Supplementary Information

S100A12 triggers NETosis to aggravate myocardial infarction injury via the Annexin A5-calcium axis

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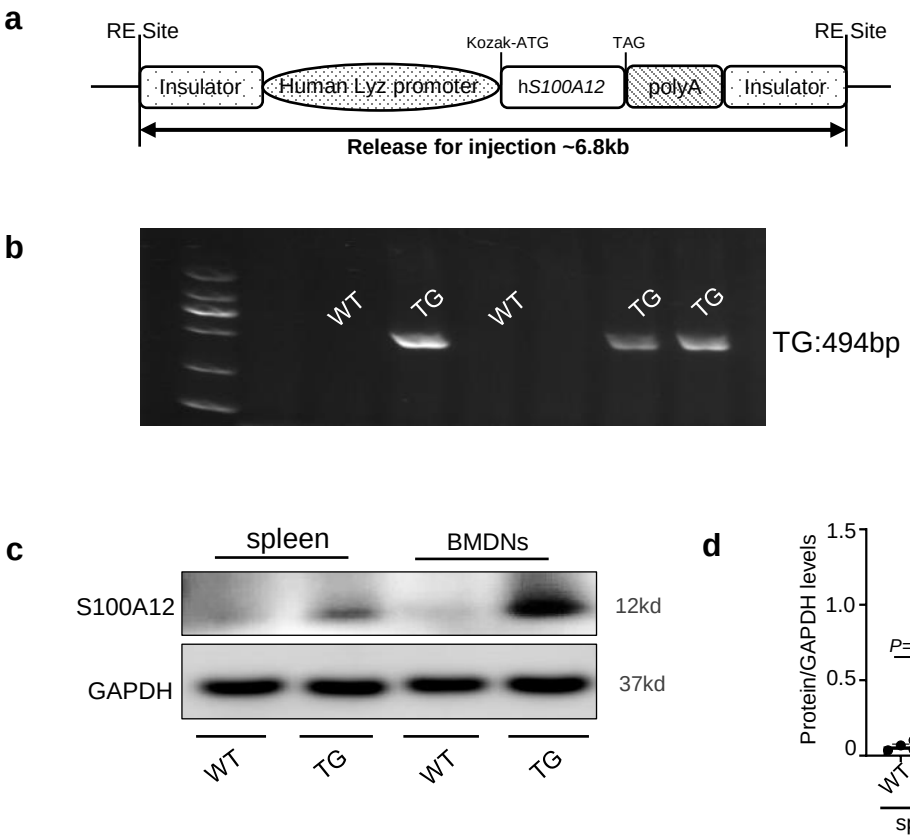
** These authors jointly supervised this work: Chenghui Yan, Yaling Han.*

Supplementary Table 1. List of the primers used in this study

Gene	Forward primers（5’→3’）	Reverse primers（5’→3’）
h-*S100A12*	TCTTGGAAGTAGTGCTATGCCCCAA	CCTGTTCATCTTGATTAGCATCCAGG
h-*MPO*	GAGCAGGACAAATACCGCACCA	AGAGAAGCCGTCCTCATACTCC
h-*NE*	TGCGCCCAACTTCGTCATGTCG	CGTAGCCGTTTTCGAAGATGCG
h-*ANXA5*	GTGGCTCTGATGAAACCCTCTC	GGCTCTCAGTTCTTCAGGTGTC
h-*HIF1α*	TATGAGCCAGAAGAACTTTTAGGC	CACCTCTTTTGGCAAGCATCCTG
h-*GAPDH*	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
m-*Gapdh*	CATCACTGCCACCCAGAAGACTG	ATGCCAGTGAGCTTCCCGTTCAGG

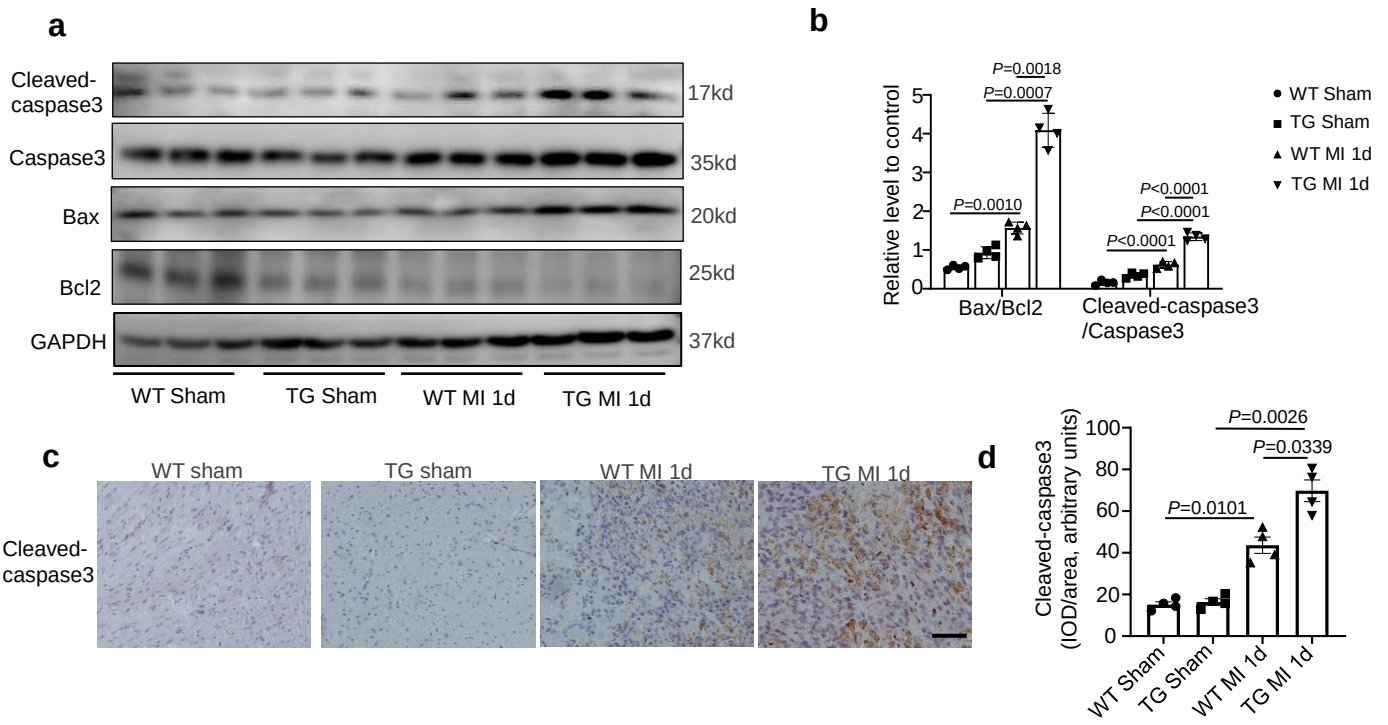
Supplementary Table 2. List of the siRNAs used in this study

Gene	Sense strand（5’→3’）	Antisense strand（5’→3’）
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
si-S100A12	CCCAUUACCACACCCACAATT	UUGUGGGUGUGGUAAUGGGTT
si-ANXA5	GCCUCAUCAGUUUCAUCAUTT	AUGAUGAAACUGAUGAGGCTT
si-HIF1α	CCUGAAGAGUGUCUGAAUUTT	AAUUUCAGACACUCUUCAGGTT



