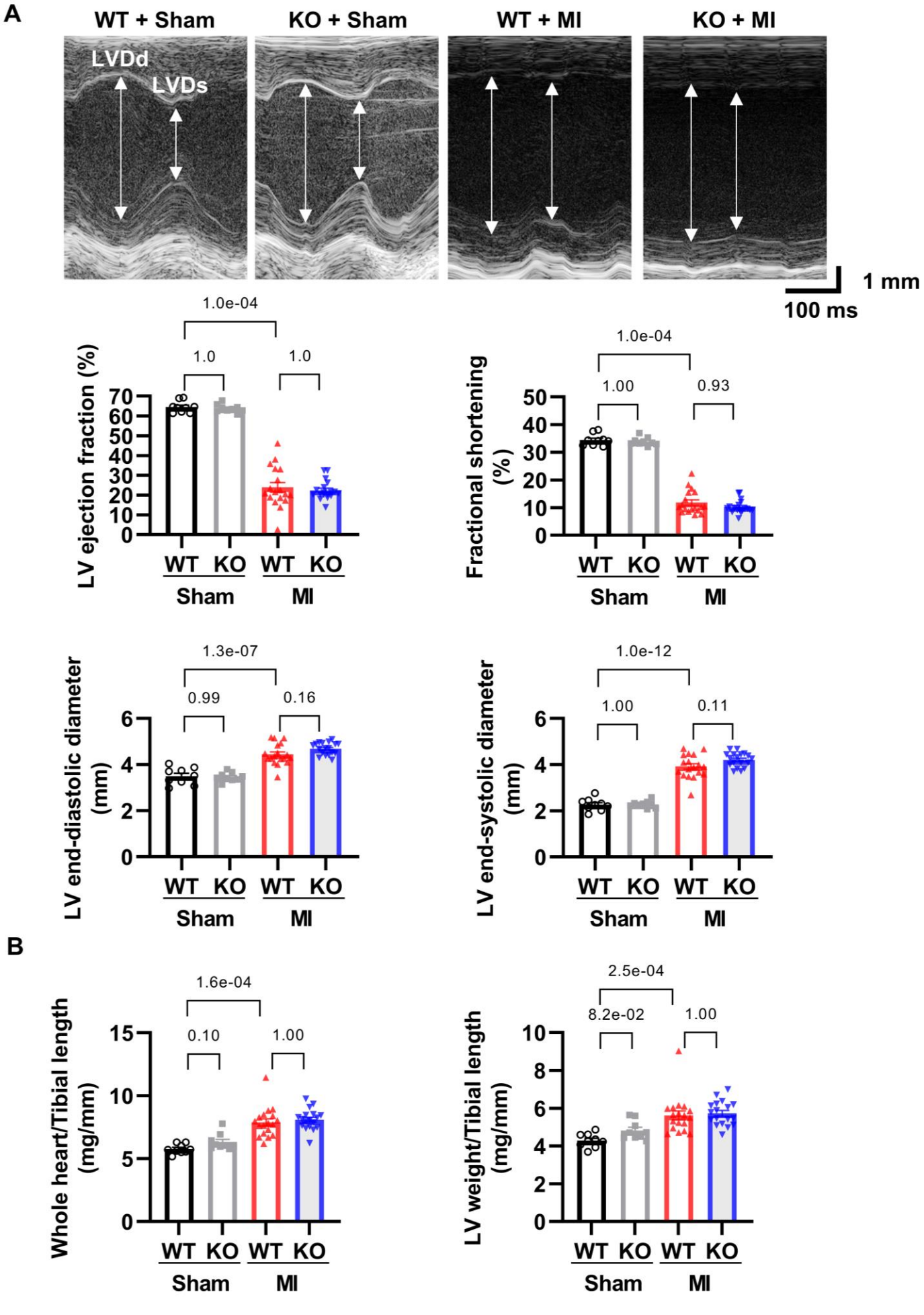

A
WT + Sham
KO + Sham
WT + MI
KO + MI
LVDd
LVDs
1 mm
100 ms
LV ejection fraction (%)
1.0e-04
1.0
1.0
Fractional shortening (%)
1.00
0.93
LV end-diastolic diameter (mm)
1.3e-07
0.99
0.16
LV end-systolic diameter (mm)
1.0e-12
1.00
0.11
WT KO WT KO
Sham MI
B
Whole heart/Tibial length (mg/mm)
1.6e-04
0.10
1.00
LV weight/Tibial length (mg/mm)
2.5e-04
8.2e-02
1.00

Figure S15. ZBP1 knockout does not change cardiac dysfunction and remodeling in early phase after MI.

(A) The representative echocardiographic images of ZBP1 knockout (KO) and wild type mice hearts 3 days after left anterior descending artery (LAD) ligation (n = 18) or sham operation (n = 9). Long two-way arrows and short two-way arrows indicate left ventricular end-diastolic diameter (LVDd) and left ventricular end-systolic diameter (LVDs), respectively. (B) Heart weight to tibial length (TL) ratio and left ventricle (LV) weight to TL ratio in each group (n = 18 for LAD ligation and 9 for sham operation). Error bars denote standard errors. Data were analyzed using the Wilcoxon rank sum test (A LVDd and LVDs; adjust = 3), and two-way ANOVA followed by Tukey multiple comparisons test (A LV ejection fraction, fractional shortening, and B).