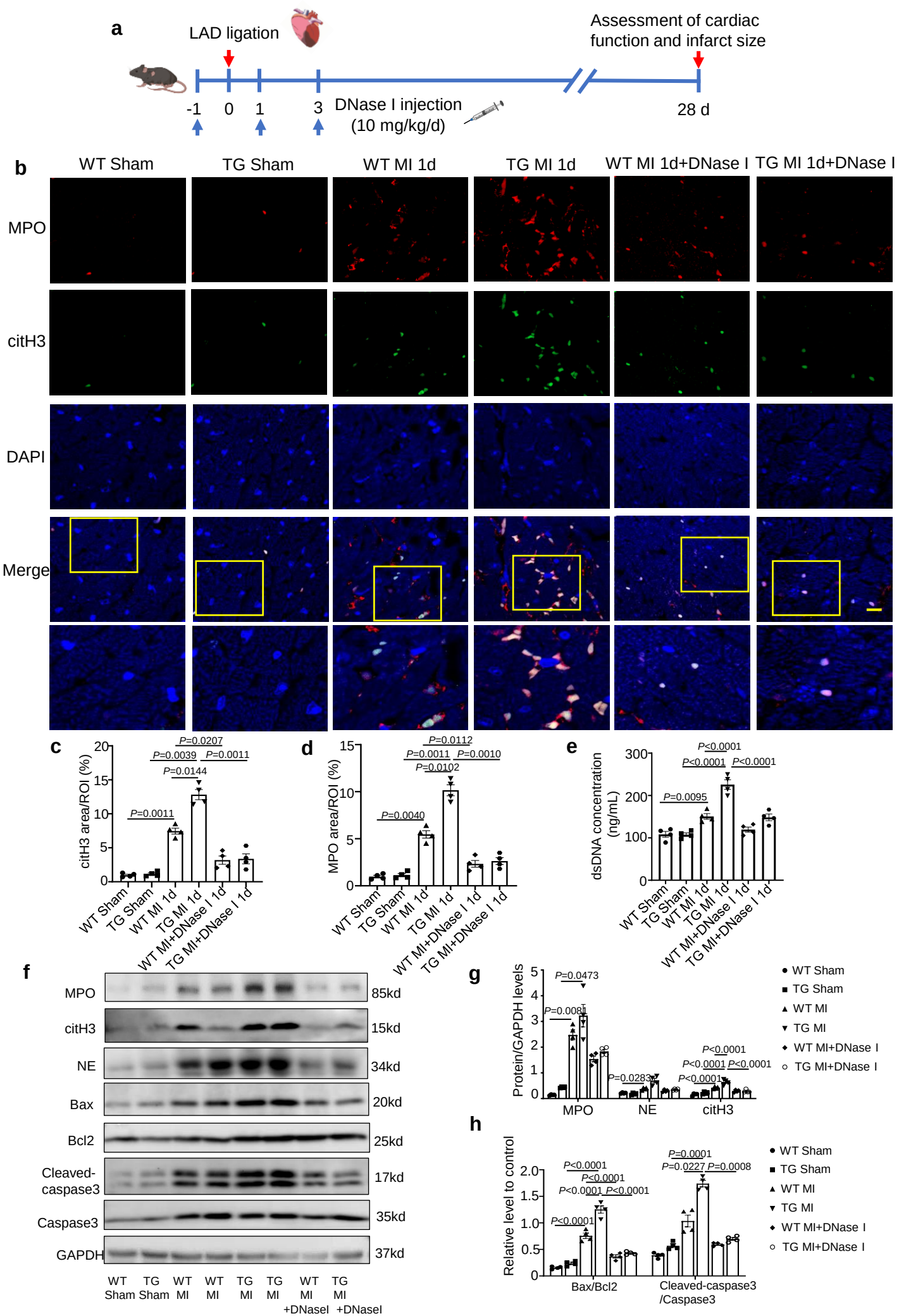
Supplementary Fig. 1. Generation of S100A12 TG mice.

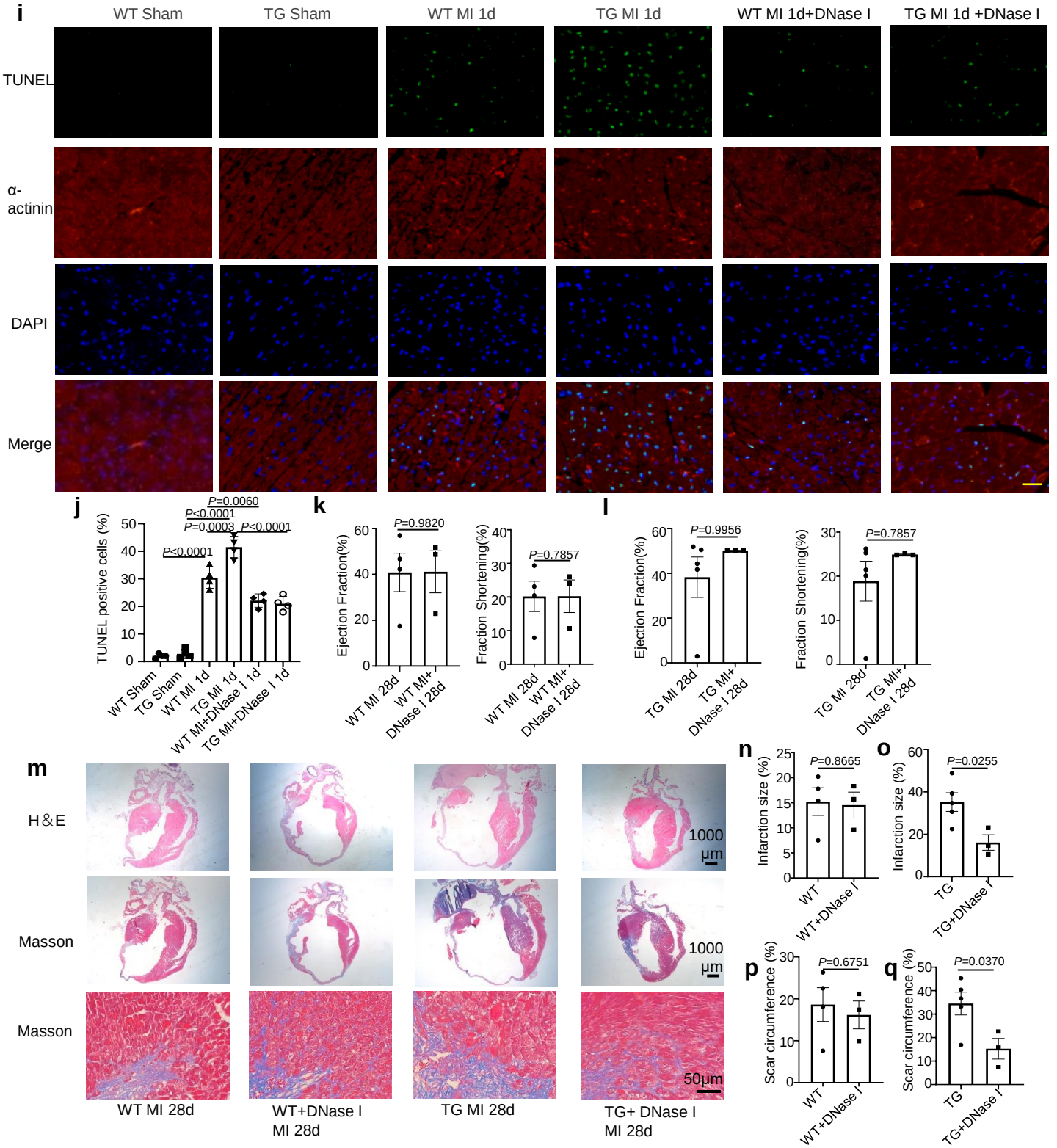
a A schematic diagram of the TG mice generation. **b** TG mice were identified using PCR analysis. **c, d** Representative blots and quantification of S100A12 protein expression in the spleen and bone marrow-derived neutrophils (BMDNs) (*n* = 4 biological replicates). Data are expressed as mean ± SEM. Statistical analyses were carried out using Brown-Forsythe and Welch's ANOVA test for (**d**). Source data are provided as a Source Data file.



Supplementary Fig. 2. S100A12 significantly increased in the apoptosis of myocardial tissue in TG mice during the early stages after MI.

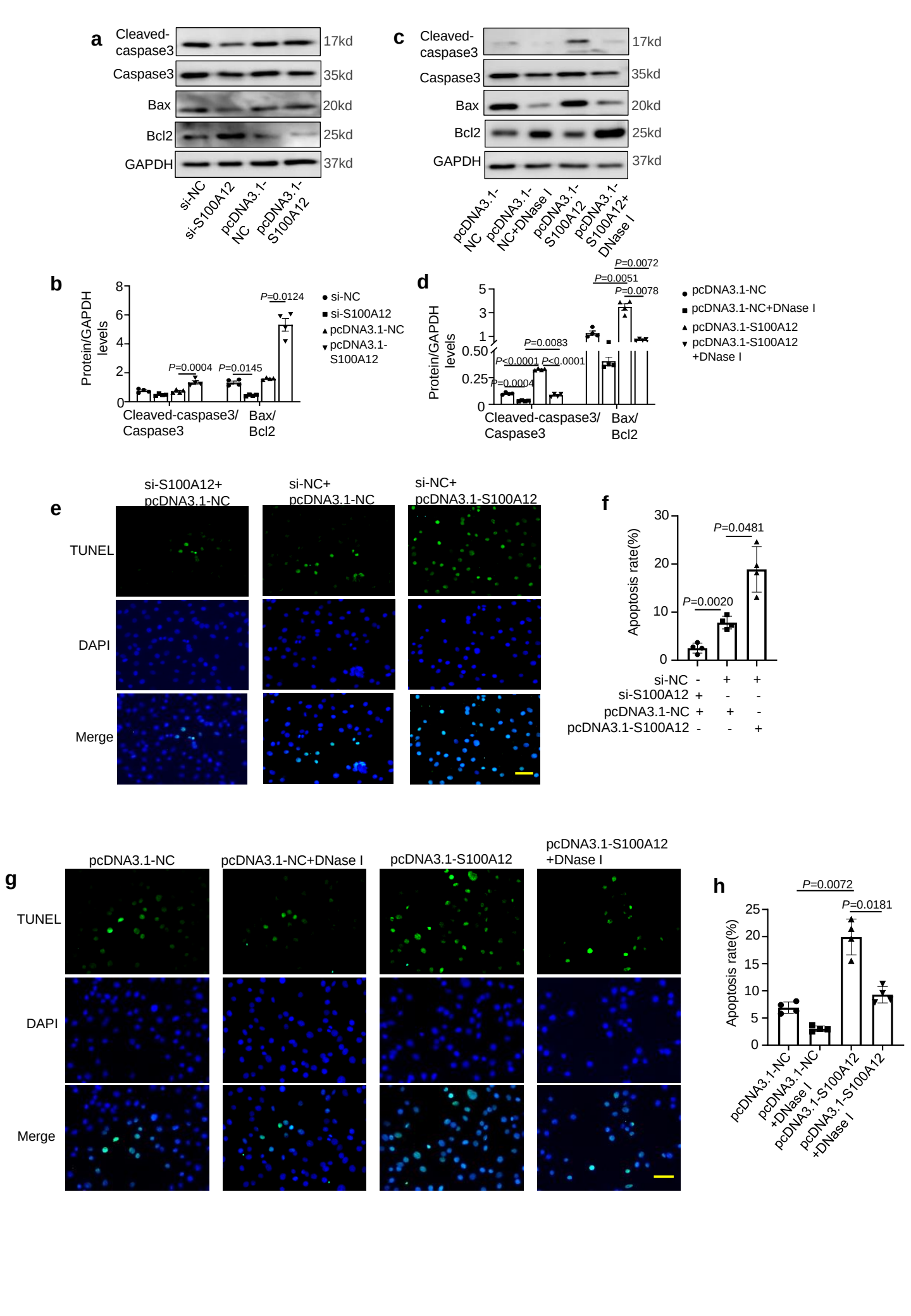
a, b Representative blots of apoptosis-related proteins (Bax, Bcl2, Cleaved-caspase3, and Caspase3) and quantification in WT and TG mice at Day 1 after MI. One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of Cleaved-caspase3/Caspase3,and Brown-Forsythe and Welch's ANOVA test is used for analyses of Bax/Bcl2 (n = 4 biological replicates). **c, d** Representative IHC staining of Cleaved-caspase3 and quantification in WT and TG mice at Day 1 post MI (n = 4 biological replicates). Brown-Forsythe and Welch's ANOVA test is used. Scale bar, 50 μm. Data are expressed as mean ± SEM. Source data are provided as a Source Data file.


a
LAD ligation
Assessment of cardiac function and infarct size
-1
0
1
3
DNase I injection (10 mg/kg/d)
28 d
b
WT Sham
TG Sham
WT MI 1d
TG MI 1d
WT MI 1d+DNase I
TG MI 1d+DNase I
MPO
citH3
DAPI
Merge
c
citH3 area/ROI (%)
P=0.0207
P=0.0039
P=0.0011
P=0.0144
P=0.0011
WT Sham
TG Sham
WT MI 1d
TG MI 1d
WT MI+DNase I 1d
TG MI+DNase I 1d
d
MPO area/ROI (%)
P=0.0112
P=0.0011
P=0.0010
P=0.0102
P=0.0040
e
dsDNA concentration (ng/mL)
P<0.0001
P<0.0001
P<0.0001
P=0.0095
f
MPO
citH3
NE
Bax
Bcl2
Cleaved-caspase3
Caspase3
GAPDH
85kd
15kd
34kd
20kd
25kd
17kd
35kd
37kd
WT Sham
TG Sham
WT MI
WT MI +DNaseI
TG MI
TG MI +DNaseI
g
Protein/GAPDH levels
P=0.0473
P=0.0081
P=0.0283
P<0.0001
P<0.0001
P<0.0001
P<0.0001
MPO
NE
citH3
WT Sham
TG Sham
WT MI
TG MI
WT MI+DNase I
TG MI+DNase I
h
Relative level to control
P<0.0001
P<0.0001
P<0.0001
P<0.0001
P<0.0001
P=0.0001
P=0.0227
P=0.0008
Bax/Bcl2
Cleaved-caspase3 /Caspase3
WT Sham
TG Sham
WT MI
TG MI
WT MI+DNase I
TG MI+DNase I


i
WT Sham
TG Sham
WT MI 1d
TG MI 1d
WT MI 1d+DNase I
TG MI 1d +DNase I
TUNEL
α-actinin
DAPI
Merge
j
TUNEL positive cells (%)
P=0.0060
P<0.0001
P=0.0003
P<0.0001
P<0.0001
WT Sham
TG Sham
WT MI 1d
TG MI 1d
WT MI+DNase I 1d
TG MI+DNase I 1d
k
Ejection Fraction(%)
P=0.9820
Fraction Shortening(%)
P=0.7857
WT MI 28d
WT MI+ DNase I 28d
l
Ejection Fraction(%)
P=0.9956
Fraction Shortening(%)
P=0.7857
TG MI 28d
TG MI+ DNase I 28d
m
H&E
Masson
Masson
1000 μm
1000 μm
50μm
WT MI 28d
WT+DNase I MI 28d
TG MI 28d
TG+ DNase I MI 28d
n
Infarction size (%)
P=0.8665
WT
WT+DNase I
o
Infarction size (%)
P=0.0255
TG
TG+DNase I
p
Scar circumference (%)
P=0.6751
WT
WT+DNase I
q
Scar circumference (%)
P=0.0370
TG
TG+DNase I

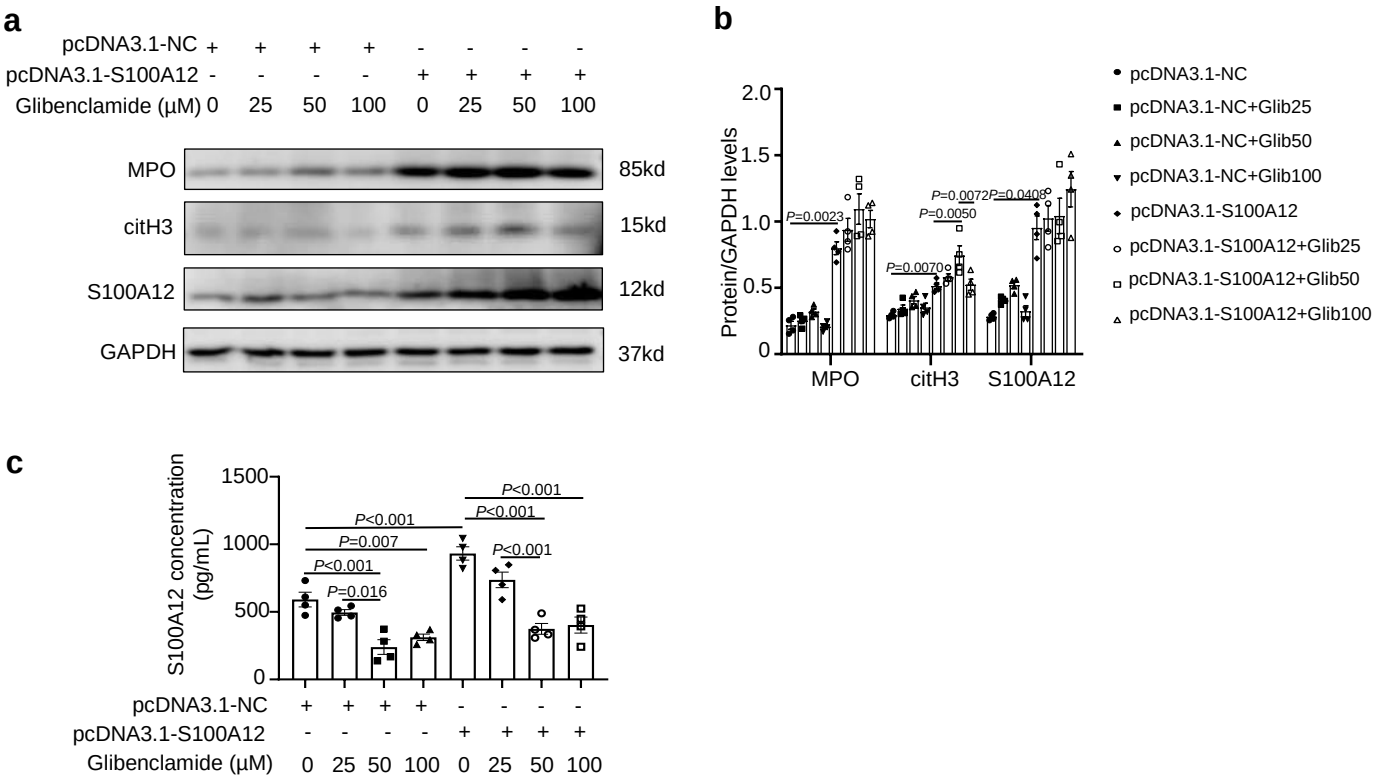
Supplementary Fig. 3. S100A12 promoted neutrophil extracellular traps (NETs)-induced cardiomyocyte apoptosis after myocardial infarction (MI).

a Mice were injected intraperitoneally with saline or DNase I (10 mg/kg/day) every other day from Day 1 before MI to Day 3 after MI. Image drawn using Figdraw software, publication licence ID: YIUAS06558. **b-d** Immunofluorescence double staining and quantifications of MPO and citH3 in the border area of DNase I-injected TG and WT mice at Day 1 after MI (n = 4 biological replicates). Scale bar, 10 μm. **e** Measurements of plasma dsDNA concentration in DNase I-injected WT and TG mice at Day 1 after MI (n = 4 biological replicates). **f–h** Representative blots of NETs- and apoptosis-related proteins and quantification in WT and TG mice at Day 1 after MI. One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of citH3, Bax/Bcl2 and Brown-Forsythe and Welch's ANOVA test is used for analyses of MPO, NE, Cleaved-caspase3/Caspase3 (n = 4 biological replicates). (**i–j**) Representative images of TdT-mediated dUTP Nick-End Labelling (TUNEL) and cardiomyocyte specific marker (α-actinin) co-staining and analysis of TUNEL positive apoptotic cells in WT and TG mice at day 1 post MI (n = 4 biological replicates). Scale bar, 50 μm. **k, l** Echocardiographic parameters were analyzed in WT and TG mice at Day 28 MI (n = 3 biological replicates for WT+DNase I and TG+DNase I group, n = 4 biological replicates for WT MI group, and n = 5 biological replicates for TG MI group). **m** Representative Masson and H＆E staining of cardiac tissue at Day 28 after MI. **n–q** Quantitative analysis of scar circumference and infarct size. Data are expressed as mean ± SEM. Statistical analyses were carried out using two-tailed unpaired t-test with Welch's correction for (**k**), (**n**), (**o**), (**p**), one-way ANOVA with Tukey's multiple comparisons test for (**e**), (**q**), Brown-Forsythe and Welch's ANOVA test for (**c**), (**d**), and Kruskal-Wallis test with Dunn's multiple-comparison test for **(l).** Source data are provided as a Source Data file.


a
Cleaved-caspase3
Caspase3
Bax
Bcl2
GAPDH
17kd
35kd
20kd
25kd
37kd
si-NC
si-S100A12
pcDNA3.1-NC
pcDNA3.1-S100A12
c
pcDNA3.1-NC
pcDNA3.1-NC+DNase I
pcDNA3.1-S100A12
pcDNA3.1-S100A12+DNase I
b
Protein/GAPDH levels
P=0.0004
P=0.0145
P=0.0124
Cleaved-caspase3/Caspase3
Bax/Bcl2
si-NC
si-S100A12
pcDNA3.1-NC
pcDNA3.1-S100A12
d
P=0.0072
P=0.0051
P=0.0078
P=0.0083
P<0.0001
P<0.0001
P=0.0004
pcDNA3.1-NC
pcDNA3.1-NC+DNase I
pcDNA3.1-S100A12
pcDNA3.1-S100A12+DNase I
e
si-S100A12+pcDNA3.1-NC
si-NC+pcDNA3.1-NC
si-NC+pcDNA3.1-S100A12
TUNEL
DAPI
Merge
f
Apoptosis rate(%)
P=0.0020
P=0.0481
si-NC
si-S100A12
pcDNA3.1-NC
pcDNA3.1-S100A12
g
pcDNA3.1-NC
pcDNA3.1-NC+DNase I
pcDNA3.1-S100A12
pcDNA3.1-S100A12+DNase I
TUNEL
DAPI
Merge
h
P=0.0072
P=0.0181
Apoptosis rate(%)
pcDNA3.1-NC
pcDNA3.1-NC+DNase I
pcDNA3.1-S100A12
pcDNA3.1-S100A12+DNase I

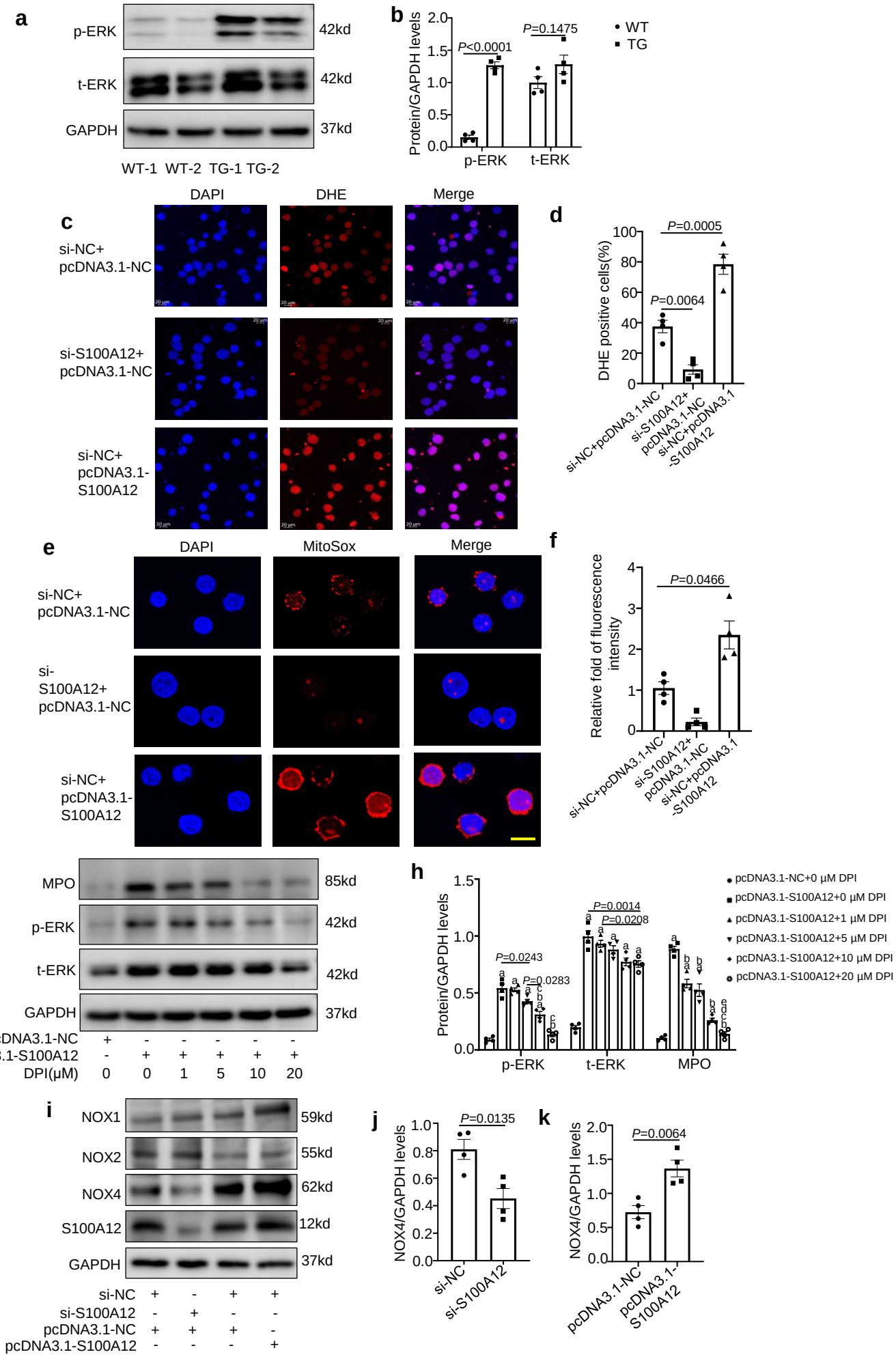
Supplementary Fig. 4. S100A12 expression in neutrophils affected the apoptosis level of co-cultured cardiomyocytes.

Cultured dHL-60 cells were transfected with si-S100A12 or pcDNA3.1-S100A12. **a, b** Western blotting was conducted to detect the protein expression ratio of cleaved-caspase3/Caspase3 and Bax/Bcl2, and statistical results. One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of Cleaved-caspase3/Caspase3, and Brown-Forsythe and Welch's ANOVA test is used for analyses of Bax/Bcl2 (n = 4 biological replicates). **c, d** dHL-60 cells transfected with pcDNA3.1-S100A12 or pcDNA3.1-NC were meanwhile treated with 50 μg/mL DNase I or PBS for 24 h. Western blotting was conducted to detect the protein expression ratio of cleaved-caspase3/Caspase3 and Bax/Bcl2, and statistical results (n = 4 biological replicates). **e, f** Representative images of TdT-mediated dUTP Nick-End Labelling (TUNEL) staining and analysis of the apoptosis rate in dHL-60 co-cultured cardiomyocytes (n = 4 biological replicates). Scale bar, 50 μm. **g, h** Representative images of TUNEL staining and analysis of apoptosis rate in dHL-60 co-cultured cardiomyocytes (n = 4 biological replicates). Scale bar, 50 μm. Data are expressed as mean ± SEM. Statistical analyses were carried out using Brown-Forsythe and Welch's ANOVA test for (**d**), (**f**) and (**h**). Source data are provided as a Source Data file.



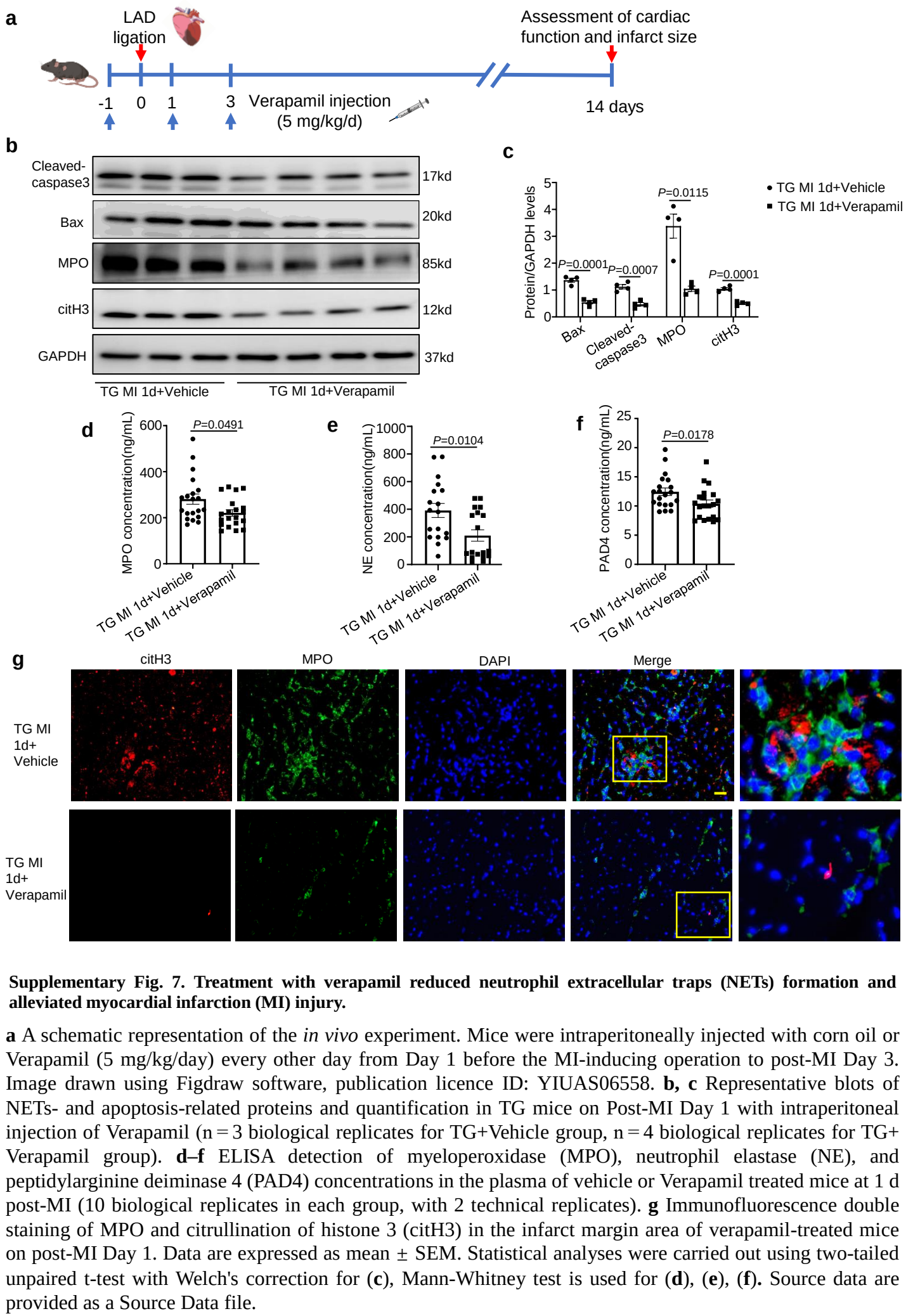
Supplementary Fig. 5. The effect of S100A12 exocrine inhibitor (Glibenclamide) on the formation of neutrophil extracellular traps (NETs).

a, b Western blotting was performed to detect the protein expression of citrullination of histone 3 (citH3), myeloperoxidase (MPO) and statistical results for dHL-60 cells treated with pcDNA3.1-S100A12 and different concentrations of Glibenclamide (Glib). One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of citH3, and Brown-Forsythe and Welch's ANOVA test is used for analyses of MPO and S100A12 (n = 4 biological replicates). **c** The expression of S100A12 in the supernatant of cultured dHL-60 cells treated with pcDNA3.1-S100A12 and different concentrations of Glib was detected using ELISA (n = 4 biological replicates). Statistical analyses were carried out using one-way ANOVA with Tukey's multiple comparisons test. Data are expressed as mean ± SEM. Source data are provided as a Source Data file.


a
p-ERK
t-ERK
GAPDH
42kd
42kd
37kd
WT-1 WT-2 TG-1 TG-2
b
Protein/GAPDH levels
P<0.0001
P=0.1475
WT
TG
p-ERK
t-ERK
c
DAPI
DHE
Merge
si-NC+ pcDNA3.1-NC
si-S100A12+ pcDNA3.1-NC
si-NC+ pcDNA3.1-S100A12
d
DHE positive cells(%)
P=0.0005
P=0.0064
si-NC+pcDNA3.1-NC
si-S100A12+ pcDNA3.1-NC
si-NC+pcDNA3.1 -S100A12
e
DAPI
MitoSox
Merge
si-NC+ pcDNA3.1-NC
si- S100A12+ pcDNA3.1-NC
si-NC+ pcDNA3.1- S100A12
f
Relative fold of fluorescence intensity
P=0.0466
si-NC+pcDNA3.1-NC
si-S100A12+ pcDNA3.1-NC
si-NC+pcDNA3.1 -S100A12
g
MPO
p-ERK
t-ERK
GAPDH
85kd
42kd
42kd
37kd
pcDNA3.1-NC + - - - - -
pcDNA3.1-S100A12 - + + + + +
DPI(μM) 0 0 1 5 10 20
h
Protein/GAPDH levels
P=0.0243
P=0.0283
P=0.0014
P=0.0208
p-ERK
t-ERK
MPO
pcDNA3.1-NC+0 μM DPI
pcDNA3.1-S100A12+0 μM DPI
pcDNA3.1-S100A12+1 μM DPI
pcDNA3.1-S100A12+5 μM DPI
pcDNA3.1-S100A12+10 μM DPI
pcDNA3.1-S100A12+20 μM DPI
i
NOX1
NOX2
NOX4
S100A12
GAPDH
59kd
55kd
62kd
12kd
37kd
si-NC + - + +
si-S100A12 - + - -
pcDNA3.1-NC + + + -
pcDNA3.1-S100A12 - - - +
j
NOX4/GAPDH levels
P=0.0135
si-NC
si-S100A12
k
NOX4/GAPDH levels
P=0.0064
pcDNA3.1-NC
pcDNA3.1-S100A12

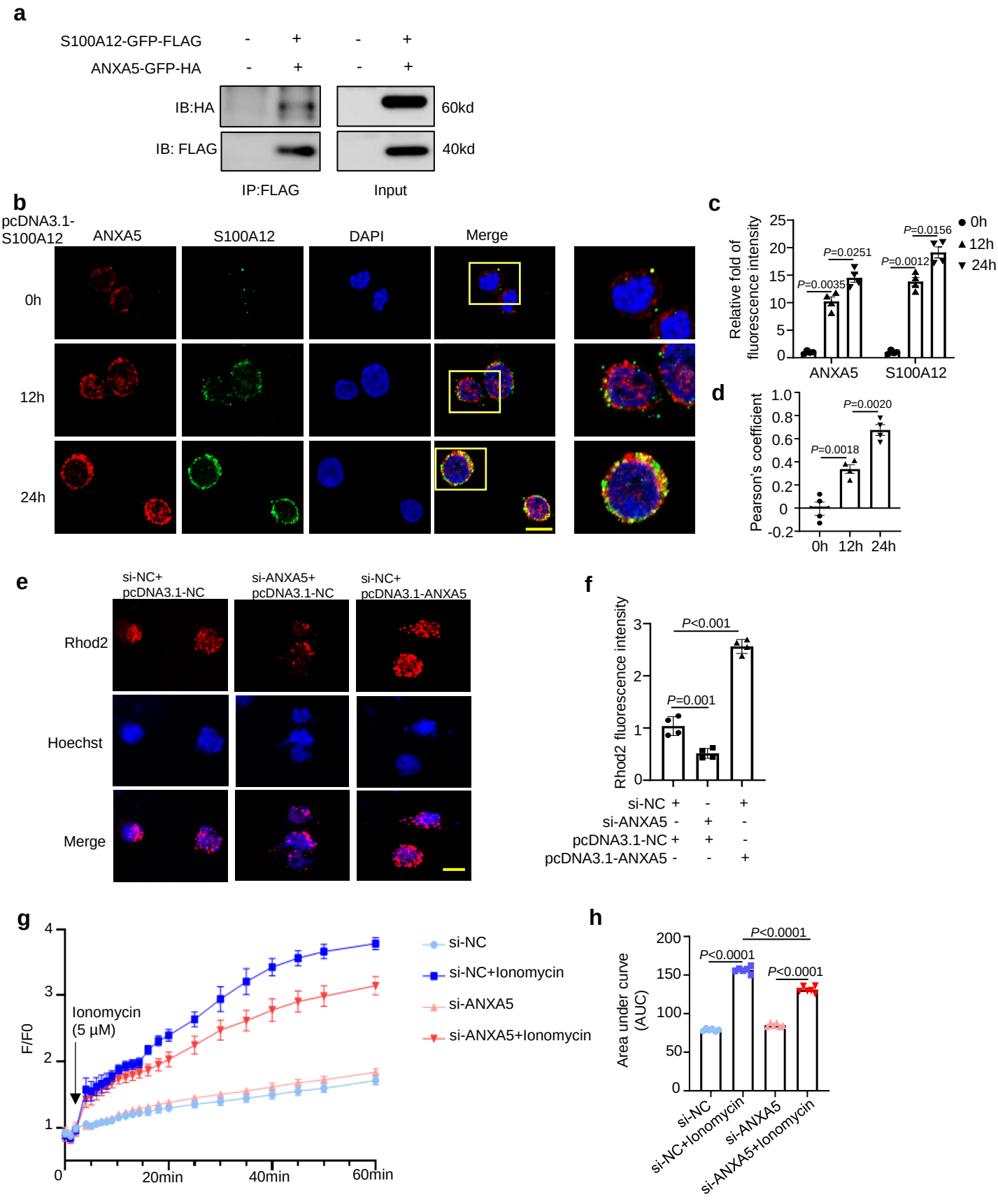
Supplementary Fig. 6. Endogenous S100A12 promotes suicide NETosis by the NADPH oxidase 4 (NOX4)-reactive oxygen species (ROS)-pERK pathway.

a, b Representative blots of p-ERK and t-ERK in TG and WT primary neutrophils (n = 4 biological replicates). **c, d** Detection and quantification of ROS by dihydroethidium (DHE) staining in dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12 (n = 4 biological replicates). **e, f** Representative MitoSOX staining of neutrophils. DAPI was used to stain the nuclei of dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12 (n = 4 biological replicates). Scale bar, 10 μm. **g, h** The ROS inhibitor Diphenyleineiodonium (DPI) reduced NETs formation in neutrophils transfected with pcDNA3.1-S100A12 (n = 4 biological replicates). **i–k** Representative blots of NOX1, NOX2, and NOX4 in dHL-60 cells transfected with si-S100A12 or pcDNA3.1-S100A12 (n = 4 biological replicates). a: $P < 0.001$ vs pcDNA3.1-NC+0 μM DPI, b: $P < 0.001$ vs pcDNA3.1-S100A12+0 μM DPI, c: $P < 0.001$ vs pcDNA3.1-S100A12+1 μM DPI, d: $P < 0.001$ vs pcDNA3.1-S100A12+5 μM DPI, e: $P < 0.001$ vs pcDNA3.1-S100A12+10 μM DPI. Data are expressed as mean ± SEM. Statistical analyses were carried out using two-tailed unpaired t-test with Welch's correction for (**b**), (**j**), (**k**), one-way ANOVA with Tukey's multiple comparisons test for (**d**), (**f**), (**h**)**.** Source data are provided as a Source Data file.



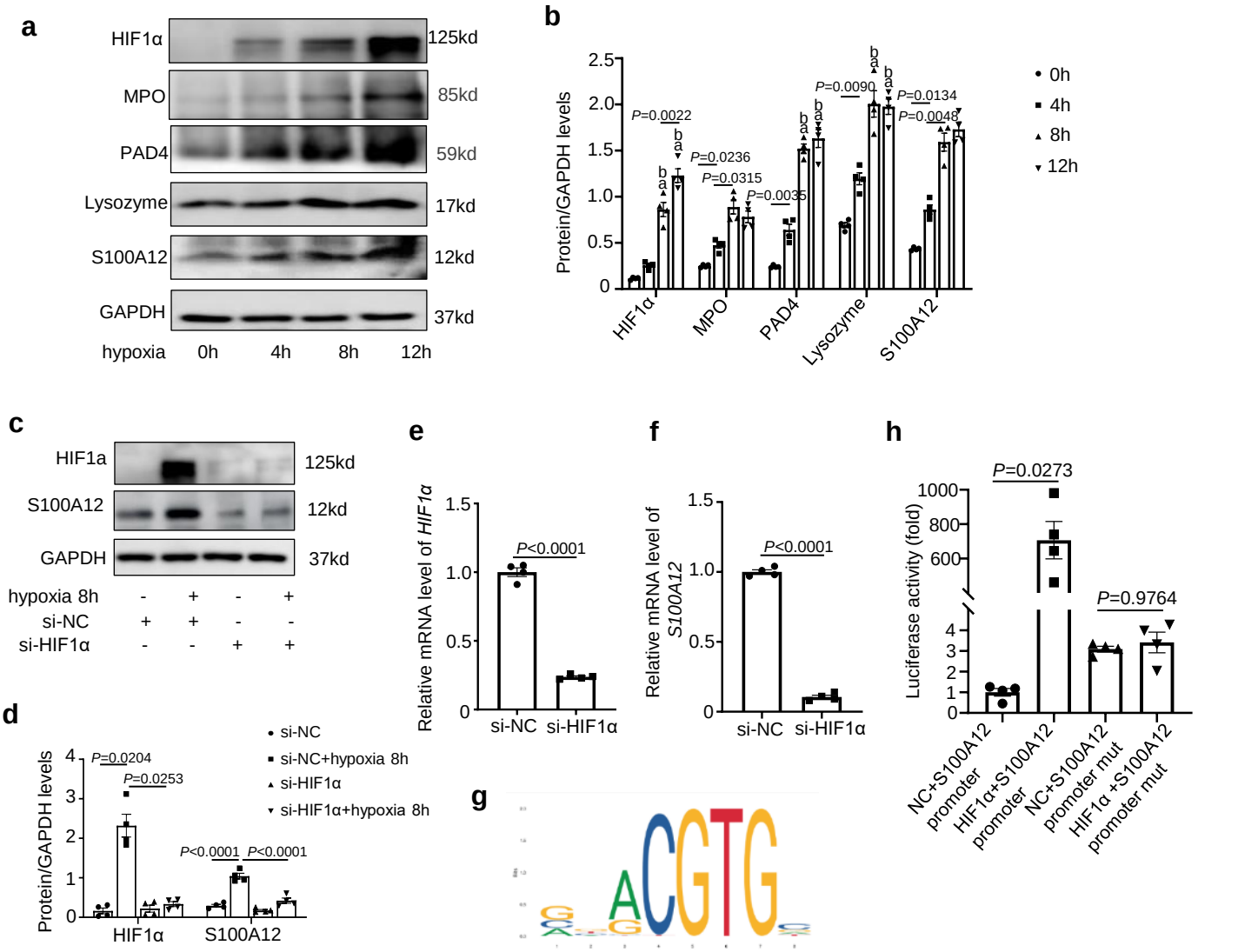
Supplementary Fig. 7. Treatment with verapamil reduced neutrophil extracellular traps (NETs) formation and alleviated myocardial infarction (MI) injury.

a A schematic representation of the *in vivo* experiment. Mice were intraperitoneally injected with corn oil or Verapamil (5 mg/kg/day) every other day from Day 1 before the MI-inducing operation to post-MI Day 3. Image drawn using Figdraw software, publication licence ID: YIUAS06558. **b, c** Representative blots of NETs- and apoptosis-related proteins and quantification in TG mice on Post-MI Day 1 with intraperitoneal injection of Verapamil (n = 3 biological replicates for TG+Vehicle group, n = 4 biological replicates for TG+ Verapamil group). **d–f** ELISA detection of myeloperoxidase (MPO), neutrophil elastase (NE), and peptidylarginine deiminase 4 (PAD4) concentrations in the plasma of vehicle or Verapamil treated mice at 1 d post-MI (10 biological replicates in each group, with 2 technical replicates). **g** Immunofluorescence double staining of MPO and citrullination of histone 3 (citH3) in the infarct margin area of verapamil-treated mice on post-MI Day 1. Data are expressed as mean ± SEM. Statistical analyses were carried out using two-tailed unpaired t-test with Welch's correction for (**c**), Mann-Whitney test is used for (**d**), (**e**), (**f**). Source data are provided as a Source Data file.


a
S100A12-GFP-FLAG - + - +
ANXA5-GFP-HA - + - +
IB:HA
IB: FLAG
60kd
40kd
IP:FLAG
Input
b
pcDNA3.1-S100A12
ANXA5
S100A12
DAPI
Merge
0h
12h
24h
c
Relative fold of fluorescence intensity
P=0.0035
P=0.0251
P=0.0012
P=0.0156
0h
12h
24h
ANXA5
S100A12
d
Pearson's coefficient
P=0.0018
P=0.0020
0h 12h 24h
e
si-NC+ pcDNA3.1-NC
si-ANXA5+ pcDNA3.1-NC
si-NC+ pcDNA3.1-ANXA5
Rhod2
Hoechst
Merge
f
Rhod2 fluorescence intensity
P<0.001
P=0.001
si-NC + - +
si-ANXA5 - + -
pcDNA3.1-NC + + -
pcDNA3.1-ANXA5 - - +
g
F/F0
Ionomycin (5 μM)
20min
40min
60min
si-NC
si-NC+Ionomycin
si-ANXA5
si-ANXA5+Ionomycin
h
Area under curve (AUC)
P<0.0001
P<0.0001
P<0.0001
si-NC
si-NC+Ionomycin
si-ANXA5
si-ANXA5+Ionomycin

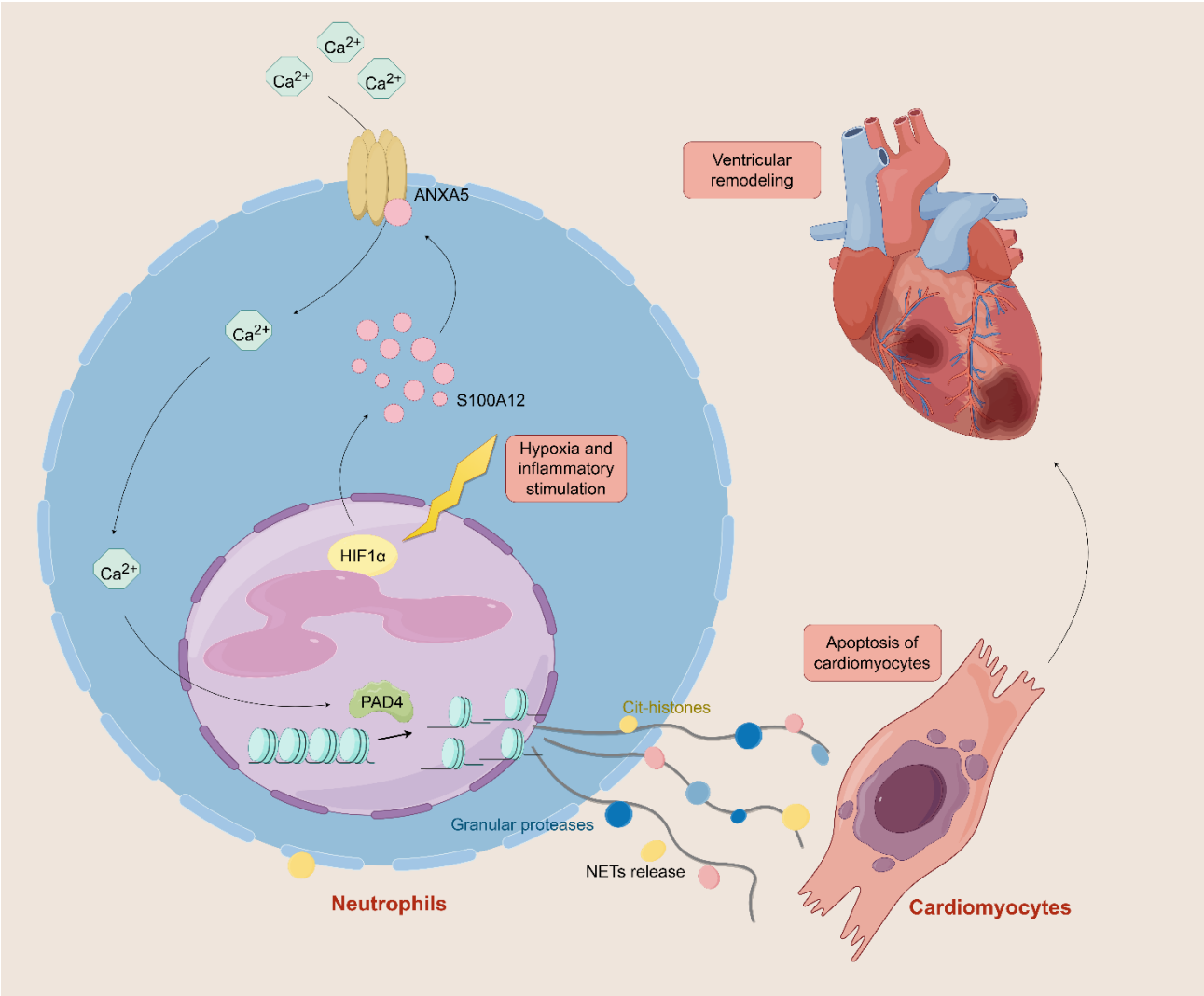
Supplementary Fig. 8. S100A12 interacted with Annexin A5 (ANXA5) to activate Ca^{2+}-peptidylarginine deiminase 4 (PAD4)-induced neutrophil extracellular traps (NETs) release.

a Co-immunoprecipitation assay showing the interaction between S100A12 and ANXA5 in dHL-60 cells. **b–d** Immunofluorescent co-staining of S100A12 and ANXA5 in cells transfected with pcDNA3.1-S100A12 at different time points. The degree of colocalization of S100A12 and ANXA5 was assessed using Pearson's coefficient (n = 4 biological replicates). Scale bar, 10 μm. **e, f** Intracellular calcium concentrations detected by confocal Rhod2 imaging, and statistical results after transfection with si-ANXA5 or pcDNA3.1-ANXA5 (n = 4 biological replicates). Scale bar, 10 μm. **g** Neutrophils transfected with or without si-ANXA5 were preloaded with the calcium probe Fluo-4 AM dye and stimulated with ionomycin (5 μM). The time course of the fluorescent signal was recorded at excitation and emission wavelengths of 488 and 526 nm, respectively. The Area under curve (AUC) was estimated using sums-of-squares method (n = 8 biological replicates). Data are expressed as mean ± SEM. Statistical analyses were carried out using Brown-Forsythe and Welch's ANOVA test for (**c**), one-way ANOVA with Tukey's multiple comparisons test for (**d**), (**f**), (**h**)**.** Source data are provided as a Source Data file.



Supplementary Fig. 9. Hypoxia-inducible factor-1α (HIF1α) regulated S100A12 expression under hypoxia.

a, b Time series of protein expression of HIF1α, Lysozyme, S100A12, myeloperoxidase (MPO), and peptidylarginine deiminase 4 (PAD4), and statistical results for dHL-60 cells under hypoxia stimulation (n = 4 biological replicates). One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of HIF1α, Lysozyme, PAD4, and Brown-Forsythe and Welch's ANOVA test is used for analyses of MPO and S100A12. a: $P < 0.001$ vs 0 h. b: $P < 0.001$ vs 4 h. **c, d** Western blotting was conducted to detect the protein expression of S100A12, and statistical results for dHL-60 cells treated with si-HIF1α under hypoxia stimulation. One-way ANOVA with Tukey's multiple comparisons test is used for statistical analyses of S100A12, and Brown-Forsythe and Welch's ANOVA test is used for analyses of HIF1α (n = 4 biological replicates). **e, f** The relative mRNA levels of *HIF1α* and *S100A12* in dHL-60 cells transfected with si-HIF1α (n = 4 biological replicates). **g** Sequence logo of the potential S100A12 binding site in JASPAR. **h** The dual-luciferase reporter assay was performed in 293T cells to verify that S100A12 was a sponged target of HIF1α. Data are expressed as mean ± SEM. Statistical analyses were carried out using Brown-Forsythe and Welch's ANOVA test for (**h**), unpaired t-test with Welch's correction for (**e**), (**f**). Source data are provided as a Source Data file.



Supplementary Fig. 10. Schematic picture.
Hypoxia-induced endogenous S100A12 expression in neutrophils stimulated the generation of neutrophil extracellular traps (NETs) by increasing the Annexin A5 (ANXA5) activated Ca^{2+}-peptidylarginine deiminase 4 (PAD4) axis, which exacerbated myocardial infarction (MI), and the blockage of ANXA5 effectively attenuated the impairment of heart function after acute myocardial infarction (AMI). Image drawn using Figdraw software, publication licence ID: YIUAS06558.